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IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF NEW JERSEY

IMMUNEX CORPORATION; AMGEN MANUFACTURING, LIMITED; and HOFFMANN-LA ROCHE INC.;	Civil Action No. _____
Plaintiffs,	**COMPLAINT & DEMAND FOR JURY**
v.	
SANDOZ INC.; SANDOZ INTERNATIONAL GMBH; SANDOZ GMBH;	***Confidential - Filed Under Seal***
Defendants.	

COMPLAINT

Plaintiffs Immunex Corporation; Amgen Manufacturing, Limited; and Hoffmann-La Roche Inc., by and through their undersigned attorneys, for their Complaint against Defendants

Sandoz Inc.; Sandoz International GmbH; and Sandoz GmbH (collectively, "Defendants") allege as follows:

I. THE PARTIES

A. Plaintiffs

1. Immunex Corporation ("Immunex") is a corporation organized and existing under the laws of the State of Washington with its principal place of business at One Amgen Center Drive, Thousand Oaks, California 91320. Amgen Inc. acquired Immunex in July 2002, and Immunex became a wholly-owned subsidiary of Amgen Inc.

2. Amgen Manufacturing, Limited ("AML") is a corporation existing under the laws of the Territory of Bermuda, with its principal place of business at Road 31 km 24.6, Juncos, Puerto Rico 00777. AML is a wholly-owned subsidiary of Amgen Inc.

3. Hoffmann-La Roche Inc. ("Roche") is a corporation organized and existing under the laws of the State of New Jersey with its principal place of business at 150 Clove Road, Suite 8, Little Falls, New Jersey 07424.

B. Defendants

4. On information and belief, Sandoz Inc. is a corporation organized and existing under the laws of the State of Colorado, with its principal place of business at 100 College Road West, Princeton, New Jersey 08540. Upon information and belief, acting in concert with each of the other Defendants, Sandoz Inc. is in the business of developing, manufacturing, and marketing biopharmaceutical products that are distributed and sold in the State of New Jersey and throughout the United States. Upon information and belief, Sandoz Inc. is also the United States agent for Sandoz International GmbH and Sandoz GmbH for purposes including, but not limited to, filing regulatory submissions to and corresponding with the Food and Drug Administration ("FDA").

5. Upon information and belief, Sandoz International GmbH is a corporation existing under the laws of the Federal Republic of Germany with its principal place of business at Industriestraße 25, 83607 Holzkirchen, Germany. Upon information and belief, acting in concert with each of the other Defendants, Sandoz International GmbH is in the business of developing, manufacturing, and marketing biopharmaceutical products that are distributed and sold in the State of New Jersey and throughout the United States.

6. Upon information and belief, Sandoz GmbH is a corporation existing under the laws of the Republic of Austria with its principal place of business at Biochemiestraße 10, 6250 Kundl, Austria. Upon information and belief, acting in concert with each of the other Defendants, Sandoz GmbH is in the business of developing, manufacturing, and marketing biopharmaceutical products that are distributed and sold in the State of New Jersey and throughout the United States.

7. Upon information and belief, Sandoz GmbH operates as a subsidiary of Sandoz International GmbH.

8. Upon information and belief, Defendants collaborate to develop, manufacture, seek regulatory approval for, import, market, distribute, and sell biopharmaceutical products (including products intended to be sold as biosimilar versions of successful biopharmaceutical products developed by others) in the State of New Jersey and throughout the United States.

II. NATURE OF THE ACTION

9. This is an action for patent infringement arising under 35 U.S.C. § 271, including § 271(e)(2)(C), which was enacted in 2010 as part of the Biologics Price Competition and Innovation Act ("the BPCIA"). This action involves patents that cover etanercept (the active ingredient of the biologic drug product, ENBREL®), its method of manufacture, certain materials used in its manufacture, and certain therapeutic uses of etanercept. Immunex and AML

(collectively, "Immunex/AML") and Roche bring this suit to enjoin Defendants from infringing their patents and to recover any damages resulting from Defendants' infringement.

10. The asserted patents are United States Patent Nos. 8,063,182 ("the '182 patent"), 8,163,522 ("the '522 patent"), 7,915,225 ("the '225 patent"), 8,119,605 ("the '605 patent"), and 8,722,631 ("the '631 patent") (collectively, "the patents-in-suit").

11. Roche is the owner of the '182 and '522 patents. Immunex is the exclusive licensee of all commercial rights in the '182 and '522 patents, including all rights to sell ENBREL®.

12. Immunex is the owner of the '225, '605, and '631 patents.

13. Immunex has granted AML an exclusive license (or, with respect to the '182 and '522 patents, an exclusive sublicense) to the asserted patents.

14. On September 29, 2015, the FDA accepted Defendants' abbreviated Biologics License Application ("aBLA") pursuant to the BPCIA, specifically 42 U.S.C. § 262(k) (also known as § 351(k) of the Public Health Service Act ("PHSA")), seeking authorization from the FDA to market a biosimilar version of Immunex's ENBREL® (etanercept) product.

15. The BPCIA created an abbreviated pathway for the approval of biosimilar versions of approved biologic drugs. The abbreviated pathway (also known as "the (k) pathway") allows a biosimilar applicant (here Sandoz Inc.) to rely on the prior licensure and approval status of the innovative biological product (here ENBREL®) that the biosimilar purports to copy. Immunex is the sponsor of the reference product, ENBREL®, which is approved by the FDA for a number of different indications (*i.e.*, therapeutic uses).

16. Defendants committed an act of infringement under 35 U.S.C. § 271(e)(2)(C) when they caused Sandoz Inc. to submit Defendants' aBLA seeking FDA approval to

commercially manufacture, use, offer for sale, sell, distribute in, or import into the United States Defendants' etanercept product prior to the expiration of the asserted patents.

17. If the FDA approves Defendants' aBLA, Defendants will also infringe one or more claims of each of the patents-in-suit, under 35 U.S.C. § 271(a), (b), or (g), should they engage in the commercial manufacture, use, offer for sale, sale, distribution in, or importation into the United States of Defendants' etanercept product.

III. JURISDICTION AND VENUE

A. Subject-matter Jurisdiction

18. This Court has subject-matter jurisdiction over Immunex/AML and Roche's patent infringement claims under 28 U.S.C. §§ 1331, 1338(a), and 2201(a).

B. Sandoz Inc.

19. This Court has personal jurisdiction over Sandoz Inc. by virtue of the fact that, on information and belief, Sandoz Inc.'s principal place of business is in the District of New Jersey.

20. Sandoz Inc., Sandoz International GmbH, and Sandoz GmbH hold themselves out as a unitary entity and have represented to the public that their activities are directed, controlled, and carried out as a single entity.

21. For example, during prior litigation brought by Sandoz Inc. concerning the '182 and '522 patents, *see Sandoz Inc. v. Amgen Inc.*, 773 F.3d 1274 (Fed. Cir. 2014), Sandoz Inc. submitted a declaration by Rüdiger Jankowsky which stated that he worked for "Sandoz." According to his LinkedIn profile, at the time Jankowsky worked for "Sandoz Biopharmaceuticals/Novartis" in Holzkirchen, Germany—the location of Sandoz International GmbH's principal place of business.

22. As another example, during the same prior litigation brought by Sandoz Inc. concerning the '182 and '522 patents, Sandoz Inc. submitted a declaration by Karsten Roth

which stated that he was employed by "Sandoz Inc." However, according to his LinkedIn profile, at the time of his declaration he was employed by "Sandoz" in the "Munich Area, Germany." Upon information and belief, Roth attended meetings with the FDA to discuss Defendants' aBLA and encouraged the FDA to approve Defendants' aBLA.

C. **Sandoz International GmbH**

23. Upon information and belief, Sandoz International GmbH collaborates with Sandoz Inc. to develop, manufacture, seek approval for, and sell FDA-approved biopharmaceutical drugs, which are being marketed, distributed, and sold in New Jersey and in the United States.

24. Upon information and belief, Sandoz International GmbH exercises considerable control over Sandoz Inc. with respect to biosimilar products, and approves significant decisions of Sandoz Inc. such as allowing Sandoz Inc. to act as the agent for Sandoz International GmbH in connection with preparing and filing Defendants' aBLA and to act as Sandoz International GmbH's agent in the United States. For example, the Sandoz Management Team includes "Richard Francis, Global Head of Sandoz," and "Peter Goldschmidt, President of Sandoz US and Head of North America." Upon information and belief, Mr. Francis is the head of Sandoz International GmbH, Mr. Goldschmidt is the President of Sandoz Inc. as well as the Head of North American Operations at Sandoz International GmbH, and Mr. Goldschmidt directly or indirectly reports to Mr. Francis.

25. Upon information and belief, employees or officers of Sandoz International GmbH, such as Mark McCamish and Ingrid Schwarzenberger, have attended meetings with the FDA to discuss Defendants' aBLA and have encouraged the FDA to approve Defendants' aBLA.

26. In addition, Sandoz International GmbH and Sandoz Inc. hold themselves out as a unitary entity and have represented to the public that the activities of Sandoz International GmbH

and Sandoz Inc. are directed, controlled, and carried out as a single entity. For example, Sandoz maintains an Internet website at the URL www.sandoz.com attached hereto as Exhibit A, which states that it is "the website of Sandoz International" and on which Sandoz states that all of the worldwide generic pharmaceutical businesses owned by Novartis operate "under one single global brand as known today: Sandoz."

27. Upon information and belief, Sandoz International GmbH is actively involved in planning Sandoz Inc.'s new products and filing Defendants' aBLA for the biosimilar product in dispute. For example, Sandoz Inc.'s President, Mr. Goldschmidt, is also the Head of North American Operations at Sandoz International GmbH.

28. Upon information and belief, Sandoz International GmbH acted in concert with Sandoz Inc. to develop a biosimilar version of ENBREL®. Upon information and belief, Sandoz International GmbH acted in concert with, directed, or authorized Sandoz Inc. to file an aBLA seeking approval from the FDA to market and sell Defendants' biosimilar product in the State of New Jersey and throughout the United States, which directly gives rise to Plaintiffs' claims of patent infringement. For example, Novartis AG, the ultimate corporate parent of both Sandoz International GmbH and Sandoz Inc., issued a press release on October 2, 2015, from Holzkirchen, Germany announcing that the FDA had accepted an application by "Sandoz" for biosimilar etanercept. *See* Press Release, Novartis, "FDA accepts Sandoz regulatory submission for a proposed biosimilar etanercept" (Oct. 2, 2015), https://www.novartis.com/news/media-releases/fda-accepts-sandoz-regulatory-submission-proposed-biosimilar-etanercept, attached hereto as Exhibit B. Upon information and belief, the press release announcing the FDA's acceptance of Defendants' aBLA, which is the subject of Plaintiffs' claims, was issued on behalf of Sandoz International GmbH.

29. Upon information and belief, the acts of Sandoz Inc. complained of herein were done, in part, for the benefit of Sandoz International GmbH. Upon information and belief, Sandoz International GmbH directly or indirectly manufactures, imports into the United States, or sells Defendants' biosimilar product that is the subject of the infringement claims in this action in New Jersey and throughout the United States.

30. Additionally, and in the alternative, Immunex/AML and Roche allege that to the extent Sandoz International GmbH is not subject to the jurisdiction of the courts of general jurisdiction of the State of New Jersey, Sandoz International GmbH likewise is not subject to the jurisdiction of the courts of general jurisdiction of any state, and accordingly is amenable to service of process based on its aggregate contacts with the United States, including but not limited to the above described contacts, as authorized by Rule 4(k)(2) of the Federal Rules of Civil Procedure.

D. **Sandoz GmbH**

31. Upon information and belief, Sandoz GmbH collaborates with Sandoz Inc. to develop, manufacture, seek approval for, and sell FDA-approved biopharmaceutical drugs, which are being marketed, distributed, and sold in New Jersey and in the United States.

32. Sandoz GmbH and Sandoz Inc. hold themselves out as a unitary entity and have represented to the public that the activities of Sandoz GmbH and Sandoz Inc. are directed, controlled, and carried out as a single entity. For example, Sandoz maintains an Internet website at the URL www.sandoz.com, attached hereto Exhibit A, which states that it is "the website of Sandoz International" and on which Sandoz states that all of the worldwide generic pharmaceutical businesses owned by Novartis operate "under one single global brand as known today: Sandoz."

33. Upon information and belief, Sandoz GmbH is actively involved with planning Sandoz Inc.'s new biosimilar etanercept products and filing Defendants' aBLA for the biosimilar product in dispute. Title 42 U.S.C. § 262(k)(2)(A)(V) provides that a biosimilar application submitted to the FDA under the § 262(k) pathway "shall include" information demonstrating "the facility in which the biological product is manufactured, processed, packed, or held meets standards designed to assure that the biological product continues to be safe, pure, and potent." Upon information and belief, Defendants' biosimilar product that is the subject of Defendants' aBLA is manufactured at Sandoz GmbH facilities. Therefore, upon information and belief, Sandoz GmbH actively participated in the preparation of Defendants' aBLA, for example by providing information regarding the facilities in which Defendants' biosimilar product is manufactured, processed, packed, or held.

34. Upon information and belief, Sandoz GmbH acted in concert with Sandoz Inc. to develop a biosimilar version of ENBREL®. Upon information and belief, Sandoz GmbH acted in concert with, directed, or authorized Sandoz Inc. to file an aBLA seeking approval from the FDA to market and sell Defendants' biosimilar product in the State of New Jersey and throughout the United States, which directly gives rise to Plaintiffs' claims of patent infringement.

35. Upon information and belief, employees or officers of Sandoz GmbH, such as Fritz Reiter and Thomas Stangler, have attended meetings with the FDA to discuss Defendants' aBLA and have encouraged the FDA to approve Defendants' aBLA.

36. Upon information and belief, employees or officers of Sandoz GmbH, such as Albrecht Ralf, have signed certifications which were executed to be included as part of Defendants' aBLA.

37. Upon information and belief, the acts of Sandoz Inc. complained of herein were done, in part, for the benefit of Sandoz GmbH. Upon information and belief, Sandoz GmbH directly or indirectly manufactures, imports into the United States, or sells Defendants' biosimilar product that is the subject of the infringement claims in this action in New Jersey and throughout the United States.

38. Additionally, and in the alternative, Plaintiffs allege that to the extent Sandoz GmbH is not subject to the jurisdiction of the courts of general jurisdiction of the State of New Jersey, Sandoz GmbH likewise is not subject to the jurisdiction of the courts of general jurisdiction of any state, and accordingly is amenable to service of process based on its aggregate contacts with the United States, including but not limited to the above described contacts, as authorized by Rule 4(k)(2) of the Federal Rules of Civil Procedure.

E. **Venue**

39. Venue is proper in this District pursuant to 28 U.S.C. § 1391(b) and (c), and 28 U.S.C. § 1400(b). On information and belief, Defendants manufacture, seek regulatory approval to market, distribute, and sell pharmaceutical products, and market, distribute, and sell pharmaceutical products for use throughout the United States, including in this District.

IV. BACKGROUND

A. **TNF and TNF Receptors**

40. Tumor necrosis factor ("TNF") is a cell signaling protein that is involved in various biological effects that include the regulation of immune response, inflammation, and other processes. It was first identified as an agent that has cytotoxic effects on tumor cells, and hence was named "tumor necrosis factor." Overproduction of TNF in the body is also implicated in various autoimmune diseases and other inflammatory disorders.

41. The biological effects of TNF can be mediated via specific receptors that are found on the membranes of certain cells. TNF receptors on the surface of the cells can specifically bind to TNF. This binding can trigger reactions inside the cell, which can give rise to a number of different responses, including inflammation, cell growth, and cell death.

42. Two cell membrane-bound receptors specific to TNF are sometimes referred to as the "p55 TNF receptor" and the "p75 TNF receptor."

B. **Immunex's Investment in ENBREL® (etanercept)**

43. The active ingredient in ENBREL® is etanercept, a genetically engineered, non-naturally occurring fusion protein that binds to and inhibits TNF from binding to a TNF receptor.

44. The etanercept fusion protein was genetically engineered to fuse the extracellular region of the human p75 version of the TNF receptor with a portion of a human immunoglobulin heavy chain (*i.e.*, a portion of a human antibody).

45. By binding to and inhibiting TNF from interacting with TNF receptors, ENBREL® can reduce certain inflammatory responses implicated in certain disorders such as rheumatoid arthritis, psoriasis, and psoriatic arthritis, and others.

46. The FDA has approved ENBREL® for the following indications: rheumatoid arthritis, polyarticular juvenile idiopathic arthritis, psoriatic arthritis, ankylosing spondylitis, and plaque psoriasis. The availability of ENBREL® represented a major advance in the treatment of these disorders.

47. Immunex conducted Phase I testing to determine whether ENBREL® was safe to administer to patients with rheumatoid arthritis; results published in 1993 indicated that it was. Immunex then conducted Phase II testing to begin determining whether ENBREL® improved symptoms of rheumatoid arthritis; results indicating that it did improve symptoms were published in 1996. Immunex conducted Phase III testing and invested a substantial amount of

time and resources testing ENBREL® to demonstrate that it was safe and effective for certain disorders.

48. Based on the results of clinical testing in rheumatoid arthritis, Immunex filed Biologic License Application ("BLA") No. 103795. As a result, in November 1998, the FDA first approved ENBREL®, pursuant to BLA No. 103795, for the treatment of moderate to severe rheumatoid arthritis. Immunex holds the rights to BLA No. 103795.

49. Other clinical testing revealed that ENBREL® was safe and effective for certain additional diseases. Based on Immunex's further clinical testing, Immunex filed supplements to BLA No. 103795, requesting that ENBREL® be approved for certain additional indications. As a result, the FDA approved ENBREL® for the treatment of polyarticular juvenile idiopathic arthritis in 1999, psoriatic arthritis in 2002, ankylosing spondylitis in 2003, and plaque psoriasis in 2004. These approvals are the direct result of very significant investments by Immunex in the development and clinical trials of ENBREL®.

C. **Defendants' Abbreviated BLA**

50. Defendants are piggybacking on the fruits of Immunex/AML and Roche's trailblazing efforts. Defendants have publicly announced that they filed their aBLA under the (k) pathway to obtain approval to commercially manufacture, use, offer to sell, and sell, and import into the United States their etanercept product that they assert is a biosimilar version of Immunex's ENBREL®.

51. Defendants have also chosen to benefit from the clinical data generated by Immunex/AML's investments demonstrating the therapeutic indications for which ENBREL® is effective. Defendants issued a press release stating that "Sandoz is seeking approval for all indications included in the label of the reference product which is used to treat a range of autoimmune diseases including rheumatoid arthritis and psoriasis affecting approx. 1.3 million

and 7.5 million people (respectively) in the US" (footnotes omitted). *See* Press Release, Novartis, "FDA accepts Sandoz regulatory submission for a proposed biosimilar etanercept" (Oct. 2, 2015), https://www.novartis.com/news/media-releases/fda-accepts-sandoz-regulatory-submission-proposed-biosimilar-etanercept, attached hereto as Exhibit B.

52. On information and belief, Defendants conducted clinical trials only for the use of their biosimilar drug product on psoriasis patients, despite the breadth of their request to the FDA for approval for other indications, such as rheumatoid arthritis, polyarticular juvenile idiopathic arthritis, psoriatic arthritis, and ankylosing spondylitis.

53. On information and belief, Defendants did not conduct any clinical trials on indications for which ENBREL® had not already been demonstrated to be safe and effective.

54. On information and belief, the amino acid sequence of Defendants' etanercept fusion protein is the same amino acid sequence of the etanercept fusion protein in ENBREL®.

55. On information and belief, Defendants have represented to the FDA that their etanercept product is biosimilar to Immunex's ENBREL®. As such, on information and belief, Defendants' etanercept product utilizes the same mechanism of action as ENBREL® for the conditions of use prescribed, recommended, or suggested in ENBREL®'s approved label. In addition, the route of administration, the dosage form, and the strength of Defendants' etanercept product are the same as those of Immunex's ENBREL®. *See* 42 U.S.C. § 262(k)(2)(A)(i).

D. **Defendants' Refusal to Comply with the BPCIA**

56. Defendants have—for the second time—tried to reap the commercial benefits provided to biosimilar manufacturers under the BPCIA while seeking to avoid the obligations in that same Act that Congress established to protect innovators such as Immunex/AML and Roche.

57. On October 19, 2015, which was, on information and belief, 20 days after the FDA notified Sandoz Inc. that its aBLA had been accepted for review, Sandoz Inc. provided

Immunex with remote access to a Sandoz-hosted database of TIFF images, modified to include added confidentiality designations, that Sandoz Inc. represented to constitute its aBLA and information relating to the manufacturing process for Defendants' biosimilar product. The manner in which this database access was provided would not have allowed Immunex local access and evaluation except after manual download of the thousands of documents included therein, along with a folder-by-folder manual reconstruction of the database's directory structure. Sandoz Inc. did not provide a local copy of the database—including the necessary database load files and associated data—and an unaltered copy of the aBLA in the same electronic format as submitted to FDA until October 28, 2015.

58. On November 9, 2015, determining that Sandoz had failed to provide complete information describing the processes used to manufacture the biological product that is the subject of Defendants' aBLA, Immunex requested that Sandoz Inc. provide further information.

59. On November 16, 2015, Sandoz Inc. provided additional documents which it represented to relate to the manufacturing process for Defendants' biosimilar product.

60. Notwithstanding issues with the timeliness and completeness of the information Sandoz Inc. had provided, in respect of 42 U.S.C. § 262(*l*)(3)(A), Immunex nevertheless provided to Sandoz Inc. on December 18, 2015 a list of patents for which a claim of infringement could be reasonably asserted based on Defendants' etanercept product.

61. On January 27, 2016, Sandoz Inc. responded to Immunex's list of patents by stating that it no longer wished to follow the strictures of the BPCIA. Specifically, Sandoz Inc. sent Immunex a 86-page letter stating its patent contentions but "agreeing" with Immunex's 42 U.S.C. § 262(*l*)(3)(A) list. Sandoz Inc. also stated it was "waiving" its right to receive a statement by Immunex pursuant to 42 U.S.C. § 262(*l*)(3)(C), and declared that negotiations

pursuant to 42 U.S.C. § 262(*l*)(4) and (5) were unnecessary. Sandoz Inc. then insisted that Immunex file an action for patent infringement pursuant to 42 U.S.C. § 262(*l*)(6) within 30 days, *i.e.*, by February 26, 2016. Also on January 27, 2016, Sandoz Inc. provided additional documents which it represented provided even more information relating to the manufacturing process for Defendants' biosimilar product.

62. On February 10, 2016, Immunex explained to Sandoz Inc. that its refusal to participate in negotiations pursuant to 42 U.S.C. § 262(*l*)(4) and (5) was contrary to the text of the statute. Immunex also requested that Sandoz Inc. withdraw its refusal to participate in the statutory process set forth in 42 U.S.C. § 262(*l*)(4) and (5), and explained that Sandoz Inc.'s failure to do so implicated 42 U.S.C. § 262(*l*)(9), which authorizes the reference product sponsor, but not the subsection (k) applicant, to file a declaratory judgment action on patents that are or would be infringed by the biosimilar applicant (Sandoz Inc.).

63. On February 17, 2016, Sandoz Inc. confirmed its refusal to participate in negotiations pursuant to 42 U.S.C. § 262(*l*)(4) and (5), and stated that it wished for patent litigation to begin as soon as possible. No list of patents for Defendants' etanercept biosimilar product was generated as described in 42 U.S.C. § 262(*l*)(4) or as described in 42 U.S.C. § 262(*l*)(5).

64. Defendants have failed to participate, and thus, have not complied with the process defined in the statute that must precede an "immediate patent infringement action" under 42 U.S.C. § 262(*l*)(6). By refusing to participate in a timely and complete manner under the BPCIA, including by seeking to extinguish Immunex's ability to consider and respond to Sandoz Inc.'s contentions regarding the patents that Immunex had properly identified and entirely evading the negotiations specified in 42 U.S.C. § 262(*l*)(4) and (5), Sandoz Inc. has repudiated

its obligations under the BPCIA. Thus, in addition to bringing an action under 35 U.S.C. § 271(e)(2)(C), Immunex—but not Defendants—pursuant to 42 U.S.C. § 262(*l*)(9) may bring a declaratory action on patents related to Defendants' biosimilar product.

V. THE PATENTS-IN-SUIT

A. The '182 and '522 Patents

65. In the late 1980s, Roche and Immunex scientists were early pioneers in isolating, characterizing, cloning, and sequencing p55 and p75 versions of the human TNF receptors, respectively.

66. Roche scientists were the first to clone and sequence the human p55 TNF receptor gene and determine the amino acid sequence of the receptor. They published the sequence of this receptor on April 20, 1990. *See* Loetscher et al., "Molecular Cloning and Expression of the Human 55 kd Tumor Necrosis Factor Receptor," *Cell* 61:351-359 (April 20, 1990).

67. Immunex scientists were the first to clone and sequence the p75 TNF receptor gene and determine the amino acid sequence of the receptor. They published the sequence of the human p75 TNF receptor later the same year. *See* Smith et al., "A Receptor for Tumor Necrosis Factor Defines an Unusual Family of Cellular and Viral Proteins," *Science* 248:1019-1023 (1989).

68. On August 31, 1990, Roche scientists filed European Patent Application No. 90116707.2, which disclosed and taught the novel concept of fusing of the extracellular fragment of the TNF receptors with a portion of the human immunoglobulin heavy chain (*i.e.*, all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain of said constant region). These Roche scientists also filed a United States patent application on September 10, 1990, which claimed priority to said European patent application.

69. The '182 and '522 patents issued from applications that claim priority to the European patent application filed on August 31, 1990.

70. The '182 patent is directed to a fusion protein incorporating a portion of the p75 TNF receptor and covers etanercept. The '522 patent is directed to nucleic acids, host cells, and methods of using such nucleic acids and host cells to make the p75 TNF receptor fusion protein.

B. **The '225, '605, and '631 Patents**

71. In developing etanercept as a therapeutic, Immunex also developed and obtained patents directed toward using etanercept to treat psoriasis and/or psoriatic arthritis. The '225 patent, the '605 patent, and the '631 patent ("the Psoriasis Patents"), owned by Immunex, disclose and claim methods of using etanercept to treat psoriasis and/or psoriatic arthritis.

72. Psoriasis is a chronic inflammatory disease of the skin and joints. It results in scaly growths on the skin of affected patients, which can be disfiguring and extremely uncomfortable.

73. Psoriatic arthritis is an inflammatory arthritis characterized by joint pain, stiffness, and swelling. It can cause to joint damage which limits daily activities.

74. In the late 1990s, there were no biologic therapies approved to treat psoriasis or psoriatic arthritis.

75. Dermatologists had used various other therapeutic approaches to treating psoriasis, such as methotrexate, psoralen and ultraviolet A radiation, and cyclosporine. However, each of these therapies was found to have serious side effects, such as liver damage, skin damage, and kidney damage, respectively, after they had been used for many years.

76. The Psoriasis Patents claim priority to a provisional application filed on August 11, 1999. The Psoriasis Patents also claim priority to non-provisional applications filed August 13, 1999, and June 23, 2000.

77. As a general matter, the Psoriasis Patents contain claims to using etanercept to treat psoriasis and/or psoriatic arthritis, and further specify certain dosage regimes to follow.

78. The manner in which ENBREL® is commonly used to treat psoriasis (or psoriasis and psoriatic arthritis) today falls within the scope of the claims of the Psoriasis Patents.

COUNT 1: INFRINGEMENT OF THE '182 PATENT UNDER 35 U.S.C. § 271(e)(2)(C)

79. Immunex/AML and Roche incorporate by reference paragraphs 1-70 as if fully set forth herein.

80. The '182 patent, titled "Human TNF Receptor Fusion Protein," was duly and legally issued on November 22, 2011 by the United States Patent and Trademark Office ("USPTO"). A true and correct copy of the '182 patent is attached to this Complaint as Exhibit C.

81. The claims of the '182 patent cover etanercept and pharmaceutical compositions that are made from etanercept.

82. Defendants have infringed the '182 patent by submitting an aBLA referencing Immunex's ENBREL® product and seeking FDA approval under 42 U.S.C. § 262(k) to manufacture, import, offer to sell, or sell within the United States the product that is the subject of that application.

83. On December 18, 2015, Immunex, as the reference product sponsor for ENBREL®, identified the '182 patent to Sandoz pursuant to 42 U.S.C. § 262(*l*)(3)(A).

84. Defendants have known of the '182 patent since at least June 2013. Despite such knowledge, Defendants nonetheless filed their aBLA with the FDA in July 2015 seeking approval of Defendants' etanercept biosimilar product, with the intent to import, offer to sell, and sell their biosimilar product within the United States before the expiration of the '182 patent and in violation of Immunex/AML and Roche's patent rights.

85. Immunex/AML and/or Roche will be irreparably harmed if Defendants are not enjoined from infringing one or more claims of the '182 patent. Immunex/AML and Roche do not have an adequate remedy at law and are entitled to injunctive relief preventing Defendants from any further infringement.

86. Defendants' commercial manufacture, use, offer for sale, or sale within the United States, or importation into the United States, upon FDA approval of Defendants' etanercept biosimilar product and before the expiration of the '182 patent, will cause injury to Immunex/AML and Roche, entitling them to damages or other monetary relief.

COUNT 2: DECLARATORY JUDGMENT OF INFRINGEMENT OF THE '182 PATENT UNDER 35 U.S.C. § 271(a)

87. Immunex/AML and Roche incorporate by reference paragraphs 1-70 as if fully set forth herein.

88. Defendants have sought FDA approval under 42 U.S.C. § 262(k) to manufacture, use, import, offer to sell, or sell within the United States Defendants' etanercept biosimilar product, a biosimilar version of ENBREL® (etanercept).

89. The FDA has publicly stated that the agency's goal is to act upon an aBLA application within 10 months of receipt. On information and belief, Defendants believe that the FDA may act upon Defendants' aBLA as soon as May 2016, and that Defendants will be able to pay the user fee prescribed under the Prescription Drug User Fee Act by that time.

90. On information and belief, Defendants intend to, and will immediately and imminently upon FDA licensure of Defendants' aBLA, notwithstanding the clear requirements of § 262(*l*)(8), import and offer to sell or sell within the United States Defendants' etanercept biosimilar product, which will infringe one or more claims of the '182 patent under 35 U.S.C. § 271(a).

91. An actual controversy has arisen and now exists between the parties concerning whether Defendants' etanercept biosimilar product has or will infringe one or more claims of the '182 patent.

92. Defendants also have failed to complete the actions required of them under 42 U.S.C. § 262(*l*)(4) and (5) by failing to engage in negotiation and exchange of patent lists under 42 U.S.C. § 262(*l*)(4) and 262(*l*)(5). No list of patents for Defendants' etanercept biosimilar product was generated as described in 42 U.S.C. § 262(l)(4) or as described in 42 U.S.C. § 262(*l*)(5).

93. Immunex/AML and Roche are entitled to a declaratory judgment that Defendants have infringed or will infringe one or more claims of the '182 patent by making, using, offering to sell, or selling within the United States, or importing into the United States Defendants' etanercept biosimilar product before the expiration of the '182 patent.

94. Immunex/AML and Roche do not have an adequate remedy at law and are entitled to injunctive relief prohibiting Defendants from making, using, offering to sell, or selling within the United States, or importing into the United States Defendants' etanercept biosimilar product before the expiration of the '182 patent.

95. Defendants' manufacture, use, offer for sale, or sale within the United States, or importation into the United States, of Defendants' etanercept biosimilar product before the expiration of the '182 patent will cause injury to Immunex/AML and Roche, entitling them to damages under 35 U.S.C. § 284.

COUNT 3: INFRINGEMENT OF THE '522 PATENT UNDER 35 U.S.C. § 271(e)(2)(C)

96. Immunex/AML and Roche incorporate by reference paragraphs 1-70 as if fully set forth herein.

97. The '522 patent, titled "Human TNF Receptor," was duly and legally issued on April 24, 2012 by the USPTO. A true and correct copy of the '522 patent is attached to this Complaint as Exhibit D.

98. The claims of the '522 patent cover, among other things, methods of making etanercept and certain materials used in such methods.

99. Defendants have infringed the '522 patent by submitting an aBLA referencing Immunex's ENBREL® product and seeking FDA approval under 42 U.S.C. § 262(k) to manufacture, import, offer to sell, or sell within the United States the product that is the subject of that application.

100. On December 18, 2015, Immunex, as the reference product sponsor for ENBREL®, identified the '522 patent to Sandoz Inc. pursuant to 42 U.S.C. § 262(*l*)(3)(A).

101. Defendants have known of the '522 patent since at least June 2013. Despite such knowledge, Defendants nonetheless filed their aBLA with the FDA in July 2015 seeking approval of Defendants' etanercept biosimilar product that was manufactured by the methods of the '522 patent, with the intent to import, offer to sell, and sell their biosimilar product within the United States before the expiration of the '522 patent and in violation of Immunex/AML and Roche's patent rights.

102. Immunex/AML and Roche will be irreparably harmed if Defendants are not enjoined from infringing one or more claims of the '522 patent. Immunex/AML and Roche do not have an adequate remedy at law and are entitled to injunctive relief preventing Defendants from any further infringement.

103. Defendants' commercial manufacture of Defendants' etanercept product, and their subsequent importation for sale within the United States, upon FDA approval of

Defendants' etanercept biosimilar product and before the expiration of the '522 patent will cause injury to Immunex/AML and Roche, entitling them to damages or other monetary relief.

COUNT 4: DECLARATORY JUDGMENT OF INFRINGEMENT OF THE '522 PATENT UNDER 35 U.S.C. § 271(g)

104. Immunex/AML and Roche incorporate by reference paragraphs 1-70 as if fully set forth herein.

105. On information and belief, Defendants intend to, and will immediately and imminently upon FDA licensure of Defendants' aBLA, manufacture Defendants' etanercept product according to the process described in their aBLA and import and offer to sell or sell within the United States Defendants' etanercept biosimilar product made by such process, which will infringe the method claims of the '522 patent under 35 U.S.C. § 271(g).

106. The FDA has publicly stated that the agency's goal is to act upon an aBLA application within 10 months of receipt. On information and belief, Defendants believe that the FDA may act upon Defendants' aBLA as soon as May 2016, and that Defendants will be able to pay the user fee prescribed under the Prescription Drug User Fee Act by that time.

107. On information and belief, Defendants intend to, and will immediately and imminently upon FDA licensure of Defendants' aBLA, notwithstanding the clear requirements of 42 U.S.C. § 262(*l*)(8), import and offer to sell or sell within the United States Defendants' etanercept biosimilar product, which will infringe one or more claims of the '522 patent under 35 U.S.C. § 271(g).

108. The etanercept made by Defendants' process that infringes the '522 patent is the essential active ingredient of Defendants' biological drug product. On information and belief, there is no subsequent process that materially changes that active ingredient, including during any fill and finish of the biological product.

109. An actual controversy has arisen and now exists between the parties concerning whether Defendants' etanercept biosimilar product has infringed or will infringe one or more claims of the '522 patent.

110. Defendants also have failed to complete the actions required of them under 42 U.S.C. § 262(*l*)(4) and (5) by failing to engage in negotiation and exchange of patent lists under 42 U.S.C. § 262(*l*)(4) and 262(*l*)(5). No list of patents for Defendants' etanercept biosimilar product was generated as described in 42 U.S.C. § 262(*l*)(4) or as described in 42 U.S.C. § 262(*l*)(5).

111. Immunex/AML and Roche are entitled to a declaratory judgment that Defendants have infringed or will infringe one or more claims of the '522 patent by making Defendants' etanercept biosimilar product and importing it into the United States for sale in the United States, before the expiration of the '522 patent.

112. Immunex/AML and Roche do not have an adequate remedy at law and are entitled to injunctive relief prohibiting Defendants from making, importing into, and selling within the United States Defendants' etanercept biosimilar product before the expiration of the '522 patent.

113. Defendants' making, importing, and selling within the United States of Defendants' etanercept biosimilar product before the expiration of the '522 patent will cause Immunex/AML and Roche injury, entitling them to damages under 35 U.S.C. § 284.

COUNT 5: INFRINGEMENT OF THE '225 PATENT UNDER 35 U.S.C. § 271(e)(2)(C)

114. Immunex/AML incorporate by reference paragraphs 1-113 as if fully set forth herein.

115. The '225 patent, titled "Soluble Tumor Necrosis Factor Receptor Treatment of Medical Disorders" was duly and legally issued on March 29, 2011 by the USPTO. A true and correct copy of the '225 patent is attached to this Complaint as Exhibit E.

116. The '225 patent is generally directed to methods of treating psoriasis and/or psoriatic arthritis by administering etanercept.

117. Defendants have infringed the '225 patent by submitting an aBLA referencing Immunex's ENBREL® product and seeking FDA approval under 42 U.S.C. § 262(k) to manufacture, import, offer to sell, or sell within the United States the product that is the subject of that application.

118. With the intent to infringe the '225 patent, Defendants submitted their aBLA seeking FDA approval under 42 U.S.C. § 262(k) to market, offer to sell, or sell within the United States Defendants' etanercept biosimilar product for the treatment of psoriasis or psoriatic arthritis. On information and belief, Defendants conducted clinical trials of their etanercept biosimilar product for the psoriasis indication only. On information and belief, Defendants copied ENBREL®'s labeling that instructs physicians and patients to administer etanercept subcutaneously for treatment of psoriasis or psoriatic arthritis in specific dosages, which is covered by the '225 patent.

119. On information and belief, Defendants knew of the '225 patent before July 2015. Despite such knowledge, Defendants nonetheless filed their aBLA with the FDA in July 2015 seeking approval of Defendants' etanercept biosimilar product, with the intent to import, market, offer to sell, and sell their biosimilar product for psoriasis and/or psoriatic arthritis within the United States before the expiration of the '225 patent and in violation of Immunex/AML's patent rights.

120. On December 18, 2015, Immunex, as the reference product sponsor for ENBREL®, identified the '225 patent to Sandoz Inc. pursuant to 42 U.S.C. § 262(*l*)(3)(A).

121. Immunex/AML will be irreparably harmed if Defendants are not enjoined from infringing one or more claims of the '225 patent. Immunex/AML do not have an adequate remedy at law and are entitled to injunctive relief preventing Defendants from any further infringement.

122. Defendants' commercial marketing, offer for sale, or sale within the United States, upon FDA approval of Defendants' etanercept biosimilar product for use with psoriasis and/or psoriatic arthritis before the expiration of the '225 patent, will cause injury to Immunex/AML, entitling Immunex/AML to damages or other monetary relief.

COUNT 6: DECLARATORY JUDGMENT OF INFRINGEMENT OF THE '225 PATENT UNDER 35 U.S.C. § 271(b)

123. Immunex/AML incorporate by reference paragraphs 1-122 as if fully set forth herein.

124. Defendants have sought FDA approval under 42 U.S.C. § 262(k) to import, market, offer to sell, or sell within the United States Defendants' etanercept biosimilar product, a biosimilar version of ENBREL® (etanercept), for treating psoriasis and psoriatic arthritis.

125. The FDA has publicly stated that the agency's goal is to act upon an aBLA application within 10 months of receipt. On information and belief, Defendants believe that the FDA may act upon Defendants' aBLA as soon as May 2016, and that Defendants will be able to pay the user fee prescribed under the Prescription Drug User Fee Act by that time.

126. On information and belief, Defendants intend to, and will immediately and imminently upon FDA licensure of Defendants' aBLA, notwithstanding the clear requirements of § 262(*l*)(8), import, market, offer to sell, or sell within the United States Defendants'

etanercept biosimilar product for treating psoriasis and/or psoriatic arthritis, which use by physicians and patients will infringe one or more claims of the '225 patent under 35 U.S.C. § 271(b).

127. An actual controversy has arisen and now exists between the parties concerning whether Defendants will induce infringement by physicians and patients of the '225 patent by their marketing and sales of their etanercept biosimilar product for psoriasis and/or psoriatic arthritis.

128. Defendants also have failed to complete the actions required of them under 42 U.S.C. § 262(*l*)(4) and (5) by failing to engage in negotiation and exchange of patent lists under 42 U.S.C. § 262(*l*)(4) and 262(*l*)(5). No list of patents for Defendants' etanercept biosimilar product was generated as described in 42 U.S.C. § 262(*l*)(4) or as described in 42 U.S.C. § 262(*l*)(5).

129. Immunex/AML are entitled to a declaratory judgment that Defendants have infringed, will infringe, or will induce infringement of one or more claims of the '225 patent by marketing, offering to sell, or selling within the United States Defendants' etanercept biosimilar product for treatment of psoriasis and/or psoriatic arthritis before the expiration of the '225 patent.

130. Immunex/AML do not have an adequate remedy at law and are entitled to injunctive relief prohibiting Defendants from making, importing into, and selling within the United States Defendants' etanercept biosimilar product before the expiration of the '225 patent.

131. Defendants' marketing, offer for sale, or sale within the United States of Defendants' etanercept biosimilar product for treating psoriasis and/or psoriatic arthritis before

the expiration of the '225 patent will cause Immunex/AML injury, entitling them to damages under 35 U.S.C. § 284.

COUNT 7: INFRINGEMENT OF THE '605 PATENT UNDER 35 U.S.C. § 271(e)(2)(C)

132. Immunex/AML incorporate by reference paragraphs 1-131 as if fully set forth herein.

133. The '605 patent, titled "Soluble Tumor Necrosis Factor Receptor Treatment of Medical Disorders" was duly and legally issued on February 21, 2012 by the USPTO. A true and correct copy of the '605 patent is attached to this Complaint as Exhibit F.

134. The '605 patent is generally directed to methods of treating psoriasis by administering etanercept.

135. Defendants have infringed the '605 patent by submitting an aBLA referencing Immunex's ENBREL® product and seeking FDA approval under 42 U.S.C. § 262(k) to manufacture, import, offer to sell, or sell within the United States the product that is the subject of that application.

136. With the intent to infringe the '605 patent, Defendants submitted their aBLA seeking FDA approval under 42 U.S.C. § 262(k) to market, offer to sell, or sell within the United States Defendants' etanercept biosimilar product for the treatment of psoriasis. On information and belief, Defendants conducted clinical trials of their etanercept biosimilar product for the psoriasis indication only. On information and belief, Defendants copied ENBREL®'s labeling that instructs physicians and patients to administer etanercept for treating psoriasis in specific dosages, which is covered by the '605 patent.

137. On information and belief, Defendants knew of the '605 patent before July 2015. Despite such knowledge, Defendants nonetheless filed their aBLA with the FDA in July 2015 seeking approval of Defendants' etanercept biosimilar product, with the intent to import, market,

offer to sell, and sell their biosimilar product for treating psoriasis within the United States before the expiration of the '605 patent and in violation of Immunex/AML's patent rights.

138. On December 18, 2015, Immunex, as the reference product sponsor for ENBREL®, identified the '605 patent to Sandoz Inc. pursuant to 42 U.S.C. § 262(*l*)(3)(A).

139. Immunex/AML will be irreparably harmed if Defendants are not enjoined from infringing one or more claims of the '605 patent. Immunex/AML do not have an adequate remedy at law and are entitled to injunctive relief preventing Defendants from any further infringement.

140. Defendants' commercial marketing, offer for sale, or sale within the United States, upon FDA approval of Defendants' etanercept biosimilar product for treating psoriasis and before the expiration of the '605 patent, will cause injury to Immunex/AML, entitling Immunex/AML to damages or other monetary relief.

COUNT 8: DECLARATORY JUDGMENT OF INFRINGEMENT OF THE '605 PATENT UNDER 35 U.S.C. § 271(b)

141. Immunex/AML incorporate by reference paragraphs 1-140 as if fully set forth herein.

142. Defendants have sought FDA approval under 42 U.S.C. § 262(k) to import, market, offer to sell, or sell within the United States Defendants' etanercept biosimilar product, a biosimilar version of ENBREL® (etanercept), for treating psoriasis.

143. The FDA has publicly stated that the agency's goal is to act upon an aBLA application within 10 months of receipt. On information and belief, Defendants believe that the FDA may act upon Defendants' aBLA as soon as May 2016, and that Defendants will be able to pay the user fee prescribed under the Prescription Drug User Fee Act by that time.

144. On information and belief, Defendants intend to, and will immediately and imminently upon FDA licensure of Defendants' aBLA, notwithstanding the clear requirements of 42 U.S.C. § 262(*l*)(8), import, market, offer to sell, or sell within the United States the Sandoz etanercept biosimilar product for treating psoriasis, which use by physicians and patients will infringe one or more claims of the '605 patent under 35 U.S.C. § 271(b).

145. An actual controversy has arisen and now exists between the parties concerning whether Sandoz will induce infringement by physicians and patients of the '605 patent by their marketing and sales of Defendants' etanercept biosimilar product for psoriasis.

146. Defendants also have failed to complete the actions required of them under 42 U.S.C. § 262(*l*)(4) and (5) by failing to engage in negotiation and exchange of patent lists under 42 U.S.C. § 262(*l*)(4) and 262(*l*)(5). No list of patents for Defendants' etanercept biosimilar product was generated as described in 42 U.S.C. § 262(*l*)(4) or as described in 42 U.S.C. § 262(*l*)(5).

147. Immunex/AML are entitled to a declaratory judgment that Defendants have infringed or will infringe one or more claims of the '605 patent by marketing, offering to sell, or selling within the United States Defendants' etanercept biosimilar product for treatment of psoriasis before the expiration of the '605 patent.

148. Immunex/AML do not have an adequate remedy at law and are entitled to injunctive relief prohibiting Defendants from making, importing into, and selling within the United States Defendants' etanercept biosimilar product before the expiration of the '605 patent.

149. Defendants' marketing, offer for sale, or sale within the United States of the Sandoz etanercept biosimilar product for treating psoriasis before the expiration of the '605 patent will cause Immunex/AML injury, entitling them to damages under 35 U.S.C. § 284.

COUNT 9: INFRINGEMENT OF THE '631 PATENT UNDER 35 U.S.C. § 271(e)(2)(C)

150. Immunex/AML incorporate by reference paragraphs 1-149 as if fully set forth herein.

151. The '631 patent, titled "Soluble Tumor Necrosis Factor Receptor Treatment of Medical Disorders" was duly and legally issued on May 13, 2014 by the USPTO. A true and correct copy of the '631 patent is attached to this Complaint as Exhibit G.

152. The '631 patent is generally directed to methods of treating psoriasis and/or psoriatic arthritis by administering etanercept.

153. Defendants have infringed the '631 patent by submitting an aBLA referencing Immunex's ENBREL® product and seeking FDA approval under 42 U.S.C. § 262(k) to manufacture, import, offer to sell, or sell within the United States the product that is the subject of that application.

154. With the intent to infringe the '631 patent, Defendants submitted their aBLA seeking FDA approval under 42 U.S.C. § 262(k) to market, offer to sell, or sell within the United States Defendants' etanercept biosimilar product for the treatment of psoriasis and psoriatic arthritis. On information and belief, Defendants conducted clinical trials of their etanercept biosimilar product for the psoriasis indication only. On information and belief, Defendants copied ENBREL®'s labeling that instructs physicians and patients to administer etanercept subcutaneously for treatment of psoriasis or psoriatic arthritis in specific dosages, which is covered by the '631 patent.

155. On information and belief, Defendants knew of the '631 patent before July 2015. Despite such knowledge, Defendants nonetheless filed their aBLA with the FDA in July 2015 seeking approval of Defendants' etanercept biosimilar product, with the intent to import, market, offer to sell, and sell their biosimilar product for psoriasis and/or psoriatic arthritis within the

United States before the expiration of the '631 patent and in violation of Immunex/AML's patent rights.

156. On December 18, 2015, Immunex, as the reference product sponsor for ENBREL®, identified the '631 patent to Sandoz Inc. pursuant to 42 U.S.C. § 262(*l*)(3)(A).

157. Immunex/AML will be irreparably harmed if Defendants are not enjoined from infringing one or more claims of the '631 patent. Immunex/AML do not have an adequate remedy at law and are entitled to injunctive relief preventing Defendants from any further infringement.

158. Defendants' commercial marketing, offer for sale, or sale within the United States, upon FDA approval of Defendants' etanercept biosimilar product for use in psoriasis or psoriatic arthritis and before the expiration of the '631 patent, will cause injury to Immunex/AML, entitling Immunex/AML to damages or other monetary relief.

COUNT 10: DECLARATORY JUDGMENT OF INFRINGEMENT OF THE '631 PATENT UNDER 35 U.S.C. § 271(b)

159. Immunex/AML incorporate by reference paragraphs 1-158 as if fully set forth herein.

160. Defendants have sought FDA approval under 42 U.S.C. § 262(k) to import, market, offer to sell, or sell within the United States Defendants' etanercept biosimilar product, a biosimilar version of ENBREL® (etanercept), for treating psoriasis and/or psoriatic arthritis.

161. The FDA has publicly stated that the agency's goal is to act upon an aBLA application within 10 months of receipt. On information and belief, Defendants believe that the FDA may act upon Defendants' aBLA as soon as May 2016, and Defendants will be able to pay the user fee prescribed under the Prescription Drug User Fee Act by that time.

162. On information and belief, Defendants intend to, and will immediately and imminently upon FDA licensure of Defendants' aBLA, notwithstanding the clear requirements of 42 U.S.C. § 262(*l*)(8), import, market, offer to sell, or sell within the United States Defendants' etanercept biosimilar product for treating psoriasis and/or psoriatic arthritis, which use by physicians and patients will infringe one or more claims of the '631 patent under 35 U.S.C. § 271(b).

163. An actual controversy has arisen and now exists between the parties concerning whether Defendants will induce infringement by physicians and patients of the '631 patent by their marketing and sales of Defendants' etanercept biosimilar product for psoriasis and/or psoriatic arthritis.

164. Defendants also have failed to complete the actions required of Defendants under 42 U.S.C. § 262(*l*)(4) and (5) by failing to engage in negotiation and exchange of patent lists under 42 U.S.C. § 262(*l*)(4) and 262(*l*)(5). No list of patents for Defendants' etanercept biosimilar product was generated as described in 42 U.S.C. § 262(*l*)(4) or as described in 42 U.S.C. § 262(*l*)(5).

165. Immunex/AML are entitled to a declaratory judgment that Defendants have infringed or will infringe one or more claims of the '631 patent by marketing, offering to sell, or selling within the United States Defendants' etanercept biosimilar product for treatment of psoriasis and/or psoriatic arthritis before the expiration of the '631 patent.

166. Immunex/AML do not have an adequate remedy at law and are entitled to injunctive relief prohibiting Defendants from making, importing into, and selling within the United States Defendants' etanercept biosimilar product before the expiration of the '631 patent.

167. Defendants' marketing, offer for sale, or sale within the United States of Defendants' etanercept biosimilar product for treating psoriasis and/or psoriatic arthritis before the expiration of the '631 patent will cause Immunex/AML injury, entitling them to damages under 35 U.S.C. § 284.

PRAYER FOR RELIEF

WHEREFORE, Roche (with respect to the '182 and '522 patents) and Immunex/AML (with respect to all patents-in-suit) respectfully request that this Court enter judgment in their favor against Defendants and grant the following relief:

A. A judgment that Defendants have infringed one or more claims of the '182 patent under 35 U.S.C. § 271(e)(2)(C), by submitting to the FDA Defendants' aBLA to obtain approval of Defendants' etanercept biosimilar product under the PHSA to engage in the commercial manufacture, use, or sale of Defendants' etanercept biosimilar product before the expiration of the '182 patent;

B. A judgment that Defendants have infringed or will infringe one or more claims of the '182 patent by engaging in the manufacture, use, offer for sale, or sale within the United States, or importation into the United States, of Defendants' etanercept biosimilar product before the expiration of the '182 patent;

C. A judgment that Defendants have infringed one or more claims of the '522 patent under 35 U.S.C. § 271(e)(2)(C), by submitting to the FDA Defendants' aBLA to obtain approval of Defendants' etanercept biosimilar product under the PHSA to engage in the commercial manufacture, use, or sale of Defendants' etanercept biosimilar product before the expiration of the '522 patent;

D. A judgment that Defendants have infringed or will infringe one or more claims of the '522 patent by engaging in the manufacture, use, offer for sale, or sale within the United

States, or importation into the United States, of Defendants' etanercept biosimilar product before the expiration of the '522 patent;

E. A judgment that Defendants have infringed one or more claims of the '225 patent under 35 U.S.C. § 271(e)(2)(C), by submitting to the FDA Defendants' aBLA to obtain approval of the Sandoz etanercept biosimilar product under the PHSA to engage in the commercial manufacture, use, or sale of Defendants' etanercept biosimilar product before the expiration of the '225 patent;

F. A judgment that Defendants have infringed, will infringe, or will induce infringement of one or more claims of the '225 patent by engaging in the manufacture, use, offer for sale, or sale within the United States, or importation into the United States, of Defendants' etanercept biosimilar product before the expiration of the '225 patent;

G. A judgment that Defendants have infringed one or more claims of the '605 patent under 35 U.S.C. § 271(e)(2)(C), by submitting to the FDA Defendants' aBLA to obtain approval of Defendants' etanercept biosimilar product under the PHSA to engage in the commercial manufacture, use, or sale of Defendants' etanercept biosimilar product before the expiration of the '605 patent;

H. A judgment that Defendants have infringed, will infringe, or will induce infringement of one or more claims of the '605 patent by engaging in the manufacture, use, offer for sale, or sale within the United States, or importation into the United States, of Defendants' etanercept biosimilar product before the expiration of the '605 patent;

I. A judgment that Defendants have infringed one or more claims of the '631 patent under 35 U.S.C. § 271(e)(2)(C), by submitting to the FDA Defendants' aBLA to obtain approval of Defendants' etanercept biosimilar product under the PHSA to engage in the commercial

manufacture, use, or sale of Defendants' etanercept biosimilar product before the expiration of the '631 patent;

J. A judgment that Defendants have infringed, will infringe, or will induce infringement of one or more claims of the '631 patent by engaging in the manufacture, use, offer for sale, or sale within the United States, or importation into the United States, of Defendants' etanercept biosimilar product before the expiration of the '631 patent;

K. A judgment compelling Defendants to pay to Immunex/AML and Roche damages or other monetary relief adequate to compensate for Defendants' infringement, in accordance with 35 U.S.C. § 271(e)(4)(C) and § 284;

L. An injunction against future infringement and future inducement of infringement by Defendants, as well as all officers, employees, agents, representatives, affiliates, assignees, successors, and affiliates of Defendants, and all persons acting on behalf of or at the direction of, or in concert with Defendants, until the date of expiration of the last of the patents-in-suit;

M. A declaration that this is an exceptional case and awarding to Immunex/AML and Roche their attorneys' fees and costs pursuant to 35 U.S.C. § 285; and

N. Such other relief as this Court may deem just and proper.

DEMAND FOR A JURY TRIAL

Immunex/AML and Roche hereby demand a jury trial on all issues so triable.

Dated: February 26, 2016

Respectfully submitted,
Plaintiffs, by counsel:

s/ Liza M. Walsh

Liza M. Walsh
Christine I. Gannon
Eleonore Ofosu-Antwi
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s/ David De Lorenzi

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Attorneys for Hoffmann-La Roche Inc.

RULE 11.2 CERTIFICATION

U.S. Patent No. 8,163,522 is the subject of a pending petition for *inter partes* review before the Patent Trial and Appeal Board of the United States Patent and Trademark Office docketed as *Coalition for Affordable Drugs V LLC et al. v. Hoffmann-La Roche Inc. et al.*, IPR2015-01792.

I hereby certify that, to the best of my knowledge, the matter in controversy is not the subject of any other pending or anticipated litigation in any court or arbitration proceeding, nor are there any non-parties known to Plaintiffs that should be joined to this action. In addition, I recognize a continuing obligation during the course of this litigation to file and to serve on all other parties and with the Court an amended certification if there is a change in the facts stated in this original certification.

Dated: February 26, 2016

Respectfully submitted,
Plaintiffs, by counsel:

s/ Liza M. Walsh

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Attorneys for Hoffmann-La Roche Inc.

RULE 201.1 CERTIFICATION

I hereby certify that the above-captioned matter is not subject to compulsory arbitration in that the Plaintiffs seek, *inter alia*, injunctive relief.

Dated: February 26, 2016

Respectfully submitted,
Plaintiffs, by counsel:

s/ Liza M. Walsh

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ABOUT US

ABOUT US
OUR COMPANY
VISION AND VALUES
DIVERSITY & INCLUSION
SANDOZ HISTORY
MANAGEMENT
INVESTOR INFORMATION
SANDOZ WORLDWIDE

SANDOZ HISTORY

Sandoz history dates back more than 120 years, during which time it has transformed itself from a small diversified chemical company to the world's second largest producer of high-quality generic pharmaceuticals and global leader in biosimilars and differentiated products. Throughout our long and distinguished history, we have been led by dedicated and visionary individuals who have always aspired to be one step ahead and who have delivered success through high-quality products and services.

Learn about our proud history and heritage by reading about our key historical milestones:

The Ear y Years 1886 – 1939

The Start of Anti-infectives 1946 – 1963

Expansion and Diversification 1964 – 1982

A New Corporate Structure 1990 – 1998

A G oba Generics Business 2000 – 2013

2000 – Novartis Generics acquires BASF Pharma's European generics business through which it gains entry to the markets of France and Italy with GNR Pharma and the UK with Lagap. Apotheccon is also acquired in the United States and Grandis in Germany.

FAQ?

FREQUENTLY ASKED QUESTIONS

Everything you wanted to know about the generics industry.
More

2001 – Sandoz enters the Argentinian market under the name of Labinca.

2002 – Sandoz acquires Lek Pharmaceuticals d.d., Slovenia's largest pharmaceutical company with a strong presence in Central and Eastern Europe.

2003 Novartis unites its generics businesses under one single global brand as known today: Sandoz. The Amifarma S.L. production plant in Palafolls, located near Barcelona, Spain is also acquired.

2004 – Through acquisition of Sabex, Sandoz establishes a new presence in Canada, the sixth largest generics market worldwide. A new operational hub in the Nordic region is founded through its acquisition of Durascan, the generic subsidiary of AstraZeneca in Denmark.

2005 – Sandoz acquires HEXAL and Eon Labs, two other premier generics companies, strengthening its market position globally and achieving a top position in key markets, particularly the U.S. and Germany.

2006 – Sandoz becomes the first generics pharmaceutical company to receive approval for a biosimilar product in the EU and US. Omnitrope®, a recombinant human growth hormone, approved by the EMEA (European Medicines Agency) also becomes the first biosimilar to receive approval from the US Food and Drug Administration (FDA).

2007 – Sandoz gains EU market approval for the world's first complex biosimilar, Binocrit®, a follow-on version of life-saving anemia medicine epoetin alfa. The approval marks a key milestone in Sandoz's efforts to bring state-of-the-art biosimilars to patients around the globe.

2008 – Sandoz receives EU market approval for its third biosimilar medicine Zarzio® (filgrastim), indicated for use in treating neutropenia, marking another important milestone in its efforts to bring affordable high-quality biopharmaceuticals to patients worldwide.

2009 – Sandoz completes acquisition of FRESENIUS Pharma's specialty generic injectables business, improving worldwide access to affordable injectable cancer medicines and paving the way for the creation of a global center of excellence in generic oncology injectables.

2010 – Sandoz acquires Oriel Therapeutics of the US, entering the market of respiratory inhalables, adding a third pillar to its differentiated value-added portfolio. Sandoz's generic enoxaparin is also approved by the US FDA as a fully substitutable generic in the US, indicating the FDA's growing confidence in the ability to designate complex non-patented molecules as interchangeable.

2011 – Sandoz Japan announces generic pharmaceutical alliance with Nipro Corporation, focusing on a broad range of cross-licensing and co-development opportunities for the Japanese generics market. Through the acquisition of Alcon's US generics business, Falcon Pharmaceuticals, Sandoz positions itself as the global #1 in generic ophthalmics.

2012 – Sandoz acquires Fougera Pharmaceuticals, positioning Sandoz as #1 in generic (topical) dermatology. With innovative products and affordable, high-quality generic medicines focused on skin conditions, Fougera has strong generic dermatology development and manufacturing expertise, particularly in the area of semi-solid forms such as creams and ointments, as well as a well-known branded business, PharmaDerm.

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a Novartis company

NOVARTIS

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FDA accepts Sandoz regulatory submission for a proposed biosimilar etanercept [1]

Publish Date: Oct 02, 2015

- *Etanercept is an anti-TNF medicine used to treat a range of immunological diseases including rheumatoid arthritis and psoriasis.*
- *Sandoz is seeking approval for all indications included in the reference product's label.*
- *Sandoz believes that the totality of evidence in its submission, including two pivotal clinical studies, will demonstrate that the proposed biosimilar is essentially the same as the reference product.*

Holzkirchen, October 2, 2015 - Sandoz, a Novartis company and the global leader in biosimilars, announced today that the US Food and Drug Administration (FDA) has accepted its Biologics License Application (BLA) under the 351 (k) pathway for its proposed biosimilar to Amgen's US-licensed Enbrel®* (etanercept) - a tumor necrosis factor alpha (TNF-alpha) inhibitor. Sandoz is seeking approval for all indications included in the label of the reference product which is used to treat a range of autoimmune diseases including rheumatoid arthritis and psoriasis affecting approx. 1.3 million[1] and 7.5 million[2] people (respectively) in the US.

Mark McCamish, M.D., Ph.D., and Head of Global Biopharmaceutical & Oncology Injectables Development at Sandoz said "anti-TNFs will continue to play a leading role in immunology treatment and the acceptance of our regulatory submission by the FDA today is a significant step towards increasing patient access to these life-changing medicines." McCamish continued "we believe we are the first company to receive FDA file acceptance of a biosimilar version of etanercept."

This is the second BLA submission by Sandoz using the 351(k) biosimilar pathway. The BLA consists of a comprehensive data package that includes data from analytical, functional, pre-clinical and clinical studies. Sandoz believes that the two pivotal clinical studies; a pharmacokinetic (PK) study in healthy volunteers (HVs) and a confirmatory safety and efficacy study in patients with chronic plaque-type psoriasis (EGALITY), will provide confirmation of similarity to the reference product established in prior analytical comparability investigations.

Sandoz has an unwavering commitment to increasing patient access to high-quality, life-enhancing biosimilars. It is the pioneer and global market leader and currently markets three biosimilars. Sandoz recently launched Zarxio™ (filgrastim-sndz) - the first biosimilar in the United States, signaling a shift toward more competition and affordability in the

healthcare system. Sandoz has a leading pipeline with several biosimilars across the various stages of development including five programs in Phase III clinical trials or registration preparation. The company plans to make ten regulatory submissions in the next three years. As part of the Novartis Group, Sandoz is uniquely positioned to lead the biosimilars industry based on its experience and capabilities in development, manufacturing and commercialization.

-End-

Disclaimer

The foregoing release contains forward-looking statements that can be identified by words such as "proposed," "seeking," "will," "step towards," "believe," "commitment," "signaling," "pipeline," "plans," or similar terms, or by express or implied discussions regarding potential marketing approvals for biosimilar etanercept, or regarding potential future revenues from biosimilar etanercept. You should not place undue reliance on these statements. Such forward-looking statements are based on the current beliefs and expectations of management regarding future events, and are subject to significant known and unknown risks and uncertainties. Should one or more of these risks or uncertainties materialize, or should underlying assumptions prove incorrect, actual results may vary materially from those set forth in the forward-looking statements. There can be no guarantee that biosimilar etanercept will be approved for sale in the United States, or at any particular time. Neither can there be any guarantee that biosimilar etanercept will be submitted or approved for sale in any additional markets, or at any particular time. Nor can there be any guarantee that biosimilar etanercept will be commercially successful in the future. In particular, management's expectations regarding biosimilar etanercept could be affected by, among other things, unexpected regulatory actions or delays or government regulation generally; the uncertainties inherent in research and development, including unexpected clinical trial results and additional analysis of existing clinical data; competition in general, including potential approval of additional versions of biosimilar etanercept; global trends toward health care cost containment, including government, industry and general public pricing pressures; unexpected litigation outcomes, including intellectual property disputes or other legal efforts to prevent or limit Sandoz from selling biosimilar etanercept; the particular prescribing preferences of physicians and patients; unexpected safety issues; unexpected manufacturing or quality issues; general economic and industry conditions, and other risks and factors referred to in Novartis AG's current Form 20-F on file with the US Securities and Exchange Commission. Novartis is providing the information in this press release as of this date and does not undertake any obligation to update any forward-looking statements contained in this press release as a result of new information, future events or otherwise.

About Sandoz

Sandoz, a Novartis company, is a global leader in generic pharmaceuticals, driving sustainable access to high-quality healthcare. Sandoz employs more than 26,000 people worldwide and supplies a broad range of affordable, primarily off-patent products to patients and customers around the globe.

The Sandoz global portfolio comprises approximately 1,100 molecules, which accounted for 2014 sales of USD 9.6 billion. Sandoz holds the global #1 position in biosimilars as well as in generic anti-infectives, ophthalmics and transplantation medicines.

Nearly half of Sandoz's portfolio is in differentiated products - products that are scientifically more difficult to develop and manufacture than standard generics.

In addition to strong organic growth since consolidating its generics businesses under the Sandoz brand name in 2003, Sandoz has consistently driven growth in selected geographies and differentiated product areas through a series of targeted acquisitions.

* Enbrel is a registered trademark of Amgen Inc.

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US008063182B1

(12) **United States Patent**
Brockhaus et al.

(10) **Patent No.: US 8,063,182 B1**
(45) **Date of Patent: Nov. 22, 2011**

(54) **HUMAN TNF RECEPTOR FUSION PROTEIN**

(75) Inventors: **Manfred Brockhaus**, Bettingen (CH); **Reiner Gentz**, Rheinfelden (DE); **Dembic Zlatko**, Basel (CH); **Werner Lesslauer**, Basel (CH); **Hansruedi Lotscher**, Mohlin (CH); **Ernst-Jurgen Schlaeger**, Efringen-Kirchen (DE)

(73) Assignee: **Hoffman-LaRoche Inc.**, Nutley, NJ (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **08/444,790**

(22) Filed: **May 19, 1995**

Related U.S. Application Data

(60) Division of application No. 08/095,640, filed on Jul. 21, 1993, now Pat. No. 5,610,279, which is a continuation of application No. 07/580,013, filed on Sep. 10, 1990, now abandoned.

(30) **Foreign Application Priority Data**

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(52) **U.S. Cl.** **530/350**; 514/12.2; 530/387.3; 536/23.5; 930/144

(58) **Field of Classification Search** 530/324, 530/350; 424/192.1; 536/23.5
See application file for complete search history.

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(57) **ABSTRACT**

The present invention is concerned with non-soluble proteins and soluble or insoluble fragments thereof, which bind TNF, in homogeneous form, as well as their physiologically compatible salts, especially those proteins having a molecular weight of about 55 or 75 kD (non-reducing SDS-PAGE conditions), a process for the isolation of such proteins, antibodies against such proteins, DNA sequences which code for non-soluble proteins and soluble or non-soluble fragments thereof, which bind TNF, as well as those which code for proteins comprising partly of a soluble fragment, which binds TNF, and partly of all domains except the first of the constant region of the heavy chain of human immunoglobulins and the recombinant proteins coded thereby as well as a process for their manufacture using transformed pro- and eukaryotic host cells.

36 Claims, 6 Drawing Sheets

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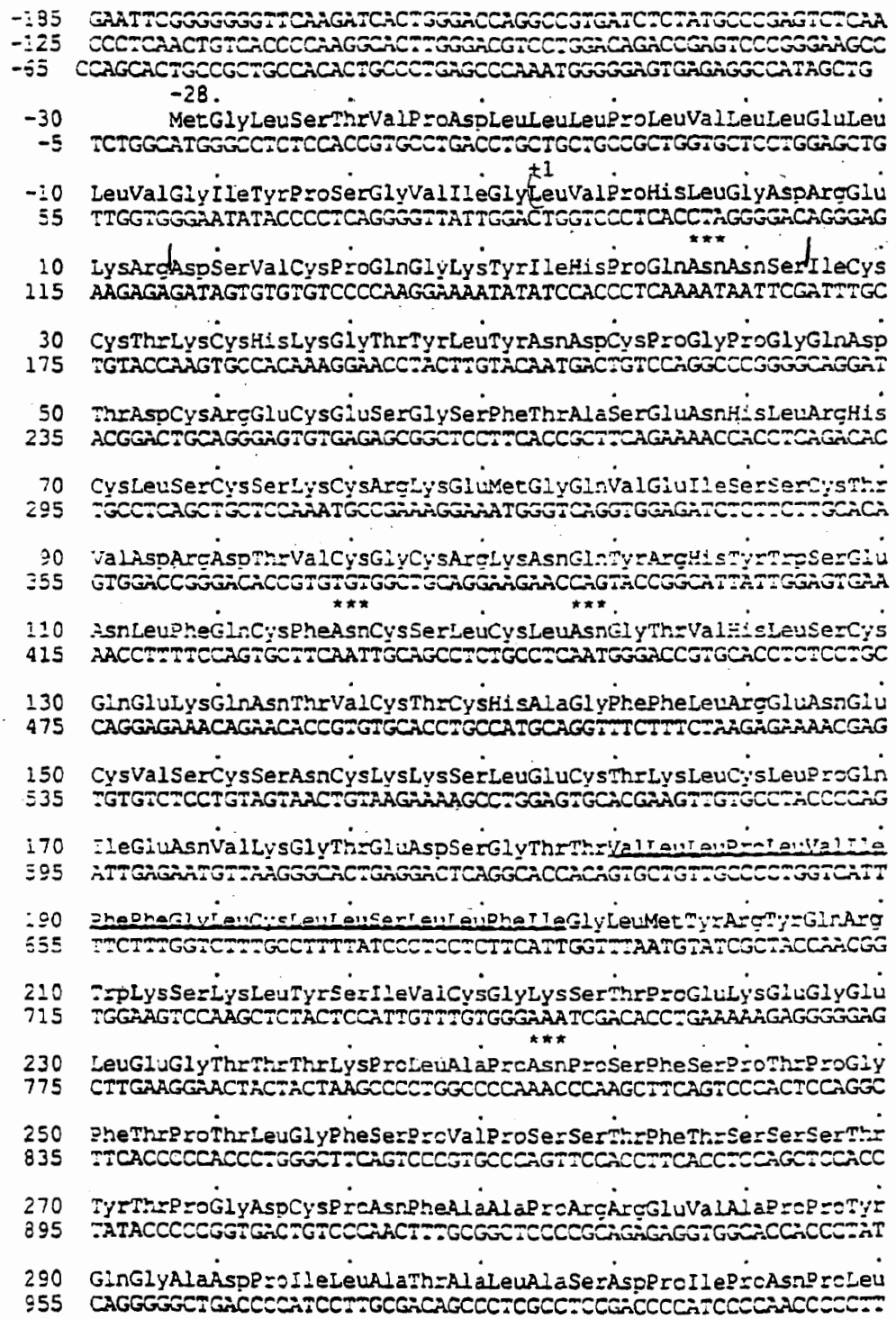
Figure 1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±1
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10 LysArgAspSerValCysProGlnGlyLysTyrIleHisProGlnAsnAsnSerIleCys
115 AAGAGAGATAGTGTGTGTCCCCAAGGAAAATATATCCACCCTCAAAATAATTCGATTTGC
30 CysThrLysCysHisLysGlyThrTyrLeuTyrAsnAspCysProGlyProGlyGlnAsp
175 TGTACCAAGTGCCACAAAGGAACCTACTTGTACAATGACTGTCCAGGCCCGGGGCAGGAT
50 ThrAspCysArgGluCysGluSerGlySerPheThrAlaSerGluAsnHisLeuArgHis
235 ACGGACTGCAGGGAGTGTGAGAGCGGCTCCTTCACCGCTTCAGAAAACCACCTCAGACAC
70 CysLeuSerCysSerLysCysArgLysGluMetGlyGlnValGluIleSerSerCysThr
295 TGCCTCAGCTGCTCCAAATGCCGAAAGGAAATGGGTCAGGTGGAGATCTCTTCTTGCACA
90 ValAspArgAspThrValCysGlyCysArgLysAsnGlnTyrArgHisTyrTrpSerGlu
355 GTGGACCGGGACACCGTGTGTGGCTGCAGGAAGAACCAGTACCGGCATTATTGGAGTGAA
*** ***
110 AsnLeuPheGlnCysPheAsnCysSerLeuCysLeuAsnGlyThrValHisLeuSerCys
415 AACCTTTTCCAGTGCTTCAATTGCAGCCTCTGCCTCAATGGGACCGTGCACCTCTCCTGC
130 GlnGluLysGlnAsnThrValCysThrCysHisAlaGlyPhePheLeuArgGluAsnGlu
475 CAGGAGAAACAGAACACCGTGTGCACCTGCCATGCAGGTTTCTTTCTAAGAGAAAACGAG
150 CysValSerCysSerAsnCysLysLysSerLeuGluCysThrLysLeuCysLeuProGln
535 TGTGTCTCCTGTAGTAACTGTAAGAAAAGCCTGGAGTGCACGAAGTTGTGCCTACCCCAG
170 IleGluAsnValLysGlyThrGluAspSerGlyThrThrValLeuLeuProLeuValIle
595 ATTGAGAATGTTAAGGGCACTGAGGACTCAGGCACCACAGTGCTGTTGCCCCTGGTCATT
190 PhePheGlyLeuCysLeuLeuSerLeuLeuPheIleGlyLeuMetTyrArgTyrGlnArg
655 TTCTTTGGTCTTTGCCTTTTATCCCTCCTCTTCATTGGTTTAATGTATCGCTACCAACGG
210 TrpLysSerLysLeuTyrSerIleValCysGlyLysSerThrProGluLysGluGlyGlu
715 TGGAAGTCCAAGCTCTACTCCATTGTTTGTGGGAAATCGACACCTGAAAAAGAGGGGGAG
***
230 LeuGluGlyThrThrThrLysProLeuAlaProAsnProSerPheSerProThrProGly
775 CTTGAAGGAACTACTACTAAGCCCCTGGCCCCAAACCCAAGCTTCAGTCCCACTCCAGGC
250 PheThrProThrLeuGlyPheSerProValProSerSerThrPheThrSerSerSerThr
835 TTCACCCCCACCCTGGGCTTCAGTCCCGTGCCCAGTTCCACCTTCACCTCCAGCTCCACC
270 TyrThrProGlyAspCysProAsnPheAlaAlaProArgArgGluValAlaProProTyr
895 TATACCCCCGGTGACTGTCCCAACTTTGCGGCTCCCCGCAGAGAGGTGGCACCACCCTAT
290 GlnGlyAlaAspProIleLeuAlaThrAlaLeuAlaSerAspProIleProAsnProLeu
955 CAGGGGGCTGACCCCATCCTTGCGACAGCCCTCGCCTCCGACCCCATCCCCAACCCCCTT

Figure 1 (cont.

```
 310  GlnLysTrpGluAspSerAlaHisLysProGlnSerLeuAspThrAspAspProAlaThr
1015  CAGAAGTGGGAGGACAGCGCCCACAAGCCACAGAGCCTAGACACTGATGACCCCGCGACG

 330  LeuTyrAlaValValGluAsnValProProLeuArgTrpLysGluPheValArgArgLeu
1075  CTGTACGCCGTGGTGGAGAACGTGCCCCCGTTGCGCTGGAAGGAATTCGTGCGGCGCCTA

 350  GlyLeuSerAspHisGluIleAspArgLeuGluLeuGlnAsnGlyArgCysLeuArgGlu
1135  GGGCTGAGCGACCACGAGATCGATCGGCTGGAGCTGCAGAACGGGCGCTGCCTGCGCGAG

 370  AlaGlnTyrSerMetLeuAlaThrTrpArgArgArgThrProArgArgGluAlaThrLeu
1195  GCGCAATACAGCATGCTGGCGACCTGGAGGCGGCGCACGCCGCGGCGCGAGGCCACGCTG

 390  GluLeuLeuGlyArgValLeuArgAspMetAspLeuLeuGlyCysLeuGluAspIleGlu
1255  GAGCTGCTGGGACGCGTGCTCCGCGACATGGACCTGCTGGGCTGCCTGGAGGACATCGAG

 410  GluAlaLeuCysGlyProAlaAlaLeuProProAlaProSerLeuLeuArg
1315  GAGGCGCTTTGCGGCCCCGCCGCCCTCCCGCCCGCGCCCAGTCTTCTCAGATGAGGCTGC
1375  GCCCCTGCGGGCAGCTCTAAGGACCGTCCTGCGAGATCGCCTTCCAACCCCACTTTTTTC
1435  TGGAAAGGAGGGGTCCTGCAGGGGCAAGCAGGAGCTAGCAGCCGCCTACTTGGTGCTAAC
1495  CCCTCGATGTACATAGCTTTTCTCAGCTGCCTGCGCGCCGCCGACAGTCAGCGCTGTGCG
1555  CGCGGAGAGAGGTGCGCCGTGGGCTCAAGAGCCTGAGTGGGTGGTTTGCGAGGATGAGGG
1615  ACGCTATGCCTCATGCCCGTTTTGGGTGTCCTCACCAGCAAGGCTGCTCGGGGGCCCCTG
1675  GTTCGTCCCTGAGCCTTTTTCACAGTGCATAAGCAGTTTTTTTTGTTTTTGTTTTGTTTT
1735  GTTTTGTTTTTAAATCAATCATGTTACACTAATAGAAACTTGGCACTCCTGTGCCCTCTG
1795  CCTGGACAAGCACATAGCAAGCTGAACTGTCCTAAGGCAGGGGCGAGCACGGAACAATGG
1855  GGCCTTCAGCTGGAGCTGTGGACTTTTGTACATACACTAAAATTCTGAAGTTAAAAAAAA
1915  AACCCGAATTC
```

Figure 2A

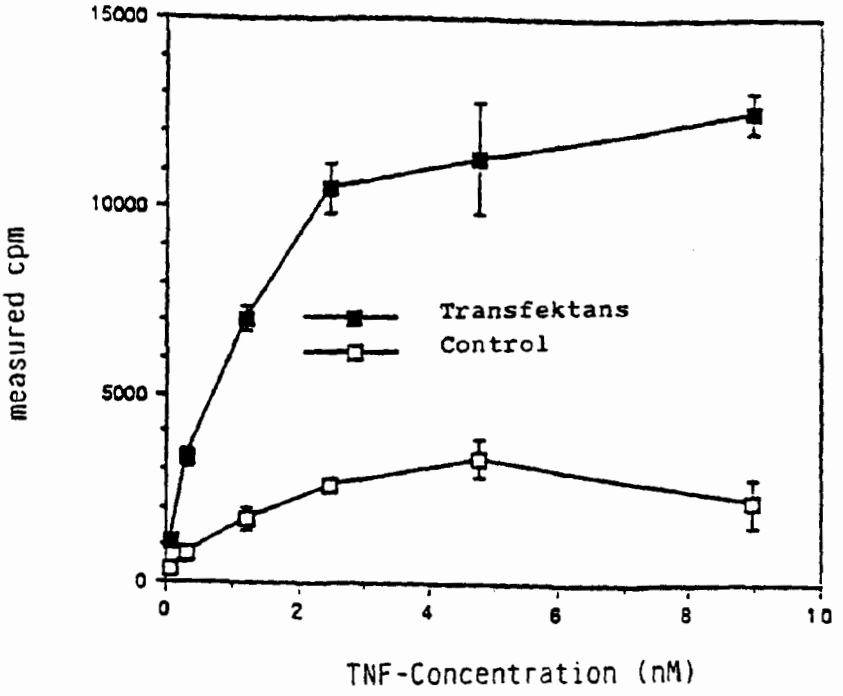

Figure 2B

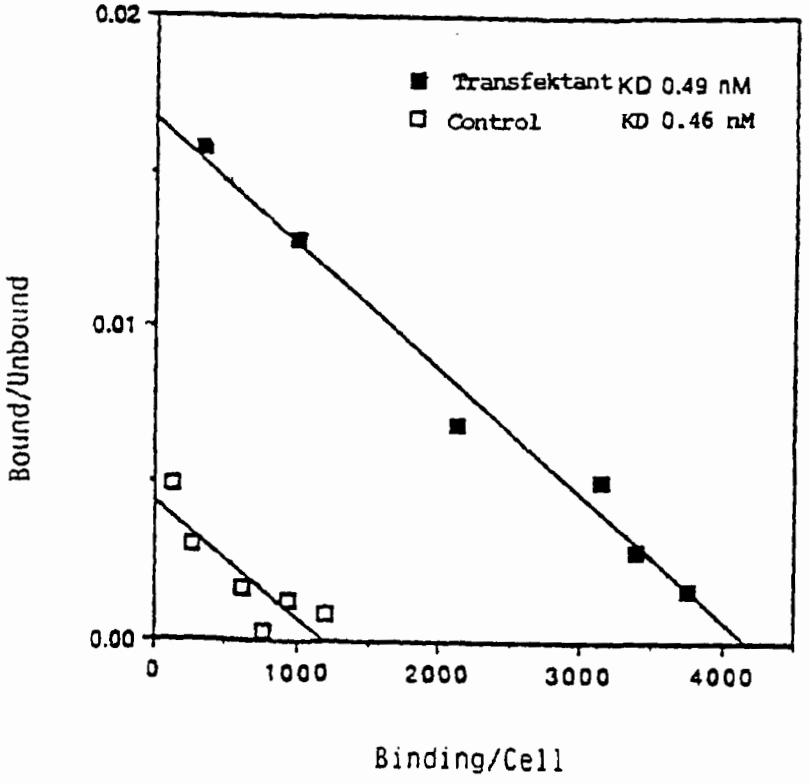

Figure 3

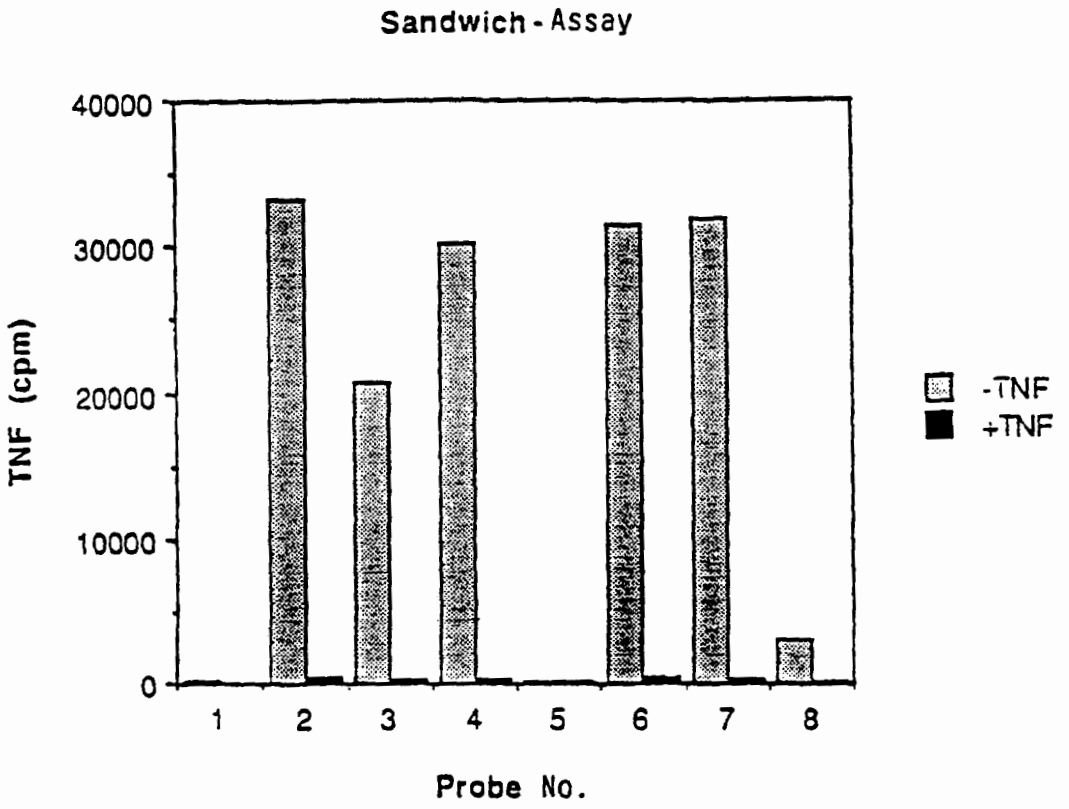
Sandwich - Assay
TNF (cpm)
40000
30000
20000
10000
0
1
2
3
4
5
6
7
8
Probe No.
-TNF
+TNF

Figure 4

```
  1  SerAspSerValCysAspSerCysGluAspSerThrTyrThrGlnLeuTrpAsnTrpVal
  1  TCGGACTCCGTGTGTGACTCCTGTGAGGACAGCACATACACCCAGCTCTGGAACTGGGTT

 21  ProGluCysLeuSerCysGlySerArgCysSerSerAspGlnValGluThrGlnAlaCys
 61  CCCGAGTGCTTGAGCTGTGGCTCCCGCTGTAGCTCTGACCAGGTGGAAACTCAAGCCTGC

 41  ThrArgGluGlnAsnArgIleCysThrCysArgProGlyTrpTyrCysAlaLeuSerLys
121  ACTCGGGAACAGAACCGCATCTGCACCTGCAGGCCCGGCTGGTACTGCGCGCTGAGCAAG

 61  GlnGluGlyCysArgLeuCysAlaProLeuProLysCysArgProGlyPheGlyValAla
181  CAGGAGGGGTGCCGGCTGTGCGCGCCGCTGCCGAAGTGCCGCCCGGGCTTCGGCGTGGCC

 81  ArgProGlyThrGluThrSerAspValValCysLysProCysAlaProGlyThrPheSer
241  AGACCAGGAACTGAAACATCAGACGTGGTGTGCAAGCCCTGTGCCCCGGGGACGTTCTCC

101  AsnThrThrSerSerThrAspIleCysArgProHisGlnIleCysAsnValValAlaIle
301  AACACGACTTCATCCACGGATATTTGCAGGCCCCACCAGATCTGTAACGTGGTGGCCATC

121  ProGlyAsnAlaSerArgAspAlaValCysThrSerThrSerProThrArgSerMetAla
361  CCTGGGAATGCAAGCAGGGATGCAGTCTGCACGTCCACGTCCCCCACCCGGAGTATGGCC

141  ProGlyAlaValHisLeuProGlnProValSerThrArgSerGlnHisThrGlnProSer
421  CCAGGGGCAGTACACTTACCCCAGCCAGTGTCCACACGATCCCAACACACGCAGCCAAGT

161  ProGluProSerThrAlaProSerThrSerPheLeuLeuProMetGlyProSerProPro
481  CCAGAACCCAGCACTGCTCCAAGCACCTCCTTCCTGCTCCCAATGGGCCCCAGCCCCCA

181  AlaGluGlySerThrGlyAspPheAlaLeuProValGlyLeuIleValGlyValThrAla
541  GCTGAAGGGAGCACTGGCGACTTCGCTCTTCCAGTTGGACTGATTGTGGGTGTGACAGCC

201  LeuGlyLeuLeuIleIleGlyValValAsnCysValIleMetThrGlnValLysLysLys
601  TTGGGTCTACTAATAATAGGAGTGGTGAACTGTGTCATCATGACCCAGGTGAAAAAGAAG

221  ProLeuCysLeuGlnArgGluAlaLysValProHisLeuProAlaAspLysAlaArgGly
661  CCCTTGTGCCTGCAGAGAGAAGCCAAGGTGCCTCACTTGCCTGCCGATAAGGCCCGGGGT

241  ThrGlnGlyProGluGlnGlnHisLeuLeuIleThrAlaProSerSerSerSerSerSer
721  ACACAGGGGCCCCGAGCAGCAGCACCTGCTGATCACAGCGCCGAGCTCCAGCAGCAGCTCC

261  LeuGluSerSerAlaSerAlaLeuAspArgArgAlaProThrArgAsnGlnProGlnAla
781  CTGGAGAGCTCGGCCAGTGCGTTGGACAGAAGGGCGCCCACTCGGAACCAGCCACAGGCA
```

Figure 4 (cont.)

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 281  ProGlyValGluAlaSerGlyAlaGlyGluAlaArgAlaSerThrGlySerSerAlaAsp
 841  CCAGGCGTGGAGGCCAGTGGGGCCGGGGAGGCCCGGGCCAGCACCGGGAGCTCAGCAGAT

 301  SerSerProGlyGlyHisGlyThrGlnValAsnValThrCysIleValAsnValCysSer
 901  TCTTCCCCTGGTGGCCATGGGACCCAGGTCAATGTCACCTGCATCGTGAACGTCTGTAGC

 321  SerSerAspHisSerSerGlnCysSerSerGlnAlaSerSerThrMetGlyAspThrAsp
 961  AGCTCTGACCACAGCTCACAGTGCTCCTCCCAAGCCAGCTCCACAATGGGAGACACAGAT

 341  SerSerProSerGluSerProLysAspGluGlnValProPheSerLysGluGluCysAla
1021  TCCAGCCCCTCGGAGTCCCCGAAGGACGAGCAGGTCCCCTTCTCCAAGGAGGAATGTGCC

 361  PheArgSerGlnLeuGluThrProGluThrLeuLeuGlySerThrGluGluLysProLeu
1081  TTTCGGTCACAGCTGGAGACGCCAGAGACCCTGCTGGGGAGCACCGAAGAGAAGCCCCTG

 381  ProLeuGlyValProAspAlaGlyMetLysProSer
1141  CCCCTTGGAGTGCCTGATGCTGGGATGAAGCCCAGTTAACCAGGCCGGTGTGGGCTGTGT
1201  CGTAGCCAAGGTGGCTGAGCCCTGGCAGGATGACCCTGCGAAGGGGCCCTGGTCCTTCCA
1261  GGCCCCCACCACTAGGACTCTGAGGCTCTTTCTGGGCCAAGTTCCTCTAGTGCCCTCCAC
1321  AGCCGCAGCCTCCCTCTGACCTGCAGGCCAAGAGCAGAGGCAGCGAGTTGTGGAAAGCCT
1381  CTGCTGCCATGGCGTGTCCCTCTCGGAAGGCTGGCTGGGCATGGACGTTCGGGGCATGCT
1441  GGGGCAAGTCCCTGAGTCTCTGTGACCTGCCCCGCCCAGCTGCACCTGCCAGCCTGGCTT
1501  CTGGAGCCCTTGGGTTTTTTGTTTGTTTGTTTGTTTGTTTGTTTCTCCCCCTGGGC
1561  TCTGCCCAGCTCTGGCTTCCAGAAAACCCCAGCATCCTTTTCTGCAGAGGGGCTTTCTGG
1621  AGAGGAGGGATGCTGCCTGAGTCACCCATGAAGACAGGACAGTGCTTCAGCCTGAGGCTG
1681  AGACTGCGGGATGGTCCTGGGGCTCTGTGCAGGGAGGAGGTGGCAGCCCTGTAGGGAACG
1741  GGGTCCTTCAAGTTAGCTCAGGAGGCTTGGAAAGCATCACCTCAGGCCAGGTGCAGTGGC
1801  TCACGCCTATGATCCCAGCACTTTGGGAGGCTGAGGCGGGTGGATCACCTGAGGTTAGGA
1861  GTTCGAGACCAGCCTGGCCAACATGGTAAAACCCCATCTCTACTAAAAATACAGAAATTA
1921  GCCGGGCGTGGTGGCGGGCACCTATAGTCCCAGCTACTCAGAAGCCTGAGGCTGGGAAAT
1981  CGTTTGAACCCGGGAAGCGGAGGTTGCAGGGAGCCGAGATCACGCCACTGCACTCCAGCC
2041  TGGGCGACAGAGCGAGAGTCTGTCTCAAAAGAAAAAAAAAAAGCACCGCCTCCAAATGCT
2101  AACTTGTCCTTTTGTACCATGGTGTGAAAGTCAGATGCCCAGAGGGCCCAGGCAGGCCAC
2161  CATATTCAGTGCTGTGGCCTGGGCAAGATAACGCACTTCTAACTAGAAATCTGCCAATTT
2221  TTTAAAAAAGTAAGTACCACTCAGGCCAACAAGCCAACGACAAAGCCAAACTCTGCCAGC
2281  CACATCCAACCCCCCACCTGCCATTTGCACCCTCCGCCTTCACTCCGGTGTGCCTGCAG
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HUMAN TNF RECEPTOR FUSION PROTEIN

This is a division of application Ser. No. 08/095,640, filed Jul. 21, 1993; now U.S. Pat. No. 5,610,279, which is a continuation application of Ser. No. 07/580,013, filed Sep. 10, 1990, now abandoned. This application claims priority under 35 U.S.C. §119 to application Serial Numbers 3319/89, 746/90 and 1347/90, filed on Sep. 12, 1989, Mar. 8, 1990 and Apr. 20, 1990, respectively, all in Switzerland. This application also claims priority under 35 U.S.C. §119 to European Patent Application Number 90116707.2—(now Patent Number EP 0417563), filed Aug. 31, 1990.

BACKGROUND OF THE INVENTION

Tumor necrosis factor α (TNFα, also cachectin), discovered as a result of its hemorragic-necrotizing activity on certain tumors, and lymphotoxin (TNFβ) are two closely related peptide factors [3] from the class of lymphokines/cytokines which are both referred to herein-after as TNF [see references 2 and 3]. TNF possesses a broad cellular spectrum of activity. For example, TNF has inhibitory or cytotoxic activity on a series of tumor cell lines [2,3], stimulates the proliferation of fibroblasts and the phagocytic/cytotoxic activity of myeloic cells [4, 5, 6], induces adhesion molecules in endothelial cells or exerts an inhibitory activity on the endothelium [7, 8, 9, 10], inhibits the synthesis of specific enzymes in adipocytes [11] and induces the expression of histo-compatibility antigens [12]. Many of these TNF activities are produced via induction of other factors or by synergistic effects with other factors such as interferons or interleukins [13-16].

TNF is involved in pathological conditions such as shock states in meningococcal sepsis [17], the development of autoimmune glomerulonephritis in mice [18] and cerebral malaria in mice [19] and human beings [41]. The toxic effects of endotoxin appear to be mediated by TNF [20]. Furthermore, TNF can trigger interleukin-1 fever [39]. On the basis of its pleiotropic functional properties, TNF in interaction with other cytokines is involved in additional pathological conditions as a mediator of immune response, inflammation, and other processes.

These biological effects are mediated by TNF via specific receptors. According to present knowledge not only TNFα, but also TNFβ bind to the same receptors [21]. Different cell types differ in their number of TNF receptors [22, 23, 24]. Generally known TNF-binding proteins (TNF-BP) have been detected by covalent bonding to radioactively labelled TNF [24-29], and the following apparent molecular weights of the TNF/TNF-BP complexes obtained have been determined to be: 95/100 kD and 75 kD [24], 95 kD and 75 kD [25], 138 kD, 90 kD, 75 kD and 54 kD [26], 100±5 kD [27], 97 kD and 70 kD [28] and 145 kD [29]. One such TNF/TNF-BP complex was isolated by anti-TNF-antibody immune affinity chromatography and preparative SDS-polyacrylamide gel electrophoreses (SDS-PAGE) [27]. The reductive cleavage of this complex and subsequent SDS-PAGE analysis gave several bands which were not tested for TNF-binding activity. Since the specific conditions which must be used for the cleavage of the complex lead to inactivation of the binding protein [31], the latter has also not been possible. The separation of soluble TNF-BP from human serum or urine by ion exchange chromatography and gel filtration (molecular weight in the region of 50 kD) was described by Olsson et al. [30].

Brockhaus et al. [32] obtained an enriched TNF-BP preparation from membrane extracts of HL_{60} cells by TNFα-ligand affinity chromatography and HPLC which, in turn, was used as an antigen preparation for the production of monoclonal

antibodies against TNF-BP. Using such an immobilized antibody (immune affinity chromatography) Loetscher and Brockhaus obtained an enriched preparation of TNF-BP [31] from an extract of human placenta using TNFα-ligand affinity chromatography and HPLC, which gave a strong broad band at 35 kD, a weak band at about 40 kD and a very weak band in the region between 55 kD and 60 kD on SDS-PAGE analysis. Moreover, the gel showed a protein background smear in the region of 33 kD to 40 kD. The significance of these protein bands was, however, not clear due to the heterogenicity of the starting material which was used (placenta tissue; combined material from several placentas). In the state of the art TNF-BP have already been characterized by a N-terminal partial sequence [European Patent Application, Publication No. 308 378], whereby this sequence differs from the N-terminal partial sequence according to formula (IA) in accordance with the invention. Moreover, the TNF-binding proteins described in the state of the art are soluble, i.e. non-membrane bound, TNF-BP and not membrane-bound, i.e. insoluble, TNF-BP isolated from urine.

SUMMARY OF THE INVENTION

This invention comprises insoluble, homogenous proteins or soluble or insoluble fragments thereof, capable of binding tumor necrosis factor-(TNF).

This invention also comprises TNF-binding proteins containing amino acid sequences of FIG. **1** or FIG. **4**, proteins containing fragments of these sequences, and proteins analagous to the sequences of FIG. **1** or FIG. **4** or to fragments thereof.

This invention % further comprises DNA sequences encoding the proteins: described above, proteins encoded by these sequences, and antibodies to any of these proteins.

This invention comprises. DNA sequences which combine two partial DNA sequences, one sequence encoding soluble fragments of TNF binding proteins and the other partial sequence encoding all domains except the first domain of the constant region of the heavy chain of human immunoglobulin IgG, IgA, IgM, or IgE, and the recombinant proteins encoded by these sequences.

This invention additionally comprises vectors containing the above DNA sequences, and host systems transfected with such vectors.

This invention finally comprises a process for the isolation of an insoluble homogenous protein capable of binding TNF.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1**. Nucleotide sequence (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) for cDNA clone derived from 55 kD TNF-BP. The 19 amino acid transmembrane region is underlined. Hypothetical glycosylation sites are identified by asterisks.

FIG. **2**. Binding analysis of COS cells transfected with plasmid pN123. Panel 2A—binding of transfected cells to ^{125}I-TNFα. Panel 2B—Scatchard plot of binding data.

FIG. **3**. Sandwich assays of cells transfected with plasmid pK19. Culture supernatants of cells transfected 30 with pK19 were incubated with anti-55 kD TNF-BP antibody followed by ^{125}I-TNFα. Columns 1, 5, and 8 are controls. Columns 2, 3, 4, 5, and 6 are five parallel transfections.

FIG. **4**. Nucleotide sequence (SEQ ID NO: 3) and deduced amino acid sequence (SEQ ID NO: 4) for cDNA clones derived from 75/6510 TNF-BP.

DETAILED DESCRIPTION OF THE INVENTION

The TNF-binding proteins of the present invention are homogenous, insoluble proteins and soluble or insoluble

fragments of such proteins which are capable of binding TNF. These proteins have the ability to bind TNF as measured by standard assays.

The TNF-binding proteins of the present invention include homogenous proteins containing the amino acid sequence depicted in FIG. **1** (SEQ ID NO: 2) or in FIG. **4** (SEQ ID NO: 4), proteins containing fragments of either sequence, and analogues of any such proteins for example proteins containing amino acid sequences analogous to the amino acid sequences of FIG. **1** (SEQ ID NO: 2) or FIG. **4** (SEQ ID NO: 4) or to fragments thereof. An analogue is a protein in which one or more amino acids of the sequences depicted in FIG. **1** (SEQ ID NO: 2) or in FIG. **4** (SEQ ID NO: 4) have had their side-groups chemically modified in a known manner, or those in which one or more amino acids have been replaced or deleted, without thereby eliminating TNF-binding ability. Such analogues may be produced by known methods of peptide chemistry, or by known methods of recombinant DNA technology, such as planned mutagenesis.

The TNF binding activity of the proteins of the present invention may be determined using the assay described in Example 1.

TNF-binding proteins of this invention are obtained as follows:

TNF binding proteins may be isolated from tissues and purified to homogeneity, or isolated from cells which contain membrane-bound TNF binding protein, and purified to homogeneity. One possible method for growing cells and isolating cell extract is described in Example 2, however, other cells types and other growth and isolation methods are well known in the art. Purification of TNF-binding proteins from cell extracts may be performed using the methods described in Examples 4, 5, and 6 in combination with the assay described in Example 1. TNF-binding proteins isolated and purified by these methods were sequenced by well-known methods, as described in Example 7. From these amino acid sequences, DNA probes were produced and used to obtain mRNA encoding TNF binding proteins from which cDNA was made, all by known methods described in Examples 8 and 11. Other well-known methods for producing cDNA are known in the art and may effectively be used. In general, any TNF-binding protein can be isolated from any cell or tissue expressing such proteins using a cDNA probe such as the probe described above, isolating mRNA and transcribing the mRNA into cDNA. Thereafter, the protein can be produced by inserting the cDNA into an expression vector as described in Example 9, such as a virus, plasmid, cosmid, or other vector, inserting the expression vector into a cell, such as the COS cell-described in Example 9 or the insect cell described in Example 10, proliferating the resulting cells, and isolating the expressed TNF-binding protein from the medium or from cell extract as described above. Alternatively, TNF-binding proteins may be chemically synthesized using the sequence described above and an amino acid synthesizer, or manual synthesis using chemical conditions well known to form peptide bonds between selected amino acids. Analogues and fragments of TNF-binding proteins may be produced by the above methods. In the case of analogues, the proteins may be chemically modified, or modified by genetic engineering as described above. These fragments and analogues may then be tested for TNF-binding activity using methods such as the assay of Example 1.

Finally, monoclonal antibodies directed against TNF-binding proteins, such as the antibodies described in Example 3, may be produced by known techniques, and used to isolate TNF-binding proteins.

In more detail, the proteins of the present invention are non-soluble proteins, i.e. for example membrane proteins or so-called receptors, and soluble or non-soluble, fragments thereof, which bind TNF (TNF-BP), in homogeneous form, as well as their physiologically compatible salts. Preferred proteins are those which according to SDS-PAGE under non-reducing conditions are characterized by apparent molecular weights of about 55 kD, 51 kD, 38 kD, 36 kD and 34 kD or 75 kD and 65 kD, especially those with about 55 kD and 75 kD. Furthermore, there are preferred those proteins which are characterized by containing at least one of the following amino acid partial sequences:

(IA) Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile (SEQ ID NO: 5)

(IB) Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys (SEQ ID NO: 6)

(IIA) Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu (SEQ ID NO: 7)

(IIB) Val-Phe-Cys-Thr (SEQ ID NO: 8)

(IIC) Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala (SEQ ID NO: 9)

(IID) Leu-pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys (SEQ ID NO: 10)

(IIE) Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu (SEQ ID NO: 11)

(IIF) Leu-Cys-Ala-Pro (SEQ ID NO: 12)

(IIG) Val-Pro-His-Leu-Pro-Ala-Asp (SEQ ID NO: 13)

(IIH) Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro (SEQ ID NO: 14)

in which X stands for an amino acid residue which could not be unequivocally determined.

A process for the isolation of the TNF-BP in accordance with the invention is also an object of the present invention. This process comprises carrying out essentially the following purification steps in sequence: production of a cell or tissue extract, immune affinity chromatography and/or single or multiple ligand affinity chromatography, high resolution liquid chromatography (HPLC) and preparative SDS-polyacrylamide gel electro phoresis (SDS-PAGE). The combination of the individual purification steps, which are known from the state of the art, is essential to the success of the process in accordance with the invention, whereby individual steps have been modified and improved having regard to the problem to be solved. Thus, for example, the original combined immune affinity chromatography/TNFα-ligand affinity chromatography step originally used for the enrichment of TNF-BP from human placenta [31] has been altered by using a BSA-Sepharose 4B pre-column. For the application of the cell or membrane extract, this pre-column was connected in series with the immune affinity column followed by the ligand affinity column. After the application of the extract the two aforementioned columns were coupled, each eluted and the TNF-BP-active fractions were purified again via a ligand affinity column. The use of a detergent-containing solvent mixture for the performance of the reversed-phase HPLC step is essential to the invention.

Further, an industrial process for the production of high cell densities of mammalian cells from which TNF-BP can be isolated is also an object of the present invention. Such a process comprises using a medium, which has been developed for the specific growth requirements of the cell line used, in combination with a perfusion apparatus as described e.g. in detail in Example 2. By means of such a process there can be produced, for example, in the case of HL-60 cells up to more than 20-fold higher cell densities than usual.

In addition thereto, the present invention is also concerned with DNA sequences coding for proteins and soluble or non-soluble fragments thereof, which bind TNF. Thereunder there are to be understood; for example, DNA sequences coding for non-soluble proteins or soluble as well as non-soluble fragments thereof, which bind TNF, such DNA sequences being selected from the following:

(a) DNA sequences as given FIG. **1** or FIG. **4** as well as their complementary strands, or those which include these sequences;

(b) DNA sequences which hybridize with sequences defined under (a) or fragments thereof;

(c) DNA sequences which, because of the degeneracy of the genetic code, do not hybridize with sequences as defined under (a) and (b), but which code for polypeptides, having exactly the same amino acid sequence.

That is to say, the present invention embraces not only allelic variants, but also those DNA sequences which result from deletions, substitutions and additions from one or more nucleotides of the sequences given in FIG. **1** or FIG. **4**, whereby in the case of the proteins coded thereby there come into consideration, just as before, TNF-BP. One sequence which results from such a deletion is described, for example, in Science 248, 1019-1023, (1990).

There are preferred first of all those DNA sequences which code for such a protein having an apparent molecular weight of about 55 kD, whereby the sequence given in FIG. **1** is especially preferred, and sequences which code for non-soluble as well as soluble fragments of such proteins. A DNA sequence which codes, for example, for such a non-soluble protein fragment extends from nucleotide −185 to 1122 of the sequence given in FIG. **1**. DNA sequences which code for soluble protein fragments are, for example, those which extend from nucleotide −185 to 633 or from nucleotide −14 to 633 of the sequence given in FIG. **1**. There are also preferred DNA sequences which code for a protein of about 75/65 kD, whereby those which contain the partial cDNA sequences shown in FIG. **4** are preferred. Especially preferred DNA sequences in this case are the sequences of the open reading frame of nucleotide 2 to 1,177. The peptides IIA, IIC, IIE, IIF, IIG and IIH are coded by the partial cDNA sequence in FIG. **4**, whereby the insignificant deviations in the experimentally determined amino acid sequences are based on the cDNA-derived sequence with highest probability from the limited resolution of the gas phase sequencing. DNA sequences which code for insoluble (deposited on Oct. 17, 2006 with the American Type Culture Collection under Accession No. PTA 7942) as well as soluble fractions of TNF-binding proteins having an apparent molecular weight of 65 kD/75 kD are also preferred. DNA sequences for such soluble fragments can be determined on the basis of the amino acid sequences derived from the nucleic acid sequences coding for such non-soluble TNF-BP.

The invention is also concerned with DNA sequences which comprise a combination of two partial DNA sequences, with one of the partial sequences coding for those soluble fragments of non-soluble proteins which bind TNF (see above) and the other partial sequence coding for all domains other than the first domain of the constant region of the heavy chain of human immunoglobulins such as IgG, IgA, IgM or IgE, in particular IgG_1 or IgG_3 subtypes.

The present invention is also concerned with the recombinant proteins coded by any of DNA sequences described above. Of course, there are thereby also included such proteins in whose amino acid sequences amino acids have been exchanged, for example by planned mutagenesis, so that the activity of the TNF-BP or fragments thereof, namely the binding of TNF or the interaction with other membrane components participating in the signal transfer, have been altered or maintained in a desirable manner. Amino acid exchanges in proteins and peptides which do not generally alter the activity of such molecules are known in the state of the art and are described, for example, by H. Neurath and R. L. Hill in "The'Proteins" (Academic Press, New York, 1979, see especially FIG. **6**, page 14). The most commonly occurring exchanges are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly as well as these in reverse. The present invention is also concerned with vectors which contain any of the DNA sequences described above in accordance with the invention and which are suitable for the transformation of suitable pro- and eukaryotic host systems, whereby there are preferred those vectors whose use leads to the expression of the proteins which are coded by any of the DNA sequences described above in accordance with the invention. Finally, the present invention is also concerned with pro- and eukaryotic host systems transformed with such vectors, as well as a process for the production of recombinant compounds in accordance with the invention by cultivating such host systems and subsequently isolating these compounds from the host systems themselves or their culture supernatants.

An object of the present invention are also pharmaceutical preparations which contain at least one of these TNF-BPs or fragments thereof, if desired in combination with other pharmaceutically active substances and/or non-toxic, inert, therapeutically compatible carrier materials.

Finally, the present invention is concerned with the use of such a TNF-BP on the one hand for the production of pharmaceutical preparations and on the other hand for the treatment of illnesses, preferably those in which TNF is involved in their course.

Starting materials for the TNF-BP in accordance with the invention are quite generally cells which contain such TNF-BP [in membrane-bound form] and which are generally accessible without restrictions to a person skilled in the art, such as, for example, HL60 [ATCC No. CCL 240], U 937 [ATCC No. CRL 1593], SW 480 [ATCC No. CCL 228] and HEp2 cells [ATCC No. CCL 23]. These cells can be cultivated according to known methods of the state of the art [40] or, in order to produce high cell densities, according to the procedure already described generally and described in detail in. Example 2 for HL60 cells. TNF-BP can then be extracted from the cells, which are centrifuged-off from the medium and washed, according to known methods of the state of the art using suitable detergents, for example Triton X-114, 1-0-n-octyl-β-D-glucopyranoside (octylglucoside) or 3-[(3-cholylamido-propyl)-dimethylammonio]-1-propane sulphonate (CHAPS), especially using Triton X-100. For the detection of such TNF-BP there can be Used the usually used detection methods for TNF-BP, for example a polyethylene glycol-1-induced precipitation of the ^{125}I-TNF/TNF-BP complex [27], especially filter-binding tests with radioactively labelled TNF according to Example 1. In order to produce the TNF-BP in accordance with the invention, the general methods of the state of the art used for the purification of proteins, especially of membrane proteins, such as, for example, ion exchange chromatography, gel filtration, affinity chromatography, HPLC and SDS-PAGE can be used. Especially preferred methods for the production of TNF-BP in accordance with the invention are affinity chromatography, especially with TNF-a as the ligand bound to the solid phase, and immune-affinity chromatography, HPLC and SDS-PAGE. The elution of TNF-BP bands which are separated

using SDS-PAGE can be effected according to known methods of protein chemistry, for example using electroelution according to Hunkapiller et al. [34], whereby according to present knowledge the electro-dialysis times given there generally have to be doubled. Thereafter, traces of SDS which still remain can then be removed in accordance with Bosserhoff et al. [50].

The thus-purified TNF-BP can be characterized by methods of peptide chemistry which are known in the state of the art, such as, for example, N-terminal amino acid sequencing of enzymatic well as chemical peptide cleavage. Fragments obtained by enzymatic or chemical cleavage can be separated according to usual methods such as, for example, HPLC and can themselves be subjected to further N-terminal sequencing. Such fragments which themselves bind TNF can be identified using the afore-mentioned detection methods for TNF-BP and are likewise objects of the present invention.

Starting from the thus-obtained amino acid sequence information or the DNA and amino acid sequences given in FIG. **1** as well as in FIG. **4** there can be produced, taking into consideration the degeneracy of the genetic code, according to methods known in the state of the art suitable oligonucleotides [51]. By means of these, again according to known methods of molecular biology [42,43], cDNA or genomic DNA banks can be searched for clones which contain nucleic acid sequences coding for TNF-BP. More-over, using the polymerase chain reaction (PCR) [49] cDNA fragments can be cloned by completely degenerating the amino acid sequence of two spaced apart relatively short segments while taking into consideration the genetic code and introducing into their complementarity suitable oligo-nucleotides as a "primer", whereby the fragment lying between these two sequences can be amplified and identified. The determination of the nucleotide sequence of a such a fragment permits an independent determination of the amino acid sequence of the protein fragment for which it codes. The cDNA fragments obtainable by PCR can also, as already described for the oligonucleotides themselves, be used according to known methods to search for clones containing nucleic acid sequences coding for TNF-BP from cDNA or genomic DNA banks. Such nucleic acid sequences can then be sequenced according to known methods [42]. On the basis of the thus-determined sequences and of the already known sequences for certain receptors, those partial sequences which code for soluble TNF-BP fragments can be determined and cut out from the complete sequence using known methods [42].

The complete sequence or such partial sequences can then be integrated using known methods into vectors described in the state of the art for their multiplication and expression in prokaryotes [42]. Suitable prokaryotic host organisms are, for example, gram-negative and gram-positive bacteria such as, for example, *E. coli* strains such as *E. coli* HB101 [ATCC No. 33 694] or *E. coli* W3110 [ATCC No. 27 325] or *B. subtilis* strains.

Furthermore, nucleic acid sequences in accordance with the invention which code for TNF-BP as well as for TNF-BP fragments can be integrated using known methods into suitable vectors for reproduction and expression in eukaryotic host cells, such as, for example, yeast, insect cells and mammalian cells. Expression of such sequences is preferably effected in mammalian and insect cells.

A typical expression vector for mammalian cells contains an efficient promoter element in order to produce a good transcription rate, the DNA sequence to be expressed and signals for an efficient termination and polyadenylation of the transcript. Additional elements which can be used are "enhancers" which lead to again intensified transcription and sequences which e.g. can bring about a longer half life of the mRNA. For the expression of nucleic acid sequences in which the endogenous sequence fragment coding for a signal peptide is missing, there can be used vectors which contain such suitable sequences which code for signal peptides of other known proteins. See, for example, the vector pLJ268 described by Cullen, B. R. in Cell 46, 973-982 (1986) as well as Sharma, S. et al. in "Current Communications in Molecular Biology", edt. by Gething, M. J., Cold Spring Harbor Lab. (1985), pages 73-78.

Most of these vectors which are used for a transient expression of a particular DNA sequence in mammalian cells contain the replication source of the SV40 virus. In cells which express the T-antigen of the virus (e.g. COS cells), these vectors are reproduced abundantly. A transient expression is, however, not limited to COS cells. In principle any transfectable mammalian cell line can be used for this purpose. Signals which can bring about a strong transcription are e.g. the early and late promoters of SV40, the promoter and enhancer of the "major immediate-early" gene of HCMV (human cytomegalovirus), the LTR's ("long terminal repeats") of retroviruses such as, for example, RSV, HIV and MMTV. There can, however, also be used signals of cellular genes such as e.g. the promoters of the actin and collagenase genes.

Alternatively, however, stable cell lines which have the specific DNA sequence integrated into the genome (chromosome) can also be obtained. For this, the DNA sequence is cotransfected together with a selectable marker, e.g. neomycin, hygromycin, dihydrofolate reductase (dhfr) or hypoxanthin guanine phosphoribosyl transferase (hgpt). The DNA sequence stably incorporated in the chromosome can also be reproduced abundantly. A suitable selection marker for this is, for example, dihydrofolate reductase (dhfr). Mammalian cells (e.g. CHO cells), which contain no intact dhfr gene, are thereby incubated with increasing amounts of methotrexate after transinfection has been effected. In this manner cell lines which contain more than a thousand copies of the desired DNA sequence can be obtained.

Mammalian cells which can be used for the expression are e.g. cells of the human cell lines Hela [ATCC CCL2] and 293 [ATCC CRL 1573] as well as 3T3 [ATCC CCL 163] and L cells, e.g. [ATCC CCL 149], (CHO) cells [ATCC CCL 61], BHK [ATCC CCL 10] cells as well as the CV 1 [ATCC CCL 70] and the COS cell lines [ATCC CRL 1650, CRL 1651].

Suitable expression vectors include, for example, vectors such as pBC12MI [ATCC 67 109], pSV2dhfr [ATCC 37 146], pSVL [Pharmacia, Uppsala, Sweden], pRSVcat [ATCC 37 152] and pMSG [Pharmacia, Uppsala, Sweden]. The vectors "pK19" and "pN123" used in Example 9 are especially preferred vectors. These can be isolated according to known methods from *E. coli* strains HB101(pK19) and HB101 (pN123) transformed with them [42]. These *E. coli* strains have been deposited on the 26 Jan. 1990 at the Deutschen Sammlung von Mikroorganismen and Zellkulturen GmbH (DSM) in Braunschweig, FRG, under DSM 5761 for HB101 (pK19) and DMS 5764 for HB101(pN123). For the expression of proteins which consist of a soluble fragment of non-soluble TNF-BP and an immunoglobulin fragment, i.e. all domains except the first of the constant region of the heavy chain, there are especially suitable pSV2-derived vectors as described, for example, by German, C. in "DNA Cloning" [Vol. II., edt. by Glover, D. M., IRL Press, Oxford, 1985]. The vectors pCD4-Hp (DSM 5315, deposited on 21 Apr. 1989), pDC4-Hy1 (DSM 5314, deposited on 21 Apr. 1989) and pCD4-Hy3 (DSM 5523, deposited on 14 Sep. 1989) which have been deposited at the Deutschen Sammlung von Mikroorganismen and Zellkulturen GmbH (DSM) in Braunsch-

weig, FRG, and which are described in detail in European Patent Application No. 90107393.2 are especially preferred vectors. This European Patent Specification and the equivalent Applications referred to in Example 11 also contain data with respect to the further use of these vectors for the expression of chimeric proteins (see also Example 11) and for the construction of vectors for the expression of such chimeric proteins with other immunoglobulin fragments.

The manner in which these cells are transfected depends on the chosen expression system and vector system. An overview of these methods is to be found e.g. in Pollard et al., "DNA Transformation of Mammalian Cells" in "Methods in Molecular Biology" [Nucleic Acids Vol. 2, 1984, Walker, J. M., ed, Humana, Clifton, N.J.]. Further methods are to be found in Chen and Okayama ["High-Efficiency Transformation of Mammalian Cells by Plasmid DNA", Molecular and Cell Biology 7, 2745-2752, 1987] and in Feigner [Feigner et al., "Lipofectin: A highly efficient, lipid-mediated. DNA-transfection procedure", Proc. Nat. Acad. Sci. USA 84, 7413-7417, 1987].

The baculovirus expression system, which has already been used successfully for the expression of a series of proteins (for an overview see Luckow and Summers, Bio/Technology 6, 47-55, 1988), can be used for the expression in insect cells. Recombinant proteins can be produced in authentic form or as fusion proteins. The thus-produced proteins can also be modified such as, for example, glycosylated (Smith et al., Proc. Nat. Acad. Sci. USA 82, 8404-8408, 1987). For the production of a recombinant baculovirus which expresses the desired protein there is used a so-called "transfer vector". Under this there is to be understood a plasmid which contains the heterologous DNA sequence under the control of a strong promoter, e.g. that of the polyhedron gene, whereby this is surrounded on both sides by viral sequences. The vectors "pN113", "pN119" and "pN124" used in Example 10 are especially preferred vectors. These can be isolated according to known methods from *E. coli* strains HB101(pN113), HB101(pN119) and HB101(pN124) transformed with them. These *E. coli* strains have been deposited on the 26 Jan. 1990 at the Deutschen Sammlung von Mikroorganismen and Zellkulturen GmbH (DSM) in Braunschweig, FRG, under DSM 5762 for HB101(pN113), DSM 5763 for HB101 (pN119) and DSM 5765 for HB101(pN124). The transfer vector is then transfected into the insect cells together with DNA of the wild type baculovirus. The recombinant viruses which result in the cells by homologous recombination can then be identified and isolated according to known methods. An overview of the baculovirus expression system and the methods used therein is to be found in Luckow and Summers [52].

Expressed TNF-BP as well as its non-soluble or soluble fractions can then be purified from the cell mass or the culture supernatants according to methods of protein chemistry which are known in the state of the art, such as, for example, the procedure already described on pages 5-6.

The TNF-BP obtained in accordance with the invention can also be used as antigens to produce polyclonal and monoclonal antibodies according to known-techniques [44, 45] or according to the procedure described in Example 3. Such antibodies, especially monoclonal antibodies against the 75 kD TNF-BP species, are also an object of the present invention. Those antibodies which are directed against the 75 kD TNF-BP can be used for the isolation of TNF-BP by modifications of the purification procedure described in detail in Examples 4-6 which are familiar to a person skilled in the art.

On the basis of the high binding affinity of TNF-BP in accordance with the invention for TNF (K_d value in the order of 10^{-9}-10^{-10} M), these or fragments thereof can be used as diagnostics for the detection of TNF in serum or other body fluids according to methods known in the state of the art, for example in solid phase binding tests or in combination with anti-TNF-BP antibodies in so-called "sandwich" tests.

Moreover, TNF-BP in accordance with the invention can be used on the one hand for the purification of TNF and on the other hand for the detection of TNF agonists and TNF antagonists according to procedures which are known in the state of the art.

The TNF-BP in accordance with the invention as well as their physiologically compatible salts, which can be manufactured according to methods which are known in the state of the art, can also be used for the production of pharmaceutical preparations, primarily those for the treatment of illnesses in which TNF is involved in their course. For this purpose, one or more of the said compounds, where desired or required in combination with other pharmaceutically active substances, can be processed in a known manner with the usually used solid or liquid carrier. materials. The dosage of such preparations can be effected having regard to the usual criteria in analogy to already used preparations of similar activity and structure.

Since the invention has been described hereinbefore in general terms, the following Examples are intended to illustrate details of the invention, but they are not intended to limit its scope in any manner.

Example 1

Detection of TNF-Binding Proteins

The TNF-BP were detected in a filter test with human radioiodinated ^{125}I-TNF. TNF (46, 47) was radioactively labelled with Na^{125}I (IMS40, Amersham, Amersham, England) and iodo gene (#28600, Pierce Eurochemie, Oud-Beijerland, Netherlands) according to Fraker and Speck [48]. For the detection of the TNF-BP, isolated membranes of the cells or their solubilized, enriched and purified fractions were applied to moist nitrocellulose filter (0.45µ, BioRad. Richmond, Calif., USA). The filters were then blocked in buffer solution with 1% skimmed milk powder and subsequently incubated with $5 \cdot 10^5$ cpm/ml of ^{125}I-TNFα (0.3-1.0·10^8 cpm/µg) in two batches with and without the addition of 5 µg/ml of non-labelled TNFα, washed and dried in the air. The bound radio-activity was detected semiquantitatively by autoradiography or counted in a gamma-counter. The specific ^{125}I-TNF-α binding was determined after correction for unspecific binding in the presence of unlabelled TNF-α in excess. The specific TNF-binding in the filter test was measured at various TNF concentrations and analyzed according to Scatchard, whereby a K_d value of ·10^{-9}-10^{-10} M was determined.

Example 2

Cell Extracts of HL-60 Cells

HL60 cells [ATCC No. CCL 240] were cultivated on an experimental laboratory scale in a RPMI 1640 medium [GIBCO catalogue No. 074-01800], which contained 2 g/l $NaHCO_3$ and 5% foetal calf serum, in a 5% CO_2 atmosphere, and subsequently centrifuged.

The following procedure was used to produce high cell densities on an industrial scale. The cultivation was carried out in a 75 l Airlift fermenter (Fa. Chemap, Switzerland) with a working volume of 58 l. For this there was used the cassette membrane system "PROSTAK" (Millipore, Switzerland)

with a membrane surface of 0.32 m^2(1 cassette) integrated into the external circulation circuit. The culture medium (see Table 1) was pumped around with a Watson-Marlow pump, Type 603U, with 5 l/min. After a steam sterilization of the installation, whereby the "PROSTAK" system was sterilized separately in autoclaves, the fermentation was started with growing HL-60 cells from a 20 l Airlift fermenter (Chemap). The cell cultivation in the inoculation fermenter was effected in a conventional batch process in the medium according to Table 1 and an initial cell titre of 2×10^5 cells/ml. After 4 days the HL60 batch was transferred with a titre of 4.9×10^6 cells/ml into the 75 l fermenter. The pH value was held at 7.1 and the pO_2 value was held at 25% saturation, whereby the oxygen introduction was effected through a microporous frit. After initial batch fermentation, on the 2nd day the perfusion at a cell titre of 4×10^6 cells/ml was started with 30 l of medium exchange per day. On the filtrate side of the medium the conditioned medium was removed and replaced by the addition of fresh medium. The added medium was fortified as follows: Primatone from 0.25% to 0.35%, glutamine from 5 mM to 6 mM and glucose from 4 g/l to 6 g/l. The perfusion rate was then increased on the 3rd and 4th day to 72 l of medium/day and on the 5th day to 100 l of medium/day. The fermentation had finished after 120 hours of continuous cultivation. Exponential cell growth up to 40×10^6 cells/ml took place under the given fermentation conditions. The duplication time of the cell population was 20-22 hours to 10×10^6 cells/ml and then increased to 30-36 hours with increasing cell density. The proportion of living cells lay at 90-95% during the entire fermentation period. The HL-60 batch was then cooled down in the fermenter to about 12° C. and the cells were harvested by centrifugation (Beckman centrifuge [Model J-6B, Rotor JS], 3000 rpm, 10 min., 4° C.).

TABLE 1

HL-60 medium

Components	Concentrations mg/l
$CaCl_2$ (anhydrous)	112.644
$Ca(NO_3)_2 \cdot 4H_2O$	20
$CuSO_4 \cdot 5H_2O$	$0.498 \cdot 10^{-3}$
$Fe(NO_3)_3 \cdot 9H_2O$	0.02
$FeSO_4 \cdot 7H_2O$	0.1668
KCl	336.72
KNO_3	0.0309
$MgCl_2$ (anhydrous)	11.444
$MgSO_4$ (anhydrous)	68.37
NaCl	5801.8
Na_2HPO_4 (anhydrous)	188.408
$NaH_2PO_4 \cdot H_2O$	75
$Na_2SeO_3 \cdot 5H_2O$	$9.6 \cdot 10^{-3}$
$ZnSO_4 \cdot 7H_2O$	0.1726
D-Glucose	4000
Glutathion (red.)	0.2
Hepes buffer	2383.2
Hypoxanthin	0.954
Linoleic acid	0.0168
Lipoic acid	0.042
Phenol Red	10.24
Putrescine 2HCl	0.0322
Na pyruvate	88
Thymidine	0.146
Biotin	0.04666
D-Ca pantothenate	2.546
Choline chloride	5.792
Folic acid	2.86
i-Inositol	11.32
Niacinamide	2.6
Nicotinamide	0.0074
para-Aminobenzoic acid	0.2

TABLE 1-continued

HL-60 medium

Components	Concentrations mg/l
Pyridoxal HCl	2.4124
Pyridoxin HCl	0.2
Riboflavin	0.2876
Thiamin HCl	2.668
Vitamin B_{12}	0.2782
L-Alanine	11.78
L-Aspartic acid	10
L-Asparagine H_2O	14.362
L-Arginine	40
L-Arginine HCl	92.6
L-Aspartate	33.32
L-Cystine 2HCl	62.04
L-Cysteine HCl • H_2O	7.024
L-Glutamic acid	36.94
L-Glutamine	730
L-Glycine	21.5
L-Histidine	3
L-Histidine HCl • H_2O	27.392
L-Hydroxypyroline	4
L-Isoleucine	73.788
L-Leucine	75.62
L-Lysine HCl	102.9
L-Methionine	21.896
L-Phenylalanine	43.592
L-Proline	26.9
L-Serine	31.3
L-Threonine	53
L-Tryptophan	11.008
L-Tyrosine • 2Na	69.76
L-Valine	62.74
Penicillin/streptomycin	100 U/ml
Insulin (human)	5 µg/ml
Tranferrin (human)	15 µg/ml
Bovine serum albumin	67 µg/ml
Primatone RL (Sheffield Products, Norwich NY, USA)	0.25%
Pluronic F68 (Serva, Heidelberg, FRG)	0.01%
Foetal calf serum	0.3-3%

The centrifugate was washed with isotonic phosphate buffer (PBS; 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 8.0 g/l NaCl, 2.16 g/l $Na_2HPO_4.7H_20$), which had been treated with 5% dimethylformamide, 10 mM benzamidine, 100 U/ml aprotinin, 10 µM leupeptin, 1 µM pepstatin, 1 mM o-phenanthroline, 5 mM iodoacetamide, 1 mM phenyl-methylsulphonyl fluoride (referred to hereinafter as PBS-M). The washed cells were extracted at a density of $2.5\cdot10^8$ cells/ml in PBS-M with Triton X-100 (final concentration 1.0%). The cell extract was clarified by centrifugation (15,000×g, 1 hour; 100,000×g, 1 hour).

Example 3

Production of Monoclonal (TNF-BP) Antibodies

A centrifugation supernatant from the cultivation of HL60 cells on an experimental laboratory scale, obtained according to Example 2, was diluted with PBS in the ratio 1:10. The diluted supernatant was applied at 4° C. (flow rate: 0.2 ml/min.) to a column which contained 2 ml of Affigel 10 (Bio Rad Catalogue No. 153-6099) to which had been coupled 20 mg of recombinant human TNF-α [Pennica, D. et al. (1984) Nature 312, 724; Shirai, T. et al. (1985) Nature 313, 803; Wang, A. M. et al. (1985) Science 228, 149] according to the recommendations of the manufacturer. The column was washed at 4° C. and a throughflow rate of 1 ml/min firstly with 20 ml of PBS which contained 0.1% Triton X 114 and there-

after with 20 ml of PBS. Thus-1-enriched TNF-BP was eluted at 22° C. and a flow rate of 2 ml/min with 4 ml of 100 mM glycine, pH 2.8, 0.1% decyl-maltoside. The eluate was concentrated to 10 µl in a Centricon 30 unit [Amicon].

10 µl of this eluate were mixed with 20 µl of complete Freund's adjuvant to give an emulsion. 10 µl of the emulsion were injected according to the procedure described by Holmdahl, R. et al. [(1985), J. Immunol. Methods 32, 379] on each of days 0, 7 and 12 into a hind paw of a narcotized Balb/c mouse.

The immunized mice were sacrificed on day 14, the popliteal lymph nodes were removed, minced and suspended by repeated pipetting in Iscove's medium (IMEM, GIBCO Catalogue No. 074-2200) which contained 2 g/l $NaHCO_3$. According to a modified procedure of De St. Groth and Scheidegger [J. Immunol. Methods (1980), 35, 1] 5×10^7 cells of the lymph nodes were fused with 5×10^7 PAI mouse myeloma cells (J. W. Stocker et al., Research Disclosure, 217, May 1982, 155-157) which were in logarithmic growth. The cells were mixed, collected by centrifugation and resuspended in 2 ml of 50% (v/v) polyethylene glycol in IMEM at room temperature by slight shaking and diluted by the slow addition of 10 ml of IMEM during careful shaking for 10 minutes. The cells were collected by centrifugation and resuspended in 200 ml of complete medium [IMEM+20% foetal calf serum, glutamine (2.0 mM), 2-mercaptoethanol (100 µl), 100 µM hypoxanthine, 0.4 µM aminopterine and 16 µM thymidine (HAT)]. The suspension was distributed on 10 tissue 10 culture dishes each containing 96 wells and incubated at 37° C. for 11 days without changing the medium in an atmosphere of 5% CO_2 and a relative humidity of 98%.

The antibodies are distinguished by their inhibitory action on the binding of TNF to HL60 cells or by their binding to antigens in the filter test according to Example 1. The following procedure was used to detect the biological activity of anti(TNF-BP) antibodies: 5×10^6 HL60 or U937 cells were incubated in complete RPMI 1640 medium together with affinity-purified monoclonal anti-(TNF-BP) antibodies or control antibodies (i.e. those which are not directed against TNF-BP) in a concentration range of 1 ng/ml to 10 µg/ml. After incubation at 37° C. for one hour the cells were collected by centrifugition and washed with 4.5 ml of PBS at 0° C. They were resuspended in 1 ml of complete RPMI 1640 medium (Example 2) which additionally contained 0.1% sodium azide and ^{125}I-TNFα (10^6 cpm/ml) with or without the addition of unlabelled TNFα (see above). The specific radioactivity of the ^{125}I-TNFα amounted to 700 Ci/mmol. The cells were incubated at 4° C. for 2 hours, collected and washed 4 times at 0° C. with 4.5 ml of PBS which contained 1% BSA and 0.001% Triton X 100 (Fluka). The radioactivity bound to the cells was measured in a γ-scintillation counter. The cell-bound radioactivity of cells which had not been treated with anti-(TNF-BP) antibodies was determined in a comparative experiment (approximately 10 000 cpm/5×10^6 cells).

Example 4

Affinity Chromatography

For the further purification, a monoclonal anti-(55 kD TNF-BP) antibody (2.8 mg/ml gel), obtained according to Example 3, TNFα (3.9 mg/ml gel) and bovine serum albumin (BSA, 8.5 mg/ml gel) were each covalently coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the directions of the manufacturer. The cell extract obtained according to Example 2 was passed through the thus-prepared columns which were connected in series in the following sequence: BSA-Sepharose pre-column, immune affinity column (anti-(55 kD-TNF-BP) antibody), TNFα-ligand affinity column. After completion of the application the two last-mentioned columns were separated and washed individually with in each case 100 ml of the following buffer solutions: (1) PBS, 1,0% Triton X-100, 10 mM benzamidine, 100 U/ml aprotinin; (2) PBS, 0.1% Triton X-100, 0.5M NaCl, 10 mM ATP, 10 mM benzamidine, 100 U/ml aprotinin; and (3) PBS, 0.1% Triton X-100, 10 mM benzamidine, 100 U/ml aprotinin. Not only the immune affinity column, but also the TNFα-ligand affinity column were then each eluted with 100 mM glycine pH 2.5, 100 mM NaCl, 0.2% decylmaltoside, 10 mM benzamidine, 100 U/ml aprotinin. The fractions of each column which were active in the filter test according to Example 1 were thereafter combined and neutralized with 1M Tris pH 8.0.

The thus-combined TNF-BP active fractions of the immune affinity chromatography on the one hand and of the TNFα-ligand affinity chromatography on the other hand were, for further purification, again applied to in each case one small TNFa-ligand affinity column. Thereafter, these two columns were washed with in each case 40 ml of (1) PBS, 1.0% Triton X-100, 10 mM benzamidine, 100 U/ml aprotinin, (2) PBS, 0.1% Triton X-100, 0.5M NaCl, 10 mM ATP, 10 mM benzamidine, 100 U/ml aprotinin, (3) PBS, 0.1% Triton X-100, (4) 50 mM Tris pH 7.5, 150 mM NaCl, 1.0% NP-40, 1.0% desoxycholate, 0.1% SDS, (5) PBS, 0.2% decyl-maltoside. Subsequently, the columns were eluted with 100 mM glycine pH 2.5, 100 mM NaCl, 0.2% decylmaltoside. Fractions of 0.5 ml from each column were collected and the fractions from each column which were active according to the filter test (Example 1) were combined and concentrated in a Centricon unit (Amicon, molecular weight exclusion 10,000).

Example 5

Separation by Means of HPLC

The active fractions obtained according to Example 4 were each applied according to their different source (immune or ligand affinity chromatography) to C1/C8 reversed phase HPLC columns (ProRPC, Pharmacia, 5×20 mm) which had been equilibrated with 0.1% trifluoroacetic acid, 0.1% octylglucoside. The columns were then eluted with a linear acetonitrile gradient (0-80%) in the same buffer at a flow of 0.5 ml/min. Fractions of 1.0 ml were collected from each column and the active fractions from each column were combined (detection according to Example 1).

Example 6

Separation by Means of SDS-PAGE

The fractions which were obtained according to Example 5 and which were active according to the filter test (Example 1) were further separated by SDS-PAGE according to [34]. For this purpose, the samples were heated to 95° C. for 3 minutes in SDS sample buffer and subsequently separated electrophoretically on a 12% acrylamide separation gel with a 5% collection gel. The following standard proteins were used as a reference for the determination of the apparent molecular weights on the SDS-PAGE gel: phosphorylase B (97.4 kD), BSA (66.2 kD), ovalbumin (42.7 kD), carboanhydrase (31.0 kD), soya trypsin inhibitor (21.5 kD) and lysozyme (14.4 kD).

Under the mentioned conditions there were obtained for samples which has been obtained according to Example 4 by TNF-α-ligand affinity chromatography of immune affinity chromatography eluates and which had been further separated by HPLC according to Example 5 two bands of 55 kD and 51 kD as well as three weaker bands of 38 kD, 36 kD and 34 kD. These bands were transferred electro-phoretically during 1 hour at 100 V in 25 mM Tris, 192 mM glycine, 20% methanol on to a PVDF membrane (Immobilon, Millipore, Bedford, Mass. USA) in a Mini Trans Blot System (BioRad, Richmond, Calif., USA). Thereafter, the PVDF membrane was either protein-stained with 0.15% Serva-Blue (Serva, Heidelberg, FRG) in methanol/water/glacial acetic acid (50/40/10 parts by volume) or blocked with skimmed milk powder and subsequently incubated with ^{125}I-TNFα according to the filter test conditions described in Example 1 in order to detect bands having TNF-BP activity. This showed that all bands produced in the protein staining bonded TNFα specifically. In the Western blot according to Towbin et al. [38] all of these bands also bonded the monoclonal anti-55 kD-TNF-BP antibody produced according to Example 3. In this case, a procedure according to that described in Example 1 with Na^{125}I radioactively-labelled, affinity-purified (mouse immuno-globulin-Sepharose-4B affinity column) rabbit-anti-mouse-immunoglobulin antibody was used for the autoradiographic detection of this antibody.

Samples which had been obtained according to Example 4 by two-fold TNF-α-ligand affinity chromatography of the throughput of the immune affinity chromatography and which had been further separated by HPLC according to Example 5 showed under the above-specified SDS-PAGE and blot transfer conditions two additional bands of 75 kD and 65 kD, both of which bonded TNF specifically in the filter test (Example 1). In the Western blot according to Towbin et al. (see above) the proteins of these two bands did not react with the anti-(55 kD TNF-BP) antibody produced according to Example 3. They reacted, however, with a monoclonal antibody which had been produced starting from the 75 kD band (anti-75 kD TNF-BP antibody) according to Example 3.

Example 7

Amino Acid Sequence Analysis

For the amino acid sequence analysis, the fractions which had been obtained according to Example 5 and which were active according to the filter test (Example 1) were separated using the SDS-PAGE conditions described in Example 6, but now reducing (SDS sample buffer with 125 mM dithiothreitol). The same bands as in Example 6 were found, but because of the reducing conditions of the SDS-PAGE in comparison to Example 6 all showed an about 1-2 kD higher molecular weight. These bands were then transferred according to Example 6 on to PVDF membranes and stained with 0.15% Serva-Blue in methanol/water/glacial acetic acid (50/400/10 parts by volume) for 1 minute, decolorized with methanol/water/glacial acetic acid (45/48/7 parts by volume), rinsed with water, dried in air and thereafter cut out. The conditions given by Hunkapiller [34] were adhered to in all steps in order to avoid N-terminal blocking. Initially, the purified TNF-BP were used unaltered for the amino acid sequencing. In order to obtain additional sequence information, the TNF-BP after reduction and S-carboxymethylation [Jones, B. N. (1986) in "Methods of Protein Micro-characterisation", J. E. Shively, ed., Humana Press, Clifton N.J., 124-125] were cleaved with cyanogen bromide (Tarr, G. E. in "Methods of Protein Micro-characterisation", 165-166, loc. cit.), trypsin and/or protease K and the peptides were separated by HPLC according to known methods of protein chemistry. Thus-prepared samples were then sequenced in an automatic gas phase microsequencing apparatus (Applied Biosystems Model 470A, ABI, Foster City, Calif., USA) with an on-line automatic HPLC PTH amino acid analyzer (Applied Biosystems Model 120, ABI see above) connected to the outlet, whereby the following amino acid sequences were determined:

1. For the 55 kD band (according to non-reducing SDS-PAGE):

Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile (SEQ ID NO: 5),

and

Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys (SEQ ID NO: 6) in which X stands for an amino acid residue which could not be determined,

2. for the 51 kD and 38 kD bands (according to non-reducing SDS-PAGE):

Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu (SEQ ID NO: 15)

3. for the 6510 band (according to non-reducing SDS-PAGE)

In the N-terminal sequencing of the 6510 band two parallel sequences were determined up to the 15th residue without interruption. Since one of the two sequences corresponded to a partial sequence of ubiquitin [36,37], the following sequence was derived for the 65 kD band:

Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys. (SEQ ID NO: 10)

in which X stands for an amino acid residue which could not be determined.

Additional peptide sequences for 75(65)kDa-TNF-BP were determined:

Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu (SEQ ID NO: 11)

and

Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu (SEQ ID NO: 7) and Val-Phe-Cys-Thr (SEQ ID NO: 8)

and

Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala (SEQ ID NO: 9) and Leu-Cys-Ala-Pro (SEQ ID NO: 12)

and

Val-Pro-His-Leu-Pro-Ala-Asp (SEQ ID NO: 13)

and

Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro (SEQ ID NO: 14), in which X stands for an amino acid residue which could not be determined.

Example 8

Determination of Base Sequences of Complementary DNA (cDNA)

Starting from the amino acid sequence according to formula IA there were synthesized having regard to the genetic code for the amino acid residues 2-7 and 17-23 corresponding completely degenerated oligonucleotides in suitable complementarity ("sense" and "antisense" oligonucleotides). Total cellular RNA was isolated from HL60 cells [42,43] and the first cDNA strand was synthesized by oligo-dT priming or by priming with the "antisense" oligonucleotide using a cDNA synthesis kit (RPN 1256, Amersham, Amersham; England) according to the instructions of the manufacturer. This cDNA strand and the two synthesized degenerate "sense" and "antisense" oligonucleotides were used in a polymerase chain reaction (PCR, Perkin Elmer Cetus, Norwalk, Conn., USA

according to the instructions of the manufacturer) to synthesize as a cDNA fragment the base sequence coding for the amino acid residues 8-16 (formula IA). The base sequence of this cDNA fragment accorded to: 5'-AGGGAGAA-GAGAGATAGTGTGTGTCCC-3' (SEQ ID NO: 16). This cDNA fragment was used as a probe in order to identify according to a known procedure a cDNA clone coding for the 55 kD TNF-BP in a Xgt11-cDNA gene bank from human placenta (42, 43). This clone was then cut according to usual methods from the X-vector and cloned in the plasmids pUC18 (Pharmacia, Uppsala, Sweden) and pUC19 (Pharmacia, Uppsala, Sweden) and in the M13 mp 18/M13 mp 19 bacteriophage (Pharmacia, Uppsala, Sweden) (42, 43). The nucleotide sequence of this cDNA clone was determined using a Sequenase kit (U.S. Biochemical, Cleveland, Ohio, USA) according to the details of the manufacturer. The nucleotide sequence and the amino acid sequence derived therefrom for the 55 kD TNF-BP and its signal peptide (amino acid "−28" to amino acid "0") is given in FIG. **1** using the abbreviations for bases such as amino acids usual in the state of the art. From sequence comparisons with other already known receptor protein sequences there can be determined a N-terminal domain containing approximately 180 amino acids and a C-terminal domain containing 220 amino acids which are separated from one another by a transmembrane region of 19 amino acids (underlined in FIG. **1**) which is typical according to the sequence comparisons. Hypothetical glycosylation sites are characterized in FIG. **1** by asterisks above the corresponding amino acid.

Essentially analogous techniques were used to identify 75/65 kD TNF-BP-coding partial cDNA sequences, whereby however, in this case genomic human DNA and completely degenerated 14-mer and 15-mer "sense" and "antisense" oligonucleotides derived from peptide IIA were used in order to produce a primary 26 by cDNA probe in a polymerase chain reaction. This cDNA probe was then used in a HL-60 cDNA library to identify cDNA clones of different lengths. This cDNA library was produced using isolated HL60 RNA and a cDNA cloning kit (Amersham) according to the details of the manufacturer. The sequence of such a cDNA clone is given in FIG. **4**, whereby repeated sequencing lead to the following correction. A threonine coded by "ACC" not "TCC", has to be at position 3 instead of the serine.

Example 9

Expression in COS 1 Cells

Vectors starting from the plasmid "pN11" were constructed for the expression in COS cells. The plasmid "pN11" contains the efficient promoter and enhancer of the "major immediate-early" gene of human cytomegalovirus ("HCMV"; Boshart et al., Cell 41, 521-530, 1985). After the promoter there is situated a short DNA sequence which contains several restriction cleavage sites, which are present only once in the plasmid ("polylinker"), inter alia the cleavage sites for HindIII, BalI, BamHI and PvuII (see sequence).

PvuII
5'-AAGCTTGGCCAGGATCCAGCTGACT-GACTGATCGCGAGATC-3' (SEQ ID NO: 17)
3'-TTCGAACCGGTCCTAGGTCGACTGACT-GACTAGCGCTCTAG-5' (SEQ ID NO: 18)

After these cleavage sites there are situated three translation stop codons in all three reading frames. After the polylinker sequence there is situated the 2nd intron and the polyadenylation signal of the preproinsulin gene of the rat (Lomedico et al., Cell 18, 545-558, 1979). The plasmid also contains the replication origin of the SV40 virus and a fragment from pBR322 which confers *E. coli*-bacteria ampicillin resistance and permits the replication of the plasmid in *E. coli*.

For the construction of the expression vector "pN123", this plasmid "pN11" was cleaved the restriction endo-nuclease PvuII and subsequently treated with alkaline phosphatase. The dephosphorylated vector was thereafter isolated from an agarose gel (V1). The 5'-projecting nucleotides of the EcoRI-cleaved 1.3 kb fragment of the 55 kD TNF-BP-cDNA (see Example 8) were filled in using Klenow enzyme. Subsequently, this fragment was isolated from an agarose gel (F1). Thereafter, V1 and F1 were joined together using T4-ligase. *E. coli* HB101 cells were then transformed with this ligation batch according to known methods [42]. By means of restriction analyses and DNA sequencing according to known methods [42] there were identified transformants which had been transformed with a plasmid and which contained the 1.3 kb EcoRI fragment of the 55 kD TNF-BP-cDNA in the correct orientation for expression via the HCMV-promoter. This vector received the designation "pN123".

The following procedure was used for the construction of the vector "pK19". A DNA fragment which contained only the cDNA coding for the extracellular part of the 55 kD TNF-BP (amino acids −28 to 182 according to FIG. **1**) was obtained by PCR technology (Saiki et al., Science 230, 1350-1354, 1985, see also Example 8). The following oligonucleotides were used in order to amplify the cDNA from "pN123" coding for the extracellular part of the 55 kD TNF-BP:

BAMHI 5'-CACAGGGATCCATAGCTGTCTG-GCATGGGCCTCTCCAC-3' (SEQ ID NO: 19)
ASP718
3'-CGTGACTCCTGAGTCCGTGGTGTAT-TATCTCTAGACCA TGGCCC-5' (SEQ ID NO: 20)

By means of these oligonucleotides there were also introduced two stop codons of the translation after amino acid 182. The thus-amplified DNA fragment was cleaved with BamHI and Asp718, the thereby resulting projecting ends were filled in using Klenow enzyme and this fragment was subsequently isolated from an agarose gel (F2). F2 was then ligated with V1 and the entire batch was used for the transformation of *E. coli* HB101, as already described. Transformants which had been transformed with a plasmid containing the DNA fragment in the correct orientation for the expression via the HCMV-promoter were identified by DNA sequencing (see above). The plasmid isolated therefrom received the designation "pK19".

Transfection of the COS cells with the plasmids "pN123" or "pK19" was carried out according to the lipofection method published by Feigner et al. (Proc. Natl. Acad. Sci. USA 84, 7413-7417, 1987). 72 hours after the transfection had been effected the cells transfected with "pN123" were analyzed for binding with ^{125}I-TNFα according to known methods. The results of the Scatchard analysis [Scatchard, G., Ann. N.Y. Acad. Sci. 51, 660, 1949] of the thus-obtained binding data (FIG. **2**A) is given in FIG. **2**B. The culture supernatants of the cells transfected with "pK19" were investigated in a "sandwich" test. For this purpose, PVC microtitre plates (Dynatech, Arlington, Va., USA) were sensitized with 100 µl/well of a rabbit-anti-mouse immunoglobulin (10 µg/ml PBS). Subsequently, the plates were washed and incubated (3 hours, 20° C.) with an anti-55 kD TNF-BP antibody which had been detected by its antigen binding and isolated according to Example 3, but which did not inhibit the TNF-binding to cells. The plates were then again washed and incubated overnight at 4° C. with 100 µl/well of the culture supernatant (diluted 1:4 with buffer A containing 1% skimmed milk powder: 50 mM Tris/HCl pH 7.4, 140 mM

NaCl, 5 mM EDTA, 0.02% Na azide). The plates were emptied and incubated at 4° C. for 2 hours with buffer A containing ^{125}I-TNFα (10^6 cpm/ml, 100 μl/well) with or without the addition of 2 μg/ml of unlabelled TNF. Thereafter, the plates were washed 4 times with PBS, the individual wells were cut out and measured in a λ-counter. The results of 5 parallel transfections (columns # 2, 3, 4, 6 and 7), of two control transfections
with the pN11 vector (columns # 1, 5) and of a control with HL60 cell lysate (column # 8) are given in FIG. **3**.

Example 10

Expression in Insect Cells

The plasmid "pVL941" (Luckow and Summers, 1989, "High Level Expression of Nonfused Foreign Genes with *Autographa california* Nuclear Polyhedrosis virus Expression Vectors", Virology 170, 31-39) was used for the expression in a baculovirus expression system and was modified as follows. The single EcoRI restriction cleavage site in "pVL941" was removed by cleaving the plasmid with EcoRI and the projecting 5'-end was filled in with Klenow enzyme. The plasmid pVL941/E obtained therefrom was digested with BamHI and Asp718 and the vector trunk was subsequently isolated from an agarose gel. This fragment was ligated with a synthetic oligonucleotide of the following sequence:

BamHI EcoRI Asp718
5'-GATCCAGAATTCATAATAG-3' (SEQ ID NO: 21)
3'-GTCTTAAGTATTATCCATG-5' (SEQ ID NO: 22)

E. coli HB101 was transformed with the ligation batch and transformants containing a plasmid in which the oligonucleotide had been incorporated correctly were identified by restriction analysis and DNA sequencing according to known methods (see above); this plasmid was named "pNR704". For the construction of the transfer vector "pN113", this plasmid "pNR704" was cleaved with EcoRI, treated with alkaline phosphatase and the thus-1-produced vector trunk (V2) was subsequently isolated from an agarose gel. The 1.3 kb fragment of the 55 kD TNF-BP-cDNA cleaved with EcoRI as above was ligated with fragment V2. Transformants obtained with this ligation batch, which contained a plasmid containing the cDNA insert in the correct orientation for the expression via the polyhedron promoter, were identified (see above). The vector isolated therefrom received the designation "pN113".

The following procedure was used for the construction of the transfer vector "pN119". The 1.3 kb EcoRI/EcoRI fragment of the 55 kD TNF-BP cDNA in the "pUC19" plasmid (see Example 8) was digested with BanI and ligated with the following synthetic oligonucleotide:

BanI Asp718
5'-GCACCACATAATAGAGATCTGGTACCGGGAA-3' (SEQ ID NO: 23)
3'-GTGTATTATCTCTAGACCATGGCCC-5' (SEQ ID NO: 24)

Two stop codons of the translation after amino acid 182 and a cleavage site for the restriction endo-nuclease Asp718 are incorporated with the above adaptor. After carrying out ligation the batch was digested with EcoRI and Asp718 and the partial 55 kD TNF-BP fragment (F3) was isolated. Furthermore, the plasmid "pNR704", likewise cleaved with Asp718 and EcoRI, was ligated with F3 and the ligation batch was transformed into *E. coli HB*101. The identification of the transformants which contained a plasmid in which the partial 55 kD TNF-BP cDNA had been correctly integrated for the expression was effected as already described. The plasmid isolated from these transformants received the name "pN119".

The following procedure was used for the construction of the transfer vector "pN124". The cDNA fragment coding for the extracellular part of the 55 kD TNF-BP, described in Example 9, was amplified with the specified oligo-nucleotides with the aid of PCR technology as described in Example 9. This fragment was cleaved with BamHI and Asp718 and isolated from an agarose gel (F4). The plasmid "pNR704" was also cleaved with BamHI and Asp718 and the vector trunk (V4) was isolated (see above). The fragments V4 and F4 were ligated, *E. coli* HB101 was transformed therewith and the recombinant transfer vector "pN124" was identified and isolated as described.

The following procedure was used for the transfection 10 of the insect cells. 3 μg of the transfer vector "pN113" were transfected with 1 μg of DNA of the *Autographa californica* nuclear polyhedrosisvirus (AcMNPV) (EP 127839) in Sf9 cells (ATCC CRL 1711). Polyhedron-negative viruses were identified and purified from "plaques" [52]. Sf9 cells were again infected with these recombinant viruses as described in [52]. After 3 days in the culture the infected cells were investigated for TNF-binding using ^{125}I-TNFα. For this purpose, the transfected cells were washed from the cell culture dish with a Pasteur pipette and resuspended at a cell density of 5×10^6 cells/ml of culture medium [52] which contained 10 ng/ml of ^{125}I-TNF-α, not only in the presence of, but also in the absence of 5 μg/ml of non-labelled TNF-α and incubated on ice for 2 hours. Thereafter, the cells were washed with pure culture medium and the cell-bound radio-activity was counted in a γ-counter (see Table 2).

TABLE 2

Cells	Cell-bound radioactivity per 10^6 cells
Non-infected cells (control)	60 cpm
Infected cells	1600 ± 330 cpm [1)]

[1)] Average and standard deviation from 4 experiments

Example 11

Analogously to the procedure described in Example 9, the cDNA fragment coding for the extracellular region of the 55 kDa TNF-BP was amplified in a polymerase chain reaction, but now using the following oligonucleotides as the primer:
Oligonucleotide 1:
Sst I 5'-TAC GAG CTC GGC CAT AGC TGT CTG GCA TG-3' (SEQ ID NO: 25)
Oligonucleotide 2:
Sst I 5'-ATA GAG CTC TGT GGT GCC TGA GTC CTC AG-3' (SEQ ID NO: 26)

This cDNA fragment was ligated in the pCD4-Hγ3 vector [DSM 5523; European Patent Application No. 90107393.2; Japanese Patent Application No. 108967/90; U.S. Pat. No. 51,077,390] from which the CD4-cDNA had been removed via the SstI restriction cleavage sites. SstI cleavage sites are situated in vector pCD4-Hγ3 not only in front of, but also behind the CD4-partial sequence fragment. The construction was transfixed in J558 myeloma cells (ATCC No. TIB6) by means of protoplast fusion according to Oi et al. (Procd. Natl. Acad. Sci. USA 80, 825-829, 1983). Transfectants were selected by adding 5 μg/ml of mycophenolic acid and 250 g/ml of xanthin (Traunecker et al., Eur. J. Immunol. 16, 851-

854 [1986]) in basic medium (Dulbecco's modified Eagle's Medium, 10% foetal calf serum, 5×10^{-5}M 2-mercaptoethanol). The expression product secreted by the transfixed cells could be purified using usual methods of protein chemistry, e.g. TNF-BP-antibody affinity chromatography. Unless not already specifically indicated, standard procedures as described e.g. by Freshney, R. I. in "Culture of Animal Cells", Alan R. Liss, Inc., New York (1983) were used for the cultivation of the cell lines employed, for the cloning, for the selection or for the expansion of the cloned cells.

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tctggcatgg gcctctccac cgtgcctgac ctgctgctgc cgctggtgct cctggagctg 240

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aacctttcc agtgcttcaa ttgcagcctc tgcctcaatg ggaccgtgca cctctcctgc 660

caggagaaac agaacaccgt gtgcacctgc catgcaggtt tctttctaag agaaaacgag 720

tgtgtctcct gtagtaactg taagaaaagc ctggagtgca cgaagttgtg cctaccccag 780

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ttctttggtc tttgcctttt atcctcctc ttcattggtt taatgtatcg ctaccaacgg 900

tggaagtcca agctctactc cattgtttgt gggaaatcga cacctgaaaa agaggggggag 960

cttgaaggaa ctactactaa gcccctggcc ccaaacccaa gcttcagtcc cactccaggc 1020

ttcaccccca ccctgggctt cagtcccgtg cccagttcca ccttcacctc cagctccacc 1080

tatacccccg gtgactgtcc caactttgcg gctccccgca gagaggtggc accaccctat 1140

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cacaagtggg aggacagcgc ccacaagcca cagagcctag acactgatga ccccgcgacg 1260

ctgtacgccg tggtggagaa cgtgcccccg ttgcgctgga aggaattcgt gcggcgccta 1320

gggctgagcg accacgagat cgatcggctg gagctgcaga acgggcgctg cctgcgcgag 1380

gcgcaataca gcatgctggc gacctggagg cggcgcacgc cgcggcgcga ggccacgctg 1440

gagctgctgg gacgcgtgct ccgcgacatg gacctgctgg gctgcctgga ggacatcgag 1500

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acgctatgcc tcatgcccgt tttgggtgtc ctcaccagca aggctgctcg ggggcccctg 1860

gttcgtccct gagccttttt cacagtgcat aagcagtttt ttttgttttt gttttgtttt 1920

gttttgtttt taaatcaatc atgttacact aatagaaact tggcactcct gtgccctctg 1980

cctggacaag cacatagcaa gctgaactgt cctaaggcag gggcgagcac ggaacaatgg 2040

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ggccttcagc tggagctgtg gacttttgta catacactaa aattctgaag ttaaaaaaaa 2100

aacccgaatt c 2111

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<211> LENGTH: 455
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

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Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu Val Pro
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His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys
35 40 45

Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys
50 55 60

Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp
65 70 75 80

Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu
85 90 95

Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val
100 105 110

Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg
115 120 125

Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe
130 135 140

Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu
145 150 155 160

Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu
165 170 175

Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr
180 185 190

Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser
195 200 205

Gly Thr Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu
210 215 220

Leu Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
225 230 235 240

Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu
245 250 255

Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn Pro Ser
260 265 270

Phe Ser Pro Thr Pro Gly Phe Thr Pro Thr Leu Gly Phe Ser Pro Val
275 280 285

Pro Ser Ser Thr Phe Thr Ser Ser Ser Thr Tyr Thr Pro Gly Asp Cys
290 295 300

Pro Asn Phe Ala Ala Pro Arg Arg Glu Val Ala Pro Pro Tyr Gln Gly
305 310 315 320

Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala Ser Asp Pro Ile Pro Asn
325 330 335

Pro Leu Gln Lys Trp Glu Asp Ser Ala His Lys Pro Gln Ser Leu Asp
340 345 350

Thr Asp Asp Pro Ala Thr Leu Tyr Ala Val Val Glu Asn Val Pro Pro
355 360 365

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Leu Arg Trp Lys Glu Phe Val Arg Arg Leu Gly Leu Ser Asp His Glu
    370                 375                 380

Ile Asp Arg Leu Glu Leu Gln Asn Gly Arg Cys Leu Arg Glu Ala Gln
385                 390                 395                 400

Tyr Ser Met Leu Ala Thr Trp Arg Arg Arg Thr Pro Arg Arg Glu Ala
                405                 410                 415

Thr Leu Glu Leu Leu Gly Arg Val Leu Arg Asp Met Asp Leu Leu Gly
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Cys Leu Glu Asp Ile Glu Glu Ala Leu Cys Gly Pro Ala Ala Leu Pro
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Pro Ala Pro Ser Leu Leu Arg
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<210> SEQ ID NO 3
<211> LENGTH: 2339
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

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actcgggaac agaaccgcat ctgcacctgc aggcccggct ggtactgcgc gctgagcaag    180

caggaggggt gccggctgtg cgcgccgctg ccgaagtgcc gcccgggctt cggcgtggcc    240

agaccaggaa ctgaaacatc agacgtggtg tgcaagccct gtgccccggg gacgttctcc    300

aacacgactt catccacgga tatttgcagg ccccaccaga tctgtaacgt ggtggccatc    360

cctgggaatg caagcaggga tgcagtctgc acgtccacgt cccccacccg gagtatggcc    420

ccaggggcag tacacttacc ccagccagtg tccacacgat cccaacacac gcagccaagt    480

ccagaaccca gcactgctcc aagcacctcc ttcctgctcc caatgggccc cagccccccа    540

gctgaaggga gcactggcga cttcgctctt ccagttggac tgattgtggg tgtgacagcc    600

ttgggtctac taataatagg agtggtgaac tgtgtcatca tgacccaggt gaaaaagaag    660

cccttgtgcc tgcagagaga agccaaggtg cctcacttgc ctgccgataa ggcccggggt    720

acacagggcc ccgagcagca gcacctgctg atcacagcgc cgagctccag cagcagctcc    780

ctggagagct cggccagtgc gttggacaga agggcgccca ctcggaacca gccacaggca    840

ccaggcgtgg aggccagtgg ggccggggag gcccgggcca gcaccgggag ctcagcagat    900

tcttcccctg gtggccatgg gacccaggtc aatgtcacct gcatcgtgaa cgtctgtagc    960

agctctgacc acagctcaca gtgctcctcc caagccagct ccacaatggg agacacagat   1020

tccagcccct cggagtcccc gaaggacgag caggtcccct tctccaagga ggaatgtgcc   1080

tttcggtcac agctggagac gccagagacc ctgctgggga gcaccgaaga gaagcccctg   1140

ccccttggag tgcctgatgc tgggatgaag cccagttaac caggccggtg tgggctgtgt   1200

cgtagccaag gtggctgagc cctggcagga tgaccctgcg aaggggccct ggtccttcca   1260

ggcccccacc actaggactc tgaggctctt tctgggccaa gttcctctag tgccctccac   1320

agccgcagcc tccctctgac ctgcaggcca agagcagagg cagcgagttg tggaaagcct   1380

ctgctgccat ggcgtgtccc tctcggaagg ctggctgggc atggacgttc ggggcatgct   1440

ggggcaagtc cctgagtctc tgtgacctgc cccgcccagc tgcacctgcc agcctggctt   1500

ctggagccct tgggtttttt gtttgtttgt ttgtttgttt gtttgtttct ccccctgggc   1560

tctgcccagc tctggcttcc agaaaacccc agcatccttt tctgcagagg ggctttctgg   1620
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agaggaggga tgctgcctga gtcacccatg aagacaggac agtgcttcag cctgaggctg   1680

agactgcggg atggtcctgg ggctctgtgc agggaggagg tggcagccct gtagggaacg   1740

gggtccttca agttagctca ggaggcttgg aaagcatcac ctcaggccag gtgcagtggc   1800

tcacgcctat gatcccagca ctttgggagg ctgaggcggg tggatcacct gaggttagga   1860

gttcgagacc agcctggcca acatggtaaa accccatctc tactaaaaat acagaaatta   1920

gccgggcgtg gtggcgggca cctatagtcc cagctactca gaagcctgag gctgggaaat   1980

cgtttgaacc cgggaagcgg aggttgcagg gagccgagat cacgccactg cactccagcc   2040

tgggcgacag agcgagagtc tgtctcaaaa gaaaaaaaaa aagcaccgcc tccaaatgct   2100

aacttgtcct tttgtaccat ggtgtgaaag tcagatgccc agagggccca ggcaggccac   2160

catattcagt gctgtggcct gggcaagata acgcacttct aactagaaat ctgccaattt   2220

tttaaaaaag taagtaccac tcaggccaac aagccaacga caaagccaaa ctctgccagc   2280

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<210> SEQ ID NO 4
<211> LENGTH: 392
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

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Trp Asn Trp Val Pro Glu Cys Leu Ser Cys Gly Ser Arg Cys Ser Ser
            20                  25                  30

Asp Gln Val Glu Thr Gln Ala Cys Thr Arg Glu Gln Asn Arg Ile Cys
        35                  40                  45

Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu Ser Lys Gln Glu Gly Cys
    50                  55                  60

Arg Leu Cys Ala Pro Leu Pro Lys Cys Arg Pro Gly Phe Gly Val Ala
65                  70                  75                  80

Arg Pro Gly Thr Glu Thr Ser Asp Val Val Cys Lys Pro Cys Ala Pro
                85                  90                  95

Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr Asp Ile Cys Arg Pro His
            100                 105                 110

Gln Ile Cys Asn Val Val Ala Ile Pro Gly Asn Ala Ser Arg Asp Ala
        115                 120                 125

Val Cys Thr Ser Thr Ser Pro Thr Arg Ser Met Ala Pro Gly Ala Val
    130                 135                 140

His Leu Pro Gln Pro Val Ser Thr Arg Ser Gln His Thr Gln Pro Ser
145                 150                 155                 160

Pro Glu Pro Ser Thr Ala Pro Ser Thr Ser Phe Leu Leu Pro Met Gly
                165                 170                 175

Pro Ser Pro Pro Ala Glu Gly Ser Thr Gly Asp Phe Ala Leu Pro Val
            180                 185                 190

Gly Leu Ile Val Gly Val Thr Ala Leu Gly Leu Leu Ile Ile Gly Val
        195                 200                 205

Val Asn Cys Val Ile Met Thr Gln Val Lys Lys Lys Pro Leu Cys Leu
    210                 215                 220

Gln Arg Glu Ala Lys Val Pro His Leu Pro Ala Asp Lys Ala Arg Gly
225                 230                 235                 240

Thr Gln Gly Pro Glu Gln Gln His Leu Leu Ile Thr Ala Pro Ser Ser
                245                 250                 255
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Ser Ser Ser Ser Leu Glu Ser Ser Ala Ser Ala Leu Asp Arg Arg Ala
            260                 265                 270

Pro Thr Arg Asn Gln Pro Gln Ala Pro Gly Val Glu Ala Ser Gly Ala
        275                 280                 285

Gly Glu Ala Arg Ala Ser Thr Gly Ser Ser Ala Asp Ser Ser Pro Gly
    290                 295                 300

Gly His Gly Thr Gln Val Asn Val Thr Cys Ile Val Asn Val Cys Ser
305                 310                 315                 320

Ser Ser Asp His Ser Ser Gln Cys Ser Ser Gln Ala Ser Ser Thr Met
                325                 330                 335

Gly Asp Thr Asp Ser Ser Pro Ser Glu Ser Pro Lys Asp Glu Gln Val
            340                 345                 350

Pro Phe Ser Lys Glu Glu Cys Ala Phe Arg Ser Gln Leu Glu Thr Pro
        355                 360                 365

Glu Thr Leu Leu Gly Ser Thr Glu Glu Lys Pro Leu Pro Leu Gly Val
    370                 375                 380

Pro Asp Ala Gly Met Lys Pro Ser
385                 390


<210> SEQ ID NO 5
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (25)..(25)
<223> OTHER INFORMATION: Xaa = unknown amino acid

<400> SEQUENCE: 5

Leu Val Pro His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro
1               5                   10                  15

Gln Gly Lys Tyr Ile His Pro Gln Xaa Asn Ser Ile
            20                  25


<210> SEQ ID NO 6
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 6

Ser Thr Pro Glu Lys Glu Gly Glu Leu Glu Gly Thr Thr Thr Lys
1               5                   10                  15


<210> SEQ ID NO 7
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 7

Ser Gln Leu Glu Thr Pro Glu Thr Leu Leu Gly Ser Thr Glu Glu Lys
1               5                   10                  15

Pro Leu


<210> SEQ ID NO 8
<211> LENGTH: 4
<212> TYPE: PRT
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<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 8

Val Phe Cys Thr
1

<210> SEQ ID NO 9
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 9

Asn Gln Pro Gln Ala Pro Gly Val Glu Ala Ser Gly Ala Gly Glu Ala
1 5 10 15

<210> SEQ ID NO 10
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Xaa = unknown amino acid

<400> SEQUENCE: 10

Leu Pro Ala Gln Val Ala Phe Xaa Pro Tyr Ala Pro Glu Pro Gly Ser
1 5 10 15

Thr Cys

<210> SEQ ID NO 11
<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Xaa = unknown amino acid

<400> SEQUENCE: 11

Ile Xaa Pro Gly Phe Gly Val Ala Tyr Pro Ala Leu Glu
1 5 10

<210> SEQ ID NO 12
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 12

Leu Cys Ala Pro
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<210> SEQ ID NO 13
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 13

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Val Pro His Leu Pro Ala Asp
1 5

<210> SEQ ID NO 14
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
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<222> LOCATION: (9)..(10)
<223> OTHER INFORMATION: Xaa = unknown amino acid
<220> FEATURE:
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<222> LOCATION: (13)..(13)
<223> OTHER INFORMATION: Xaa = unknown amino acid

<400> SEQUENCE: 14

Gly Ser Gln Gly Pro Glu Gln Gln Xaa Xaa Leu Ile Xaa Ala Pro
1 5 10 15

<210> SEQ ID NO 15
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 15

Leu Val Pro His Leu Gly Asp Arg Glu
1 5

<210> SEQ ID NO 16
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 16

agggagaaga gagatagtgt gtgtccc 27

<210> SEQ ID NO 17
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 17

aagcttggcc aggatccagc tgactgactg atcgcgagat c 41

<210> SEQ ID NO 18
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense primer

<400> SEQUENCE: 18

gatctcgcga tcagtcagtc agctggatcc tggccaagct t 41

<210> SEQ ID NO 19
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:

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<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 19

cacagggatc catagctgtc tggcatgggc ctctccac 38

<210> SEQ ID NO 20
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense primer

<400> SEQUENCE: 20

cccggtacca gatctctatt atgtggtgcc tgagtcctca gtgc 44

<210> SEQ ID NO 21
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 21

gatccagaat tcataatag 19

<210> SEQ ID NO 22
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense primer

<400> SEQUENCE: 22

gtacctatta tgaattctg 19

<210> SEQ ID NO 23
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 23

gcaccacata tagagatct ggtaccggga a 31

<210> SEQ ID NO 24
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense primer

<400> SEQUENCE: 24

cccggtacca gatctctatt atgtg 25

<210> SEQ ID NO 25
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 25

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<210> SEQ ID NO 26

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<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 26

atagagctct gtggtgcctg agtcctcag 29

The invention claimed is:

1. A protein comprising

(a) a human tumor necrosis factor (TNF)-binding soluble fragment of an insoluble human TNF receptor, wherein the insoluble human TNF receptor (i) specifically binds human TNF, (ii) has an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel, and (iii) comprises the amino acid sequence LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10); and

(b) all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain of said constant region;

wherein said protein specifically binds human TNF.

2. The protein of claim 1, wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO: 12) and VFCT (SEQ ID NO: 8).

3. The protein of claim 2, wherein the soluble fragment further comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).

4. The protein of claim 1, wherein said human immunoglobulin IgG heavy chain is IgG_1.

5. The protein of claim 4, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by pCD4-Hγ1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5314).

6. A pharmaceutical composition comprising the protein of claim 4 and a pharmaceutically acceptable carrier material.

7. The protein of claim 1, wherein the protein is purified.

8. The protein of claim 1, wherein the protein is produced by CHO cells.

9. The protein of claim 1, wherein the protein consists of (a) the soluble fragment of the receptor and (b) all of the domains of the constant region of the human immunoglobulin IgG heavy chain other than the first domain of the constant region.

10. The protein of claim 1, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by pCD4-Hγ1 vector (deposited at Deutschen Sammlung von Mikroorganismen and Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5314) or by pCD4-Hγ3 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5523).

11. The protein of claim 1, wherein the protein consists essentially of the extracellular region of the insoluble human TNF receptor and all the domains of the constant region of a human IgG_1 immunoglobulin heavy chain other than the first domain of the constant region.

12. A pharmaceutical composition comprising the protein of claim 11 and a pharmaceutically acceptable carrier material.

13. A protein comprising

(a) a human tumor necrosis factor (TNF)-binding soluble fragment of an insoluble human TNF receptor, wherein the insoluble human TNF receptor (i) specifically binds human TNF, (ii) has an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel, and (iii) comprises the amino acid sequences LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10), LCAP (SEQ ID NO: 12), VFCT (SEQ ID NO: 8), NQPQAPGVEASGAGEA (SEQ ID NO: 9) and VPHLPAD (SEQ ID NO: 13),

wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO: 12) and VFCT (SEQ ID NO: 8); and

(b) all of the domains of the constant region of a human IgG_1 heavy chain other than the first domain of the constant region;

wherein said protein specifically binds human TNF.

14. The protein of claim 13, wherein the protein is purified.

15. The protein of claim 13, wherein the protein is produced by CHO cells.

16. The protein of claim 13, wherein the protein consists of (a) the soluble fragment of the receptor and (b) all of the domains of the constant region of the human IgG_1 heavy chain other than the first domain of the constant region.

17. The protein of claim 13, wherein the soluble fragment further comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).

18. A protein encoded by a polynucleotide which comprises two nucleic acid subsequences,

(a) one of said subsequences encoding a human TNF-binding soluble fragment of an insoluble human TNF receptor protein having an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel, said soluble fragment comprising the amino acid sequence LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10), and

(b) the other of said subsequences encoding all of the domains of the constant region of the heavy chain of a human IgG immunoglobulin other than the first domain of said constant region,

wherein said protein specifically binds human TNF.

19. The protein of claim 18, wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO: 12) and VFCT (SEQ ID NO: 8).

20. The protein of any one of claim 18 or 19, wherein said human immunoglobulin heavy chain is IgG_1.

21. The protein of claim 20, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by the DNA insert of pCD4-Hγ1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5314).

22. The protein of claim **18**, wherein the protein is purified.

23. The protein of claim **18**, wherein the protein is produced by CHO cells.

24. The protein of claim **18**, wherein the protein consists of (a) the soluble fragment of the receptor and (b) all of the domains of the constant region of the human IgG_1 heavy chain other than the first domain of the constant region.

25. The protein of claim **18**, wherein said domains of the constant region of the human immunoglobulin heavy chain consist essentially of the immunoglobulin amino acid sequence encoded by pCD4-Hγ1 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5314) or by pCD4-Hγ3 vector (deposited at Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG under No. DSM 5523).

26. A protein consisting of

(a) a human tumor necrosis factor (TNF)-binding soluble fragment of an insoluble human TNF receptor, wherein the insoluble human TNF receptor (i) specifically binds human TNF, and (ii) has an apparent molecular weight of about 75 kilodaltons on a non-reducing SDS-polyacrylamide gel and (iii) comprises the amino acid sequence LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10),

wherein the soluble fragment comprises the peptides LCAP (SEQ ID NO:12) and VFCT (SEQ ID NO:8), and

(b) all of the domains of the constant region of a human IgG_1 heavy chain other than the first domain of the constant region,

wherein the protein specifically binds human TNF, and

wherein the protein is produced by CHO cells.

27. The protein of claim **26**, wherein the soluble fragment comprises the peptide LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10).

28. The protein of claim **26**, wherein the protein is purified.

29. A pharmaceutical composition comprising the protein of any of claim **1**, **18**, **26**, or **27** and a pharmaceutically acceptable carrier material.

30. A protein comprising

(a) human tumor necrosis factor (TNF) binding soluble fragment of the amino acid sequence encoded by the cDNA insert of the plasmid deposited with the ATCC on Oct. 17, 2006 under accession number PTA 7942,

(b) all of the domains of the constant region of a human immunoglobulin IgG heavy chain other than the first domain of said constant region;

wherein said protein specifically binds human TNF.

31. The protein of claim **30**, consisting of the soluble fragment and all the domains of the constant region of the human immunoglobulin IgG heavy chain other than the first domain of said constant region.

32. The protein of claim **30**, wherein the protein is expressed by a mammalian host cell.

33. The protein of claim **32**, wherein the mammalian host cell is a CHO cell.

34. The protein of claim **32**, consisting of the soluble fragment and all the domains of the constant region of the human immunoglobulin IgG heavy chain other than the first domain of said constant region.

35. The protein of claim **30**, wherein the protein consists essentially of the extracellular region of the human tumor necrosis factor (TNF) receptor amino acid sequence encoded by the cDNA insert, and all the domains of the constant region of a human IgG_1 immunoglobulin heavy chain other than the first domain of the constant region.

36. A pharmaceutical composition comprising the protein of claim **35** and a pharmaceutically acceptable carrier material.

* * * * *

Exhibit D

US008163522B1

(12) **United States Patent**
Brockhaus et al.

(10) **Patent No.: US 8,163,522 B1**
(45) **Date of Patent: Apr. 24, 2012**

(54) **HUMAN TNF RECEPTOR**

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(57) **ABSTRACT**

The present invention is concerned with non-soluble proteins and soluble or insoluble fragments thereof, which bind TNF, in homogeneous form, as well as their physiologically compatible salts, especially those proteins having a molecular weight of about 55 or 75 kD (non-reducing SDS-PAGE conditions), a process for the isolation of such proteins, antibodies against such proteins, DNA sequences which code for non-soluble proteins and soluble or non-soluble fragments thereof, which bind TNF, as well as those which code for proteins comprising partly of a soluble fragment, which binds TNF, and partly of all domains except the first of the constant region of the heavy chain of human immunoglobulins and the recombinant proteins coded thereby as well as a process for their manufacture using transformed pro- and eukaryotic host cells.

10 Claims, 16 Drawing Sheets

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Exhibit A: Memorandum by D. Urdal to S. Gillis, M. Kranda, and P. Grassam, dated Oct. 27, 1989.
Exhibit B: Correspondence from D. Urdal to L. Lauffer dated Feb. 26, 1990.
Exhibit C: Lab Notebook of E. Jeffrey, pages dated May 1990 through Jan. 1991.
Exhibit D: Correspondence from L. Lauffer to D. Urdal, dated May 21, 1990.
Exhibit E: Meeting minutes, Immunex employee (author unknown) to file, dated Jun. 25, 1990.
Exhibit F: Lab notebook of Terri Davis. pages dated Jul. 11, 1990.
Exhibit G: Letter from M. Deeley to L. Lauffer, dated Jul. 20, 1990.
Exhibit H: Meeting minutes, Immunex employee (author unknown) to file, dated Jul. 23, 1990.
Exhibit I: Correspondence from Drs. Seiler and Zeittmeissl to D. Gillis, dated Aug. 8, 1990.
Exhibit J (J1-J21): Declaration of Bruce A. Beutler, Karsten Peppel, and David F. Crawford submitted to the USPTO on Jul. 16, 1993 during the prosecution of U.S. Appl. No. 07/862,495, filed Apr. 2. 1992 (issued as US 5,447.851 naming inventors B. Beutler, K. Peppel, and D. Crawford), including exhibits J-1-J21, which were submitted with the declaration.
Exhibit K: Confirmation page from D. Urdal to P. Oquendo, dated Oct. 4, 1990.
Letter from J. Thomas to L. Lauffer dated Dec. 10, 1990.
Memo from J. Thomas to P. Baum, D. Cosman, M. Deeley, R. Goodwin, S. Gillis, H. Sassenfeld, and D. Urdal, dated Dec. 17, 1990, conveying attached facsimile received Dec. 13, 1990 from L. Lauffer to J. Thomas.
Declaration of Taruna Arora under 37 C.F.R. § 1.132 plus Exhibits A-D dated Dec. 16, 2010, filed in sister case U.S. Appl. No. 08/444,790 (which was filed on May 19, 1995, inventors M. Brockhaus, Z. Dembic, R. Gentz, W. Lesslauer, H. Loetscher, E. Schlaeger, hereinafter "U.S. Appl. No. 08/444,790").
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FIGURE 1A

Case 2:16-cv-01118-CCC-MF Document 18 Filed 02/29/16 Page 89 of 180 PageID: 575

FIGURE 1B

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                  ***                 ***.
110 AsnLeuPheGlnCysPheAsnCysSerLeuCysLeuAsnGlyThrValHisLeuSerCys
415 AACCTTTTCCAGTGCTTCAATTGCAGCCTCTGCCTCAATGGGACCGTGCACCTCTCCTGC

130 GlnGluLysGlnAsnThrValCysThrCysHisAlaGlyPhePheLeuArgGluAsnGlu
475 CAGGAGAAACAGAACACCGTGTGCACCTGCCATGCAGGTTTCTTTCTAAGAGAAAACGAG

150 CysValSerCysSerAsnCysLysLysSerLeuGluCysThrLysLeuCysLeuProGln
535 TGTGTCTCCTGTAGTAACTGTAAGAAAAGCCTGGAGTGCACGAAGTTGTGCCTACCCCAG

170 IleGluAsnValLysGlyThrGluAspSerGlyThrThrValLeuLeuProLeuValIle
595 ATTGAGAATGTTAAGGGCACTGAGGACTCAGGCACCACAGTGCTGTTGCCCCTGGTCATT

190 PhePheGlyLeuCysLeuLeuSerLeuLeuPheIleGlyLeuMetTyrArgTyrGlnArg
655 TTCTTTGGTCTTTGCCTTTTATCCCTCCTCTTCATTGGTTTAATGTATCGCTACCAACGG

210 TrpLysSerLeuTyrSerIleValCysGlyLysSerThrProGluLysGluGlyGlu
715 TGGAAGTCCAAGCTCTACTCCATTGTTTGTGGGAAATCGACACCTGAAAAAGACGGGGAG

                  ***
230 LeuGluGlyThrThrThrLysProLeuAlaProAsnProSerPheSerProThrProGly
775 CTTGAAGGAACTACTACTAAGCCCCTGGCCCCAAACCCAAGCTTCAGTCCCACTCCAGGC
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Case 2:16-cv-01118-CCC-MF Document 18 Filed 02/29/16 Page 91 of 180 PageID: 577

FIGURE 1C

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                .         .         .         .         .         .
 250  PheThrProThrLeuGlyPheSerProValProSerSerThrPheThrSerSerSerThr
 835  TTCACCCCCACCCTGGGCTTCAGTCCCGTGCCCAGTTCCACCTTCACCTCCAGCTCCACC

                .         .         .         .         .         .
 270  TyrThrProGlyAspCysProAsnPheAlaAlaProArgArgGluValAlaProProTyr
 895  TATACCCCCGGTGACTGTCCCAACTTTGCGGCTCCCCGCAGAGAGGTGGCACCACCCTAT

                .         .         .         .         .         .
 290  GlnGlyAlaAspProIleLeuAlaThrAlaLeuAlaSerAspProIleProAsnProLeu
 955  CAGGGGGCTGACCCCATCCTTGCGACAGCCCTCGCCTCCGACCCCATCCCCAACCCCCTT

                .         .         .         .         .         .
 310  GlnLysTrpGluAspSerAlaHisLysProGlnSerLeuAspThrAspAspProAlaThr
1015  CAGAAGTGGGAGGACAGCGCCCACAAGCCACAGAGCCTAGACACTGATGACCCCGCGACG

                .         .         .         .         .         .
 330  LeuTyrAlaValValGluAsnValProProLeuArgTrpLysGluPheValArgArgLeu
1075  CTGTACGCCGTGGTGGAGAACGTGCCCCCGTTGCGCTGGAAGGAATTCGTGCGGCGCCTA

                .         .         .         .         .         .
 350  GlyLeuSerAspHisGluIleAspArgLeuGluLeuGlnAsnGlyArgCysLeuArgGlu
1135  GGGCTGAGCGACCACGAGATCGATCGGCTGGAGCTGCAGAACGGGCGCTGCCTGCGCGAG

                .         .         .         .         .         .
 370  AlaGlnTyrSerMetLeuAlaThrTrpArgArgArgThrProArgArgGluAlaThrLeu
1195  GCGCAATACAGCATGCTGGCGACCTGGAGGCGGCGCACGCCGCGGCGCGAGGCCACGCTG

                .         .         .         .         .         .
 390  GluLeuLeuGlyArgValLeuArgAspMetAspLeuLeuGlyCysLeuGluAspIleGlu
1255  GAGCTGCTGGGACGCGTGCTCCGCGACATGGACCTGCTGGGCTGCCTGGAGGACATCGAG
```

FIGURE 1D

```
                .         .         .         .         .
 410 GluAlaLeuCysGlyProAlaAlaLeuProProAlaProSerLeuLeuArg
1315 GAGGCGCTTGCGGCCCCGCCCCTCCCGCCCGCCCAGTCTTCTCAGATGAGGCTGC
1375 GCCCCTGCGGGCAGCTCTAAGGACCGTCCTGCGAGATCGCCTTCCAACCCCACTTTTTTC
1435 TGGAAAGGAGGGGGTCCTGCAGGGGCAAGCAGGAGCTAGCAGCCGCCTACTTGGTGCTAAC
1495 CCCTCGATGTACATAGCTTTTCTCAGCTGCCTGCGCGCCGCCGACAGTCAGCGCTGTGCG
1555 CGCGGAGAGAGGTGCGCCGTGGGCTCAAGAGCCCTGAGTGGGTGGTTTGCGAGGATGAGGG
1615 ACGCTATGCCTCATGCCCGTTTTGGGTGTCCTCACCAGCAAGGCTGCTCGGGGGCCCCCTG
1675 GTTCGTCCCTGAGCCTTTTTTCACAGTGCATAAGCAGTTTTTTTTTGTTTTTGTTTT
1735 GTTTTGTTTTTTAAATCAATCATGTTACACTAATAGAAACTTGGCACTCCTGTGCCCTCTG
1795 CCTGGACAAGCACATAGCAAGCTGAACTGTCCTAAGGCAGGGGCGAGCACGGAACAATGG
1855 GGCCTTCAGCTGGAGCTGTGGACTTTTGTACATACACTAAAATTCTGAAGTTAAAAAAAA
1915 AACCCGAATTC
```

Figure 2A

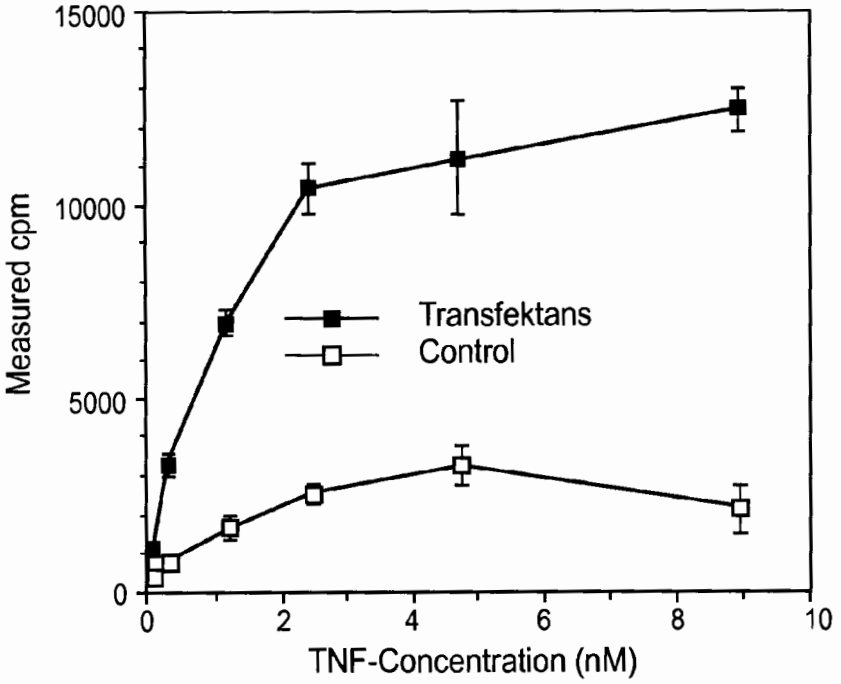


Figure 2B

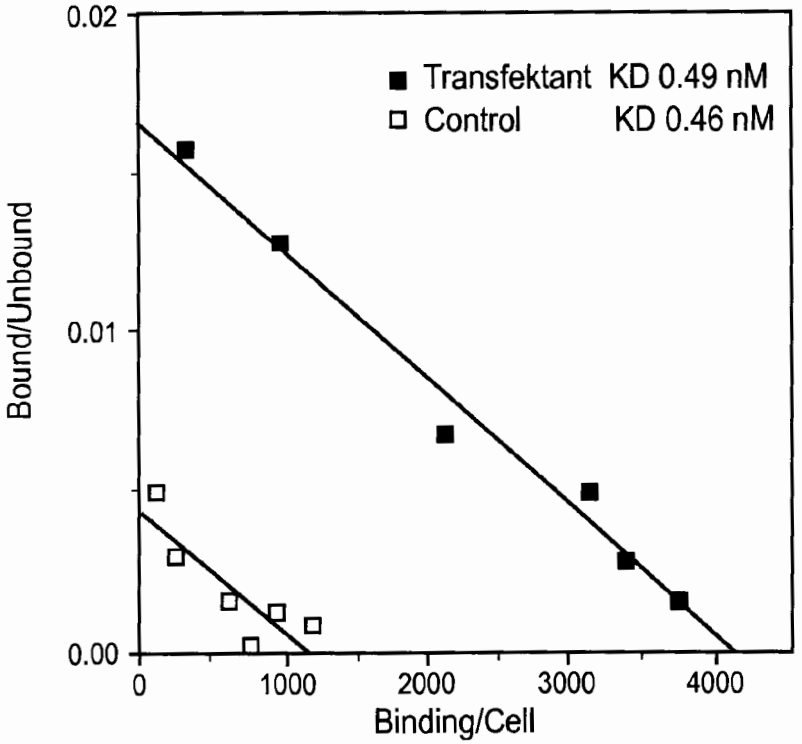
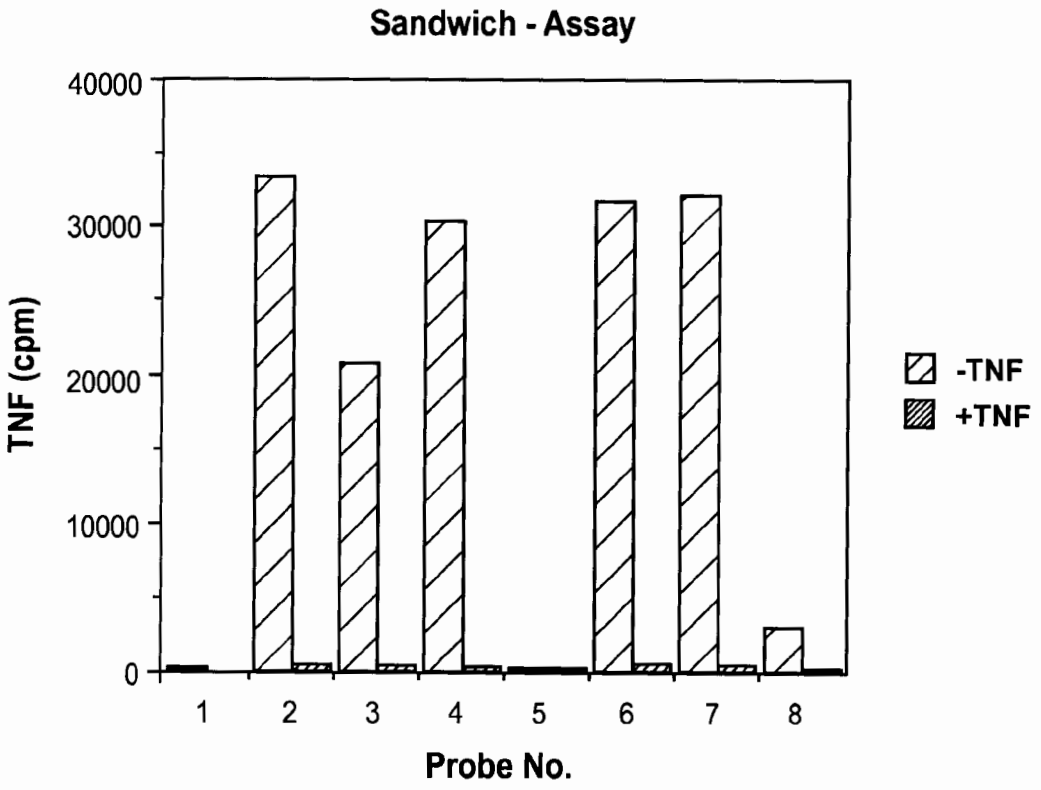


Figure 3


Sandwich - Assay
40000
30000
20000
10000
0
TNF (cpm)
1
2
3
4
5
6
7
8
Probe No.
-TNF
+TNF

Case 2:16-cv-01118-CCC-MF Document 18 Filed 02/29/16 Page 95 of 180 PageID: 581

FIGURE 4A

```
                .         .         .         .         .         .
  1 SerAspSerValCysAspSerCysGluAspSerThrTyrThrGlnLeuTrpAsnTrpVal
  1 TCGGACTCCGTGTGTGACTCCTGTGAGGACAGCACATACACCCAGCTCTGGAACTGGGTT
                .         .         .         .         .         .
 21 ProGluCysLeuSerCysGlySerArgCysSerSerAspGlnValGluThrGlnAlaCys
 61 CCCGAGTGCTTGAGCTGTGGCTCCCGCTGTAGCTCTGACCAGGTGGAAACTCAAGCCTGC
                .         .         .         .         .         .
 41 ThrArgGluGlnAsnArgIleCysThrCysArgProGlyTrpTyrCysAlaLeuSerLys
121 ACTCGGGAACAGAACCGCATCTGCACCTGCAGGCCCGGCTGGTACTGCGCGCTGAGCAAG
                .         .         .         .         .         .
 61 GlnGluGlyCysArgLeuCysAlaProLeuProLysCysArgProGlyPheGlyValAla
181 CAGGAGGGGTGCCGGCTGTGCGCGCCGCTGCCGAAGTGCCGCCCGGGCTTCGGCGTGGCC
                .         .         .         .         .         .
 81 ArgProGlyThrGluThrSerAspValValCysLysProCysAlaProGlyThrPheSer
241 AGACCAGGAACTGAAACATCAGACGTGGTGTGCAAGCCCTGTGCCCCGGGGACGTTCTCC
                .         .         .         .         .         .
101 AsnThrThrSerSerThrAspIleCysArgProHisGlnIleCysAsnValValAlaIle
301 AACACGACTTCATCCACGGATATTTGCAGGCCCCACCAGATCTGTAACGTGGTGGCCATC
                .         .         .         .         .         .
121 ProGlyAsnAlaSerArgAspAlaValCysThrSerThrSerProThrArgSerMetAla
361 CCTGGGAATGCAAGCAGGGATGCAGTCTGCACGTCCACGTCCCCCACCCGGAGTATGGCC
                .         .         .         .         .         .
141 ProGlyAlaValHisLeuProGlnProValSerThrArgSerGlnHisThrGlnProSer
421 CCAGGGGCAGTACACTTACCCCAGCCAGTGTCCACACGATCCCAACACACGCAGCCAAGT
```

FIGURE 4B

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161 ProGluProSerThrAlaProSerThrSerPheLeuLeuProMetGlyProSerProPro
481 CCAGAACCCAGCACTGCTCCAAGCACCTCCTTCCTGCTCCCAATGGGCCCCAGCCCCCCA

181 AlaGluGlySerThrGlyAspPheAlaLeuProValGlyLeuIleValGlyValThrAla
541 GCTGAAGGGAGCACTGGCGACTTCGCTCTTCCAGTTGGACTGATTGTGGGTGTGACAGCC

201 LeuGlyLeuLeuIleIleGlyValValAsnCysValIleMetThrGlnValLysLysLys
601 TTGGGTCTACTAATAATAGGAGTGGTGAACTGTGTCATCATGACCCAGGTGAAAAAGAAG

221 ProLeuCysLeuGlnArgGluAlaLysValProHisLeuProAlaAspLysAlaArgGly
661 CCCTTGTGCCTGCAGAGAAGCCAAGGTGCCTCACTTGCCTGCCGATAAGGCCCGGGGT

241 ThrGlnGlyProGluGlnGlnHisLeuLeuIleThrAlaProSerSerSerSerSerSer
721 ACACAGGGCCCCGAGCAGCACCTGCTGATCACAGCGCCGAGCTCCAGCAGCAGCTCC

261 LeuGluSerSerAlaSerAlaLeuAspArgArgAlaProThrArgAsnGlnProGlnAla
781 CTGGAGAGCTCGGCCAGTGCGTTGGACAGAAGGGCGCCCACTCGGAACCAGCCACAGGCA

281 ProGlyValGluAlaSerGlyAlaGlyGluAlaArgAlaSerThrGlySerSerAlaAsp
841 CCAGGCGTGGAGGCCAGTGGGGCCGGGGAGGCCCGGGCCAGCACCGGGAGCTCAGCAGAT

301 SerSerProGlyGlyHisGlyThrGlnValAlaAsnValThrCysIleValAsnValCysSer
901 TCTTCCCCTGGTGGCCATGGGACCCAGGTCAATGTCACCTGCATCGTGAACGTCTGTAGC
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Case 2:16-cv-01118-CCC-MF Document 18 Filed 02/29/16 Page 97 of 180 PageID: 583

FIGURE 4C

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                .         .         .         .         .         .
 321  SerSerAspHisSerSerGlnCysSerSerGlnAlaSerSerThrMetGlyAspThrAsp
 961  AGCTCTGACCACAGCTCACAGTGCTCCTCCCAAGCCAGCTCCACAATGGGAGACACAGAT
                .         .         .         .         .         .
 341  SerSerProSerGluSerProLysAspGluGlnValProPheSerLysGluGluCysAla
1021  TCCAGCCCCTCGGAGTCCCCGAAGGACGAGCAGGTCCCCTTCTCCAAGGAGGAATGTGCC
                .         .         .         .         .         .
 361  PheArgSerGlnLeuGluThrProGluThrLeuLeuGlySerThrGluGluLysProLeu
1081  TTTCGGTCACAGCTGGAGACGCCAGAGACCCTGCTGGGGAGCACCGAAGAGAAGCCCCTG
                .         .         .         .         .         .
 381  ProLeuGlyValProAspAlaGlyMetLysProSer
1141  CCCCTTGGAGTGCCTGATGCTGGGATGAAGCCCAGTTAACCAGGCCGGTGTGGGCTGTGT
1201  CGTAGCCAAGGTGGCTGAGCCCTGGCAGGATGACCCTGCGAAGGGGCCCTGGTCCTTCCA
1261  GGCCCCCACCACTAGGACTCTGAGGCTCTTTCTGGGCCAAGTTCCTCTAGTGCCCTCCAC
1321  AGCCGCAGCCTCCCTCTGACCTGCAGGCCAAGAGCAGAGGCAGCGAGTTGTGGAAAGCCT
1381  CTGCTGCCATGGCGTGTCCCTCTCGGAAGGCTGGCTGGGCATGGACGTTCGGGGCATGCT
1441  GGGGCAAGTCCCTGAGTCTCTGTGACCTGCCCCGCCCAGCTGCACCTGCCAGCCTGGCTT
1501  CTGGAGCCCTTGGGTTTTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTCTCCCCCTGGGC
1561  TCTGCCCAGCTCTGGCTTCCAGAAAACCCCAGCATCCTTTTCTGCAGAGGGGCTTTCTGG
1621  AGAGGAGGGATGCTGCCTGAGTCACCCATGAAGACAGGACAGTGCTTCAGCCTGAGGCTG
1681  AGACTGCGGGATGGTCCTGGGGCTCTGTGCAGGGAGGAGGTGGCAGCCCTGTAGGGAACG
1741  GGGTCCTTCAAGTTAGCTCAGGAGGCTTGGAAAGCATCACCTCAGGCCAGGTGCAGTGGC
1801  TCACGCCTATGATCCCAGCACTTTGGGAGGCTGAGGCGGGTGGATCACCTGAGGTTAGGA
1861  GTTCGAGACCAGCCTGGCCAACATGGTAAAACCCCATCTCTACTAAAAATACAGAAATTA
```

FIGURE 4D

```
1921  GCCGGGCGTGGTGGCGGGCACCTATAGTCCCAGCTACTCAGAAGCCTGAGGCTGGGAAAT
1981  CGTTTGAACCCGGGAAGCGGAGGTTGCAGGGAGCCGAGATCACGCCACTGCACTCCAGCC
2041  TGGGCGACAGAGCGAGAGTCTGTCTCAAAAGAAAAAAAAAAAGCACCGCCTCCAAATGCT
2101  AACTTGTCCTTTTGTACCATGGTGTGAAAGTCAGATGCCCCAGAGGGCCCAGGCAGGCCAC
2161  CATATTCAGTGCTGTGGCCTGGGCAAGATAACGCCACTTCTAACTAGAAATCTGCCAATTT
2221  TTTAAAAAAGTAAGTACCACTCAGGCCAACAAGCCCAACGACACAAAGCCCAAACTCTGCCAGC
2281  CACATCCAACCCCCCACCTGCCATTTGCACCCTCCGCCCTTCACTCCGGTGTGCCTGCAG
```

```
  1  MAPVAVWAAL AVGLELWAAA HALPAQVAFT PYAPEPGSTC RLREYYDQTA
 51  QMCCSKCSPG QHAKVFCTKT SDTVCDSCED STYTQLWNWV PECLSCGSRC
101  SSDQVETQAC TREQNRICTC RPGWYCALSK QEGCRLCAPL RKCRPGFGVA
151  RPGTETSDVV CKPCAPGTFS NTTSSTDICR PHQICNVVAI PGNASMDAVC
201  TSTSPTRSMA PGAVHLPQPV STRSQHTQPT PEPSTAPSTS FLLPMGPSPP
251  AEGSTGDFAL PVGLIVGVTA LGLLIIGVVN CVIMTQVKKK PLCLQREAKV
301  PHLPADKARG TQGPEQQHLL ITAPSSSSSS LESSASALDR RAPTRNQPQA
351  PGVEASGAGE ARASTGSSDS SPGGHGTQVN VTCIVNVCSS SDHSSQCSSQ
401  ASSTMGDTDS SPSESPKDEQ VPFSKEECAF RSQLETPETL LGSTEEKPLP
451  LGVPDAGMKP S
```

FIGURE 5

Case 2:16-cv-01118-CCC-MF Document 18 Filed 02/29/16 Page 100 of 180 PageID: 586

FIGURE 6A

```
  1 S  D  T  V  C  D  S  C  E  D  S  T  Y  T  Q  L  W  N  W  V
  1 tcggacaccgtgtgtgactcctgtgaggacagcacatacacccagctctggaactgggtt
  1          10         20         30         40         50

 21 P  E  C  L  S  C  G  S  R  C  S  S  D  Q  V  E  T  Q  A  C
 61 cccgagtgcttgagctgtggctcccgctgtagctctgaccaggtggaaactcaagcctgc
 61          70         80         90         100        110

 41 T  R  E  Q  N  R  I  C  T  C  R  P  G  W  Y  C  A  L  S  K
121 actcgggaacagaaccgcatctgcacctgcaggcccggctggtactgcgcgctgagcaag
121         130        140        150        160        170

 61 Q  E  G  C  R  L  C  A  P  L  P  K  C  R  P  G  F  G  V  A
181 caggaggggtgccggctgtgcgcgccgctgccgaagtgccgcccgggcttcggcgtggcc
181         190        200        210        220        230

 81 R  P  G  T  E  T  S  D  V  V  C  K  P  C  A  P  G  T  F  S
241 agaccaggaactgaaacatcagacgtggtgtgcaagccctgtgccccggggacgttctcc
241         250        260        270        280        290

101 N  T  T  S  S  T  D  I  C  R  P  H  Q  I  C  N  V  V  A  I
301 aacacgacttcatccacggatatttgcaggccccaccagatctgtaacgtggtggccatc
301         310        320        330        340        350
```

FIGURE 6B

```
121 P  G  N  A  S  R  D  A  V  C  T  S  T  S  P  T  R  S  M  A
361 cctgggaatgcaagcagggatgcagtctgcacgtccacgtcccccacccggagtatggcc
361          370        380        390        400        410

141 P  G  A  V  H  L  P  Q  P  V  S  T  R  S  Q  H  T  Q  P  S
421 ccagggcagtacacttacccagccagtgtccacacgatcccaacacacgcagccaagt
421          430        440        450        460        470

161 P  E  P  S  T  A  P  S  T  S  F  L  L  P  M  G  P  S  P  P
481 ccagaacccagcactgctccaagcacctccttcctgctcccaatgggccccagccccca
481          490        500        510        520        530

181 A  E  G  S  T  G  D  F  A  L  P  V  G  L  I  V  G  V  T  A
541 gctgaagggagcactggcgacttcgctcttccagttgactgattgtgggtgtgacagcc
541          550        560        570        580        590

201 L  G  L  L  I  I  G  V  V  V  N  C  V  I  M  T  Q  V  K  K  K
601 ttgggtctactaataataggagtggtgaactgtgtcatcatgacccaggtgaaaaagaag
601          610        620        630        640        650

221 P  L  C  L  Q  R  E  A  K  V  P  H  L  P  A  D  K  A  R  G
661 cccttgtgcctgcagagagaagccaaggtgcctcacttgccgataaggcccgggggt
661          670        680        690        700        710
```

Case 2:16-cv-01118-CCC-MF Document 18 Filed 02/29/16 Page 102 of 180 PageID: 588

FIGURE 6C

```
 241 T  Q  G  P  E  Q  Q  H  L  L  I  T  A  P  S  S  S  S  S  S
 721 acacagggccccgagcagcagcacctgctgatcacagcgccgagctccagcagcagctcc
 721          730        740        750        760        770

 261 L  E  S  S  A  S  A  L  D  R  R  A  P  T  R  N  Q  P  Q  A
 781 ctggagagctcggccagtgcgttggacagaagggcgcccactcggaaccagccacaggca
 781          790        800        810        820        830

 281 P  G  V  E  A  S  G  A  G  E  A  R  A  S  T  G  S  S  A  D
 841 ccaggcgtggaggccagtggggccggggaggcccgggccagcaccgggagctcagcagat
 841          850        860        870        880        890

 301 S  S  P  G  G  H  G  T  Q  V  N  V  T  C  I  V  N  V  C  S
 901 tcttcccctggtggccatgggacccaggtcaatgtcacctgcatcgtgaacgtctgtagc
 901          910        920        930        940        950

 321 S  S  D  H  S  S  Q  C  S  S  Q  A  S  S  T  M  G  D  T  D
 961 agctctgaccacagctcacagtgctcctcccaagccagctccacaatgggagacacagat
 961          970        980        990        1000       1010

 341 S  S  P  S  E  S  P  K  D  E  Q  V  P  F  S  K  E  E  C  A
1021 tccagcccctcggagtccccgaaggacgagcaggtccccttctccaaggaggaatgtgcc
1021          1030       1040       1050       1060       1070
```

FIGURE 6D

```
 361 F  R  S  Q  L  E  E  T  P  E  T  L  L  L  G  S  T  E  E  K  P  L
1081 ttcggtcacagctggagacgccagagaccctgctggggagcaccgaagagaagccctg
1081          1090      1100      1110      1120      1130

 381 P  L  G  V  P  D  A  G  M  K  P  S
1141 cccttggagtgcctgatgctgggatgaagcccagttaaccaggccggtgtgggctgtgt
1141          1150      1160      1170      1180      1190
1201 cgtagccaaggtggctgagccctggcaggatgaccctgcgaaggggcccctggtccttcca
1201          1210      1220      1230      1240      1250
1261 ggccccccaccactaggactctgaggctctttctgggccaagttcctctagtgcccctccac
1261          1270      1280      1290      1300      1310
1321 agccgcagcctccctctgacctgcaggccaagagcagaggcagcgagttgtgggaaagcct
1321          1330      1340      1350      1360      1370
1381 ctgctgccatggcgtgtccctctcggaaggctgggcatgggacgttcgggggcatgct
1381          1390      1400      1410      1420      1430
1441 ggggcaagtccctgagtctctgtgacctgccccgcccagctgcacctgccagccctggctt
1441          1450      1460      1470      1480      1490
1501 ctggagcccttgggtttttttgtttgtttgtttgtttctcccccctgggc
1501          1510      1520      1530      1540      1550
1561 tctgcccagctctggcttccagaaaaccccagcatccttttctgcagaggggctttctgg
1561          1570      1580      1590      1600      1610
1621 agaggagggatgctgcctgagtcacccatgaagacaggacagtgcttcagcctgaggctg
1621          1630      1640      1650      1660      1670
```

FIGURE 6E

```
1681 agactgcgggatggtcctgggctctgtgcagggaggaggtggcagccctgtagggaacg
1681          1690      1700      1710      1720      1730
1741 gggtccttcaagttagctcaggaggcttggaaagcatcacctcaggccaggtgcagtggc
1741          1750      1760      1770      1780      1790
1801 tcacgcctatgatcccagcactttgggaggctgaggcgggtggatcacctgaggttagga
1801          1810      1820      1830      1840      1850
1861 gttcgagaccagcctggccaacatggtaaaaccccatctctactaaaaatacagaaatta
1861          1870      1880      1890      1900      1910
1921 gccgggcgtggtggcgggcacctatagtcccagctactcagaaggcctgaggctgggaaat
1921          1930      1940      1950      1960      1970
1981 cgtttgaacccgggaagcggaggttgcagggagccgagatcacgccactgcactccagcc
1981          1990      2000      2010      2020      2030
2041 tgggcgacagagcgagagtctgtctcaaaagaaaaaaaaaaagcaccgcctcccaaatgct
2041          2050      2060      2070      2080      2090
2101 aacttgtccttttgtaccatggtgtgaaagtcagatgcccagagggcccaggccaggccac
2101          2110      2120      2130      2140      2150
2161 catattcagtgctgtggccctgggcaagataacgcgcacttctaactagaaatctgccaattt
2161          2170      2180      2190      2200      2210
2221 tttaaaaaagtaagtaccactcaggccaacaaggccaacgacaaagccaaactctgccagc
2221          2230      2240      2250      2260      2270
2281 cacatccaaccccccacctgccatttgcaccctcccgccttcactcccggtgtgcctgcag
2281          2290      2300      2310      2320      2330
```

HUMAN TNF RECEPTOR

This is a division of application Ser. No. 08/095,640, filed Jul. 21, 1993; now U.S. Pat. No. 5,610,279, which is a continuation application of Ser. No. 07/580,013, filed Sep. 10, 1990, now abandoned. This application claims priority under 35 U.S.C. §119 to application Ser. Nos. 3319/89, 746/90 and 1347/90, filed on Sep. 12, 1989, Mar. 8, 1990 and Apr. 20, 1990, respectively, all in Switzerland. This application also claims priority under 35 U.S.C. §119 to European Patent Application Number 90116707.2 (now Patent Number EP 0417563), filed Aug. 31, 1990.

BACKGROUND OF THE INVENTION

Tumor necrosis factor α (TNFα, also cachectin), discovered as a result of its haemorragic-necrotizing activity on certain tumors, and lymphotoxin (TNFβ) are two closely related peptide factors [3] from the class of lymphokines/cytokines which are both referred to hereinafter as TNF [see references 2 and 3]. TNF possesses a broad cellular spectrum of activity. For example, TNF has inhibitory or cytotoxic activity on a series of tumor cell lines [2, 3], stimulates the proliferation of fibroblasts and the phagocytic/cytotoxic activity of myeloic cells [4, 5, 6], induces adhesion molecules in endothelial cells or exerts an inhibitory activity on the endothelium [7, 8, 9, 10], inhibits the synthesis of specific enzymes in adipocytes [11] and induces the expression of histocompatibility antigens [12]. Many of these TNF activities are produced via induction of other factors or by synergistic effects with other factors such as interferons or interleukins [13-16].

TNF is involved in pathological conditions such as shock states in meningococcal sepsis [17], the development of autoimmune glomerulonephritis in mice [18] and cerebral malaria in mice [19] and human beings [41]. The toxic effects of endotoxin appear to be mediated by TNF [20]. Furthermore, TNF can trigger interleukin-1 fever [39]. On the basis of its pleiotropic functional properties, TNF in interaction with other cytokines is involved in additional pathological conditions as a mediator of immune response, inflammation, and other processes.

These biological effects are mediated by TNF via specific receptors. According to present knowledge not only TNFα, but also TNFβ bind to the same receptors [21]. Different cell types differ in their number of TNF receptors [22, 23, 24]. Generally known TNF-binding proteins (TNF-BP) have been detected by covalent bonding to radioactively labelled TNF [24-29], and the following apparent molecular weights of the TNF/TNF-BP complexes obtained have been determined to be: 95/100 kD and 75 kD [24], 95 kD and 75 kD [25], 138 kD, 90 kD, 75 kD and 54 kD [26], 100±5 kD [27], 97 kD and 70 kD [28] and 145 kD [29]. One such TNF/TNF-BP complex was isolated by anti-TNF-antibody immune affinity chromatography and preparative SDS-polyacrylamide gel electrophoreses (SDS-PAGE) [27]. The reductive cleavage of this complex and subsequent SDS-PAGE analysis gave several bands which were not tested for TNF-binding activity. Since the specific conditions which must be used for the cleavage of the complex lead to inactivation of the binding protein [31], the latter has also not been possible. The separation of soluble TNF-BP from human serum or urine by ion exchange chromatography and gel filtration (molecular weight in the region of 50 kD) was described by Olsson et al. [30].

Brockhaus et al. [32] obtained an enriched TNF-BP preparation from membrane extracts of HL_{60} cells by TNFα-ligand affinity chromatography and HPLC which, in turn, was used as an antigen preparation for the production of monoclonal antibodies against TNF-BP. Using such an immobilized antibody (immune affinity chromatography) Loetscher and Brockhaus obtained an enriched preparation of TNF-BP [31] from an extract of human placenta using TNFα-ligand affinity chromatography and HPLC, which gave a strong broad band at 35 kD, a weak band at about 40 kD and a very weak band in the region between 55 kD and 60 kD on SDS-PAGE analysis. Moreover, the gel showed a protein background smear in the region of 33 kD to 40 kD. The significance of these protein bands was, however, not clear due to the heterogenicity of the starting material which was used (placenta tissue; combined material from several placentas). In the state of the art TNF-BP have already been characterized by a N-terminal partial sequence [European Patent Application, Publication No. 308 378], whereby this sequence differs from the N-terminal partial sequence according to formula (IA) in accordance with the invention. Moreover, the TNF-binding proteins described in the state of the art are soluble, i.e. non-membrane bound, TNF-BP and not membrane-bound, i.e. insoluble, TNF-BP isolated from urine.

SUMMARY OF THE INVENTION

This invention comprises insoluble, homogenous proteins or soluble or insoluble fragments thereof, capable of binding tumor necrosis factor-(TNF).

This invention also comprises TNF-binding proteins containing amino acid sequences of FIG. **1** or FIG. **4**, proteins containing fragments of these sequences, and proteins analagous to the sequences of FIG. **1** or FIG. **4** or to fragments thereof.

This invention further comprises DNA sequences encoding the proteins described above, proteins encoded by these sequences, and antibodies to any of these proteins.

This invention comprises DNA sequences which combine two partial DNA sequences, one sequence encoding soluble fragments of TNF binding proteins and the other partial sequence encoding all domains except the first domain of the constant region of the heavy chain of human immunoglobulin IgG, IgA, IgM, or IgE, and the recombinant proteins encoded by these sequences.

This invention additionally comprises vectors containing the above DNA sequences, and host systems transfected with such vectors.

This invention finally comprises a process for the isolation of an insoluble homogenous protein capable of binding TNF.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1A-1D**. Nucleotide sequence (SEQ ID NO: 1) and deduced amino acid sequence (SEQ ID NO: 2) for cDNA clone derived from 55 kD TNF-BP. The 19 amino acid transmembrane region is underlined. Hypothetical glycosylation sites are identified by asterisks.

FIG. **2**. Binding analysis of COS cells transfected with plasmid pN123. Panel 2A—binding of transfected cells to ^{125}I-TNFα. Panel 2B—Scatchard plot of binding data.

FIG. **3**. Sandwich assays of cells transfected with plasmid pK19. Culture supernatants of cells transfected with pK19 were incubated with anti-55 kD TNF-BP antibody followed by ^{125}I-TNFα. Columns 1, 5, and 8 are controls. Columns 2, 3, **4**, 5, and 6 are five parallel transfections.

FIG. **4A-4D**. Nucleotide sequence (SEQ ID NO: 28) and deduced amino acid sequence (SEQ ID NO: 29) for cDNA clones derived from 75/65 kD TNF-BP.

FIG. **5**. Deduced amino acid sequence (SEQ ID NO: 27) for a 75/65 kD TNF-BP cDNA clone described in Smith et al., Science 248, 1019-1023, (1990). The leader region is singly underlined, the transmembrane domain is shown boxed, and potential N-linked glycosylation sites are doubly underlined.

FIGS. **6**A-**6**E: Corrected nucleotide sequence (SEQ ID NO: 3) and deduced amino acid sequence (SEQ ID NO: 4) of FIG. **4** after repeated sequencing, showing a threonine coded by "ACC" at position 3 instead of a serine coded by "TCC".

DETAILED DESCRIPTION OF THE INVENTION

The TNF-binding proteins of the present invention are homogenous, insoluble proteins and soluble or insoluble fragments of such proteins which are capable of binding TNF. These proteins have the ability to bind TNF as measured by standard assays.

The TNF-binding proteins of the present invention include homogenous proteins containing the amino acid sequence depicted in FIG. **1** (SEQ ID NO: 2) or in FIG. **4** (SEQ ID NO: 4), proteins containing fragments of either sequence, and analogues of any such proteins for example proteins containing amino acid sequences analogous to the amino acid sequences of FIG. **1** (SEQ ID NO: 2) or FIG. **4** (SEQ ID NO: 4) or to fragments thereof. An analogue is a protein in which one or more amino acids of the sequences depicted in FIG. **1** (SEQ ID NO: 2) or in FIG. **4** (SEQ ID NO: 4) have had their side-groups chemically modified in a known manner, or those in which one or more amino acids have been replaced or deleted, without thereby eliminating TNF-binding ability. Such analogues may be produced by known methods of peptide chemistry, or by known methods of recombinant DNA technology, such as planned mutagenesis.

The TNF binding activity of the proteins of the present invention may be determined using the assay described in Example 1.

TNF-binding proteins of this invention are obtained as follows:

TNF binding proteins may be isolated from tissues and purified to homogeneity, or isolated from cells which contain membrane-bound TNF binding protein, and purified to homogeneity. One possible method for growing cells and isolating cell extract is described in Example 2, however, other cells types and other growth and isolation methods are well known in the art. Purification of TNF-binding proteins from cell extracts may be performed using the methods described in Examples 4, 5, and 6 in combination with the assay described in Example 1. TNF-binding proteins isolated and purified by these methods were sequenced by well-known methods, as described in Example 7. From these amino acid sequences, DNA probes were produced and used to obtain mRNA encoding TNF binding proteins from which cDNA was made, all by known methods described in Examples 8 and 11. Other well-known methods for producing cDNA are known in the art and may effectively be used. In general, any TNF-binding protein can be isolated from any cell or tissue expressing such proteins using a cDNA probe such as the probe described above, isolating mRNA and transcribing the mRNA into cDNA. Thereafter, the protein can be produced by inserting the cDNA into an expression vector as described in Example 9, such as a virus, plasmid, cosmid, or other vector, inserting the expression vector into a cell, such as the COS cell described in Example 9 or the insect cell described in Example 10, proliferating the resulting cells, and isolating the expressed TNF-binding protein from the medium or from cell extract as described above. Alternatively, TNF-binding proteins may be chemically synthesized using the sequence described above and an amino acid synthesizer, or manual synthesis using chemical conditions well known to form peptide bonds between selected amino acids. Analogues and fragments of TNF-binding proteins may be produced by the above methods. In the case of analogues, the proteins may be chemically modified, or modified by genetic engineering as described above. These fragments and analogues may then be tested for TNF-binding activity using methods such as the assay of Example 1.

Finally, monoclonal antibodies directed against TNF-binding proteins, such as the antibodies described in Example 3, may be produced by known techniques, and used to isolate TNF-binding proteins.

In more detail, the proteins of the present invention are non-soluble proteins, i.e. for example membrane proteins or so-called receptors, and soluble or non-soluble fragments thereof, which bind TNF (TNF-BP), in homogeneous form, as well as their physiologically compatible salts. Preferred proteins are those which according to SDS-PAGE under non-reducing conditions are characterized by apparent molecular weights of about 55 kD, 51 kD, 38 kD, 36 kD and 34 kD or 75 kD and 65 kD, especially those with about 55 kD and 75 kD. Furthermore, there are preferred those proteins which are characterized by containing at least one of the following amino acid partial sequences:

(IA) Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile (SEQ ID NO: 5)

(IB) Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys (SEQ ID NO: 6)

(IIA) Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu (SEQ ID NO: 7)

(IIB) Val-Phe-Cys-Thr (SEQ ID NO: 8)

(IIC) Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala (SEQ ID NO: 9)

(IID) Leu-pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys (SEQ ID NO: 9)

(IIE) Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu (SEQ ID NO: 11)

(IIF) Leu-Cys-Ala-Pro (SEQ ID NO: 12)

(IIG) Val-Pro-His-Leu-Pro-Ala-Asp (SEQ ID NO: 13)

(IIH) Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro (SEQ ID NO: 14)

in which X stands for an amino acid residue which could not be unequivocally determined.

A process for the isolation of the TNF-BP in accordance with the invention is also an object of the present invention. This process comprises carrying out essentially the following purification steps in sequence: production of a cell or tissue extract, immune affinity chromatography and/or single or multiple ligand affinity chromatography, high resolution liquid chromatography (HPLC) and preparative SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The combination of the individual purification steps, which are known from the state of the art, is essential to the success of the process in accordance with the invention, whereby individual steps have been modified and improved having regard to the problem to be solved. Thus, for example, the original combined immune affinity chromatography/TNFα-ligand affinity chromatography step originally used for the enrichment of TNF-BP from human placenta [31] has been altered by using a BSA-Sepharose 4B pre-column. For the application of the cell or membrane extract, this pre-column was connected in series with the immune affinity column followed by the ligand affinity column. After the application of the extract the two aforementioned columns were coupled, each eluted and the TNF-BP-active fractions were purified again via a ligand affinity

column. The use of a detergent-containing solvent mixture for the performance of the reversed-phase HPLC step is essential to the invention.

Further, an industrial process for the production of high cell densities of mammalian cells from which TNF-BP can be isolated is also an object of the present invention. Such a process comprises using a medium, which has been developed for the specific growth requirements of the cell line used, in combination with a perfusion apparatus as described e.g. in detail in Example 2. By means of such a process there can be produced, for example, in the case of HL-60 cells up to more than 20-fold higher cell densities than usual.

In addition thereto, the present invention is also concerned with DNA sequences coding for proteins and soluble or non-soluble fragments thereof, which bind TNF. Thereunder there are to be understood, for example, DNA sequences coding for non-soluble proteins or soluble as well as non-soluble fragments thereof, which bind TNF, such DNA sequences being selected from the following:

(a) DNA sequences as given FIG. **1** or FIG. **4** as well as their complementary strands, or those which include these sequences;

(b) DNA sequences which hybridize with sequences defined under (a) or fragments thereof;

(c) DNA sequences which, because of the degeneracy of the genetic code, do not hybridize with sequences as defined under (a) and (b), but which code for polypeptides having exactly the same amino acid sequence.

That is to say, the present invention embraces not only allelic variants, but also those DNA sequences which result from deletions, substitutions and additions from one or more nucleotides of the sequences given in FIG. **1** or FIG. **4**, whereby in the case of the proteins coded thereby there come into consideration, just as before, TNF-BP. One sequence which results from such a deletion is described, for example, in Smith et al., Science 248, 1019-1023, (1990), which is incorporated by reference herein. FIG. **5** (a reproduction of FIG. **3**B of Smith et al.) shows the deduced amino acid sequence (SEQ ID NO: 27) of the cDNA coding region of a human TNF receptor cDNA clone. The leader region is singly underlined, the transmembrane domain is shown boxed, and potential N-linked glycosylation sites are doubly underlined. The entire nucleotide sequence is available upon request and has been deposited at Genbank under Accession Number M32315.

There are preferred first of all those DNA sequences which code for such a protein having an apparent molecular weight of about 55 kD, whereby the sequence given in FIG. **1** is especially preferred, and sequences which code for non-soluble as well as soluble fragments of such proteins. A DNA sequence which codes, for example, for such a non-soluble protein fragment extends from nucleotide −185 to 1122 of the sequence given in FIG. **1**. DNA sequences which code for soluble protein fragments are, for example, those which extend from nucleotide −185 to 633 or from nucleotide −14 to 633 of the sequence given in FIG. **1**. There are also preferred DNA sequences which code for a protein of about 75/65 kD, whereby those which contain the partial cDNA sequences shown in FIG. **4** are preferred. Especially preferred DNA sequences in this case are the sequences of the open reading frame of nucleotide 2 to 1,177. The peptides IIA, IIC, IIE, IIF, IIG and IIH are coded by the partial cDNA sequence in FIG. **4**, whereby the insignificant deviations in the experimentally determined amino acid sequences are based on the cDNA-derived sequence with highest probability from the limited resolution of the gas phase sequencing. DNA sequences which code for insoluble (deposited on Oct. 17, 2006 with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Md. 20852, under Accession No. PTA 7942) as well as soluble fractions of TNF-binding proteins having an apparent molecular weight of 65 kD/75 kD are also preferred. DNA sequences for such soluble fragments can be determined on the basis of the amino acid sequences derived from the nucleic acid sequences coding for such non-soluble TNF-BP.

The invention is also concerned with DNA sequences which comprise a combination of two partial DNA sequences, with one of the partial sequences coding for those soluble fragments of non-soluble proteins which bind TNF (see above) and the other partial sequence coding for all domains other than the first domain of the constant region of the heavy chain of human immunoglobulins such as IgG, IgA, IgM or IgE, in particular IgG_1 or IgG_3 subtypes.

The present invention is also concerned with the recombinant proteins coded by any of DNA sequences described above. Of course, there are thereby also included such proteins in whose amino acid sequences amino acids have been exchanged, for example by planned mutagenesis, so that the activity of the TNF-BP or fragments thereof, namely the binding of TNF or the interaction with other membrane components participating in the signal transfer, have been altered or maintained in a desirable manner. Amino acid exchanges in proteins and peptides which do not generally alter the activity of such molecules are known in the state of the art and are described, for example, by H. Neurath and R. L. Hill in "The Proteins" (Academic Press, New York, 1979, see especially FIG. **6**, page 14). The most commonly occurring exchanges are: Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly as well as these in reverse. The present invention is also concerned with vectors which contain any of the DNA sequences described above in accordance with the invention and which are suitable for the transformation of suitable pro- and eukaryotic host systems, whereby there are preferred those vectors whose use leads to the expression of the proteins which are coded by any of the DNA sequences described above in accordance with the invention. Finally, the present invention is also concerned with pro- and eukaryotic host systems transformed with such vectors, as well as a process for the production of recombinant compounds in accordance with the invention by cultivating such host systems and subsequently isolating these compounds from the host systems themselves or their culture supernatants.

An object of the present invention are also pharmaceutical preparations which contain at least one of these TNF-BPs or fragments thereof, if desired in combination with other pharmaceutically active substances and/or non-toxic, inert, therapeutically compatible carrier materials.

Finally, the present invention is concerned with the use of such a TNF-BP on the one hand for the production of pharmaceutical preparations and on the other hand for the treatment of illnesses, preferably those in which TNF is involved in their course.

Starting materials for the TNF-BP in accordance with the invention are quite generally cells which contain such TNF-BP in membrane-bound form and which are generally accessible without restrictions to a person skilled in the art, such as, for example, HL60 [ATCC No. CCL 240], U 937 [ATCC No. CRL 1593], SW 480 [ATCC No. CCL 228] and HEp2 cells [ATCC No. CCL 23]. These cells can be cultivated according to known methods of the state of the art [40] or, in order to produce high cell densities, according to the procedure already described generally and described in detail in

Example 2 for HL60 cells. TNF-BP can then be extracted from the cells, which are centrifuged-off from the medium and washed, according to known methods of the state of the art using suitable detergents, for example Triton X-114, 1-0-n-octyl-β-D-glucopyranoside (octylglucoside) or 3-[(3-cholylamido-propyl)-dimethylammonio]-1-propane sulphonate (CHAPS), especially using Triton X-100. For the detection of such TNF-BP there can be used the usually used detection methods for TNF-BP, for example a polyethylene glycol-induced precipitation of the ^{125}I-TNF/TNF-BP complex [27], especially filter-binding tests with radioactively labelled TNF according to Example 1. In order to produce the TNF-BP in accordance with the invention, the general methods of the state of the art used for the purification of proteins, especially of membrane proteins, such as, for example, ion exchange chromatography, gel filtration, affinity chromatography, HPLC and SDS-PAGE can be used. Especially preferred methods for the production of TNF-BP in accordance with the invention are affinity chromatography, especially with TNF-α as the ligand bound to the solid phase, and immune affinity chromatography, HPLC and SDS-PAGE. The elution of TNF-BP bands which are separated using SDS-PAGE can be effected according to known methods of protein chemistry, for example using electroelution according to Hunkapiller et al. [34], whereby according to present knowledge the electro-dialysis times given there generally have to be doubled. Thereafter, traces of SDS which still remain can then be removed in accordance with Bosserhoff et al. [50].

The thus-purified TNF-BP can be characterized by methods of peptide chemistry which are known in the state of the art, such as, for example, N-terminal amino acid sequencing or enzymatic as well as chemical peptide cleavage. Fragments obtained by enzymatic or chemical cleavage can be separated according to usual methods such as, for example, HPLC and can themselves be subjected to further N-terminal sequencing. Such fragments which themselves bind TNF can be identified using the aforementioned detection methods for TNF-BP and are likewise objects of the present invention.

Starting from the thus-obtained amino acid sequence information or the DNA and amino acid sequences given in FIG. **1** as well as in FIG. **4** there can be produced, taking into consideration the degeneracy of the genetic code, according to methods known in the state of the art suitable oligonucleotides [51]. By means of these, again according to known methods of molecular biology [42, 43], cDNA or genomic DNA banks can be searched for clones which contain nucleic acid sequences coding for TNF-BP. Moreover, using the polymerase chain reaction (PCR) [49] cDNA fragments can be cloned by completely degenerating the amino acid sequence of two spaced apart relatively short segments while taking into consideration the genetic code and introducing into their complementarity suitable oligo-nucleotides as a "primer", whereby the fragment lying between these two sequences can be amplified and identified. The determination of the nucleotide sequence of a such a fragment permits an independent determination of the amino acid sequence of the protein fragment for which it codes. The cDNA fragments obtainable by PCR can also, as already described for the oligonucleotides themselves, be used according to known methods to search for clones containing nucleic acid sequences coding for TNF-BP from cDNA or genomic DNA banks. Such nucleic acid sequences can then be sequenced according to known methods [42]. On the basis of the thus-determined sequences and of the already known sequences for certain receptors, those partial sequences which code for soluble TNF-BP fragments can be determined and cut out from the complete sequence using known methods [42].

The complete sequence or such partial sequences can then be integrated using known methods into vectors described in the state of the art for their multiplication and expression in prokaryotes [42]. Suitable prokaryotic host organisms are, for example, gram-negative and gram-positive bacteria such as, for example, *E. coli* strains such as *E. coli* HB101 [ATCC No. 33 694] or *E. coli* W3110 [ATCC No. 27 325] or *B. subtilis* strains.

Furthermore, nucleic acid sequences in accordance with the invention which code for TNF-BP as well as for TNF-BP fragments can be integrated using known methods into suitable vectors for reproduction and expression in eukaryotic host cells, such as, for example, yeast, insect cells and mammalian cells. Expression of such sequences is preferably effected in mammalian and insect cells.

A typical expression vector for mammalian cells contains an efficient promoter element in order to produce a good transcription rate, the DNA sequence to be expressed and signals for an efficient termination and polyadenylation of the transcript. Additional elements which can be used are "enhancers" which lead to again intensified transcription and sequences which e.g. can bring about a longer half life of the mRNA. For the expression of nucleic acid sequences in which the endogenous sequence fragment coding for a signal peptide is missing, there can be used vectors which contain such suitable sequences which code for signal peptides of other known proteins. See, for example, the vector pLJ268 described by Cullen, B. R. in Cell 46, 973-982 (1986) as well as Sharma, S. et al. in "Current Communications in Molecular Biology", edt. by Gething, M. J., Cold Spring Harbor Lab. (1985), pages 73-78.

Most of these vectors which are used for a transient expression of a particular DNA sequence in mammalian cells contain the replication source of the SV40 virus. In cells which express the T-antigen of the virus (e.g. COS cells), these vectors are reproduced abundantly. A transient expression is, however, not limited to COS cells. In principle any transfectable mammalian cell line can be used for this purpose. Signals which can bring about a strong transcription are e.g. the early and late promoters of SV40, the promoter and enhancer of the "major immediate-early" gene of HCMV (human cytomegalovirus), the LTR's ("long terminal repeats") of retroviruses such as, for example, RSV, HIV and MMTV. There can, however, also be used signals of cellular genes such as e.g. the promoters of the actin and collagenase genes.

Alternatively, however, stable cell lines which have the specific DNA sequence integrated into the genome (chromosome) can also be obtained. For this, the DNA sequence is cotransfected together with a selectable marker, e.g. neomycin, hygromycin, dihydrofolate reductase (dhfr) or hypoxanthin guanine phosphoribosyl transferase (hgpt). The DNA sequence stably incorporated in the chromosome can also be reproduced abundantly. A suitable selection marker for this is, for example, dihydrofolate reductase (dhfr). Mammalian cells (e.g. CHO cells), which contain no intact dhfr gene, are thereby incubated with increasing amounts of methotrexate after transinfection has been effected. In this manner cell lines which contain more than a thousand copies of the desired DNA sequence can be obtained.

Mammalian cells which can be used for the expression are e.g. cells of the human cell lines Hela [ATCC CCL2] and 293 [ATCC CRL 1573] as well as 3T3 [ATCC CCL 163] and L cells, e.g. [ATCC CCL 149], (CHO) cells [ATCC CCL 61],

BHK [ATCC CCL 10] cells as well as the CV 1 [ATCC CCL 70] and the COS cell lines [ATCC CRL 1650, CRL 1651].

Suitable expression vectors include, for example, vectors such as pBC12MI [ATCC 67 109], pSV2dhfr [ATCC 37 146], pSVL [Pharmacia, Uppsala, Sweden], pRSVcat [ATCC 37 152] and pMSG [Pharmacia, Uppsala, Sweden]. The vectors "pK19" and "pN123" used in Example 9 are especially preferred vectors. These can be isolated according to known methods from *E. coli* strains HB101(pK19) and HB101 (pN123) transformed with them [42]. These *E. coli* strains have been deposited on the 26 Jan. 1990 at the Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG, under DSM 5761 for HB101 (pK19) and DMS 5764 for HB101(pN123). For the expression of proteins which consist of a soluble fragment of non-soluble TNF-BP and an immunoglobulin fragment, i.e. all domains except the first of the constant region of the heavy chain, there are especially suitable pSV2-derived vectors as described, for example, by German, C. in "DNA Cloning" [Vol. II., edt. by Glover, D. M., IRL Press, Oxford, 1985]. The vectors pCD4-Hμ (DSM 5315), pDC4-Hγ1 (DSM 5314) and pCD4-Hγ3 (DSM 5523) which have been deposited at the Deutschen Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM) in Braunschweig, FRG, and which are described in detail in European Patent Application No. 90107393.2 are especially preferred vectors. This European Patent Specification and the equivalent Applications referred to in Example 11 also contain data with respect to the further use of these vectors for the expression of chimeric proteins (see also Example 11) and for the construction of vectors for the expression of such chimeric proteins with other immunoglobulin fragments.

The manner in which these cells are transfected depends on the chosen expression system and vector system. An overview of these methods is to be found e.g. in Pollard et al., "DNA Transformation of Mammalian Cells" in "Methods in Molecular Biology" [Nucleic Acids Vol. 2, 1984, Walker, J. M., ed, Humana, Clifton, N. J.]. Further methods are to be found in Chen and Okayama ["High-Efficiency Transformation of Mammalian Cells by Plasmid DNA", Molecular and Cell Biology 7, 2745-2752, 1987] and in Feigner [Feigner et al., "Lipofectin: A highly efficient, lipid-mediated DNA-transfection procedure", Proc. Nat. Acad. Sci. USA 84, 7413-7417, 1987].

The baculovirus expression system, which has already been used successfully for the expression of a series of proteins (for an overview see Luckow and Summers, Bio/Technology 47-55, 1988), can be used for the expression in insect cells. Recombinant proteins can be produced in authentic form or as fusion proteins. The thus-produced proteins can also be modified such as, for example, glycosylated (Smith et al., Proc. Nat. Acad. Sci. USA 82, 8404-8408, 1987). For the production of a recombinant baculovirus which expresses the desired protein there is used a so-called "transfer vector". Under this there is to be understood a plasmid which contains the heterologous DNA sequence under the control of a strong promoter, e.g. that of the polyhedron gene, whereby this is surrounded on both sides by viral sequences. The vectors "pN113", "pN119" and "pN124" used in Example 10 are especially preferred vectors. These can be isolated according to known methods from *E. coli* strains HB101(pN113), HB101(pN119) and HB101(pN124) transformed with them. These *E. coli* strains have been deposited on the 26 Jan. 1990 at the Deutschen Sammlung von Mikroorganismen and Zellkulturen GmbH (DSM) in Braunschweig, FRG, under DSM 5762 for HB101(pN113), DSM 5763 for HB101 (pN119) and DSM 5765 for HB101(pN124). The transfer vector is then transfected into the insect cells together with DNA of the wild type baculovirus. The recombinant viruses which result in the cells by homologous recombination can then be identified and isolated according to known methods. An overview of the baculovirus expression system and the methods used therein is to be found in Luckow and Summers [52].

Expressed TNF-BP as well as its non-soluble or soluble fractions can then be purified from the cell mass or the culture supernatants according to methods of protein chemistry which are known in the state of the art, such as, for example, the procedure already described on pages 5-6.

The TNF-BP obtained in accordance with the invention can also be used as antigens to produce polyclonal and monoclonal antibodies according to known techniques [44, 45] or according to the procedure described in Example 3. Such antibodies, especially monoclonal antibodies against the 75 kD TNF-BP species, are also an object of the present invention. Those antibodies which are directed against the 75 kD TNF-BP can be used for the isolation of TNF-BP by modifications of the purification procedure described in detail in Examples 4-6 which are familiar to a person skilled in the art.

On the basis of the high binding affinity of TNF-BP in accordance with the invention for TNF (K_d value in the order of 10^{-9}-10^{-10}M), these or fragments thereof can be used as diagnostics for the detection of TNF in serum or other body fluids according to methods known in the state of the art, for example in solid phase binding tests or in combination with anti-TNF-BP antibodies in so-called "sandwich" tests.

Moreover, TNF-BP in accordance with the invention can be used on the one hand for the purification of TNF and on the other hand for the detection of TNF agonists and TNF antagonists according to procedures which are known in the state of the art.

The TNF-BP in accordance with the invention as well as their physiologically compatible salts, which can be manufactured according to methods which are known in the state of the art, can also be used for the production of pharmaceutical preparations, primarily those for the treatment of illnesses in which TNF is involved in their course. For this purpose, one or more of the said compounds, where desired or required in combination with other pharmaceutically active substances, can be processed in a known manner with the usually used solid or liquid carrier materials. The dosage of such preparations can be effected having regard to the usual criteria in analogy to already used preparations of similar activity and structure.

Since the invention has been described hereinbefore in general terms, the following Examples are intended to illustrate details of the invention, but they are not intended to limit its scope in any manner.

Example 1

Detection of TNF-Binding Proteins

The TNF-BP were detected in a filter test with human radioiodinated ^{125}I-TNF. TNF (46, 47) was radioactively labelled with Na^{125} I (IMS40, Amersham, Amersham, England) and iodo gene (#28600, Pierce Eurochemie, Oud-Beijerland, Netherlands) according to Fraker and Speck [48]. For the detection of the TNF-BP, isolated membranes of the cells or their solubilized, enriched and purified fractions were applied to moist nitrocellulose filter (0.45μ, BioRad, Richmond, Calif., USA). The filters were then blocked in buffer solution with 1% skimmed milk powder and subsequently incubated with $5 \cdot 10^5$ cpm/ml of ^{125}I-TNFα ($0.3\text{-}1.0 \cdot 10^8$ cpm/

μg) in two batches with and without the addition of 5 μg/ml of non-labelled TNFα, washed and dried in the air. The bound radioactivity was detected semiquantitatively by autoradiography or counted in a gamma-counter. The specific ^{125}I-TNF-α binding was determined after correction for unspecific binding in the presence of unlabelled TNF-α in excess. The specific TNF-binding in the filter test was measured at various TNF concentrations and analyzed according to Scatchard, whereby a K_d value of $\cdot 10^{-9}$-10^{-10}M was determined.

Example 2

Cell Extracts of HL-60 Cells

HL60 cells [ATCC No. CCL 240] were cultivated on an experimental laboratory scale in a RPMI 1640 medium [GIBCO catalogue No. 074-01800], which contained 2 g/l $NaHCO_3$ and 5% foetal calf serum, in a 5% CO_2 atmosphere and subsequently centrifuged.

The following procedure was used to produce high cell densities on an industrial scale. The cultivation was carried out in a 75 l Airlift fermenter (Fa. Chemap, Switzerland) with a working volume of 58 l. For this there was used the cassette membrane system "PROSTAK" (Millipore, Switzerland) with a membrane surface of 0.32 m^2 (1 cassette) integrated into the external circulation circuit. The culture medium (see Table 1) was pumped around with a Watson-Marlow pump, Type 603U, with 5 l/min. After a steam sterilization of the installation, whereby the "PROSTAK" system was sterilized separately in autoclaves, the fermentation was started with growing HL-60 cells from a 20 l Airlift fermenter (Chemap). The cell cultivation in the inoculation fermenter was effected in a conventional batch process in the medium according to Table 1 and an initial cell titre of 2×10^5 cells/ml. After 4 days the HL60 batch was transferred with a titre of 4.9×10^6 cells/ml into the 75 l fermenter. The pH value was held at 7.1 and the pO_2 value was held at 25% saturation, whereby the oxygen introduction was effected through a microporous frit. After initial batch fermentation, on the 2nd day the perfusion at a cell titre of 4×10^6 cells/ml was started with 30 l of medium exchange per day. On the filtrate side of the medium the conditioned medium was removed and replaced by the addition of fresh medium. The added medium was fortified as follows: Primatone from 0.25% to 0.35%, glutamine from 5 mM to 6 mM and glucose from 4 g/l to 6 g/l. The perfusion rate was then increased on the 3rd and 4th day to 72 l of medium/day and on the 5th day to 100 l of medium/day. The fermentation had finished after 120 hours of continuous cultivation. Exponential cell growth up to 40×10^6 cells/ml took place under the given fermentation conditions. The duplication time of the cell population was 20-22 hours to 10×10^6 cells/ml and then increased to 30-36 hours with increasing cell density. The proportion of living cells lay at 90-95% during the entire fermentation period. The HL-60 batch was then cooled down in the fermenter to about 12° C. and the cells were harvested by centrifugation (Beckman centrifuge [Model J-6B, Rotor JS], 3000 rpm, 10 min., 4° C.).

TABLE 1

HL-60 medium

Components	Concentrations mg/l
$CaCl_2$ (anhydrous)	112.644
$Ca(NO_3)_2 \cdot 4H_2O$	20
$CuSO_4 \cdot 5H_2O$	$0.498\cdot10^{-3}$
$Fe(NO_3)_3 \cdot 9H_2O$	0.02
$FeSO_4 \cdot 7H_2O$	0.1668
KCl	336.72
KNO_3	0.0309
$MgCl_2$ (anhydrous)	11.444
$MgSO_4$ (anhydrous)	68.37
NaCl	5801.8
Na_2HPO_4 (anhydrous)	188.408
$NaH_2PO_4 \cdot H_2O$	75
$Na_2SeO_3 \cdot 5H_2O$	$9.6\cdot10^{-3}$
$ZnSO_4 \cdot 7H_2O$	0.1726
D-Glucose	4000
Glutathion (red.)	0.2
Hepes buffer	2383.2
Hypoxanthin	0.954
Linoleic acid	0.0168
Lipoic acid	0.042
Phenol Red	10.24
Putrescine 2HCl	0.0322
Na pyruvate	88
Thymidine	0.146
Biotin	0.04666
D-Ca pantothenate	2.546
Choline chloride	5.792
Folic acid	2.86
i-Inositol	11.32
Niacinamide	2.6
Nicotinamide	0.0074
para-Aminobenzoic acid	0.2
Pyridoxal HCl	2.4124
Pyridoxin HCl	0.2
Riboflavin	0.2876
Thiamin HCl	2.668
Vitamin B_{12}	0.2782
L-Alanine	11.78
L-Aspartic acid	10
L-Asparagine H_2O	14.362
L-Arginine	40
L-Arginine HCl	92.6
L-Aspartate	33.32
L-Cystine 2HCl	62.04
L-Cysteine $HCl \cdot H_2O$	7.024
L-Glutamic acid	36.94
L-Glutamine	730
L-Glycine	21.5
L-Histidine	3
L-Histidine $HCl \cdot H_2O$	27.392
L-Hydroxypyroline	4
L-Isoleucine	73.788
L-Leucine	75.62
L-Lysine HCl	102.9
L-Methionine	21.896
L-Phenylalanine	43.592
L-Proline	26.9
L-Serine	31.3
L-Threonine	53
L-Tryptophan	11.008
L-Tyrosine•2Na	69.76
L-Valine	62.74
Penicillin/streptomycin	100 U/ml
Insulin (human)	5 μg/ml
Tranferrin (human)	15 pg/ml
Bovine serum albumin	67 pg/ml
Primatone RL (Sheffield Products, Norwich NY, USA)	0.25%
Pluronic F68 (Serva, Heidelberg, FRG)	0.01%
Foetal calf serum	0.3-3%

The centrifugate was washed with isotonic phosphate buffer (PBS; 0.2 g/l KCl, 0.2 g/l KH_2PO_4, 8.0 g/l NaCl, 2.16 g/l $Na_2HPO_4.7H_2O$), which had been treated with 5% dim-

ethylformamide, 10 mM benzamidine, 100 U/ml aprotinin, 10 μM leupeptin, 1 μM pepstatin, 1 mM o-phenanthroline, 5 mM iodoacetamide, 1 mM phenyl-methylsulphonyl fluoride (referred to hereinafter as PBS-M). The washed cells were extracted at a density of $2.5 \cdot 10^8$ cells/ml in PBS-M with Triton X-100 (final concentration 1.0%). The cell extract was clarified by centrifugation (15,000×g, 1 hour; 100,000×g, 1 hour).

Example 3

Production of Monoclonal (TNF-BP) Antibodies

A centrifugation supernatant from the cultivation of HL60 cells on an experimental laboratory scale, obtained according to Example 2, was diluted with PBS in the ratio 1:10. The diluted supernatant was applied at 4° C. (flow rate: 0.2 ml/min.) to a column which contained 2 ml of Affigel 10 (Bio Rad Catalogue No. 153-6099) to which had been coupled 20 mg of recombinant human TNF-α [Pennica, D. et al. (1984) Nature 312, 724; Shirai, T. et al. (1985) Nature 313, 803; Wang, A. M. et al. (1985) Science 228, 149] according to the recommendations of the manufacturer. The column was washed at 4° C. and a throughflow rate of 1 ml/min firstly with 20 ml of PBS which contained 0.1% Triton X 114 and thereafter with 20 ml of PBS. Thus-enriched TNF-BP was eluted at 22° C. and a flow rate of 2 ml/min with 4 ml of 100 mM glycine, pH 2.8, 0.1% decylmaltoside. The eluate was concentrated to 10 μl in a Centricon 30 unit [Amicon].

10 μl of this eluate were mixed with 20 μl of complete Freund's adjuvant to give an emulsion. 10 μl of the emulsion were injected according to the procedure described by Holmdahl, R. et al. [(1985), J. Immunol. Methods 83, 379] on each of days 0, 7 and 12 into a hind paw of a narcotized Balb/c mouse.

The immunized mice were sacrificed on day 14, the popliteal lymph nodes were removed, minced and suspended by repeated pipetting in Iscove's medium (IMEM, GIBCO Catalogue No. 074-2200) which contained 2 g/l $NaHCO_3$. According to a modified procedure of De St. Groth and Scheidegger [J. Immunol. Methods (1980), 35, 1] 5×10^7 cells of the lymph nodes were fused with 5×10^7 PAI mouse myeloma cells (J. W. Stocker et al. Research Disclosure, 217, May 1982, 155-157) which were in logarithmic growth. The cells were mixed, collected by centrifugation and resuspended in 2 ml of 50% (v/v) polyethylene glycol in IMEM at room temperature by slight shaking and diluted by the slow addition of 10 ml of IMEM during careful shaking for 10 minutes. The cells were collected by centrifugation and resuspended in 200 ml of complete medium [IMEM+20% foetal calf serum, glutamine (2.0 mM), 2-mercaptoethanol (100 μM), 100 μM hypoxanthine, 0.4 μM aminopterine and 16 μM thymidine (HAT)]. The suspension was distributed on 10 tissue culture dishes each containing 96 wells and incubated at 37° C. for 11 days without changing the medium in an atmosphere of 5% CO_2 and a relative humidity of 98%.

The antibodies are distinguished by their inhibitory action on the binding of TNF to HL60 cells or by their binding to antigens in the filter test according to Example 1. The following procedure was used to detect the biological activity of anti(TNF-BP) antibodies: 5×10^6 HL60 or U937 cells were incubated in complete RPMI 1640 medium together with affinity-purified monoclonal anti-(TNF-BP) antibodies or control antibodies (i.e. those which are not directed against TNF-BP) in a concentration range of 1 ng/ml to 10 μg/ml. After incubation at 37° C. for one hour the cells were collected by centrifugation and washed with 4.5 ml of PBS at 0° C. They were resuspended in 1 ml of complete RPMI 1640 medium (Example 2) which additionally contained 0.1% sodium azide and ^{125}I-TNFα (10^6 cpm/ml) with or without the addition of unlabelled TNFα (see above). The specific radioactivity of the ^{125}I-TNFα amounted to 700 Ci/mmol. The cells were incubated at 4° C. for 2 hours, collected and washed 4 times at 0° C. with 4.5 ml of PBS which contained 1% BSA and 0.001% Triton X 100 (Fluke). The radioactivity bound to the cells was measured in a γ-scintillation counter. The cell-bound radioactivity of cells which had not been treated with anti-(TNF-BP) antibodies was determined in a comparative experiment (approximately 10 000 cpm/5×10^6 cells).

Example 4

Affinity Chromatography

For the further purification, a monoclonal anti-(55 kD TNF-BP) antibody (2.8 mg/ml gel), obtained according to Example 3, TNFα (3.9 mg/ml gel) and bovine serum albumin (BSA, 8.5 mg/ml gel) were each covalently coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the directions of the manufacturer. The cell extract obtained according to Example 2 was passed through the thus-prepared columns which were connected in series in the following sequence: BSA-Sepharose pre-column, immune affinity column [anti-(55 kD-TNF-BP) antibody], TNFα-ligand affinity column. After completion of the application the two last-mentioned columns were separated and washed individually with in each case 100 ml of the following buffer solutions: (1) PBS, 1.0% Triton X-100, 10 mM benzamidine, 100 U/ml aprotinin; (2) PBS, 0.1% Triton X-100, 0.5M NaCl, 10 mM ATP, 10 mM benzamidine, 100 U/ml aprotinin; and (3) PBS, 0.1% Triton X-100, 10 mM benzamidine, 100 U/ml aprotinin. Not only the immune affinity column, but also the TNFα-ligand affinity column were then each eluted with 100 mM glycine pH 2.5, 100 mM NaCl, 0.2% decylmaltoside, 10 mM benzamidine, 100 U/ml aprotinin. The fractions of each column which were active in the filter test according to Example 1 were thereafter combined and neutralized with 1M Tris pH 8.0.

The thus-combined TNF-BP active fractions of the immune affinity chromatography on the one hand and of the TNFα-ligand affinity chromatography on the other hand were, for further purification, again applied to in each case one small TNFα-ligand affinity column. Thereafter, these two columns were washed with in each case 40 ml of (1) PBS, 1.0% Triton X-100, 10 mM benzamidine, 100 U/ml aprotinin, (2) PBS, 0.1% Triton X-100, 0.5M NaCl, 10 mM ATP, 10 mM benzamidine, 100 U/ml aprotinin, (3) PBS, 0.1% Triton X-100, (4) 50 mM Tris pH 7.5, 150 mM NaCl, 1.0% NP-40, 1.0% desoxycholate, 0.1% SDS, (5) PBS, 0.2% decylmaltoside. Subsequently, the columns were eluted with 100 mM glycine pH 2.5, 100 mM NaCl, 0.2% decylmaltoside. Fractions of 0.5 ml from each column were collected and the fractions from each column which were active according to the filter test (Example 1) were combined and concentrated in a Centricon unit (Amicon, molecular weight exclusion 10,000).

Example 5

Separation by Means of HPLC

The active fractions obtained according to Example 4 were each applied according to their different source (immune or

ligand affinity chromatography) to C1/C8 reversed phase HPLC-columns (ProRPC, Pharmacia, 5×20 mm) which had been equilibrated with 0.1% trifluoroacetic acid, 0.1% octylglucoside. The columns were then eluted with a linear acetonitrile gradient (0-80%) in the same buffer at a flow of 0.5 ml/min. Fractions of 1.0 ml were collected from each column and the active fractions from each column were combined (detection according to Example 1).

Example 6

Separation by Means of SDS-PAGE

The fractions which were obtained according to Example 5 and which were active according to the filter test (Example 1) were further separated by SDS-PAGE according to [34]. For this purpose, the samples were heated to 95° C. for 3 minutes in SDS sample buffer and subsequently separated electrophoretically on a 12% acrylamide separation gel with a 5% collection gel. The following standard proteins were used as a reference for the determination of the apparent molecular weights on the SDS-PAGE gel: phosphorylase B (97.4 kD), BSA (66.2 kD), ovalbumin (42.7 kD), carboanhydrase (31.0 kD), soya trypsin inhibitor (21.5 kD) and lysozyme (14.4 kD).

Under the mentioned conditions there were obtained for samples which has been obtained according to Example 4 by TNF-α-ligand affinity chromatography of immune affinity chromatography eluates and which had been further separated by HPLC according to Example 5 two bands of 55 kD and 51 kD as well as three weaker bands of 38 kD, 36 kD and 34 kD. These bands were transferred electrophoretically during 1 hour at 100 V in 25 mM Tris, 192 mM glycine, 20% methanol on to a PVDF membrane (Immobilon, Millipore, Bedford, Mass. USA) in a Mini Trans Blot System (BioRad, Richmond, Calif., USA). Thereafter, the PVDF membrane was either protein-stained with 0.15% Serva-Blue (Serva, Heidelberg, FRG) in methanol/water/glacial acetic acid (50/40/10 parts by volume) or blocked with skimmed milk powder and subsequently incubated with ^{125}I-TNFα according to the filter test conditions described in Example 1 in order to detect bands having TNF-BP activity. This showed that all bands produced in the protein staining bonded TNFα specifically. In the Western blot according to Towbin et al. [38] all of these bands also bonded the monoclonal anti-55 kD-TNF-BP antibody produced according to Example 3. In this case, a procedure according to that described in Example 1 with Na^{125}I radioactively-labelled, affinity-purified (mouse immunoglobulin-Sepharose-4B affinity column) rabbit-anti-mouse-immunoglobulin antibody was used for the autoradiographic detection of this antibody.

Samples which had been obtained according to Example 4 by two-fold TNF-α-ligand affinity chromatography of the throughput of the immune affinity chromatography and which had been further separated by HPLC according to Example 5 showed under the above-specified SDS-PAGE and blot transfer conditions two additional bands of 75 kD and 65 kD, both of which bonded TNF specifically in the filter test (Example 1). In the Western blot according to Towbin et al. (see above) the proteins of these two bands did not react with the anti-(55 kD TNF-BP) antibody produced according to Example 3. They reacted, however, with a monoclonal antibody which had been produced starting from the 75 kD band (anti-75 kD TNF-BP antibody) according to Example 3.

Example 7

Amino Acid Sequence Analysis

For the amino acid sequence analysis, the fractions which had been obtained according to Example 5 and which were active according to the filter test (Example 1) were separated using the SDS-PAGE conditions described in Example 6, but now reducing (SDS sample buffer with 125 mM dithiothreitol). The same bands as in Example 6 were found, but because of the reducing conditions of the SDS-PAGE in comparison to Example 6 all showed an about 1-2 kD higher molecular weight. These bands were then transferred according to Example 6 on to PVDF membranes and stained with 0.15% Serva-Blue in methanol/water/glacial acetic acid (50/400/10 parts by volume) for 1 minute, decolorized with methanol/water/glacial acetic acid (45/48/7 parts by volume), rinsed with water, dried in air and thereafter cut out. The conditions given by Hunkapiller [34] were adhered to in all steps in order to avoid N-terminal blocking. Initially, the purified TNF-BP were used unaltered for the amino acid sequencing. In order to obtain additional sequence information, the TNF-BP after reduction and S-carboxymethylation [Jones, B. N. (1986) in "Methods of Protein Micro-characterisation", J. E. Shively, ed., Humana Press, Clifton N. J., 124-125] were cleaved with cyanogen bromide (Tarr, G. E. in "Methods of Protein Micro-characterisation", 165-166, loc. cit.), trypsin and/or proteinase K and the peptides were separated by HPLC according to known methods of protein chemistry. Thus-prepared samples were then sequenced in an automatic gas phase microsequencing apparatus (Applied Biosystems Model 470A, ABI, Foster City, Calif., USA) with an on-line automatic HPLC PTH amino acid analyzer (Applied Biosystems Model 120, ABI see above) connected to the outlet, whereby the following amino acid sequences were determined:

1. For the 55 kD band (according to non-reducing SDS-PAGE):

   Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu-Lys-Arg-Asp-Ser-Val-Cys-Pro-Gln-Gly-Lys-Tyr-Ile-His-Pro-Gln-X-Asn-Ser-Ile (SEQ ID NO: 5),

   and

   Ser-Thr-Pro-Glu-Lys-Glu-Gly-Glu-Leu-Glu-Gly-Thr-Thr-Thr-Lys (SEQ ID NO: 6) in which X stands for an amino acid residue which could not be determined,

2. for the 5110 and 38 kD bands (according to non-reducing SDS-PAGE):

   Leu-Val-Pro-His-Leu-Gly-Asp-Arg-Glu (SEQ ID NO: 15)

3. for the 65 kD band (according to non-reducing SDS-PAGE)

   In the N-terminal sequencing of the 65 kD band two parallel sequences were determined up to the 15th residue without interruption. Since one of the two sequences corresponded to a partial sequence of ubiquitin [36, 37], the following sequence was derived for the 65 kD band:

   Leu-Pro-Ala-Gln-Val-Ala-Phe-X-Pro-Tyr-Ala-Pro-Glu-Pro-Gly-Ser-Thr-Cys. (SEQ ID NO: 16)

   in which X stands for an amino acid residue which could not be determined.

Additional peptide sequences for 75(65) kDa-TNF-BP were determined:

Ile-X-Pro-Gly-Phe-Gly-Val-Ala-Tyr-Pro-Ala-Leu-Glu (SEQ ID NO: 11)

and

Ser-Gln-Leu-Glu-Thr-Pro-Glu-Thr-Leu-Leu-Gly-Ser-Thr-Glu-Glu-Lys-Pro-Leu (SEQ ID NO: 7) and Val-Phe-Cys-Thr (SEQ ID NO: 8)

and

Asn-Gln-Pro-Gln-Ala-Pro-Gly-Val-Glu-Ala-Ser-Gly-Ala-Gly-Glu-Ala (SEQ ID NO: 9) and Leu-Cys-Ala-Pro (SEQ ID NO: 12)

and

Val-Pro-His-Leu-Pro-Ala-Asp SEQ ID NO: 13)

and

Gly-Ser-Gln-Gly-Pro-Glu-Gln-Gln-X-X-Leu-Ile-X-Ala-Pro (SEQ ID NO: 14),

in which X stands for an amino acid residue which could not be determined.

Example 8

Determination of Base Sequences of Complementary DNA (cDNA)

Starting from the amino acid sequence according to formula IA there were synthesized having regard to the genetic code for the amino acid residues 2-7 and 17-23 corresponding completely degenerated oligonucleotides in suitable complementarity ("sense" and "antisense" oligonucleotides). Total cellular RNA was isolated from HL60 cells [42, 43] and the first cDNA strand was synthesized by oligo-dT priming or by priming with the "antisense" oligonucleotide using a cDNA synthesis kit (RPN 1256, Amersham, Amersham, England) according to the instructions of the manufacturer. This cDNA strand and the two synthesized degenerate "sense" and "antisense" oligonucleotides were used in a polymerase chain reaction (PCR, Perkin Elmer Cetus, Norwalk, Conn., USA according to the instructions of the manufacturer) to synthesize as a cDNA fragment the base sequence coding for the amino acid residues 8-16 (formula IA). The base sequence of this cDNA fragment accorded to: 5'-AGGGAGAAGAGAGATAGTGTGTGTCCC-3' (SEQ ID NO: 16). This cDNA fragment was used as a probe in order to identify according to a known procedure a cDNA clone coding for the 55 kD TNF-BP in a λgt11-cDNA gene bank from human placenta (42, 43). This clone was then cut according to usual methods from the λ-vector and cloned in the plasmids pUC18 (Pharmacia, Uppsala, Sweden) and pUC19 (Pharmacia, Uppsala, Sweden) and in the M13 mp18/M13 mp19 bacteriophage (Pharmacia, Uppsala, Sweden) (42, 43). The nucleotide sequence of this cDNA clone was determined using a Sequenase kit (U.S. Biochemical, Cleveland, Ohio, USA) according to the details of the manufacturer. The nucleotide sequence and the amino acid sequence derived therefrom for the 55 kD TNF-BP and its signal peptide (amino acid "−28" to amino acid "O") is given in FIG. **1** using the abbreviations for bases such as amino acids usual in the state of the art. From sequence comparisons with other already known receptor protein sequences there can be determined a N-terminal domain containing approximately 180 amino acids and a C-terminal domain containing 220 amino acids which are separated from one another by a transmembrane region of 19 amino acids (underlined in FIG. **1**) which is typical according to the sequence comparisons. Hypothetical glycosylation sites are characterized in FIG. **1** by asterisks above the corresponding amino acid.

Essentially analogous techniques were used to identify 75/65 kD TNF-BP-coding partial cDNA sequences, whereby, however, in this case genomic human DNA and completely degenerated 14-mer and 15-mer "sense" and "antisense" oligonucleotides derived from peptide IIA were used in order to produce a primary 26 by cDNA probe in a polymerase chain reaction. This cDNA probe was then used in a HL-60 cDNA library to identify cDNA clones of different lengths. This cDNA library was produced using isolated HL60 RNA and a cDNA cloning kit (Amersham) according to the details of the manufacturer. The sequence of such a cDNA clone is given in FIG. **4** (SEQ ID NO: 28), whereby repeated sequencing lead to the following correction as depicted in FIG. **6** (SEQ ID NO: 3). A threonine coded by "ACC" not "TCC", has to be at position 3 instead of the serine.

Example 9

Expression in COS1 Cells

Vectors starting from the plasmid "pN11" were constructed for the expression in COS cells. The plasmid "pN11" contains the efficient promotor and enhancer of the "major immediate-early" gene of human cytomegalovirus ("HCMV"; Boshart et al., Cell 41, 521-530, 1985). After the promotor there is situated a short DNA sequence which contains several restriction cleavage sites, which are present only once in the plasmid ("polylinker"), inter alia the cleavage sites for HindIII, BalI, BamHI and PvuII (see sequence).

PvuII

5'-AAGCTTGGCCAGGATCCAGCTGACT-
GACTGATCGCGAGATC-3' (SEQ ID NO: 17)
3'-TTCGAACCGGTCCTAGGTCGACTGACT-
GACTAGCGCTCTAG-5' (SEQ ID NO: 18)

After these cleavage sites there are situated three translation stop codons in all three reading frames. After the polylinker-sequence there is situated the 2nd intron and the polyadenylation signal of the preproinsulin gene of the rat (Lomedico et al., Cell 18, 545-558, 1979). The plasmid also contains the replication origin of the SV40 virus and a fragment from pBR322 which confers *E. coli*-bacteria ampicillin resistance and permits the replication of the plasmid in *E. coli*.

For the construction of the expression vector "pN123", this plasmid "pN11" was cleaved the restriction endonuclease PvuII and subsequently treated with alkaline phosphatase. The dephosphorylated vector was thereafter isolated from an agarose gel (V1). The 5'-projecting nucleotides of the EcoRI-cleaved 1.3 kb fragment of the 55 kD TNF-BP-cDNA (see Example 8) were filled in using Klenow enzyme. Subsequently, this fragment was isolated from an agarose gel (F1). Thereafter, V1 and F1 were joined together using T4-ligase. *E. coli* HB101 cells were then transformed with this ligation batch according to known methods [42]. By means of restriction analyses and DNA sequencing according to known methods [42] there were identified transformants which had been transformed with a plasmid and which contained the 1.3 kb EcoRI fragment of the 55 kD TNF-BP-cDNA in the correct orientation for expression via the HCMV-promoter. This vector received the designation "pN123".

The following procedure was used for the construction of the vector "pK19". A DNA fragment which contained only the cDNA coding for the extracellular part of the 55 kD TNF-BP (amino acids −28 to 182 according to FIG. **1**) was obtained by PCR technology (Saiki et al., Science 230, 1350-1354, 1985, see also Example 8). The following oligonucleotides were used in order to amplify the cDNA from "pN123" coding for the extracellular part of the 55 kD TNF-BP:

BAMHI

5'-CACAGGGATCCATAGCTGTCTG-
GCATGGGCCTCTCCAC-3' (SEQ ID NO: 19)

ASP718

3'-CGTGACTCCTGAGTCCGTGGTGTAT-
TATCTCTAGACCA TGGCCC-5' (SEQ ID NO: 20)

By means of these oligonucleotides there were also introduced two stop codons of the translation after amino acid 182. The thus-amplified DNA fragment was cleaved with BamHI and Asp718, the thereby resulting projecting ends were filled in using Klenow enzyme and this fragment was subsequently isolated from an agarose gel (F2). F2 was then ligated with V1 and the entire batch was used for the transformation of *E. coli* HB101, as already described. Transformants which had been transformed with a plasmid containing the DNA fragment in the correct orientation for the expression via the HCMV-

promoter were identified by DNA sequencing (see above). The plasmid isolated therefrom received the designation "pK19".

Transfection of the COS cells with the plasmids "pN123" or "pK19" was carried out according to the lipofection method published by Felgner et al. (Proc. Natl. Acad. Sci. USA 84, 7413-7417, 1987). 72 hours after the transfection had been effected the cells transfected with "pN123" were analyzed for binding with ^{125}I-TNFα according to known methods. The results of the Scatchard analysis [Scatchard, G., Ann. N.Y. Acad. Sci. 51, 660, 1949] of the thus-obtained binding data (FIG. **2**A) is given in FIG. **2**B. The culture supernatants of the cells transfected with "pK19" were investigated in a "sandwich" test. For this purpose, PVC microtitre plates (Dynatech, Arlington, Va., USA) were sensitized with 100 µl/well of a rabbit-anti-mouse immunoglobulin (10 µg/ml PBS). Subsequently, the plates were washed and incubated (3 hours, 20° C.) with an anti-55 kD TNF-BP antibody which had been detected by its antigen binding and isolated according to Example 3, but which did not inhibit the TNF-binding to cells. The plates were then again washed and incubated overnight at 4° C. with 100 µl/well of the culture supernatant (diluted 1:4 with buffer A containing 1% skimmed milk powder: 50 mM Tris/HCl pH 7.4, 140 mM NaCl, 5 mM EDTA, 0.02% Na azide). The plates were emptied and incubated at 4° C. for 2 hours with buffer A containing ^{125}I-TNFα (10^6 cpm/ml, 100 µl/well) with or without the addition of 2 µg/ml of unlabelled TNF. Thereafter, the plates were washed 4 times with PBS, the individual wells were cut out and measured in a λ-counter. The results of 5 parallel transfections (columns #2, 3, 4, 6 and 7), of two control transfections with the pN11 vector (columns #1, 5) and of a control with HL60 cell lysate (column #8) are given in FIG. **3**.

Example 10

Expression in Insect Cells

The plasmid "pVL941" (Luckow and Summers, 1989, "High Level Expression of Nonfused Foreign Genes with *Autographa california* Nuclear Polyhedrosis virus Expression Vectors", Virology 170, 31-39) was used for the expression in a baculovirus expression system and was modified as follows. The single EcoRI restriction cleavage site in "pVL941" was removed by cleaving the plasmid with EcoRI and the projecting 5'-end was filled in with Klenow enzyme. The plasmid pVL941/E obtained therefrom was digested with BamHI and Asp718 and the vector trunk was subsequently isolated from an agarose gel. This fragment was ligated with a synthetic oligonucleotide of the following sequence:

BamHI EcoRI Asp718

5'-GATCCAGAATTCATAATAG-3' (SEQ ID NO: 21)

3'-GTCTTAAGTATTATCCATG-5' (SEQ ID NO: 22)

E. coli HB101 was transformed with the ligation batch and transformants containing a plasmid in which the oligonucleotide had been incorporated correctly were identified by restriction analysis and DNA sequencing according to known methods (see above); this plasmid was named "pNR704". For the construction of the transfer vector "pN113", this plasmid "pNR704" was cleaved with EcoRI, treated with alkaline phosphatase and the thus-produced vector trunk (V2) was subsequently isolated from an agarose gel. The 1.3 kb fragment of the 55 kD TNF-BP-cDNA cleaved with EcoRI as above was ligated with fragment V2. Transformants obtained with this ligation batch, which contained a plasmid containing the cDNA insert in the correct orientation for the expression via the polyhedron promoter, were identified (see above). The vector isolated therefrom received the designation "pN113".

The following procedure was used for the construction of the transfer vector "pN119". The 1.3 kb EcoRI/EcoRI fragment of the 55 kD TNF-BP cDNA in the "pUC19" plasmid (see Example 8) was digested with BanI and ligated with the following synthetic oligonucleotide:

BanI Asp718

5'-GCACCACATAATAGAGATCTGGTACCGGGAA-3' (SEQ ID NO: 23)

3'-GTGTATTATCTCTAGACCATGGCCC-5' (SEQ ID NO: 24)

Two stop codons of the translation after amino acid 182 and a cleavage site for the restriction endonuclease Asp718 are incorporated with the above adaptor. After carrying out ligation the batch was digested with EcoRI and Asp718 and the partial 55 kD TNF-BP fragment (F3) was isolated. Furthermore, the plasmid "pNR704", likewise cleaved with Asp718 and EcoRI, was ligated with F3 and the ligation batch was transformed into *E. coli* HB101. The identification of the transformants which contained a plasmid in which the partial 55 kD TNF-BP cDNA had been correctly integrated for the expression was effected as already described. The plasmid isolated from these transformants received the name "pN119".

The following procedure was used for the construction of the transfer vector "pN124". The cDNA fragment coding for the extracellular part of the 55 kD TNF-BP, described in Example 9, was amplified with the specified oligonucleotides with the aid of PCR technology as described in Example 9. This fragment was cleaved with BamHI and Asp718 and isolated from an agarose gel (F4). The plasmid "pNR704" was also cleaved with BamHI and Asp718 and the vector trunk (V4) was isolated (see above). The fragments V4 and F4 were ligated, *E. coli* HB101 was transformed therewith and the recombinant transfer vector "pN124" was identified and isolated as described.

The following procedure was used for the transfection of the insect cells. 3 µg of the transfer vector "pN113" were transfected with 1 µg of DNA of the *Autographa californica* nuclear polyhedrosisvirus (AcMNPV) (EP 127839) in Sf9 cells (ATCC CRL 1711). Polyhedron-negative viruses were identified and purified from "plaques" [52]. Sf9 cells were again infected with these recombinant viruses as described in [52]. After 3 days in the culture the infected cells were investigated for TNF-binding using ^{125}I-TNFα. For this purpose, the transfected cells were washed from the cell culture dish with a Pasteur pipette and resuspended at a cell density of 5×10^6 cells/ml of culture medium [52] which contained 10 ng/ml of ^{125}I-TNF-α, not only in the presence of, but also in the absence of 5 µg/ml of non-labelled TNF-α and incubated on ice for 2 hours. Thereafter, the cells were washed with pure culture medium and the cell-bound radioactivity was counted in a γ-counter (see Table 2).

TABLE 2

Cells	Cell-bound radioactivity per 10^6 cells
Non-infected cells (control)	60 cpm
Infected cells	1600 ± 330 cpm [1)]

[1)] Average and standard deviation from 4 experiments

Example 11

Analogously to the procedure described in Example 9, the cDNA fragment coding for the extracellular region of the 55

kDa TNF-BP was amplified in a polymerase chain reaction, but now using the following oligonucleotides as the primer:

Oligonucleotide 1:

Sst I

5'-TAC GAG CTC GGC CAT AGC TGT CTG GCA TG-3' (SEQ ID NO: 25)

Oligonucleotide 2:

Sst I

5'-ATA GAG CTC TGT GGT GCC TGA GTC CTC AG-3' (SEQ ID NO: 26)

This cDNA fragment was ligated in the pCD4-Hγ3 vector [DSM 5523; European Patent Application No. 90107393.2; Japanese Patent Application No. 108967/90; U.S. patent application Ser. No. 51/077,390] from which the CD4-cDNA had been removed via the SstI restriction cleavage sites. SstI cleavage sites are situated in vector pCD4-Hγ3 not only in front of, but also behind the CD4-partial sequence fragment. The construction was transfixed in J558 myeloma cells (ATCC No. TIB6) by means of protoplast fusion according to Oi et al. (Procd. Natl. Acad. Sci. USA 80, 825-829, 1983). Transfectants were selected by adding 5 μg/ml of mycophenolic acid and 250 μg/ml of xanthin (Traunecker et al., Eur. J. Immunol. 16, 851-854 [1986]) in basic medium (Dulbecco's modified Eagle's Medium, 10% foetal calf serum, 5×10^{-5}M 2-mercaptoethanol). The expression product secreted by the transfixed cells could be purified using usual methods of protein chemistry, e.g. TNF-BP-antibody affinity chromatography. Unless not already specifically indicated, standard procedures as described e.g. by Freshney, R. I. in "Culture of Animal Cells", Alan R. Liss, Inc., New York (1983) were used for the cultivation of the cell lines employed, for the cloning, for the selection or for the expansion of the cloned cells.

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tctggcatgg gcctctccac cgtgcctgac ctgctgctgc cgctggtgct cctggagctg 240

ttggtgggaa tatacccctc aggggttatt ggactggtcc ctcacctagg ggacagggag 300

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acggactgca gggagtgtga gagcggctcc ttcaccgctt cagaaaacca cctcagacac 480

tgcctcagct gctccaaatg ccgaaaggaa atgggtcagg tggagatctc ttcttgcaca 540

gtggaccggg acaccgtgtg tggctgcagg aagaaccagt accggcatta ttggagtgaa 600

aacctttcc agtgcttcaa ttgcagcctc tgcctcaatg ggaccgtgca cctctcctgc 660

caggagaaac agaacaccgt gtgcacctgc catgcaggtt tctttctaag agaaaacgag 720

tgtgtctcct gtagtaactg taagaaaagc ctggagtgca cgaagttgtg cctaccccag 780

attgagaatg ttaagggcac tgaggactca ggcaccacag tgctgttgcc cctggtcatt 840

ttctttggtc tttgcctttt atccctcctc ttcattggtt taatgtatcg ctaccaacgg 900

tggaagtcca agctctactc cattgtttgt gggaaatcga cacctgaaaa agaggggggag 960

cttgaaggaa ctactactaa gcccctggcc ccaaacccaa gcttcagtcc cactccaggc 1020

ttcaccccca ccctgggctt cagtcccgtg cccagttcca ccttcacctc cagctccacc 1080

tatacccccg gtgactgtcc caactttgcg gctccccgca gagaggtggc accaccctat 1140

caggggggctg accccatcct tgcgacagcc ctcgcctccg accccatccc caaccccctt 1200

cagaagtggg aggacagcgc ccacaagcca cagagcctag acactgatga ccccgcgacg 1260

ctgtacgccg tggtggagaa cgtgcccccg ttgcgctgga aggaattcgt gcggcgccta 1320

gggctgagcg accacgagat cgatcggctg gagctgcaga acgggcgctg cctgcgcgag 1380

gcgcaataca gcatgctggc gacctggagg cggcgcacgc cgcggcgcga ggccacgctg 1440

gagctgctgg gacgcgtgct ccgcgacatg gacctgctgg gctgcctgga ggacatcgag 1500

gaggcgcttt gcggccccgc cgccctcccg cccgcgccca gtcttctcag atgaggctgc 1560

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gcccctgcgg gcagctctaa ggaccgtcct gcgagatcgc cttccaaccc cacttttttc 1620

tggaaaggag gggtcctgca ggggcaagca ggagctagca gccgcctact tggtgctaac 1680

ccctcgatgt acatagcttt tctcagctgc ctgcgcgccg ccgacagtca gcgctgtgcg 1740

cgcggagaga ggtgcgccgt gggctcaaga gcctgagtgg gtggtttgcg aggatgaggg 1800

acgctatgcc tcatgcccgt tttgggtgtc ctcaccagca aggctgctcg ggggcccctg 1860

gttcgtcccc gagcctttt cacagtgcat aagcagtttt ttttgttttt gttttgtttt 1920

gttttgtttt taaatcaatc atgttacact aatagaaact tggcactcct gtgccctctg 1980

cctggacaag cacatagcaa gctgaactgt cctaaggcag gggcgagcac ggaacaatgg 2040

ggccttcagc tggagctgtg gacttttgta catacactaa aattctgaag ttaaaaaaaa 2100

aacccgaatt c 2111

<210> SEQ ID NO 2
<211> LENGTH: 455
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 2

Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu Leu
1 5 10 15

Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu Val Pro
20 25 30

His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys
35 40 45

Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr Lys Cys His Lys
50 55 60

Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp
65 70 75 80

Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr Ala Ser Glu Asn His Leu
85 90 95

Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val
100 105 110

Glu Ile Ser Ser Cys Thr Val Asp Arg Asp Thr Val Cys Gly Cys Arg
115 120 125

Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe
130 135 140

Asn Cys Ser Leu Cys Leu Asn Gly Thr Val His Leu Ser Cys Gln Glu
145 150 155 160

Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu
165 170 175

Asn Glu Cys Val Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr
180 185 190

Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser
195 200 205

Gly Thr Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu
210 215 220

Leu Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
225 230 235 240

Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu
245 250 255

Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn Pro Ser
260 265 270

Phe Ser Pro Thr Pro Gly Phe Thr Pro Thr Leu Gly Phe Ser Pro Val

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        275                 280                 285

Pro Ser Ser Thr Phe Thr Ser Ser Ser Thr Tyr Thr Pro Gly Asp Cys
    290                 295                 300

Pro Asn Phe Ala Ala Pro Arg Arg Glu Val Ala Pro Pro Tyr Gln Gly
305                 310                 315                 320

Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala Ser Asp Pro Ile Pro Asn
                325                 330                 335

Pro Leu Gln Lys Trp Glu Asp Ser Ala His Lys Pro Gln Ser Leu Asp
            340                 345                 350

Thr Asp Asp Pro Ala Thr Leu Tyr Ala Val Val Glu Asn Val Pro Pro
        355                 360                 365

Leu Arg Trp Lys Glu Phe Val Arg Arg Leu Gly Leu Ser Asp His Glu
    370                 375                 380

Ile Asp Arg Leu Glu Leu Gln Asn Gly Arg Cys Leu Arg Glu Ala Gln
385                 390                 395                 400

Tyr Ser Met Leu Ala Thr Trp Arg Arg Arg Thr Pro Arg Arg Glu Ala
                405                 410                 415

Thr Leu Glu Leu Leu Gly Arg Val Leu Arg Asp Met Asp Leu Leu Gly
            420                 425                 430

Cys Leu Glu Asp Ile Glu Glu Ala Leu Cys Gly Pro Ala Ala Leu Pro
        435                 440                 445

Pro Ala Pro Ser Leu Leu Arg
    450                 455


<210> SEQ ID NO 3
<211> LENGTH: 2339
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

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cccgagtgct tgagctgtgg ctcccgctgt agctctgacc aggtggaaac tcaagcctgc    120

actcgggaac agaaccgcat ctgcacctgc aggcccggct ggtactgcgc gctgagcaag    180

caggaggggt gccggctgtg cgcgccgctg ccgaagtgcc gcccgggctt cggcgtggcc    240

agaccaggaa ctgaaacatc agacgtggtg tgcaagccct gtgccccggg gacgttctcc    300

aacacgactt catccacgga tatttgcagg ccccaccaga tctgtaacgt ggtggccatc    360

cctgggaatg caagcaggga tgcagtctgc acgtccacgt cccccacccg gagtatggcc    420

ccaggggcag tacacttacc ccagccagtg tccacacgat cccaacacac gcagccaagt    480

ccagaaccca gcactgctcc aagcacctcc ttcctgctcc caatgggccc cagcccccca    540

gctgaaggga gcactggcga cttcgctctt ccagttggac tgattgtggg tgtgacagcc    600

ttgggtctac taataatagg agtggtgaac tgtgtcatca tgacccaggt gaaaaagaag    660

cccttgtgcc tgcagagaga agccaaggtg cctcacttgc ctgccgataa ggcccggggt    720

acacagggcc ccgagcagca gcacctgctg atcacagcgc cgagctccag cagcagctcc    780

ctggagagct cggccagtgc gttggacaga agggcgccca ctcggaacca gccacaggca    840

ccaggcgtgg aggccagtgg ggccggggag gcccgggcca gcaccgggag ctcagcagat    900

tcttcccctg gtggccatgg gacccaggtc aatgtcacct gcatcgtgaa cgtctgtagc    960

agctctgacc acagctcaca gtgctcctcc caagccagct ccacaatggg agacacagat   1020

tccagccccc cggagtcccc gaaggacgag caggtccccc tctccaagga ggaatgtgcc   1080

tttcggtcac agctggagac gccagagacc ctgctgggga gcaccgaaga gaagcccctg   1140
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cccttggag tgcctgatgc tgggatgaag cccagttaac caggccggtg tgggctgtgt   1200

cgtagccaag gtggctgagc cctggcagga tgaccctgcg aaggggccct ggtccttcca   1260

ggcccccacc actaggactc tgaggctctt tctgggccaa gttcctctag tgccctccac   1320

agccgcagcc tccctctgac ctgcaggcca agagcagagg cagcgagttg tggaaagcct   1380

ctgctgccat ggcgtgtccc tctcggaagg ctggctgggc atggacgttc ggggcatgct   1440

ggggcaagtc cctgagtctc tgtgacctgc cccgcccagc tgcacctgcc agcctggctt   1500

ctggagccct tgggtttttt gtttgtttgt ttgtttgttt gtttgtttct ccccctgggc   1560

tctgcccagc tctggcttcc agaaaacccc agcatccttt tctgcagagg ggctttctgg   1620

agaggaggga tgctgcctga gtcacccatg aagacaggac agtgcttcag cctgaggctg   1680

agactgcggg atggtcctgg ggctctgtgc agggaggagg tggcagccct gtagggaacg   1740

gggtccttca agttagctca ggaggcttgg aaagcatcac ctcaggccag gtgcagtggc   1800

tcacgcctat gatcccagca ctttgggagg ctgaggcggg tggatcacct gaggttagga   1860

gttcgagacc agcctggcca acatggtaaa accccatctc tactaaaaat acagaaatta   1920

gccgggcgtg gtggcgggca cctatagtcc cagctactca gaagcctgag gctgggaaat   1980

cgtttgaacc cgggaagcgg aggttgcagg gagccgagat cacgccactg cactccagcc   2040

tgggcgacag agcgagagtc tgtctcaaaa gaaaaaaaaa aagcaccgcc tccaaatgct   2100

aacttgtcct tttgtaccat ggtgtgaaag tcagatgccc agagggccca ggcaggccac   2160

catattcagt gctgtggcct gggcaagata acgcacttct aactagaaat ctgccaattt   2220

tttaaaaaag taagtaccac tcaggccaac aagccaacga caaagccaaa ctctgccagc   2280

cacatccaac cccccacctg ccatttgcac cctccgcctt cactccggtg tgcctgcag    2339
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<210> SEQ ID NO 4
<211> LENGTH: 392
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

```
Ser Asp Thr Val Cys Asp Ser Cys Glu Asp Ser Thr Tyr Thr Gln Leu
1               5                   10                  15

Trp Asn Trp Val Pro Glu Cys Leu Ser Cys Gly Ser Arg Cys Ser Ser
            20                  25                  30

Asp Gln Val Glu Thr Gln Ala Cys Thr Arg Glu Gln Asn Arg Ile Cys
        35                  40                  45

Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu Ser Lys Gln Glu Gly Cys
    50                  55                  60

Arg Leu Cys Ala Pro Leu Pro Lys Cys Arg Pro Gly Phe Gly Val Ala
65                  70                  75                  80

Arg Pro Gly Thr Glu Thr Ser Asp Val Val Cys Lys Pro Cys Ala Pro
                85                  90                  95

Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr Asp Ile Cys Arg Pro His
            100                 105                 110

Gln Ile Cys Asn Val Val Ala Ile Pro Gly Asn Ala Ser Arg Asp Ala
        115                 120                 125

Val Cys Thr Ser Thr Ser Pro Thr Arg Ser Met Ala Pro Gly Ala Val
    130                 135                 140

His Leu Pro Gln Pro Val Ser Thr Arg Ser Gln His Thr Gln Pro Ser
145                 150                 155                 160

Pro Glu Pro Ser Thr Ala Pro Ser Thr Ser Phe Leu Leu Pro Met Gly
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                165                 170                 175

Pro Ser Pro Pro Ala Glu Gly Ser Thr Gly Asp Phe Ala Leu Pro Val
            180                 185                 190

Gly Leu Ile Val Gly Val Thr Ala Leu Gly Leu Leu Ile Ile Gly Val
        195                 200                 205

Val Asn Cys Val Ile Met Thr Gln Val Lys Lys Lys Pro Leu Cys Leu
    210                 215                 220

Gln Arg Glu Ala Lys Val Pro His Leu Pro Ala Asp Lys Ala Arg Gly
225                 230                 235                 240

Thr Gln Gly Pro Glu Gln Gln His Leu Leu Ile Thr Ala Pro Ser Ser
                245                 250                 255

Ser Ser Ser Ser Leu Glu Ser Ser Ala Ser Ala Leu Asp Arg Arg Ala
            260                 265                 270

Pro Thr Arg Asn Gln Pro Gln Ala Pro Gly Val Glu Ala Ser Gly Ala
        275                 280                 285

Gly Glu Ala Arg Ala Ser Thr Gly Ser Ser Ala Asp Ser Ser Pro Gly
    290                 295                 300

Gly His Gly Thr Gln Val Asn Val Thr Cys Ile Val Asn Val Cys Ser
305                 310                 315                 320

Ser Ser Asp His Ser Ser Gln Cys Ser Ser Gln Ala Ser Ser Thr Met
                325                 330                 335

Gly Asp Thr Asp Ser Ser Pro Ser Glu Ser Pro Lys Asp Glu Gln Val
            340                 345                 350

Pro Phe Ser Lys Glu Glu Cys Ala Phe Arg Ser Gln Leu Glu Thr Pro
        355                 360                 365

Glu Thr Leu Leu Gly Ser Thr Glu Glu Lys Pro Leu Pro Leu Gly Val
    370                 375                 380

Pro Asp Ala Gly Met Lys Pro Ser
385                 390


<210> SEQ ID NO 5
<211> LENGTH: 28
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (25)..(25)
<223> OTHER INFORMATION: Xaa = unknown amino acid

<400> SEQUENCE: 5

Leu Val Pro His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro
1               5                   10                  15

Gln Gly Lys Tyr Ile His Pro Gln Xaa Asn Ser Ile
            20                  25


<210> SEQ ID NO 6
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 6

Ser Thr Pro Glu Lys Glu Gly Glu Leu Glu Gly Thr Thr Thr Lys
1               5                   10                  15


<210> SEQ ID NO 7
<211> LENGTH: 18
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<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 7

Ser Gln Leu Glu Thr Pro Glu Thr Leu Leu Gly Ser Thr Glu Glu Lys
1 5 10 15

Pro Leu

<210> SEQ ID NO 8
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 8

Val Phe Cys Thr
1

<210> SEQ ID NO 9
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 9

Asn Gln Pro Gln Ala Pro Gly Val Glu Ala Ser Gly Ala Gly Glu Ala
1 5 10 15

<210> SEQ ID NO 10
<211> LENGTH: 18
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: Xaa = unknown amino acid

<400> SEQUENCE: 10

Leu Pro Ala Gln Val Ala Phe Xaa Pro Tyr Ala Pro Glu Pro Gly Ser
1 5 10 15

Thr Cys

<210> SEQ ID NO 11
<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (2)..(2)
<223> OTHER INFORMATION: Xaa = unknown amino acid

<400> SEQUENCE: 11

Ile Xaa Pro Gly Phe Gly Val Ala Tyr Pro Ala Leu Glu
1 5 10

<210> SEQ ID NO 12
<211> LENGTH: 4
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:

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<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 12

Leu Cys Ala Pro
1

<210> SEQ ID NO 13
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 13

Val Pro His Leu Pro Ala Asp
1 5

<210> SEQ ID NO 14
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (9)..(10)
<223> OTHER INFORMATION: Xaa = unknown amino acid
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (13)..(13)
<223> OTHER INFORMATION: Xaa = unknown amino acid

<400> SEQUENCE: 14

Gly Ser Gln Gly Pro Glu Gln Gln Xaa Xaa Leu Ile Xaa Ala Pro
1 5 10 15

<210> SEQ ID NO 15
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic peptide

<400> SEQUENCE: 15

Leu Val Pro His Leu Gly Asp Arg Glu
1 5

<210> SEQ ID NO 16
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 16

agggagaaga gagatagtgt gtgtccc 27

<210> SEQ ID NO 17
<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 17

aagcttggcc aggatccagc tgactgactg atcgcgagat c 41

<210> SEQ ID NO 18

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<211> LENGTH: 41
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense primer

<400> SEQUENCE: 18

gatctcgcga tcagtcagtc agctggatcc tggccaagct t 41

<210> SEQ ID NO 19
<211> LENGTH: 38
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 19

cacagggatc catagctgtc tggcatgggc ctctccac 38

<210> SEQ ID NO 20
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense primer

<400> SEQUENCE: 20

cccggtacca gatctctatt atgtggtgcc tgagtcctca gtgc 44

<210> SEQ ID NO 21
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 21

gatccagaat tcataatag 19

<210> SEQ ID NO 22
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense primer

<400> SEQUENCE: 22

gtacctatta tgaattctg 19

<210> SEQ ID NO 23
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 23

gcaccacata tagagatct ggtaccggga a 31

<210> SEQ ID NO 24
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Antisense primer

<400> SEQUENCE: 24

cccggtacca gatctctatt atgtg 25

<210> SEQ ID NO 25
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 25

tacgagctcg gccatagctg tctggcatg 29

<210> SEQ ID NO 26
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial sequence
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic primer

<400> SEQUENCE: 26

atagagctct gtggtgcctg agtcctcag 29

<210> SEQ ID NO 27
<211> LENGTH: 461
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 27

Met Ala Pro Val Ala Val Trp Ala Ala Leu Ala Val Gly Leu Glu Leu
1 5 10 15

Trp Ala Ala Ala His Ala Leu Pro Ala Gln Val Ala Phe Thr Pro Tyr
20 25 30

Ala Pro Glu Pro Gly Ser Thr Cys Arg Leu Arg Glu Tyr Tyr Asp Gln
35 40 45

Thr Ala Gln Met Cys Cys Ser Lys Cys Ser Pro Gly Gln His Ala Lys
50 55 60

Val Phe Cys Thr Lys Thr Ser Asp Thr Val Cys Asp Ser Cys Glu Asp
65 70 75 80

Ser Thr Tyr Thr Gln Leu Trp Asn Trp Val Pro Glu Cys Leu Ser Cys
85 90 95

Gly Ser Arg Cys Ser Ser Asp Gln Val Glu Thr Gln Ala Cys Thr Arg
100 105 110

Glu Gln Asn Arg Ile Cys Thr Cys Arg Pro Gly Trp Tyr Cys Ala Leu
115 120 125

Ser Lys Gln Glu Gly Cys Arg Leu Cys Ala Pro Leu Arg Lys Cys Arg
130 135 140

Pro Gly Phe Gly Val Ala Arg Pro Gly Thr Glu Thr Ser Asp Val Val
145 150 155 160

Cys Lys Pro Cys Ala Pro Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr
165 170 175

Asp Ile Cys Arg Pro His Gln Ile Cys Asn Val Val Ala Ile Pro Gly
180 185 190

Asn Ala Ser Met Asp Ala Val Cys Thr Ser Thr Ser Pro Thr Arg Ser
195 200 205

Met Ala Pro Gly Ala Val His Leu Pro Gln Pro Val Ser Thr Arg Ser
210 215 220

Gln His Thr Gln Pro Thr Pro Glu Pro Ser Thr Ala Pro Ser Thr Ser
225 230 235 240

Phe Leu Leu Pro Met Gly Pro Ser Pro Pro Ala Glu Gly Ser Thr Gly

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               245                 250                 255

Asp Phe Ala Leu Pro Val Gly Leu Ile Val Gly Val Thr Ala Leu Gly
            260                 265                 270

Leu Leu Ile Ile Gly Val Val Asn Cys Val Ile Met Thr Gln Val Lys
        275                 280                 285

Lys Lys Pro Leu Cys Leu Gln Arg Glu Ala Lys Val Pro His Leu Pro
    290                 295                 300

Ala Asp Lys Ala Arg Gly Thr Gln Gly Pro Glu Gln Gln His Leu Leu
305                 310                 315                 320

Ile Thr Ala Pro Ser Ser Ser Ser Ser Ser Leu Glu Ser Ser Ala Ser
                325                 330                 335

Ala Leu Asp Arg Arg Ala Pro Thr Arg Asn Gln Pro Gln Ala Pro Gly
            340                 345                 350

Val Glu Ala Ser Gly Ala Gly Glu Ala Arg Ala Ser Thr Gly Ser Ser
        355                 360                 365

Asp Ser Ser Pro Gly Gly His Gly Thr Gln Val Asn Val Thr Cys Ile
    370                 375                 380

Val Asn Val Cys Ser Ser Ser Asp His Ser Ser Gln Cys Ser Ser Gln
385                 390                 395                 400

Ala Ser Ser Thr Met Gly Asp Thr Asp Ser Ser Pro Ser Glu Ser Pro
                405                 410                 415

Lys Asp Glu Gln Val Pro Phe Ser Lys Glu Glu Cys Ala Phe Arg Ser
            420                 425                 430

Gln Leu Glu Thr Pro Glu Thr Leu Leu Gly Ser Thr Glu Glu Lys Pro
        435                 440                 445

Leu Pro Leu Gly Val Pro Asp Ala Gly Met Lys Pro Ser
    450                 455                 460
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<210> SEQ ID NO 28
<211> LENGTH: 2339
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 28

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cccgagtgct tgagctgtgg ctcccgctgt agctctgacc aggtggaaac tcaagcctgc    120

actcgggaac agaaccgcat ctgcacctgc aggcccggct ggtactgcgc gctgagcaag    180

caggaggggt gccggctgtg cgcgccgctg ccgaagtgcc gcccgggctt cggcgtggcc    240

agaccaggaa ctgaaacatc agacgtggtg tgcaagccct gtgccccggg gacgttctcc    300

aacacgactt catccacgga tatttgcagg ccccaccaga tctgtaacgt ggtggccatc    360

cctgggaatg caagcaggga tgcagtctgc acgtccacgt cccccacccg gagtatggcc    420

ccaggggcag tacacttacc ccagccagtg tccacacgat cccaacacac gcagccaagt    480

ccagaaccca gcactgctcc aagcacctcc ttcctgctcc caatgggccc cagccccccа    540

gctgaaggga gcactggcga cttcgctctt ccagttggac tgattgtggg tgtgacagcc    600

ttgggtctac taataatagg agtggtgaac tgtgtcatca tgacccaggt gaaaaagaag    660

cccttgtgcc tgcagagaga agccaaggtg cctcacttgc ctgccgataa ggcccggggt    720

acacagggcc ccgagcagca gcacctgctg atcacagcgc cgagctccag cagcagctcc    780

ctggagagct cggccagtgc gttggacaga agggcgccca ctcggaacca gccacaggca    840

ccaggcgtgg aggccagtgg ggccggggag gcccgggcca gcaccgggag ctcagcagat    900

tcttcccctg gtggccatgg gacccaggtc aatgtcacct gcatcgtgaa cgtctgtagc    960
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agctctgacc acagctcaca gtgctcctcc caagccagct ccacaatggg agacacagat 1020

tccagcccct cggagtcccc gaaggacgag caggtcccct tctccaagga ggaatgtgcc 1080

tttcggtcac agctggagac gccagagacc ctgctgggga gcaccgaaga gaagcccctg 1140

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385                 390
```

The invention claimed is:

1. A method comprising the steps of:

(a) culturing a host cell comprising a polynucleotide, wherein the polynucleotide encodes a protein consisting of:

(i) the extracellular region of an insoluble human TNF receptor, wherein the insoluble human TNF receptor has an apparent molecular weight of about 75 kilodaltons as determined on a non-reducing SDS-polyacrylamide gel and comprises the amino acid sequence LPAQVAFX-PYAPEPGSTC (SEQ ID NO: 10), and

(ii) all of the domains of the constant region of a human IgG immunoglobulin heavy chain other than the first domain of said constant region, and

(b) purifying an expression product of the polynucleotide from the cell mass or the culture medium.

2. The method of claim **1**, wherein the host cell is a CHO cell.

3. The method of claim **1**, wherein the IgG heavy chain is an IgG_1 heavy chain.

4. A polynucleotide encoding a protein consisting of:

(a) the extracellular region of an insoluble human TNF receptor,

wherein the insoluble human TNF receptor (i) has an apparent molecular weight of about 75 kilodaltons as determined on a non-reducing SDS-polyacrylamide gel and (ii) comprises the amino acid sequence LPAQVAFXPYAPEPGSTC (SEQ ID NO: 10), and

(b) all of the domains of the constant region of a human IgG_1 immunoglobulin heavy chain other than the first domain of said constant region.

5. A vector comprising the polynucleotide of claim **4**.

6. A mammalian host cell comprising the polynucleotide of claim **4**.

7. A method comprising the steps of:

(a) culturing a host cell comprising a polynucleotide, wherein the polynucleotide encodes a protein consisting of:

(i) the extracellular region of an insoluble human TNF receptor, wherein the insoluble human TNF receptor comprises the amino acid sequence of SEQ ID NO:27 and

(ii) all of the domains of the constant region of a human IgG immunoglobulin heavy chain other than the first domain of said constant region, and

(b) purifying an expression product of the polynucleotide from the cell mass or the culture medium.

8. The method of claim **7**, wherein the human IgG immunoglobulin heavy chain is an IgG_1 heavy chain.

9. The method of claim **7**, wherein the host cell is a CHO cell.

10. The method of claim **8**, wherein the host cell is a CHO cell.

* * * * *

Exhibit E

US007915225B2

(12) **United States Patent**
Finck

(10) **Patent No.: US 7,915,225 B2**
(45) **Date of Patent: *Mar. 29, 2011**

(54) **SOLUBLE TUMOR NECROSIS FACTOR RECEPTOR TREATMENT OF MEDICAL DISORDERS**

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(73) Assignee: **Immunex Corporation**, Thousand Oaks, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **12/394,962**

(22) Filed: **Feb. 27, 2009**

(65) **Prior Publication Data**

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Related U.S. Application Data

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(60) Provisional application No. 60/164,676, filed on Nov. 10, 1999, provisional application No. 60/184,864, filed on Feb. 25, 2000, provisional application No. 60/130,074, filed on Apr. 19, 1999, provisional application No. 60/134,320, filed on May 14, 1999, provisional application No. 60/143,959, filed on Jul. 15, 1999, provisional application No. 60/148,234, filed on Aug. 11, 1999.

(51) **Int. Cl.**
A61K 38/16 (2006.01)
C07K 14/715 (2006.01)
C07K 14/525 (2006.01)

(52) **U.S. Cl.** **514/21.2**; 514/18.7; 514/169; 514/863; 552/588; 607/94; 530/350; 530/866

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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Primary Examiner — David S Romeo
(74) *Attorney, Agent, or Firm* — Rosemary Sweeney

(57) **ABSTRACT**

The invention pertains to methods and compositions for treating medical disorders characterized by elevated levels or abnormal expression of TNFα by administering a TNFα antagonist, such as recombinant TNFR:Fc.

20 Claims, No Drawings

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SOLUBLE TUMOR NECROSIS FACTOR RECEPTOR TREATMENT OF MEDICAL DISORDERS

This application is a divisional of U.S. application Ser. No. 10/853,479, filed May 25, 2004, now pending; which is a divisional of U.S. application Ser. No. 09/602,351, filed Jun. 23, 2000, now abandoned, which claims benefit of U.S. Provisional Application Nos. 60/164,676, filed Nov. 10, 1999, and 60/184,864, filed Feb. 25, 2000, and which is a continuation-in-part of U.S. application Ser. No. 09/373,828, filed Aug. 13, 1999, now abandoned, which claims the benefit of U.S. Provisional Application Nos. 60/130,074, filed Apr. 19, 1999, 60/134,320, filed May 14, 1999, 60/143,959, filed Jul. 15, 1999, and 60/148,234, filed Aug. 11, 1999; all of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The invention pertains to methods for treating various medical disorders that are characterized by abnormal or excessive TNFα levels by administering a TNFα antagonist, preferably a soluble TNFα. The TNFα inhibitor may be administered in combination with other biologically active molecules.

BACKGROUND OF THE INVENTION

The pleiotropic cytokine tumor necrosis factor alpha (TNFα) is associated with inflammation and binds to cells through membrane receptor molecules, including two molecules having molecular weights of approximately 55 kDa and 75 kDa (p55 and p75). In addition to binding TNFα, the p55 and p75 TNF receptors mediate the binding to cells of homotrimers of TNFβ, which is another cytokine associated with inflammation and which shares structural similarities with TNFα (e.g., see Cosman, *Blood Cell Biochem* 7:51-77, 1996). TNFβ is also known as lymphotoxin-α (LTα).

It has been proposed that a systemic or localized excess of TNFα contributes to the progression of numerous medical disorders. For example, patients with chronic heart failure have elevated levels of serum TNFα, which have been shown to increase with disease progression (see, for example, Levine et al., *N Eng J Med* 323:236-241, 1990). A variety of other diseases are associated with elevated levels of TNFα (see, for example, Feldman et al., *Transplantation Proceedings* 30:4126-4127, 1998).

Psoriatic arthritis (PsA) is a chronic autoimmune condition that shares some features with both rheumatoid arthritis (RA) and the inflammatory skin disease psoriasis (for review, see Breathnach, In Klippel and Dieppe eds. *Rheumatology,* 2nd Ed., Mosby, 1998. 22.1-22.4). Psoriasis is characterized by epidermal keratinocyte hyperproliferation, accompanied by neutrophil and T cell infiltration, and is associated with elevated levels of inflammatory cytokines, including TNFα, IL-6 and TGFβ (see, for example, Bonifati et al., *Clin Exp Dermatol* 19:383-387, 1994). Psoriasis and PsA are different clinical entities, and are associated with somewhat different MHC haplotypes (Gladman, *Rheum Dis Clin NA.* 18:247-256, 1992; Breathnach, 1998). The overall prognosis for PsA is far worse than for ordinary psoriasis. Nonetheless, treatments used for the psoriatic lesions of PsA generally are similar to those used to treat psoriasis.

Psoriatic skin lesions are present in patients with PsA, although only a minority of psoriasis sufferers actually have PsA. Ordinary psoriasis occasionally is accompanied by joint pain, but does not involve the extreme pain and often deforming degeneration of joints and bone that occurs in PsA patients.

Treatments that sometimes are effective in treating ordinary psoriasis include topical medications (e.g., steroids, coal tar, anthralin, Dead Sea salts, various natural oils, vitamin D3 and its analogs, sunshine, topical retinoids), phototherapy (e.g., ultraviolet light, photochemotherapy (PUVA)), and internal medications (e.g., methotrexate, systemic steroids, oral retinoids, cyclosporine, or a rotating regimen of these three). In addition, it has been proposed that psoriasis could be treated with TNF-derived peptides, quinolinesulfonamides, pyrrolidinone derivatives, catechol diether compounds, isoxazoline compounds, matrix metalloproteinase inhibitors or mercapto alkyl peptidyl compounds, all of which inhibit either TNFα production or its release from cultured cells (see, for example, U.S. Pat. No. 5,691,382, U.S. Pat. No. 5,834,485, U.S. Pat. No. 5,420,154, U.S. Pat. No. 5,563,143, U.S. Pat. No. 5,869,511 and U.S. Pat. No. 5,872,146), as well as with various combination therapies involving TNFα antagonists (for example, see U.S. Pat. No. 5,888,511 or U.S. Pat. No. 5,958,413).

Conflicting results have been reported regarding the role of TNFα in psoriasis. Some investigators have proposed that overproduction of TNFα contributes to the pathology of psoriasis (e.g., Pigatto et al., *J. Invest Dermatol* 94:372-376, 1990; Sagawa et al., *Dermatol* 187:81-83, 1993; Ameglio et al., *Dermatol* 189:359-363, 1994). One group reported some improvement after treatment with pentoxifylline, a drug that can inhibit the release of TNFα, but which exerts many of its physiological effects by inhibiting cyclic AMP phosphodiesterase (Omulecki et al., *J Am Acad Dermatol* 34:714-715, 1996; Centola et al., *J Androl* 16:136-142, 1995; Elferinck et al., *Biochem Pharmacol* 54:475-480, 1997). However, other reports have cast doubt on the hypothesis that overproduction of TNFα exacerbates psoriasis. For example, some investigators have reported that treatment with TNFα itself actually can mitigate psoriasis (see, e.g., Takematsu et al., *Br J Dermatol* 124:209-210, 1991; Creaven et al., *J Am Acad Dermatol* 24:735-737, 1991).

In addition to psoriatic lesions, PsA is characterized by distal interphalangeal joint (DIP) involvement, enthesopathy, nail lesions, spondylitis and dactylitis. The histopathogenesis of PsA and the more well-studied rheumatoid arthritis share certain features. In both RA and in active PsA, patients exhibit increased levels of HLA-DR$^+$ T cells and MHC class II antigens in their synovial membranes and synovial fluid, as well as increased expression of the cytokine TNFα. In addition, both diseases are associated with prominent synovial vascular changes.

The discovery of rheumatoid factor in the serum of RA patients provided an important tool for differentiating PsA from RA, but the realization that RA and PsA are distinct diseases was based primarily on their many clinical differences (e.g., Helliwell and Wright, In Klippel and Dieppe eds. *Rheumatology,* 2nd Ed., Mosby, 1998, 21.1-21.8). Studies have shown that levels of TNFα, Il-1β, Il-8 as well as TNFα receptors in synovial fluids were higher in PsA patients than in osteoarthritis patients, though they were lower than in RA patients (Partsch et al., *J Rheumatol* 24:518-523, 1997; Partsch et al., *J Rheumatol* 25:105-110, 1998; Partsch et al., *Ann Rheum Dis* 57:691-693, 1998). PsA is distinguished from RA also by radiographic appearance, a notably higher degree of synovial membrane vascularity as well as differences in the levels of various cytokines in the synovial fluids (Ritchlin et al., *J Rheumatol* 25:1544-52, 1998; Veale et al., *Arth Rheum* 36:893-900, 1993). Veale et al. noted differences

in synovial membrane adhesion molecules and numbers of macrophages when they compared RA and PsA patients, as well as observing a minimal degree of hyperplasia and hypertrophy of synoviocytes in PsA as compared with RA patients. Because of such differences, coupled with the association of PsA but not RA with class I MHC antigens, Ritchlin et al. have suggested that PsA must be triggered by different mechanisms than those underlying RA. Veale et al. suggested for similar reasons that different cytokines were likely to be interacting in the synovium of PsA and RA patients.

Most of the drugs used for treating the arthritic aspects of PsA are similar to those used in RA (Salvarini et al., *Curr Opin Rheumatol* 10:229-305, 1998), for example the non-steroidal antiinflammatories (NSAIDs), which may be used alone or in combination with the disease-modifying anti-rheumatic drugs, or "DMARDs." However, one group found that long-term administration of the DMARD methotrexate failed to slow the progression of joint damage in PsA patients (Abu-Shakra et al., *J Rheumatol* 22:241-45, 1995), and another group reported very little improvement in PsA patients who had received methotrexate (Willkens et al., *Arthr Rheum* 27:376-381, 1984). Similarly, Clegg et al. found only a slight improvement over placebo in PsA patients treated with sulfasalazine, another drug classified as a DMARD (Clegg et al., *Arthritis Rheum* 39: 2013-20, 1996). Some studies have indicated that the immunosuppressor cyclosporine is effective in treating PsA (reviewed in Salvarini et al., 1998), though this drug has severe side effects. In addition, others have proposed that PsA could be treated with truncated TNFα receptors or with a combination of methotrexate and antibodies against TNFα (WO 98/01555; WO 98/0537).

A recent meta-analysis of a number of PsA treatment studies concluded that PsA and RA differed not only in their response to treatment with specific drugs, but in the relative magnitudes of improvement in the placebo arms of the studies (Jones et al., *Br J Rheumatol* 36:95-99, 1997). As an example, PsA patients responded better to gold salt therapy than did RA patients, though the gold did not affect the psoriatic skin lesions (Dorwart et al., *Arthritis Rheum* 21:515-513, 1978).

It has been suggested that the suppression of TNFα might be beneficial in patients suffering from various disorders characterized by abnormal or excessive TNFα expression. However, although progress has been made in devising effective treatment for such diseases, improved medicaments and methods of treatment are needed.

SUMMARY OF THE INVENTION

Provided herein are methods for treating a number of medical disorders characterized by abnormal TNFα expression by repeatedly administering an antagonist of TNFα, such as a soluble TNFα receptor, for a period of time sufficient to induce a sustained improvement in the patient's condition.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides compounds, compositions and methods for treating a mammalian patient, including a human patient, who is suffering from a medical disorder that is characterized by abnormal or elevated expression of TNFα. For purposes of this disclosure, the terms "illness," "disease," "medical condition," "abnormal condition" and the like are used interchangeably with the term "medical disorder."

The subject methods involve administering to the patient a soluble TNFα antagonist that is capable of reducing the effective amount of endogenous biologically active TNFα, such as by reducing the amount of TNFα produced, or by preventing the binding of TNFα to its cell surface receptor (TNFR). Antagonists capable of inhibiting this binding include receptor-binding peptide fragments of TNFα, antibodies directed against TNFα, and recombinant proteins comprising all or portions of receptors for TNFα or modified variants thereof, including genetically-modified muteins, multimeric forms and sustained-release formulations. Other compounds suitable for treating the diseases described herein include thalidomide and pentoxifylline.

Preferred embodiments of the invention utilize soluble TNFRs as the TNFα antagonist. Soluble forms of TNFRs may include monomers, fusion proteins (also called "chimeric proteins"), dimers, trimers or higher order multimers. In certain embodiments of the invention, the soluble TNFR derivative is one that mimics the 75 kDa TNFR or the 55 kDa TNFR and that binds to TNFα in the patient's body. The soluble TNFR mimics of the present invention may be derived from TNFRs p55 or p75 or fragments thereof. TNFRs other than p55 and p75 also are useful for deriving soluble compounds for treating the various medical disorders described herein, such for example the TNFR described in WO 99/04001. Soluble TNFR molecules used to construct TNFR mimics include, for example, analogs or fragments of native TNFRs having at least 20 amino acids, that lack the transmembrane region of the native TNFR, and that are capable of binding TNFα. Antagonists derived from TNFRs compete for TNFα with the receptors on the cell surface, thus inhibiting TNFα from binding to cells, thereby preventing it from manifesting its biological activities. Binding of soluble TNFRs to TNFα or LTα can be assayed using ELISA or any other convenient assay. This invention provides for the use of soluble TNFα receptors in the manufacture of medicaments for the treatment of numerous diseases.

The soluble TNFR polypeptides or fragments of the invention may be fused with a second polypeptide to form a chimeric protein. The second polypeptide may promote the spontaneous formation by the chimeric protein of a dimer, trimer or higher order muimer that is capable of binding a TNFα and/or LTα molecule and preventing it from binding to cell-bound receptors. Chimeric proteins used as antagonists include, for example, molecules derived from an antibody molecule and a TNFR. Such molecules are referred to herein as TNFR-Ig fusion proteins. A preferred TNFR-Ig fusion protein suitable for treating diseases in humans and other mammals is recombinant TNFR:Fc, a term which as used herein refers to "etanercept," which is a dimer of two molecules of the extracellular portion of the p75 TNFα receptor, each molecule consisting of a 235 amino acid TNFR-derived polypeptide that is fused to a 232 amino acid Fc portion of human IgG_1. Etanercept is currently sold by Immunex Corporation under the trade name ENBREL.® Because the p75 receptor protein that it incorporates binds not only to TNFα, but also to the inflammatory cytokine LTα, etanercept can act as a competitive inhibitor not only of TNFα, but also of LTα. This is in contrast to antibodies directed against TNFα, which cannot inhibit LTα. Also encompassed by the invention are treatments using a compound that comprises the extracellular portion of the 55 kDa TNFR fused to the Fc portion of IgG, as well as compositions and combinations containing such a molecule. Encompassed also are therapeutic methods involving the administration of TNFR-Ig proteins derived the extracellular regions of TNFα receptor molecules other than the p55 and p75 TNFRs, such as for example the TNFR described in WO 99/04001.

In one preferred embodiment of the invention, sustained-release forms of soluble TNFRs are used, including sus-

tained-release forms of TNFR:Fc. Sustained-release forms suitable for use in the disclosed methods include, but are not limited to, TNFRs that are encapsulated in a slowly-dissolving biocompatible polymer (such as the alginate microparticles described in U.S. Pat. No. 6,036,978), admixed with such a polymer (including topically applied hydrogels), and or encased in a biocompatible semi-permeable implant. In addition, the soluble TNFR may be conjugated with polyethylene glycol (pegylated) to prolong its serum half-life or to enhance protein delivery.

In accord with this invention, medical disorders characterized by abnormal or excess expression of TNFα are administered a therapeutically effective amount of a TNFα inhibitor. The TNFα inhibitor may be a TNFα-binding soluble TNFα receptor, preferably TNFR:Fc. As used herein, the phrase "administering a therapeutically effective amount" of a therapeutic agent means that the patient is treated with the agent in an amount and for a time sufficient to induce a sustained improvement in at least one indicator that reflects the severity of the disorder. An improvement is considered "sustained" if the patient exhibits the improvement on at least two occasions separated by one or more weeks. The degree of improvement is determined based on signs or symptoms, and determinations may also employ questionnaires that are administered to the patient, such as quality-of-life questionnaires.

Various indicators that reflect the extent of the patient's illness may be assessed for determining whether the amount and time of the treatment is sufficient. The baseline value for the chosen indicator or indicators is established by examination of the patient prior to administration of the first dose of the etanercept or other TNFα inhibitor. Preferably, the baseline examination is done within about 60 days of administering the first dose. If the TNFα antagonist is being administered to treat acute symptoms, such as for example to treat a traumatic knee injury, the first dose is administered as soon as practically possible after the injury has occurred.

Improvement is induced by administering TNFR:Fc or other TNFα antagonist until the patient manifests an improvement over baseline for the chosen indicator or indicators. In treating chronic conditions, this degree of improvement is obtained by repeatedly administering this medicament over a period of at least a month or more, e.g., for one, two, or three months or longer, or indefinitely. A period of one to six weeks, or even a single dose, often is sufficient for treating acute conditions. For injuries or acute conditions, a single dose may be sufficient.

Although the extent of the patient's illness after treatment may appear improved according to one or more indicators, treatment may be continued indefinitely at the same level or at a reduced dose or frequency. Once treatment has been reduced or discontinued, it later may be resumed at the original level if symptoms should reappear.

Any efficacious route of administration may be used to therapeutically administer TNFR:Fc or other TNFα antagonists. If injected, TNFR:Fc can be administered, for example, via intra-articular, intravenous, intramuscular, intralesional, intraperitoneal or subcutaneous routes by bolus injection or by continuous infusion. Other suitable means of administration include sustained release from implants, aerosol inhalation, eyedrops, oral preparations, including pills, syrups, lozenges or chewing gum, and topical preparations such as lotions, gels, sprays, ointments or other suitable techniques. Alternatively, proteinaceous TNFα inhibitors, such as a soluble TNFR, may be administered by implanting cultured cells that express the protein, for example, by implanting cells that express TNFR:Fc. In one embodiment, the patient's own cells are induced to produce TNFR:Fc by transfection in vivo or ex vivo with a DNA that encodes TNFR:Fc. This DNA can be introduced into the patient's cells, for example, by injecting naked DNA or liposome-encapsulated DNA that encodes TNFR:Fc, or by other means of transfection. When TNFR:Fc is administered in combination with one or more other biologically active compounds, these may be administered by the same or by different routes, and may be administered simultaneously, separately or sequentially.

TNFR:Fc or other soluble TNFRs preferably are administered in the form of a physiologically acceptable composition comprising purified recombinant protein in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers are nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the TNFα antagonist with buffers, antioxidants such as ascorbic acid, low molecular weight polypeptides (such as those having fewer than 10 amino acids), proteins, amino acids, carbohydrates such as glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents. In accordance with appropriate industry standards, preservatives may also be added, such as benzyl alcohol. TNFR:Fc preferably is formulated as a lyophilizate using appropriate excipient solutions (e.g., sucrose) as diluents. Appropriate dosages can be determined in standard dosing trials, and may vary according to the chosen route of administration. The amount and frequency of administration will depend, of course, on such factors as the nature and severity of the indication being treated, the desired response, the age and condition of the patient, and so forth.

In one embodiment of the invention, TNFR:Fc is administered one time per week to treat the various medical disorders disclosed herein, in another embodiment is administered at least two times per week, and in another embodiment is administered at least three times per week. An adult patient is a person who is 18 years of age or older. If injected, the effective amount of TNFR:Fc per adult dose ranges from 1-20 mg/m^2, and preferably is about 5-12 mg/m^2. Alternatively, a flat dose may be administered, whose amount may range from 5-100 mg/dose. Exemplary dose ranges for a flat dose to be administered by subcutaneous injection are 5-25 mg/dose, 25-50 mg/dose and 50-100 mg/dose. In one embodiment of the invention, the various indications described below are treated by administering a preparation acceptable for injection containing TNFR:Fc at 25 mg/dose, or alternatively, containing 50 mg per dose. The 25 mg or 50 mg dose may be administered repeatedly, particularly for chronic conditions. If a route of administration other than injection is used, the dose is appropriately adjusted in accord with standard medical practices. In many instances, an improvement in a patient's condition will be obtained by injecting a dose of about 25 mg of TNFR:Fc one to three times per week over a period of at least three weeks, or a dose of 50 mg of TNFR:Fc one or two times per week for at least three weeks, though treatment for longer periods may be necessary to induce the desired degree of improvement. For incurable chronic conditions, the regimen may be continued indefinitely, with adjustments being made to dose and frequency if such are deemed necessary by the patient's physician.

For pediatric patients (age 4-17), a suitable regimen involves the subcutaneous injection of 0.4 mg/kg, up to a maximum dose of 25 mg of TNFR:Fc, administered by subcutaneous injection one or more times per week.

The invention further includes the administration of TNFR:Fc concurrently with one or more other drugs that are

administered to the same patient in combination with the TNFR:Fc, each drug being administered according to a regimen suitable for that medicament. "Concurrent administration" encompasses simultaneous or sequential treatment with the components of the combination, as well as regimens in which the drugs are alternated, or wherein one component is administered long-term and the other(s) are administered intermittently. Components may be administered in the same or in separate compositions, and by the same or different routes of administration. Examples of drugs to be administered concurrently include but are not limited to antivirals, antibiotics, analgesics, corticosteroids, antagonists of inflammatory cytokines, DMARDs and non-steroidal anti-inflammatories. DMARDs that can be administered in combination with the subject TNFα inhibitors such as TNFR:Fc include azathioprine, cyclophosphamide, cyclosporine, hydroxychloroquine sulfate, methotrexate, leflunomide, minocycline, penicillamine, sulfasalazine and gold compounds such as oral gold, gold sodium thiomalate and aurothioglucose. Additionally, TNFR:Fc may be combined with a second TNFα antagonist, including an antibody against TNFα or TNFR, a TNFα-derived peptide that acts as a competitive inhibitor of TNFα (such as those described in U.S. Pat. No. 5,795,859), a TNFR-IgG fusion protein other than etanercept, such as one containing the extracellular portion of the p55 TNFα receptor, a soluble TNFR other than an IgG fusion protein, or other molecules that reduce endogenous TNFα levels, such as inhibitors of the TNFα converting enzyme (see e.g., U.S. Pat. No. 5,594,106). In further embodiments of this invention, TNFR:Fc is administered in combination with pentoxifylline or thalidomide.

If an antibody against TNFα is used as the TNFα inhibitor, a preferred dose range is 0.1 to 20 mg/kg, and more preferably is 1-10 mg/kg. Another preferred dose range for anti-TNFα antibody is 0.75 to 7.5 mg/kg of body weight. Humanized antibodies are preferred, that is, antibodies in which only the antigen-binding portion of the antibody molecule is derived from a non-human source. such antibodies may be injected or administered intravenously.

In one preferred embodiment of the invention, the various medical disorders disclosed herein as being treatable with inhibitors such as TNFR:Fc are treated in combination with another cytokine or cytokine inhibitor. For example, TNFR:Fc may be administered in a composition that also contains a compound that inhibits the interaction of other inflammatory cytokines with their receptors. Examples of cytokine inhibitors used in combination with TNFR:Fc include, for example, antagonists of TGFβ, Il-6 or Il-8. TNFα inhibitors such as TNFR:Fc also may be administered in combination with the cytokines GM-CSF, IL-2 and inhibitors of protein kinase A type 1 to enhance T cell proliferation in HIV-infected patients who are receiving anti-retroviral therapy. Other combinations for treating the herein described diseases include TNFR:Fc administered concurrently with compounds that block the binding of RANK and RANK-ligand, such as antagonistic antibodies against RANK or RANK-ligand, soluble forms of RANK-ligand that do not trigger RANK, osteoprotegerin or soluble forms of RANK, including RANK:Fc. Soluble forms of RANK suitable for these combinations are described, for example, in U.S. Pat. No. 6,017,729. The concurrent administration of TNFR:Fc and RANK:Fc or TNFR:Fc and osteoprotegerin is useful for preventing bone destruction in various settings including but not limited to various rheumatic disorders, osteoporosis, multiple myeloma or other malignancies that cause bone degeneration, or anti-tumor therapy aimed at preventing metastasis to bone, or bone destruction associated with prosthesis wear debris or with periodontitis.

The present invention also relates to the use of the disclosed TNFα inhibitors, such as TNFR:Fc, in the manufacture of a medicament for the prevention or therapeutic treatment of each medical disorder disclosed herein.

The disclosed TNFα inhibitors, compositions and combination therapies described herein are useful in medicines for treating bacterial, viral or protozoal infections, and complications resulting therefrom. One such disease is *Mycoplasma pneumonia*. In addition, provided herein is the use of TNFR:Fc to treat AIDS and related conditions, such as AIDS dementia complex, AIDS associated wasting, lipidistrophy due to antiretroviral therapy; and Kaposi's sarcoma. Provided herein is the use of TNFR:Fc for treating protozoal diseases, including malaria and schistosomiasis. Additionally provided is the use of TNFR:Fc to treat erythema nodosum leprosum; bacterial or viral meningitis; tuberculosis, including pulmonary tuberculosis; and pneumonitis secondary to a bacterial or viral infection. Provided also herein is the use of TNFR:Fc to prepare medicaments for treating louse-borne relapsing fevers, such as that caused by *Borrelia recurrentis*. TNFR:Fc can also be used to prepare a medicament for treating conditions caused by Herpes viruses, such as herpetic stromal keratitis, corneal lesions, and virus-induced corneal disorders. In addition, TNFR:Fc can be used in treating human papillomavirus infections. TNFR:Fc is used also to prepare medicaments to treat influenza.

Cardiovascular disorders are treatable with the disclosed TNFα inhibitors, pharmaceutical compositions or combination therapies, including aortic aneurisms; arteritis; vascular occlusion, including cerebral artery occlusion; complications of coronary by-pass surgery; ischemia/reperfusion injury; heart disease, including atherosclerotic heart disease, myocarditis, including chronic autoimmune myocarditis and viral myocarditis; heart failure, including chronic heart failure (CHF), cachexia of heart failure; myocardial infarction; restenosis after heart surgery; silent myocardial ischemia; post-implantation complications of left ventricular assist devices; Raynaud's phenomena; thrombophlebitis; vasculitis, including Kawasaki's vasculitis; giant cell arteritis, Wegener's granulomatosis; and Schoenlein-Henoch purpura.

TNFα and IL-8 have been implicated as chemotactic factors in athersclerotic abdominal aortic aneurism (Szekanecz et al., *Pathobiol* 62:134-139 (1994)). Abdominal aortic aneurism may be treated in human patients by administering a soluble TNFR, such as TNFR:Fc, which may be administered in combination with an inhibitor of IL-8, such treatment having the effect of reducing the pathological neovascularization associated with this condition.

A combination of a TNFα inhibitor and one or more other anti-angiogenesis factors may be used to treat solid tumors, thereby reducing the vascularization that nourishes the tumor tissue. Suitable anti-angiogenic factors for such combination therapies include IL-8 inhibitors, angiostatin, endostatin, kringle 5, inhibitors of vascular endothelial growth factor (such as antibodies against vascular endothelial growth factor), angiopoietin-2 or other antagonists of angiopoietin-1, antagonists of platelet-activating factor and antagonists of basic fibroblast growth factor

In addition, the subject TNFα inhibitors, compositions and combination therapies are used to treat chronic pain conditions, such as chronic pelvic pain, including chronic prostatitis/pelvic pain syndrome. As a further example, TNFR:Fc and the compositions and combination therapies of the invention are used to treat post-herpetic pain.

Provided also are methods for using TNFα inhibitors, compositions or combination therapies to treat various disorders of the endocrine system. For example, the TNFα inhibitors are used to treat juvenile onset diabetes (includes autoimmune and insulin-dependent types of diabetes) and also to treat maturity onset diabetes (includes non-insulin dependent and obesity-mediated diabetes). In addition, the subject compounds, compositions and combination therapies are used to treat secondary conditions associated with diabetes, such as diabetic retinopathy, kidney transplant rejection in diabetic patients, obesity-mediated insulin resistance, and renal failure, which itself may be associated with proteinurea and hypertension. Other endocrine disorders also are treatable with these compounds, compositions or combination therapies, including polycystic ovarian disease, X-linked adrenoleukodystrophy, hypothyroidism and thyroiditis, including Hashimoto's thyroiditis (i.e., autoimmune thyroiditis).

Conditions of the gastrointestinal system also are treatable with TNFα inhibitors, compositions or combination therapies, including coeliac disease. In addition, the compounds, compositions and combination therapies of the invention are used to treat Crohn's disease; ulcerative colitis; idiopathic gastroparesis; pancreatitis, including chronic pancreatitis and lung injury associated with acute pancreatitis; and ulcers, including gastric and duodenal ulcers.

Included also are methods for using the subject TNFα inhibitors, compositions or combination therapies for treating disorders of the genitourinary system, such as glomerulonephritis, including autoimmune glomerulonephritis, glomerulonephritis due to exposure to toxins or glomerulonephritis secondary to infections with haemolytic streptococci or other infectious agents. Also treatable with the compounds, compositions and combination therapies of the invention are uremic syndrome and its clinical complications (for example, renal failure, anemia, and hypertrophic cardiomyopathy), including uremic syndrome associated with exposure to environmental toxins, drugs or other causes. Further conditions treatable with the compounds, compositions and combination therapies of the invention are complications of hemodialysis; prostate conditions, including benign prostatic hypertrophy, nonbacterial prostatitis and chronic prostatitis; and complications of hemodialysis.

Also provided herein are methods for using TNFα inhibitors, compositions or combination therapies to treat various hematologic and oncologic disorders. For example, TNFR:Fc is used to treat various forms of cancer, including acute myelogenous leukemia, Epstein-Barr virus-positive nasopharyngeal carcinoma, glioma, colon, stomach, prostate, renal cell, cervical and ovarian cancers, lung cancer (SCLC and NSCLC), including cancer-associated cachexia, fatigue, asthenia, paraneoplastic syndrome of cachexia and hypercalcemia. Additional diseases treatable with the subject TNFα inhibitors, compositions or combination therapies are solid tumors, including sarcoma, osteosarcoma, and carcinoma, such as adenocarcinoma (for example, breast cancer) and squamous cell carcinoma. In addition, the subject compounds, compositions or combination therapies are useful for treating leukemia, including acute myelogenous leukemia, chronic or acute lymphoblastic leukemia and hairy cell leukemia. Other malignancies with invasive metastatic potential can be treated with the subject compounds, compositions and combination therapies, including multiple myeloma. In addition, the disclosed TNFα inhibitors, compositions and combination therapies can be used to treat anemias and hematologic disorders, including anemia of chronic disease, aplastic anemia, including Fanconi's aplastic anemia; idiopathic thrombocytopenic purpura (ITP); myelodysplastic syndromes (including refractory anemia, refractory anemia with ringed sideroblasts, refractory anemia with excess blasts, refractory anemia with excess blasts in transformation); myelofibrosis/myeloid metaplasia; and sickle cell vasoocclusive crisis.

Various lymphoproliferative disorders also are treatable with the disclosed TNFα inhibitors, compositions or combination therapies. These include, but are not limited to autoimmune lymphoproliferative syndrome (ALPS), chronic lymphoblastic leukemia, hairy cell leukemia, chronic lymphatic leukemia, peripheral T-cell lymphoma, small lymphocytic lymphoma, mantle cell lymphoma, follicular lymphoma, Burkitt's lymphoma, Epstein-Barr virus-positive T cell lymphoma, histiocytic lymphoma, Hodgkin's disease, diffuse aggressive lymphoma, acute lymphatic leukemias, T gamma lymphoproliferative disease, cutaneous B cell lymphoma, cutaneous T cell lymphoma (i.e., mycosis fungoides) and Sézary syndrome.

In addition, the subject TNFα inhibitors, compositions and combination therapies are used to treat hereditary conditions such as Gaucher's disease, Huntington's disease, linear IgA disease, and muscular dystrophy.

Other conditions treatable by the disclosed TNFα inhibitors, compositions and combination therapies include those resulting from injuries to the head or spinal cord, and including subdural hematoma due to trauma to the head.

The disclosed TNFα inhibitors, compositions and combination therapies are further used to treat conditions of the liver such as hepatitis, including acute alcoholic hepatitis, acute drug-induced or viral hepatitis, hepatitis A, B and C, sclerosing cholangitis and inflammation of the liver due to unknown causes.

In addition, the disclosed TNFα inhibitors, compositions and combination therapies are used to treat various disorders that involve hearing loss and that are associated with abnormal TNFα expression. One of these is inner ear or cochlear nerve-associated hearing loss that is thought to result from an autoimmune process, i.e., autoimmune hearing loss. This condition currently is treated with steroids, methotrexate and/or cyclophosphamide, which may be administered concurrently with the TNFR:Fc or other TNFα inhibitor. Also treatable with the disclosed TNFα inhibitors, compositions and combination therapies is cholesteatoma, a middle ear disorder often associated with hearing loss.

In addition, the subject invention provides TNFα inhibitors, compositions and combination therapies for the treatment of non-arthritic medical conditions of the bones and joints. This encompasses osteoclast disorders that lead to bone loss, such as but not limited to osteoporosis, including post-menopausal osteoporosis, periodontitis resulting in tooth loosening or loss, and prosthesis loosening after joint replacement (generally associated with an inflammatory response to wear debris). This latter condition also is called "orthopedic implant osteolysis." Another condition treatable by administering TNFRα inhibitors, such as TNFR:Fc, is temporal mandibular joint dysfunction (TMJ).

A number of pulmonary disorders also can be treated with the disclosed TNFα inhibitors, compositions and combination therapies. One such condition is adult respiratory distress syndrome (ARDS), which is associated with elevated TNFα, and may be triggered by a variety of causes, including exposure to toxic chemicals, pancreatitis, trauma or other causes. The disclosed compounds, compositions and combination therapies of the invention also are useful for treating bronchopulmonary dysplasia (BPD); lymphangioleiomyomatosis; and chronic fibrotic lung disease of preterm infants. In addition, the compounds, compositions and combination thera-

pies of the invention are used to treat occupational lung diseases, including asbestosis, coal worker's pneumoconiosis, silicosis or similar conditions associated with long-term exposure to fine particles. In other aspects of the invention, the disclosed compounds, compositions and combination therapies are used to treat pulmonary disorders, including chronic obstructive pulmonary disease (COPD) associated with chronic bronchitis or emphysema; fibrotic lung diseases, such as cystic fibrosis, idiopathic pulmonary fibrosis and radiation-induced pulmonary fibrosis; pulmonary sarcoidosis; and allergies, including allergic rhinitis, contact dermatitis, atopic dermatitis and asthma.

Cystic fibrosis is an inherited condition characterized primarily by the accumulation of thick mucus, predisposing the patient to chronic lung infections and obstruction of the pancreas, which results in malabsorption of nutrients and malnutrition. TNFR:Fc may be administered to treat cystic fibrosis. If desired, treatment with TNFR:Fc may be administered concurrently with corticosteroids, mucus-thinning agents such as inhaled recombinant deoxyribonuclease I (such as PULMOZYME®; Genentech, Inc.) or inhaled tobramycin (TOBI®; Pathogenesis, Inc.). TNFR:Fc also may be administered concurrently with corrective gene therapy, drugs that stimulate cystic fibrosis cells to secrete chloride or other yet-to-be-discovered treatments. Sufficiency of treatment may be assessed, for example, by observing a decrease in the number of pathogenic organisms in sputum or lung lavage (such as *Haemophilus influenzae, Stapholococcus aureus*, and *Pseudomonas aeruginosa*), by monitoring the patient for weight gain, by detecting an increase in lung capacity or by any other convenient means.

TNFR:Fc or TNFR:Fc combined with the cytokine IFNγ-1b (such as ACTIMMUNE®; InterMune Pharmaceuticals) may be used for treating cystic fibrosis or fibrotic lung diseases, such as idiopathic pulmonary fibrosis, radiation-induced pulmonary fibrosis and bleomycin-induced pulmonary fibrosis. In addition, this combination is useful for treating other diseases characterized by organ fibrosis, including systemic sclerosis (also called "scleroderma"), which often involves fibrosis of the liver. For treating cystic fibrosis, TNFR:Fc and IFNγ-1b may be combined with PULMOZYMEL® or TOBI® or other treatments for cystic fibrosis.

TNFR:Fc alone or in combination with IFNγ-1b may be administered together with other treatments presently used for treating fibrotic lung disease. Such additional treatments include glucocorticoids, azathioprine, cyclophosphamide, penicillamine, colchisicine, supplemental oxygen and so forth. Patients with fibrotic lung disease, such as IPF, often present with nonproductive cough, progressive dyspnea, and show a restrictive ventilatory pattern in pulmonary function tests. Chest radiographs reveal fibrotic accumulations in the patient's lungs. When treating fibrotic lung disease in accord with the disclosed methods, sufficiency of treatment may be detected by observing a decrease in the patient's coughing (when cough is present), or by using standard lung function tests to detect improvements in total lung capacity, vital capacity, residual lung volume or by administering a arterial blood gas determination measuring desaturation under exercising conditions, and showing that the patient's lung function has improved according to one or more of these measures. In addition, patient improvement may be determined through chest radiography results showing that the progression of fibrosis in the patient's lungs has become arrested or reduced.

In addition, TNF inhibitors (including soluble TNFRs or antibodies against TNFα or TNFR) are useful for treating organ fibrosis when administered in combination with relaxin, a hormone that down-regulates collagen production thus inhibiting fibrosis, or when given in combination with agents that block the fibrogenic activity of TGF-β. Combination therapies using TNFR:Fc and recombinant human relaxin are useful, for example, for treating systemic sclerosis or fibrotic lung diseases, including cystic fibrosis, idiopathic pulmonary fibrosis, radiation-induced pulmonary fibrosis and bleomycin-induced pulmonary fibrosis.

Other embodiments provide methods for using the disclosed TNFα inhibitors, compositions or combination therapies to treat a variety of rheumatic disorders. These include: adult and juvenile rheumatoid arthritis; systemic lupus erythematosus; gout; osteoarthritis; polymyalgia rheumatica; seronegative spondylarthropathies, including ankylosing spondylitis; and Reiter's disease. The subject TNFα inhibitors, compositions and combination therapies are used also to treat psoriatic arthritis and chronic Lyme arthritis. Also treatable with these compounds, compositions and combination therapies are Still's disease and uveitis associated with rheumatoid arthritis. In addition, the compounds, compositions and combination therapies of the invention are used in treating disorders resulting in inflammation of the voluntary muscle, including dermatomyositis and polymyositis. Moreover, the compounds, compositions ant combinations disclosed herein are useful for treating sporadic inclusion body myositis, as TNFα may play a significant role in the progression of this muscle disease. In addition, the compounds, compositions and combinations disclosed herein are used to treat multicentric reticulohistiocytosis, a disease in which joint destruction and papular nodules of the face and hands are associated with excess production of proinflammatory cytokines by multinucleated giant cells.

For purposes of this invention, patients are defined as having psoriatic arthrisis (PsA) if they have one or more swollen joints or one or more painful or tender joints, and also manifest at least one psoriatic lesion of the skin or nails. The psoriatic lesions may appear before or after the onset of swollen or tender joints. It is understood that prior to treatment, manifestations of PsA may have persisted over time, e.g., for several months or years, and may involve several joints. According to one classification system (reviewed in Alonso et al., 1991), PsA patients can be categorized based on their arthritic symptoms into five clinical subgroups: 1) DIP; 2) mutilans arthritis; 3) symmetrical polyarthritis; 4) oligoarticular arthritis; and 5) ankylosing spondylitis-like. The disclosed therapies, compounds and compositions are suitable for treating all five forms of PsA.

The TNFα inhibitors, compositions and combination therapies of the invention may be used to inhibit hypertrophic scarring, a phenomenon believed to result in part from excessive TNFα secretion. TNF inhibitors may be administered alone or concurrently with other agents that inhibit hypertrophic scarring, such as inhibitors of TGF-α.

Cervicogenic headache is a common form of headache arising from dysfunction in the neck area, and which is associated with elevated levels of TNFα, which are believed to mediate an inflammatory condition that contributes to the patient's discomfort (Martelletti, *Clin Exp Rheumatol* 18(2 Suppl 19):S33-8 (March-April, 2000)). Cervicogenic headache may be treated by administering an inhibitor of TNFα as disclosed herein, thereby reducing the inflammatory response and associated headache pain.

The TNFα inhibitors, compositions and combination therapies of the invention are useful for treating primary amyloidosis. In addition, the secondary amyloidosis that is characteristic of various conditions also are treatable with

TNFα inhibitors such as TNFR:Fc, and the compositions and combination therapies described herein. Such conditions include: Alzheimer's disease, secondary reactive amyloidosis; Down's syndrome; and dialysis-associated amyloidosis. Also treatable with the compounds, compositions and combination therapies of the invention are inherited periodic fever syndromes, including familial Mediterranean fever, hyperimmunoglobulin D and periodic fever syndrome and TNF-receptor associated periodic syndromes (TRAPS).

Disorders associated with transplantation also are treatable with the disclosed TNFα inhibitors, compositions or combination therapies, such as graft-versus-host disease, and complications resulting from solid organ transplantation, including transplantion of heart, liver, lung, skin, kidney or other organs. TNFR:Fc may be administered, for example, to prevent or inhibit the development of bronchiolitis obliterans after lung transplantation. Patients undergoing autologous hematopoietic stem cell transplantation in the form of peripheral blood stem cell transplantation may develop "engraftment syndrome," or "ES," which is an adverse and generally self-limited response that occurs about the time of hematopoietic engraftment and which can result in pulmonary deterioration. ES may be treated with inhibitors of either IL-8 or TNFα (such as TNFR:Fc), or with a combination of inhibitors against both of these cytokines.

Ocular disorders also are treatable with the disclosed TNFα inhibitors, compositions or combination therapies, including rhegmatogenous retinal detachment, and inflammatory eye disease, and inflammatory eye disease associated with smoking and macular degeneration.

TNFα inhibitors such as TNFR:Fc and the disclosed compositions and combination therapies also are useful for treating disorders that affect the female reproductive system. Examples include, but are not limited to, multiple implant failure/infertility; fetal loss syndrome or IV embryo loss (spontaneous abortion); preeclamptic pregnancies or eclampsia; and endometriosis.

In addition, the disclosed TNFα inhibitors, compositions and combination therapies are useful for treating obesity, including treatment to bring about a decrease in leptin formation. Also, the compounds, compositions and combination therapies of the invention are used to treat sciatica, symptoms of aging, severe drug reactions (for example, Il-2 toxicity or bleomycin-induced pneumopathy and fibrosis), or to suppress the inflammatory response prior, during or after the transfusion of allogeneic red blood cells in cardiac or other surgery, or in treating a traumatic injury to a limb or joint, such as traumatic knee injury. Various other medical disorders treatable with the disclosed TNFα inhibitors, compositions and combination therapies include; multiple sclerosis; Behcet's syndrome; Sjogren's syndrome; autoimmune hemolytic anemia; beta thalassemia; amyotrophic lateral sclerosis (Lou Gehrig's Disease); Parkinson's disease; and tenosynovitis of unknown cause, as well as various autoimmune disorders or diseases associated with hereditary deficiencies.

The disclosed TNFα inhibitors, compositions and combination therapies furthermore are useful for treating acute polyneuropathy; anorexia nervosa; Bell's palsy; chronic fatigue syndrome; transmissible dementia, including Creutzfeld-Jacob disease; demyelinating neuropathy; Guillain-Barre syndrome; vertebral disc disease; Gulf war syndrome; myasthenia gravis; silent cerebral ischemia; sleep disorders, including narcolepsy and sleep apnea; chronic neuronal degeneration; and stroke, including cerebral ischemic diseases.

Disorders involving the skin or mucous membranes also are treatable using the disclosed TNFα inhibitors, compositions or combination therapies. Such disorders include acantholytic diseases, including Darier's disease, keratosis follicularis and pemphigus vulgaris. Also treatable with the subject TNFα inhibitors, compositions and combination therapies are acne; acne rosacea; alopecia areata; aphthous stomatitis; bullous pemphigoid; burns; eczema; erythema, including erythema multiforme and erythema multiforme bullosum (Stevens-Johnson syndrome); inflammatory skin disease; lichen planus; linear IgA bullous disease (chronic bullous dermatosis of childhood); loss of skin elasticity; mucosal surface ulcers; neutrophilic dermatitis (Sweet's syndrome); pityriasis rubra pilaris; psoriasis; pyoderma gangrenosum; and toxic epidermal necrolysis.

Patients are defined as having ordinary psoriasis if they lack the more serious symptoms of PsA (e.g., distal interphalangeal joint DIP involvement, enthesopathy, spondylitis and dactylitis) but have one of the following: 1) inflamed swollen skin lesions covered with silvery white scale (plaque psoriasis or psoriasis vulgaris); 2) small red dots appearing on the trunk, arms or legs (guttate psoriasis); 3) smooth inflamed lesions without scaling in the flexural surfaces of the skin (inverse psoriasis); 4) widespread reddening and exfoliation of fine scales, with or without itching and swelling (erythrodermic psoriasis); 5) blister-like lesions (pustular psoriasis); 6) elevated inflamed scalp lesions covered by silvery white scales (scalp psoriasis); 7) pitted fingernails, with or without yellowish discoloration, crumbling nails, or inflammation and detachment of the nail from the nail bed (nail psoriasis).

Ordinary psoriasis may be treated by administering to a human patient compositions containing a therapeutically effective amount of a TNFα inhibitor such as a soluble TNF receptor or an antibody against TNFα.

In one preferred embodiment, the therapeutic agent is a soluble TNF receptor, and preferably is a TNFR-Ig. In a preferred embodiment, the TNFR-Ig is TNFR:Fc, which may be administered in the form of a pharmaceutically acceptable composition as described herein. Psoriasis may be treated by administering TNFR:Fc one or more times per week by subcutaneous injection, although other routes of administration may be used if desired. In one exemplary regimen for treating adult human patients, 25 mg of TNFR:Fc is administered by subcutaneous injection two times per week or three times per week for one or more weeks, and preferably for four or more weeks. Alternatively, a dose of 5-12 mg/m^2 or a flat dose of 50 mg is injected subcutaneously one time or two times per week for one or more weeks. In other embodiments, psoriasis is treated with TNFR:Fc in a sustained-release form, such as TNFR:Fc that is encapsulated in a biocompatible polymer, TNFR:Fc that is admixed with a biocompatible polymer (such as topically applied hydrogels), and TNFR:Fc that is encased in a semi-permeable implant.

Various other medicaments used to treat ordinary psoriasis may also be administered concurrently with compositions comprising TNFα inhibitors, such as TNFR:Fc. Such medicaments include: NSAIDs; DMARDs; analgesics; topical steroids; systemic steroids (e.g., prednisone); cytokines; antagonists of inflammatory cytokines; antibodies against T cell surface proteins; anthralin; coal tar; vitamin D3 and its analogs; topical retinoids; oral retinoids; salicylic acid; and hydroxyurea. Suitable analgesics for such combinations include: acetaminophen, codeine, propoxyphene napsylate, oxycodone hydrochloride, hydrocodone bitartrate and tramadol. DMARDs suitable for such combinations include: azathioprine, cyclophosphamide, cyclosporine, hydroxychloroquine sulfate, methotrexate, leflunomide, minocycline, penicillamine, sulfasalazine, oral gold, gold sodium thiomalate and aurothioglucose. In addition, the TNFR:Fc or other

TNFR mimic may be administered in combination with antimalarials or colchicine. NSAIDs suitable for the subject combination treatments of psoriasis include: salicylic acid (aspirin) and salicylate derivatives; ibuprofen; indomethacin; celecoxib; rofecoxib; ketorolac; nambumetone; piroxicam; naproxen; oxaprozin; sulindac; ketoprofen; diclofenac; and other COX-1 and COX-2 inhibitors, propionic acid derivatives, acetic acid derivatives, fumaric acid derivatives, carboxylic acid derivatives, butyric acid derivatives, oxicams, pyrazoles and pyrazolones, including newly developed anti-inflammatories.

If an antagonist against an inflammatory cytokine is administered concurrently with TNFR:Fc to treat psoriasis, suitable targets for such antagonists include TGFβ, Il-6 and Il-8.

In addition, TNFR:Fc may be used to treat psoriasis in combination with topical steroids, systemic steroids, antagonists of inflammatory cytokines, antibodies against T cell surface proteins, anthralin, coal tar, vitamin D3 and its analogs (including 1,25-dihydroxy vitamin D3 and calcipotriene), topical retinoids, oral retinoids (including but not limited to etretinate, acitretin and isotretinoin), topical salicylic acid, methotrexate. cyclosporine, hydroxyurea and sulfasalazine. In addition, TNFR:Fc may be administered to treat psoriasis in combination with one or more of the following compounds; minocycline; misoprostol; oral collagen; 6-mercaptopurine; nitrogen mustard; gabapentin; bromocriptine; somatostatin; peptide T; anti-CD4 monoclonal antibody; fumaric acid; polyunsaturated ethyl ester lipids; zinc; and other drugs that may be used to treat psoriasis. TNFR:Fc may also be used to treat psoriasis in combination with the use of various oils, including fish oils, nut oils and vegetable oils; aloe vera; jojoba; Dead Sea salts; capsaicin; milk thistle; witch hazel; moisturizers; and Epsom salts. In addition, psoriasis may be treated with compositions containing TNFR:Fc in combination with the following therapies: plasmapheresis; phototherapy with ultraviolet light B; psoralen combined with ultraviolet light A (PUVA); and sunbathing.

For determining the sufficiency of treatment when treating ordinary psoriasis in accord with the invention, the TNFR:Fc (or other TNFα inhibitor) is administered in an amount and for a time sufficient to induce an improvement in an indicator such as psoriasis area and severity index (PASI) or an improvement in Target Lesion Assessment score, which is an index for assessing the severity of individual skin lesions. In one embodiment, the treatment is regarded as sufficient when the patient exhibits an at least 50% improvement in his or her PASI score, and in another embodiment, when the patient exhibits an at least 75% improvement in PASI score. The sufficiency of treatment for psoriasis may also be determined by evaluating individual psoriatic lesions for improvement in severity (Psoriasis Target Lesion Assessment Score), and continuing treatment until an improvement is noted according to this scoring system. This scoring system involves determining for an individual lesion whether improvement has occurred in plaque elevation, amount and degree of scaling or degree of erythema, and target lesion response to treatment, each of which is separately scored. Psoriasis Target Lesion Assessment Score is determined by adding together the separate scores for all four of the aforementioned indicia.

In addition to human patients, inhibitors of TNFα are useful in the treatment of autoimmune and inflammatory conditions in non-human animals, such as pets (dogs, cats, birds, primates, etc.), domestic farm animals (horses cattle, sheep, pigs, birds, etc.), or any animal that suffers from a TNFα-mediated inflammatory or arthritic condition. In such instances, an appropriate dose may be determined according to the animal's body weight. For example, a dose of 0.2-1 mg/kg may be used. Alternatively, the dose is determined according to the animal's surface area, an exemplary dose ranging from 0.1-20 mg/m^2, or more preferably, from 5-12 mg/m^2. For small animals, such as dogs or cats, a suitable dose is 0.4 mg/kg. In a preferred embodiment, TNFR:Fc (preferably constructed from genes derived from the same species as the patient), or another soluble TNFR mimic, is administered by injection or other suitable route one or more times per week until the animal's condition is improved, or it may be administered indefinitely.

Example

Evaluation of TNFR:Fc in Patients with Psoriatic Arthritis

Sixty patients with active psoriatic arthritis (PsA) were enrolled in a Phase II double-blind, randomized, placebo controlled study to determine whether the subcutaneous biweekly administration of etanercept (recombinant TNFR:Fc) was safe in this patient population and whether efficacy could be documented for both the arthritic and psoriatic aspects of this disease.

In this study, a flat dose of 25 mg of TNFR:Fc was injected subcutaneously two times a week. After 12 weeks, patients who completed the study were eligible for continuation into a 24 week open-label extension of the study, with assessments made at weeks 16, 36 and 30 days post-study. All patients participating in the study extension received etanercept, including those patients who had received placebo during the blinded portion of the study.

In order to qualify for enrollment, subjects had to have at least one of the following forms of PsA: 1) DIP involvement; 2) polyarticular arthritis, absence of rheumatoid nodules and presence of psoriasis; 3) arthritis mutilans; 4) asymmetric peripheral arthritis; or 5) ankylosing spondylitis-like PsA. Subjects furthermore had to exhibit three or more swollen joints and three or more tender or painful joints at the time of enrollment, and to have exhibited an inadequate response to NSAID therapy. Subjects who were on other medications, including methotrexate, NSAIDs or oral corticosteroids were permitted to continue these other treatments at the same dose so long as the investigator considered these other treatments to inadequately control the patient's disease. Methotrexate was concurrently taken by 47% of the etanercept group, and 47% of the placebo group, NSAIDs were concurrently taken by 67% of the etanercept and 77% of the placebos and oral corticosteroids by 40% of the etanercept and 20% of the placebo patients. Pain medications, including acetaminophen, codeine, propoxyphene napsylate, oxycodone hydrochloride, hydrocodone bitartrate and tramadol, also were permitted during the study, as well as the use of topical tar compounds.

To qualify as having PsA, patients had to have experienced at least one psoriatic lesion of the skin or nails. Patients were evaluated at baseline (day 1 of treatment) as follows: 1) complete joint assessment; 2) psoriasis assessment; 3) duration of morning stiffness; 4) health assessment (quality of life) questionnaire, visual analog scale (HAQ/VAS); 5) patient global assessment; 6) erythrocyte sedimentation rate (ESR, Westergren); 7) C-reactive protein (CRP); and 8) urinalysis. At weeks 4 and 8, patients were evaluated as follows: 1) complete joint assessment; 2) psoriasis assessment; 3) duration of morning stiffness; 4) HAQ/VAS; 5) patient global assessment. At the end of 12 weeks, subjects were evaluated as follows: 1) complete joint assessment; 2) psoriasis assess-

ment; 3) focused physical exam; 4) duration of morning stiffness; 5) HAQ/VAS; 6) patient global assessment; 6) hematology profile; 7) chemistry profile; 8) ESR; 9) CRP; 10) urinalysis; 11) serum tested for antibody to TNFR:Fc. Only those patients whose psoriasis was stable and covered ≧3% of body area were evaluated for psoriasis response during this trial, although patients whose psoriasis was inactive or covered less area were permitted to enroll.

A primary endpoint for clinical improvement or worsening of PsA was the Psoriatic Arthritis Response score, which is a composite score based on the following four measures: 1) patient self-assessment; 2) physician assessment; 3) joint pain or tenderness: 4) joint swelling. Both self- and physician assessments, i.e., overall assessment of disease status, were measured according to a five point Likert scale, in which a patient was considered as "improved" if his or her score decreased by one category, or as "worse" if his or her score increased by one category. Joint pain or tenderness was measured on a 5-point scale, wherein 1=none and 5=severe (withdrawal on examination). Joint swelling was evaluated on a 4-point scale in which 1=none; 2=mild (detectable synovial thickening without loss of bony contour); 3=moderate (loss of distinctness of bony contours); and 4=severe (bulging synovial proliferation with cystic characteristics). For this last measure, a decrease in swelling of ≧30% was scored as an "improvement," and an increase in swelling of ≧30% was scored as a "worsening." Patients were classified as "improved" under the Psoriatic Arthritis Response scoring system if they exhibited an improvement in at least two of the four measures described above, provided that one of the improved areas was joint pain or joint tenderness, and where there was no worsening in any of the four measures.

In addition, a secondary endpoint used for assessing psoriatic arthritis was a modified version of the American College of Rheumatology Preliminary Definition of Improvement in Rheumatoid Arthritis (modified ACR 20 response) (Felson et al., 1995). To qualify as "improved" according to this measure, a patient must have exhibited ≧20% improvement in both tender joint count (78 joints assessed) and swollen joint count (76 joints assessed), and also must have shown an improvement in three of the following five: 1) subject pain assessment; 2) subject global assessment; 3) physician global assessment; 4) subject self-assessed disability; 5) acute-phase reactant (Westergreen erythrocyte sedimentation rate or C-reactive protein level). The joint count was done by scoring several different aspects of tenderness, such as pressure and joint manipulation on physical examination, wherein each joint was scored as "tender" or "nontender." Similarly, each joint is scored after physical examination as "swollen" or "not swollen." The subject's pain assessment was based on a horizontal visual analog scale (usually 10 cm) or Likert scale. The subject's and physician's global assessments of the subject's current disease status was based on an anchored horizontal visual analog scale (usually 10 cm), or Likert scale response. The subject's self-assessment of disability was based on any of the following measures, all of which have been validated in RA trials: Arthritis Impact Measurement Scale (AIMS); Health Assessment Questionnaire; the Quality (or Index) of Well Being Scale; the McMaster Health Inventory Questionnaire (MHIQ); and the McMaster-Toronto Arthritis patient preference questionnaire (MACTAR).

A primary endpoint used to assess the psoriatic aspects of PsA was the standard psoriasis area and severity index (PASI) (Fredriksson and Petersson, *Dermatologica* 157:238-244, 1978). For this study, a positive treatment response was defined as an at least 50% or an at least 75% improvement in a patient's PASI score. For assessing area and severity, the body is divided into four regions: head (10%); trunk (30%); upper extremities (20%); and lower extremities (40%). Each quadrant also was scored for the severity of erythema (E), infiltration (I) and desquamation (D), using a four point scale, in which 0=no symptoms present; 1=slight symptoms; 2=moderate symptoms; 3=striking symptoms; 4=exceptionally striking symptoms. Using a 6-point scale, each region was scored also for the percent of total area that was involved in the psoriatic manifestations of the disease, wherein 0=no involvement; 1=<10% involvement; 2=10-<30% involvement; 3=30-<50% involvement; 4=50-<70% involvement; 5=70-<90% involvement; 6=90-100% involvement. PASI scores were calculated according to the formula given below, in which E=severity score for erythrema, I=severity score for infiltration, D=severity score for desquamation and A=total area involved. In this formula, the letters "h," "t," "u" and "l" represent, respectively, the scores in each of the four body regions, i.e., head, trunk, upper extremities and lower extremities. The PASI score varies in steps of 0.1 units from 0.0 (no psoriatic lesions at all) to 72.0 (complete erythroderma of the severest possible degree).

$$PASI=0.1(Eh+Ih+Dh)Ah+0.3(Et+It+Dt)At+0.2(Eu+Iu+Du)Au+0.4(El+Il+Dl)Al$$

A secondary endpoint used for the psoriatic aspect of psoriatic arthritis was the Target Lesion Assessment Score. This score was determined for a single target lesion that was selected to be monitored throughout the trial. This measurement is a composite of four different evaluations: 1) plaque evaluation; 2) scaling; 3) erythrema; and 4) target lesion response to treatment. The following scale was used for the plaque elevation: 0=none (no evidence of plaque above normal skin level); 1=mild (slight but definite elevation above normal skin level); 2=moderate (moderate elevation with rounded or sloped edges to plaque); 3=severe (hard, marked elevation with sharp edges to plaque); 4=very severe (very marked elevation with very hard sharp edges to plaque). For the scaling assessment: 0=none (no scaling on the lesion); 1=mild (mainly fine scales, with some of the lesion at least partially covered); 2=moderate (somewhat coarser scales, most of the lesion at least partially covered); 3=severe (coarse, thick scales, virtually all the lesion covered, rough surface); 4=very severe (very coarse thick scales, all the lesions covered, very rough surface). For the erythema evaluation: 0=none (no erythema); 1=mild (light red coloration); 2=moderate (red coloration); 3=severe (very red coloration); 4=very severe (extreme red coloration). For target lesion response to treatment score: 0=completely cleared; 1=almost cleared (~90% improvement); 2=marked response (~75% improvement); 3=moderate response (~50% improvement); 4=slight response (~25% improvement); 5=condition unchanged; 6=condition worsened. The patient's Target Lesion Assessment Score was determined by summing the plaque, scaling, erythema and target lesion response scores for the monitored lesion. If the monitored lesion worsened, the percentage change from baseline was recorded as a negative number.

Treatment and placebo groups were compared in accord with the measurements described above, as well as for demographic and background characteristics; premature discontinuation rate; pain medication requirements; toxicities; serious adverse events; side effects reported by patients; number of weeks on drug until subjects met criteria for improvement, and response according to PsA subtype. Results were analyzed using standard statistical methods.

Dosing Regimen

Recombinant human TNFR:Fc (etanercept) from Immunex Corporation was used in this study. The gene fragments encoding the etanercept polypeptides were expressed in a Chinese hamster ovary (CHO) expression vector.

TNFR:Fc was supplied as a sterile lyophilized powder containing 10 mg or 25 mg TNFR:Fc; 40 mg mannitol, USP; 10 mg sucrose, NF; and 1.2 mg tromethamine (TRIS), USP per vial. Patients received either a dose of 25 mg of etanercept or a placebo. Vials of etanercept or identically-appearing placebo were reconstituted by aseptic injection of 1.0 mL Bacteriostatic Water for Injection, USP, (containing 0.9% benzyl alcohol), and was not filtered during preparation or prior to administration. If storage was required, the reconstituted solutions were stored at 2-8° C. (36-46° F.) in the original vial or in a plastic syringe for a period of no longer than 28 days. Dose was not changed during the study. Study drug was given twice weekly at approximately the same time of day.

Results

Study drug was well tolerated in all patients, and adverse events were consistent with this population and were equally distributed among both treatment groups. As illustrated in Tables 1-4, etanercept induced a significant improvement as compared with the placebo group in Psoriatic Arthritis Response (Table 1), ACR20 (Table 2), ACR50 (Table 3), PASI score, 50% improvement (Table 4), PASI score, 75% improvement (Table 5) and improvement in Target Lesion Assessment Score (Table 6). The fractions shown in Tables 1-5 represent numbers of patients. For example, the first entry in Table 1, which is "4/30," indicates that 4 of 30 patients in the placebo group scored as "improved" according to the Psoriatic Arthritis Response measurements. The tables include P-values for the differences between the two study groups, the groups being labeled as "PLACEBO" and "TNFR:Fc." All of the tables include data calculated after the first four weeks of the open label extension portion of the study ("EXTENSION"), during which all of the patients in both study groups received etanercept.

Table 1 shows the number of patients in each treatment group who scored as "improved" according to the Psoriatic Arthritis Response scoring system described above. By four weeks, there was a highly significant difference between etanercept and placebo groups. Moreover, after being switched to etanercept during the extension, those patients who had received placebo during the blinded portion of the study were seen to exhibit an improvement over baseline (Table 1, Placebo, EXTENSION). These results indicate that etanercept acts rapidly to alleviate many aspects of psoriatic arthritis.

TABLE 1

Psoriatic Arthritis Response

	Placebo	TNFR:Fc	P-value
4 weeks	4/30 (13%)	23/30 (77%)	0.000
8 weeks	7/30 (23%)	25/30 (83%)	0.000
12 weeks	6/30 (20%)	26/30 (87%)	0.000
EXTENSION	17/23 (74%)	21/25 (84%)	0.356

Tables 2 and 3, respectively, illustrate the study results for the ACR20 and ACR50 endpoints. For either measure, a significant difference between etanercept and placebo groups was observed at all three time points during the blinded portion of the study. Given the differences between test and placebo groups after only four weeks of treatment (P=0.000 for ACR20 and P=0.011 for ACR50), these data suggest that notable improvement in ACR scores occurred within the etanercept group very soon after treatment was initiated, possibly after a single dose of etanercept. During the 4 week extension period, during which all of the patients received etanercept, a striking improvement in both ACR20 and ACR50 was seen in those patients who had received placebo during the first 12 weeks (Tables 2 and 3).

TABLE 2

ACR20 Response

	Placebo	TNFR:Fc	P-value
4 weeks	1/30 (3%)	18/30 (60%)	0.000
8 weeks	3/30 (10%)	19/30 (63%)	0.000
12 weeks	4/30 (13%)	22/30 (73%)	0.000
EXTENSION	11/23 (48%)	18/25 (72%)	0.093

TABLE 3

ACR50 Response

	Placebo	TNFR:Fc	P-value
4 weeks	0/30 (0%)	6/30 (20%)	0.011
8 weeks	1/30 (3%)	11/30 (37%)	0.001
12 weeks	1/30 (3%)	15/30 (50%)	0.000
EXTENSION	7/23 (30%)	11/25 (44%)	0.316

The results of the psoriasis evaluations are presented in Tables 4-6. Tables 4 and 5, respectively, present the numbers and percentages of patients in each group who exhibited a 50% or 75% improvement in PASI score, while Table 6 presents Target Lesion Assessment scores, these latter being denoted as percent improvement over baseline. The data in Tables 4-6 clearly indicate that etanercept induced an improvement in psoriasis for a large percentage of the patients who received it. When single lesions were evaluated (Table 6), the improvement in psoriasis was even more apparent than when PASI scores were used (Tables 4 and 5). It is notable also that, for either PASI scores (Tables 4 and 5) or Psoriasis Target Lesion Assessment Score (Table 6), the scores of the placebo group improved after these patients were switched to etanercept during the extension.

Though not shown in Table 6, Target Lesion Assessment Scores for patients who were concurrently receiving methotrexate (14 of the 30 patients in the etanercept group, and 14 patients in the placebo group) were compared with the scores of those patients who did not take methotrexate. Little difference in this index was noted between the patients who received methotrexate and those who did not receive it.

TABLE 4

PASI Score - 50% Improvement

	Placebo	TNFR:Fc	P-value
4 weeks	0/19 (0%)	4/19 (21%)	0.037
8 weeks	1/19 (5%)	7/19 (37%)	0.019
12 weeks	4/19 (21%)	8/19 (42%)	0.165
EXTENSION	6/16 (38%)	6/15 (40%)	0.856

TABLE 5

PASI Response Rate 75% Improvement

	Placebo	TNFR:Fc	P-value
4 weeks	0/19 (0%)	1/19 (5%)	0.264
8 weeks	0/19 (0%)	2/19 (11%)	0.153
12 weeks	0/19 (0%)	4/19 (21%)	0.037
EXTENSION	1/16 (6%)	4/15 (27%)	0.113

TABLE 6

Psoriasis Target Lesion Assessment
(Percent Improvement or Worsening Compared with Baseline)

		Placebo	TNFR:Fc	P-value
4 weeks	Mean (SD)	2.7 (27.6)	21.2 (35.2)	0.120
	Median	0.0	14.3	
	MIN--MAX	−50.0-50.0	−33.3-100.0	
	N	19	19	
8 weeks	Mean (SD)	−7.5 (25.3)	28.5 (34.1)	0.003
	Median	0.0	29.2	
	MIN--MAX	−50.0-20.0	−33.3-100.0	
	N	17	18	
12 weeks	Mean (SD)	9.5 (23.2)	45.7 (31.6)	0.001
	Median	0.0	50.0	
	MIN--MAX	−25.0-50.0	−16.7-100.0	
	N	16	19	
EXTENSION	Mean (SD)	28.9 (41.2)	47.1 (35.8)	0.263
	Median	36.7	50.0	
	MIN--MAX	−100.0-66.7	−33.3-100.0	
	N	16	15	

What is claimed is:

1. A method for treating a patient having psoriasis comprising administering to the patient a therapeutically effective dose of TNFR:Fc, wherein the patient attains at least fifty percent improvement in PASI score.

2. The method of claim **1**, wherein the dose of TNFR:Fc administered is either 50 mg once per week or 25 mg twice per week.

3. The method of claim **1**, wherein the dose of TNFR:Fc administered is 50 mg twice per week.

4. The method of claim **1**, wherein the TNFR:Fc is administered by subcutaneous injection.

5. The method of claim **1**, wherein

(a) a dose of 50 mg of TNFR:Fc is administered two times per week for at least two months and then

(b) TNFR:Fc is administered at a reduced dose or at a reduced frequency.

6. The method of claim **5**, wherein the administration of (b) is at a dose 25 mg of TNFR:Fc twice per week.

7. The method of claim **5**, wherein the administration of (b) is at a dose of 50 mg once per week.

8. The method of claim **5**, wherein the TNFR:Fc is administered by subcutaneous injection.

9. The method of claim **1**, wherein the patient exhibits at least seventy five percent improvement in PASI score after three months of treatment.

10. The method of claim **1**, wherein a corticosteroid is administered concurrently.

11. The method of claim **1**, wherein methotrexate is administered concurrently.

12. A method for treating a patient having psoriasis and psoriatic arthritis comprising administering to the patient a therapeutically effective dose of TNFR:Fc, wherein the patient attains at least fifty percent improvement in PASI score.

13. The method of claim **12**, wherein the dose of TNFR:Fc administered is either 50 mg once per week or 25 mg twice per week.

14. The method of claim **12**, wherein a corticosteroid is administered concurrently.

15. The method of claim **12**, wherein methotrexate is administered concurrently.

16. A method for treating a patient having psoriasis comprising

(a) administering to the patient TNFR:Fc subcutaneously at a dose of 50 mg twice per week for at least two months and then

(b) administering TNFR:Fc subcutaneously at a dose of 50 mg once per week or at a dose of 25 mg twice per week.

17. The method of claim **1**, wherein cyclosporine is administered concurrently.

18. The method of claim **1**, wherein acitretin is administered concurrently.

19. The method of claim **1**, wherein ultraviolet light B phototherapy or psoralen combined with ultraviolet light A (PUVA) phototherapy is administered concurrently.

20. The method of claim **16**, wherein the patient attains at least fifty percent improvement in PASI score.

* * * * *

Exhibit F

(12) **United States Patent**
Finck

(10) **Patent No.: US 8,119,605 B2**
(45) **Date of Patent: *Feb. 21, 2012**

(54) **SOLUBLE TUMOR NECROSIS FACTOR RECEPTOR TREATMENT OF MEDICAL DISORDERS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

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(60) Division of application No. 12/394,962, filed on Feb. 27, 2009, now Pat. No. 7,915,225, which is a division of application No. 10/853,479, filed on May 25, 2004, now abandoned, which is a division of application No. 09/602,351, filed on Jun. 23, 2000, now abandoned, and a continuation-in-part of application No. 09/373,828, filed on Aug. 13, 1999, now abandoned.

(60) Provisional application No. 60/164,676, filed on Nov. 10, 1999, provisional application No. 60/184,864, filed on Feb. 25, 2000, provisional application No. 60/130,074, filed on Apr. 19, 1999, provisional application No. 60/134,320, filed on May 14, 1999, provisional application No. 60/143,959, filed on Jul. 15, 1999, provisional application No. 60/148,234, filed on Aug. 11, 1999.

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(52) **U.S. Cl.** **514/21.2**; 514/18.7; 514/169; 514/863; 552/588; 607/94; 530/350; 530/866

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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(57) **ABSTRACT**

The invention pertains to methods and compositions for treating medical disorders characterized by elevated levels or abnormal expression of TNFα by administering a TNFα antagonist, such as recombinant TNFR:Fc.

13 Claims, No Drawings

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SOLUBLE TUMOR NECROSIS FACTOR RECEPTOR TREATMENT OF MEDICAL DISORDERS

This application is a continuation of U.S. application Ser. No. 12/394,962, filed Feb. 27, 2009, now allowed; which is a divisional of U.S. application Ser. No. 10/853,479, filed May 25, 2004, now abandoned; which is a divisional of U.S. application Ser. No. 09/602,351, filed Jun. 23, 2000, now abandoned, which claims benefit of U.S. Provisional Application Nos. 60/164,676, filed Nov. 10, 1999, now abandoned, and 60/184,864, filed Feb. 25, 2000, now abandoned; and which is a continuation-in-part of U.S. application Ser. No. 09/373,828, filed Aug. 13, 1999, now abandoned, which claims the benefit of U.S. Provisional Application Nos. 60/130,074, filed Apr. 19, 1999, now abandoned, 60/134,320, filed May 14, 1999, now abandoned, 60/143,959, filed Jul. 15, 1999, now abandoned, and 60/148,234, filed Aug. 11, 1999, now abandoned; all of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The invention pertains to methods for treating various medical disorders that are characterized by abnormal or excessive TNFα levels by administering a TNFα antagonist, preferably a soluble TNFα. The TNFα inhibitor may be administered in combination with other biologically active molecules.

BACKGROUND OF THE INVENTION

The pleiotropic cytokine tumor necrosis factor alpha (TNFα) is associated with inflammation and binds to cells through membrane receptor molecules, including two molecules having molecular weights of approximately 55 kDa and 75 kDa (p55 and p75). In addition to binding TNFα, the p55 and p75 TNF receptors mediate the binding to cells of homotrimers of TNFβ, which is another cytokine associated with inflammation and which shares structural similarities with TNFα (e.g., see Cosman, *Blood Cell Biochem* 7:51-77, 1996). TNFβ is also known as lymphotoxin-α (LTα).

It has been proposed that a systemic or localized excess of TNFα contributes to the progression of numerous medical disorders. For example, patients with chronic heart failure have elevated levels of serum TNFα, which have been shown to increase with disease progression (see, for example, Levine et al., *N Eng J Med* 323:236-241, 1990). A variety of other diseases are associated with elevated levels of TNFα (see, for example, Feldman et al., *Transplantation Proceedings* 30:4126-4127, 1998).

Psoriatic arthritis (PsA) is a chronic autoimmune condition that shares some features with both rheumatoid arthritis (RA) and the inflammatory skin disease psoriasis (for review, see Breathnach, In Klippel and Dieppe eds. *Rheumatology*, 2nd Ed., Mosby, 1998, 22.1-22.4). Psoriasis is characterized by epidermal keratinocyte hyperproliferation, accompanied by neutrophil and T cell infiltration, and is associated with elevated levels of inflammatory cytokines, including TNFα, IL-6 and TGFβ (see, for example, Bonifati et al., *Clin Exp Dermatol* 19:383-387, 1994). Psoriasis and PsA are different clinical entities, and are associated with somewhat different MHC haplotypes (Gladman, *Rheum Dis Clin NA.* 18:247-256, 1992; Breathnach, 1998). The overall prognosis for PsA is far worse than for ordinary psoriasis. Nonetheless, treatments used for the psoriatic lesions of PsA generally are similar to those used to treat psoriasis.

Psoriatic skin lesions are present in patients with PsA, although only a minority of psoriasis sufferers actually have PsA. Ordinary psoriasis occasionally is accompanied by joint pain, but does not involve the extreme pain and often deforming degeneration of joints and bone that occurs in PsA patients.

Treatments that sometimes are effective in treating ordinary psoriasis include topical medications (e.g., steroids, coal tar, anthralin, Dead Sea salts, various natural oils, vitamin D3 and its analogs, sunshine, topical retinoids), phototherapy (e.g., ultraviolet light, photochemotherapy (PUVA)), and internal medications (e.g., methotrexate, systemic steroids, oral retinoids, cyclosporine, or a rotating regimen of these three). In addition, it has been proposed that psoriasis could be treated with TNF-derived peptides, quinolinesulfonamides, pyrrolidinone derivatives, catechol diether compounds, isoxazoline compounds, matrix metalloproteinase inhibitors or mercapto alkyl peptidyl compounds, all of which inhibit either TNFα production or its release from cultured cells (see, for example, U.S. Pat. No. 5,691,382, U.S. Pat. No. 5,834,485, U.S. Pat. No. 5,420,154, U.S. Pat. No. 5,563,143, U.S. Pat. No. 5,869,511 and U.S. Pat. No. 5,872,146), as well as with various combination therapies involving TNFα antagonists (for example, see U.S. Pat. No. 5,888,511 or U.S. Pat. No. 5,958,413).

Conflicting results have been reported regarding the role of TNFα in psoriasis. Some investigators have proposed that overproduction of TNFα contributes to the pathology of psoriasis (e.g., Pigatto et al., *J Invest Dermatol* 94:372-376, 1990; Sagawa et al., *Dermatol* 187:81-83, 1993; Ameglio et al., *Dermatol* 189:359-363, 1994). One group reported some improvement after treatment with pentoxifylline, a drug that can inhibit the release of TNFα, but which exerts many of its physiological effects by inhibiting cyclic AMP phosphodiesterase (Omulecki et al., *J Am Acad Dermatol* 34:714-715, 1996; Centola et al., *J Androl* 16:136-142, 1995; Elferinck et al., *Biochem Pharmacol* 54:475-480, 1997). However, other reports have cast doubt on the hypothesis that overproduction of TNFα exacerbates psoriasis. For example, some investigators have reported that treatment with TNFα itself actually can mitigate psoriasis (see, e.g., Takematsu et al., *Br J Dermatol* 124:209-210, 1991; Creaven et al., *J Am Acad Dermatol* 24:735-737, 1991).

In addition to psoriatic lesions, PsA is characterized by distal interphalangeal joint (DIP) involvement, enthesopathy, nail lesions, spondylitis and dactylitis. The histopathogenesis of PsA and the more well-studied rheumatoid arthritis share certain features. In both RA and in active PsA, patients exhibit increased levels of HLA-DR$^+$ T cells and MHC class II antigens in their synovial membranes and synovial fluid, as well as increased expression of the cytokine TNFα. In addition, both diseases are associated with prominent synovial vascular changes.

The discovery of rheumatoid factor in the serum of RA patients provided an important tool for differentiating PsA from RA, but the realization that RA and PsA are distinct diseases was based primarily on their many clinical differences (e.g., Helliwell and Wright, In Klippel and Dieppe eds. *Rheumatology*, 2nd Ed., Mosby, 1998, 21.1-21.8). Studies have shown that levels of TNFα, Il-1β, Il-8 as well as TNFα receptors in synovial fluids were higher in PsA patients than in osteoarthritis patients, though they were lower than in RA patients (Partsch et al., *J Rheumatol* 24:518-523, 1997; Partsch et al., *J Rheumatol* 25:105-110, 1998; Partsch et al., *Ann Rheum Dis* 57:691-693, 1998). PsA is distinguished from RA also by radiographic appearance, a notably higher degree of synovial membrane vascularity as well as differ-

ences in the levels of various cytokines in the synovial fluids (Ritchlin et al., *J Rheumatol* 25:1544-52, 1998; Veale et al., *Arth Rheum* 36:893-900, 1993). Veale et al. noted differences in synovial membrane adhesion molecules and numbers of macrophages when they compared RA and PsA patients, as well as observing a minimal degree of hyperplasia and hypertrophy of synoviocytes in PsA as compared with RA patients. Because of such differences, coupled with the association of PsA but not RA with class I MHC antigens, Ritchlin et al. have suggested that PsA must be triggered by different mechanisms than those underlying RA. Veale et al. suggested for similar reasons that different cytokines were likely to be interacting in the synovium of PsA and RA patients.

Most of the drugs used for treating the arthritic aspects of PsA are similar to those used in RA (Salvarini et al., *Curr Opin Rheumatol* 10:229-305, 1998), for example the non-steroidal antiinflammatories (NSAIDs), which may be used alone or in combination with the disease-modifying anti-rheumatic drugs, or "DMARDs." However, one group found that long-term administration of the DMARD methotrexate failed to slow the progression of joint damage in PsA patients (Abu-Shakra et al., *J Rheumatol* 22:241-45, 1995), and another group reported very little improvement in PsA patients who had received methotrexate (Willkens et al., *Arthr Rheum* 27:376-381, 1984). Similarly, Clegg et al. found only a slight improvement over placebo in PsA patients treated with sulfasalazine, another drug classified as a DMARD (Clegg et al., *Arthritis Rheum* 39: 2013-20, 1996). Some studies have indicated that the immunosuppressor cyclosporine is effective in treating PsA (reviewed in Salvarini et al., 1998), though this drug has severe side effects. In addition, others have proposed that PsA could be treated with truncated TNFα receptors or with a combination of methotrexate and antibodies against TNFα (WO 98/01555; WO 98/0537).

A recent meta-analysis of a number of PsA treatment studies concluded that PsA and RA differed not only in their response to treatment with specific drugs, but in the relative magnitudes of improvement in the placebo arms of the studies (Jones et al., *Br J Rheumatol* 36:95-99, 1997). As an example, PsA patients responded better to gold salt therapy than did RA patients, though the gold did not affect the psoriatic skin lesions (Dorwart et al., *Arthritis Rheum* 21:515-513, 1978).

It has been suggested that the suppression of TNFα might be beneficial in patients suffering from various disorders characterized by abnormal or excessive TNFα expression. However, although progress has been made in devising effective treatment for such diseases, improved medicaments and methods of treatment are needed.

SUMMARY OF THE INVENTION

Provided herein are methods for treating a number of medical disorders characterized by abnormal TNFα expression by repeatedly administering an antagonist of TNFα, such as a soluble TNFα receptor, for a period of time sufficient to induce a sustained improvement in the patient's condition.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides compounds, compositions and methods for treating a mammalian patient, including a human patient, who is suffering from a medical disorder that is characterized by abnormal or elevated expression of TNFα. For purposes of this disclosure, the terms "illness," "disease," "medical condition," "abnormal condition" and the like are used interchangeably with the term "medical disorder."

The subject methods involve administering to the patient a soluble TNFα antagonist that is capable of reducing the effective amount of endogenous biologically active TNFα, such as by reducing the amount of TNFα produced, or by preventing the binding of TNFα to its cell surface receptor (TNFR). Antagonists capable of inhibiting this binding include receptor-binding peptide fragments of TNFα, antibodies directed against TNFα, and recombinant proteins comprising all or portions of receptors for TNFα or modified variants thereof, including genetically-modified muteins, multimeric forms and sustained-release formulations. Other compounds suitable for treating the diseases described herein include thalidomide and pentoxifylline.

Preferred embodiments of the invention utilize soluble TNFRs as the TNFα antagonist. Soluble forms of TNFRs may include monomers, fusion proteins (also called "chimeric proteins), dimers, trimers or higher order multimers. In certain embodiments of the invention, the soluble TNFR derivative is one that mimics the 75 kDa TNFR or the 55 kDa TNFR and that binds to TNFα in the patient's body. The soluble TNFR mimics of the present invention may be derived from TNFRs p55 or p75 or fragments thereof. TNFRs other than p55 and p75 also are useful for deriving soluble compounds for treating the various medical disorders described herein, such for example the TNFR described in WO 99/04001. Soluble TNFR molecules used to construct TNFR mimics include, for example, analogs or fragments of native TNFRs having at least 20 amino acids, that lack the transmembrane region of the native TNFR, and that are capable of binding TNFα. Antagonists derived from TNFRs compete for TNFα with the receptors on the cell surface, thus inhibiting TNFα from binding to cells, thereby preventing it from manifesting its biological activities. Binding of soluble TNFRs to TNFα or LTα can be assayed using ELISA or any other convenient assay. This invention provides for the use of soluble TNFα receptors in the manufacture of medicaments for the treatment of numerous diseases.

The soluble TNFR polypeptides or fragments of the invention may be fused with a second polypeptide to form a chimeric protein. The second polypeptide may promote the spontaneous formation by the chimeric protein of a dimer, trimer or higher order muimer that is capable of binding a TNFα and/or LTα molecule and preventing it from binding to cell-bound receptors. Chimeric proteins used as antagonists include, for example, molecules derived from an antibody molecule and a TNFR. Such molecules are referred to herein as TNFR-Ig fusion proteins. A preferred TNFR-Ig fusion protein suitable for treating diseases in humans and other mammals is recombinant TNFR:Fc, a term which as used herein refers to "etanercept," which is a dimer of two molecules of the extracellular portion of the p75 TNFα receptor, each molecule consisting of a 235 amino acid TNFR-derived polypeptide that is fused to a 232 amino acid Fc portion of human IgG_1. Etanercept is currently sold by Immunex Corporation under the trade name ENBREL.® Because the p75 receptor protein that it incorporates binds not only to TNFα, but also to the inflammatory cytokine LTα, etanercept can act as a competitive inhibitor not only of TNFα, but also of LTα. This is in contrast to antibodies directed against TNFα, which cannot inhibit LTα. Also encompassed by the invention are treatments using a compound that comprises the extracellular portion of the 55 kDa TNFR fused to the Fc portion of IgG, as well as compositions and combinations containing such a molecule. Encompassed also are therapeutic methods involving the administration of TNFR-Ig proteins derived the extra-

cellular regions of TNFα receptor molecules other than the p55 and p75 TNFRs, such as for example the TNFR described in WO 99/04001.

In one preferred embodiment of the invention, sustained-release forms of soluble TNFRs are used, including sustained-release forms of TNFR:Fc. Sustained-release forms suitable for use in the disclosed methods include, but are not limited to, TNFRs that are encapsulated in a slowly-dissolving biocompatible polymer (such as the alginate microparticles described in U.S. Pat. No. 6,036,978), admixed with such a polymer (including topically applied hydrogels), and or encased in a biocompatible semi-permeable implant. In addition, the soluble TNFR may be conjugated with polyethylene glycol (pegylated) to prolong its serum half-life or to enhance protein delivery.

In accord with this invention, medical disorders characterized by abnormal or excess expression of TNFα are administered a therapeutically effective amount of a TNFα inhibitor. The TNFα inhibitor may be a TNFα-binding soluble TNFα receptor, preferably TNFR:Fc. As used herein, the phrase "administering a therapeutically effective amount" of a therapeutic agent means that the patient is treated with the agent in an amount and for a time sufficient to induce a sustained improvement in at least one indicator that reflects the severity of the disorder. An improvement is considered "sustained" if the patient exhibits the improvement on at least two occasions separated by one or more weeks. The degree of improvement is determined based on signs or symptoms, and determinations may also employ questionnaires that are administered to the patient, such as quality-of-life questionnaires.

Various indicators that reflect the extent of the patient's illness may be assessed for determining whether the amount and time of the treatment is sufficient. The baseline value for the chosen indicator or indicators is established by examination of the patient prior to administration of the first dose of the etanercept or other TNFα inhibitor. Preferably, the baseline examination is done within about 60 days of administering the first dose. If the TNFα antagonist is being administered to treat acute symptoms, such as for example to treat a traumatic knee injury, the first dose is administered as soon as practically possible after the injury has occurred.

Improvement is induced by administering TNFR:Fc or other TNFα antagonist until the patient manifests an improvement over baseline for the chosen indicator or indicators. In treating chronic conditions, this degree of improvement is obtained by repeatedly administering this medicament over a period of at least a month or more, e.g., for one, two, or three months or longer, or indefinitely. A period of one to six weeks, or even a single dose, often is sufficient for treating acute conditions. For injuries or acute conditions, a single dose may be sufficient.

Although the extent of the patient's illness after treatment may appear improved according to one or more indicators, treatment may be continued indefinitely at the same level or at a reduced dose or frequency. Once treatment has been reduced or discontinued, it later may be resumed at the original level if symptoms should reappear.

Any efficacious route of administration may be used to therapeutically administer TNFR:Fc or other TNFα antagonists. If injected, TNFR:Fc can be administered, for example, via intra-articular, intravenous, intramuscular, intralesional, intraperitoneal or subcutaneous routes by bolus injection or by continuous infusion. Other suitable means of administration include sustained release from implants, aerosol inhalation, eyedrops, oral preparations, including pills, syrups, lozenges or chewing gum, and topical preparations such as lotions, gels, sprays, ointments or other suitable techniques. Alternatively, proteinaceous TNFα inhibitors, such as a soluble TNFR, may be administered by implanting cultured cells that express the protein, for example, by implanting cells that express TNFR:Fc. In one embodiment, the patient's own cells are induced to produce TNFR:Fc by transfection in vivo or ex vivo with a DNA that encodes TNFR:Fc. This DNA can be introduced into the patient's cells, for example, by injecting naked DNA or liposome-encapsulated DNA that encodes TNFR:Fc, or by other means of transfection. When TNFR:Fc is administered in combination with one or more other biologically active compounds, these may be administered by the same or by different routes, and may be administered simultaneously, separately or sequentially.

TNFR:Fc or other soluble TNFRs preferably are administered in the form of a physiologically acceptable composition comprising purified recombinant protein in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers are nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the TNFα antagonist with buffers, antioxidants such as ascorbic acid, low molecular weight polypeptides (such as those having fewer than 10 amino acids), proteins, amino acids, carbohydrates such as glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents. In accordance with appropriate industry standards, preservatives may also be added, such as benzyl alcohol. TNFR:Fc preferably is formulated as a lyophilizate using appropriate excipient solutions (e.g., sucrose) as diluents. Appropriate dosages can be determined in standard dosing trials, and may vary according to the chosen route of administration. The amount and frequency of administration will depend, of course, on such factors as the nature and severity of the indication being treated, the desired response, the age and condition of the patient, and so forth.

In one embodiment of the invention, TNFR:Fc is administered one time per week to treat the various medical disorders disclosed herein, in another embodiment is administered at least two times per week, and in another embodiment is administered at least three times per week. An adult patient is a person who is 18 years of age or older. If injected, the effective amount of TNFR:Fc per adult dose ranges from 1-20 mg/m^2, and preferably is about 5-12 mg/m^2. Alternatively, a flat dose may be administered, whose amount may range from 5-100 mg/dose. Exemplary dose ranges for a flat dose to be administered by subcutaneous injection are 5-25 mg/dose, 25-50 mg/dose and 50-100 mg/dose. In one embodiment of the invention, the various indications described below are treated by administering a preparation acceptable for injection containing TNFR:Fc at 25 mg/dose, or alternatively, containing 50 mg per dose. The 25 mg or 50 mg dose may be administered repeatedly, particularly for chronic conditions. If a route of administration other than injection is used, the dose is appropriately adjusted in accord with standard medical practices. In many instances, an improvement in a patient's condition will be obtained by injecting a dose of about 25 mg of TNFR:Fc one to three times per week over a period of at least three weeks, or a dose of 50 mg of TNFR:Fc one or two times per week for at least three weeks, though treatment for longer periods may be necessary to induce the desired degree of improvement. For incurable chronic conditions, the regimen may be continued indefinitely, with adjustments being made to dose and frequency if such are deemed necessary by the patient's physician.

For pediatric patients (age 4-17), a suitable regimen involves the subcutaneous injection of 0.4 mg/kg, up to a maximum dose of 25 mg of TNFR:Fc, administered by subcutaneous injection one or more times per week.

The invention further includes the administration of TNFR:Fc concurrently with one or more other drugs that are administered to the same patient in combination with the TNFR:Fc, each drug being administered according to a regimen suitable for that medicament. "Concurrent administration" encompasses simultaneous or sequential treatment with the components of the combination, as well as regimens in which the drugs are alternated, or wherein one component is administered long-term and the other(s) are administered intermittently. Components may be administered in the same or in separate compositions, and by the same or different routes of administration. Examples of drugs to be administered concurrently include but are not limited to antivirals, antibiotics, analgesics, corticosteroids, antagonists of inflammatory cytokines, DMARDs and non-steroidal anti-inflammatories. DMARDs that can be administered in combination with the subject TNFα inhibitors such as TNFR:Fc include azathioprine, cyclophosphamide, cyclosporine, hydroxychloroquine sulfate, methotrexate, leflunomide, minocycline, penicillamine, sulfasalazine and gold compounds such as oral gold, gold sodium thiomalate and aurothioglucose. Additionally, TNFR:Fc may be combined with a second TNFα antagonist, including an antibody against TNFα or TNFR, a TNFα-derived peptide that acts as a competitive inhibitor of TNFα (such as those described in U.S. Pat. No. 5,795,859), a TNFR-IgG fusion protein other than etanercept, such as one containing the extracellular portion of the p55 TNFα receptor, a soluble TNFR other than an IgG fusion protein, or other molecules that reduce endogenous TNFα levels, such as inhibitors of the TNFα converting enzyme (see e.g., U.S. Pat. No. 5,594,106). In further embodiments of this invention, TNFR:Fc is administered in combination with pentoxifylline or thalidomide.

If an antibody against TNFα is used as the TNFα inhibitor, a preferred dose range is 0.1 to 20 mg/kg, and more preferably is 1-10 mg/kg. Another preferred dose range for anti-TNFα antibody is 0.75 to 7.5 mg/kg of body weight. Humanized antibodies are preferred, that is, antibodies in which only the antigen-binding portion of the antibody molecule is derived from a non-human source. such antibodies may be injected or administered intravenously.

In one preferred embodiment of the invention, the various medical disorders disclosed herein as being treatable with inhibitors such as TNFR:Fc are treated in combination with another cytokine or cytokine inhibitor. For example, TNFR:Fc may be administered in a composition that also contains a compound that inhibits the interaction of other inflammatory cytokines with their receptors. Examples of cytokine inhibitors used in combination with TNFR:Fc include, for example, antagonists of TGFβ, Il-6 or Il-8. TNFα inhibitors such as TNFR:Fc also may be administered in combination with the cytokines GM-CSF, IL-2 and inhibitors of protein kinase A type 1 to enhance T cell proliferation in HIV-infected patients who are receiving anti-retroviral therapy. Other combinations for treating the hereindescribed diseases include TNFR:Fc administered concurrently with compounds that block the binding of RANK and RANK-ligand, such as antagonistic antibodies against RANK or RANK-ligand, soluble forms of RANK-ligand that do not trigger RANK, osteoprotegerin or soluble forms of RANK, including RANK:Fc. Soluble forms of RANK suitable for these combinations are described, for example, in U.S. Pat. No. 6,017,729. The concurrent administration of TNFR:Fc and RANK:Fc or TNFR:Fc and osteoprotegerin is useful for preventing bone destruction in various settings including but not limited to various rheumatic disorders, osteoporosis, multiple myeloma or other malignancies that cause bone degeneration, or anti-tumor therapy aimed at preventing metastasis to bone, or bone destruction associated with prosthesis wear debris or with periodontitis.

The present invention also relates to the use of the disclosed TNFα inhibitors, such as TNFR:Fc, in the manufacture of a medicament for the prevention or therapeutic treatment of each medical disorder disclosed herein.

The disclosed TNFα inhibitors, compositions and combination therapies described herein are useful in medicines for treating bacterial, viral or protozoal infections, and complications resulting therefrom. One such disease is *Mycoplasma pneumonia*. In addition, provided herein is the use of TNFR:Fc to treat AIDS and related conditions, such as AIDS dementia complex, AIDS associated wasting, lipidistrophy due to antiretroviral therapy; and Kaposi's sarcoma. Provided herein is the use of TNFR:Fc for treating protozoal diseases, including malaria and schistosomiasis. Additionally provided is the use of TNFR:Fc to treat erythema nodosum leprosum; bacterial or viral meningitis; tuberculosis, including pulmonary tuberculosis; and pneumonitis secondary to a bacterial or viral infection. Provided also herein is the use of TNFR:Fc to prepare medicaments for treating louse-borne relapsing fevers, such as that caused by *Borrelia recurrentis*. TNFR:Fc can also be used to prepare a medicament for treating conditions caused by Herpes viruses, such as herpetic stromal keratitis, corneal lesions, and virus-induced corneal disorders. In addition, TNFR:Fc can be used in treating human papillomavirus infections. TNFR:Fc is used also to prepare medicaments to treat influenza.

Cardiovascular disorders are treatable with the disclosed TNFα inhibitors, pharmaceutical compositions or combination therapies, including aortic aneurisms; arteritis; vascular occlusion, including cerebral artery occlusion; complications of coronary by-pass surgery; ischemia/reperfusion injury; heart disease, including atherosclerotic heart disease, myocarditis, including chronic autoimmune myocarditis and viral myocarditis; heart failure, including chronic heart failure (CHF), cachexia of heart failure; myocardial infarction; restenosis after heart surgery; silent myocardial ischemia; post-implantation complications of left ventricular assist devices; Raynaud's phenomena; thrombophlebitis; vasculitis, including Kawasaki's vasculitis; giant cell arteritis, Wegener's granulomatosis; and Schoenlein-Henoch purpura.

TNFα and IL-8 have been implicated as chemotactic factors in atherosclerotic abdominal aortic aneurism (Szekanecz et al., *Pathobiol* 62:134-139 (1994)). Abdominal aortic aneurism may be treated in human patients by administering a soluble TNFR, such as TNFR:Fc, which may be administered in combination with an inhibitor of IL-8, such treatment having the effect of reducing the pathological neovascularization associated with this condition.

A combination of a TNFα inhibitor and one or more other anti-angiogenesis factors may be used to treat solid tumors, thereby reducing the vascularization that nourishes the tumor tissue. Suitable anti-angiogenic factors for such combination therapies include IL-8 inhibitors, angiostatin, endostatin, kringle 5, inhibitors of vascular endothelial growth factor (such as antibodies against vascular endothelial growth factor), angiopoietin-2 or other antagonists of angiopoietin-1, antagonists of platelet-activating factor and antagonists of basic fibroblast growth factor

In addition, the subject TNFα inhibitors, compositions and combination therapies are used to treat chronic pain condi-

tions, such as chronic pelvic pain, including chronic prostatitis/pelvic pain syndrome. As a further example, TNFR:Fc and the compositions and combination therapies of the invention are used to treat post-herpetic pain.

Provided also are methods for using TNFα inhibitors, compositions or combination therapies to treat various disorders of the endocrine system. For example, the TNFα inhibitors are used to treat juvenile onset diabetes (includes autoimmune and insulin-dependent types of diabetes) and also to treat maturity onset diabetes (includes non-insulin dependent and obesity-mediated diabetes). In addition, the subject compounds, compositions and combination therapies are used to treat secondary conditions associated with diabetes, such as diabetic retinopathy, kidney transplant rejection in diabetic patients, obesity-mediated insulin resistance, and renal failure, which itself may be associated with proteinurea and hypertension. Other endocrine disorders also are treatable with these compounds, compositions or combination therapies, including polycystic ovarian disease, X-linked adrenoleukodystrophy, hypothyroidism and thyroiditis, including Hashimoto's thyroiditis (i.e., autoimmune thyroiditis).

Conditions of the gastrointestinal system also are treatable with TNFα inhibitors, compositions or combination therapies, including coeliac disease. In addition, the compounds, compositions and combination therapies of the invention are used to treat Crohn's disease; ulcerative colitis; idiopathic gastroparesis; pancreatitis, including chronic pancreatitis and lung injury associated with acute pancreatitis; and ulcers, including gastric and duodenal ulcers.

Included also are methods for using the subject TNFα inhibitors, compositions or combination therapies for treating disorders of the genitourinary system, such as glomerulonephritis, including autoimmune glomerulonephritis, glomerulonephritis due to exposure to toxins or glomerulonephritis secondary to infections with haemolytic streptococci or other infectious agents. Also treatable with the compounds, compositions and combination therapies of the invention are uremic syndrome and its clinical complications (for example, renal failure, anemia, and hypertrophic cardiomyopathy), including uremic syndrome associated with exposure to environmental toxins, drugs or other causes. Further conditions treatable with the compounds, compositions and combination therapies of the invention are complications of hemodialysis; prostate conditions, including benign prostatic hypertrophy, nonbacterial prostatitis and chronic prostatitis; and complications of hemodialysis.

Also provided herein are methods for using TNFα inhibitors, compositions or combination therapies to treat various hematologic and oncologic disorders. For example, TNFR:Fc is used to treat various forms of cancer, including acute myelogenous leukemia, Epstein-Barr virus-positive nasopharyngeal carcinoma, glioma, colon, stomach, prostate, renal cell, cervical and ovarian cancers, lung cancer (SCLC and NSCLC), including cancer-associated cachexia, fatigue, asthenia, paraneoplastic syndrome of cachexia and hypercalcemia. Additional diseases treatable with the subject TNFα inhibitors, compositions or combination therapies are solid tumors, including sarcoma, osteosarcoma, and carcinoma, such as adenocarcinoma (for example, breast cancer) and squamous cell carcinoma. In addition, the subject compounds, compositions or combination therapies are useful for treating leukemia, including acute myelogenous leukemia, chronic or acute lymphoblastic leukemia and hairy cell leukemia. Other malignancies with invasive metastatic potential can be treated with the subject compounds, compositions and combination therapies, including multiple myeloma. In addition, the disclosed TNFα inhibitors, compositions and combination therapies can be used to treat anemias and hematologic disorders, including anemia of chronic disease, aplastic anemia, including Fanconi's aplastic anemia; idiopathic thrombocytopenic purpura (ITP); myelodysplastic syndromes (including refractory anemia, refractory anemia with ringed sideroblasts, refractory anemia with excess blasts, refractory anemia with excess blasts in transformation); myelofibrosis/myeloid metaplasia; and sickle cell vasoocclusive crisis.

Various lymphoproliferative disorders also are treatable with the disclosed TNFα inhibitors, compositions or combination therapies. These include, but are not limited to autoimmune lymphoproliferative syndrome (ALPS), chronic lymphoblastic leukemia, hairy cell leukemia, chronic lymphatic leukemia, peripheral T-cell lymphoma, small lymphocytic lymphoma, mantle cell lymphoma, follicular lymphoma, Burkitt's lymphoma, Epstein-Barr virus-positive T cell lymphoma, histiocytic lymphoma, Hodgkin's disease, diffuse aggressive lymphoma, acute lymphatic leukemias, T gamma lymphoproliferative disease, cutaneous B cell lymphoma, cutaneous T cell lymphoma (i.e., mycosis fungoides) and Sézary syndrome.

In addition, the subject TNFα inhibitors, compositions and combination therapies are used to treat hereditary conditions such as Gaucher's disease, Huntington's disease, linear IgA disease, and muscular dystrophy.

Other conditions treatable by the disclosed TNFα inhibitors, compositions and combination therapies include those resulting from injuries to the head or spinal cord, and including subdural hematoma due to trauma to the head.

The disclosed TNFα inhibitors, compositions and combination therapies are further used to treat conditions of the liver such as hepatitis, including acute alcoholic hepatitis, acute drug-induced or viral hepatitis, hepatitis A, B and C, sclerosing cholangitis and inflammation of the liver due to unknown causes.

In addition, the disclosed TNFα inhibitors, compositions and combination therapies are used to treat various disorders that involve hearing loss and that are associated with abnormal TNFα expression. One of these is inner ear or cochlear nerve-associated hearing loss that is thought to result from an autoimmune process, i.e., autoimmune hearing loss. This condition currently is treated with steroids, methotrexate and/or cyclophosphamide, which may be administered concurrently with the TNFR:Fc or other TNFα inhibitor. Also treatable with the disclosed TNFα inhibitors, compositions and combination therapies is cholesteatoma, a middle ear disorder often associated with hearing loss.

In addition, the subject invention provides TNFα inhibitors, compositions and combination therapies for the treatment of non-arthritic medical conditions of the bones and joints. This encompasses osteoclast disorders that lead to bone loss, such as but not limited to osteoporosis, including post-menopausal osteoporosis, periodontitis resulting in tooth loosening or loss, and prosthesis loosening after joint replacement (generally associated with an inflammatory response to wear debris). This latter condition also is called "orthopedic implant osteolysis." Another condition treatable by administering TNFRα inhibitors, such as TNFR:Fc, is temporal mandibular joint dysfunction (TMJ).

A number of pulmonary disorders also can be treated with the disclosed TNFα inhibitors, compositions and combination therapies. One such condition is adult respiratory distress syndrome (ARDS), which is associated with elevated TNFα, and may be triggered by a variety of causes, including exposure to toxic chemicals, pancreatitis, trauma or other causes. The disclosed compounds, compositions and combination

therapies of the invention also are useful for treating bronchopulmonary dysplasia (BPD); lymphangioleiomyomatosis; and chronic fibrotic lung disease of preterm infants. In addition, the compounds, compositions and combination therapies of the invention are used to treat occupational lung diseases, including asbestosis, coal worker's pneumoconiosis, silicosis or similar conditions associated with long-term exposure to fine particles. In other aspects of the invention, the disclosed compounds, compositions and combination therapies are used to treat pulmonary disorders, including chronic obstructive pulmonary disease (COPD) associated with chronic bronchitis or emphysema; fibrotic lung diseases, such as cystic fibrosis, idiopathic pulmonary fibrosis and radiation-induced pulmonary fibrosis; pulmonary sarcoidosis; and allergies, including allergic rhinitis, contact dermatitis, atopic dermatitis and asthma.

Cystic fibrosis is an inherited condition characterized primarily by the accumulation of thick mucus, predisposing the patient to chronic lung infections and obstruction of the pancreas, which results in malabsorption of nutrients and malnutrition. TNFR:Fc may be administered to treat cystic fibrosis. If desired, treatment with TNFR:Fc may be administered concurrently with corticosteroids, mucus-thinning agents such as inhaled recombinant deoxyribonuclease I (such as PULMOZYME®; Genentech, Inc.) or inhaled tobramycin (TOBI®; Pathogenesis, Inc.). TNFR:Fc also may be administered concurrently with corrective gene therapy, drugs that stimulate cystic fibrosis cells to secrete chloride or other yet-to-be-discovered treatments. Sufficiency of treatment may be assessed, for example, by observing a decrease in the number of pathogenic organisms in sputum or lung lavage (such as *Haemophilus influenzae, Stapholococcus aureus*, and *Pseudomonas aeruginosa*), by monitoring the patient for weight gain, by detecting an increase in lung capacity or by any other convenient means.

TNFR:Fc or TNFR:Fc combined with the cytokine IFNγ-1b (such as ACTIMMUNE®; InterMune Pharmaceuticals) may be used for treating cystic fibrosis or fibrotic lung diseases, such as idiopathic pulmonary fibrosis, radiation-induced pulmonary fibrosis and bleomycin-induced pulmonary fibrosis. In addition, this combination is useful for treating other diseases characterized by organ fibrosis, including systemic sclerosis (also called "scleroderma"), which often involves fibrosis of the liver. For treating cystic fibrosis, TNFR:Fc and IFNγ-1b may be combined with PULMOZYME® or TOBI® or other treatments for cystic fibrosis.

TNFR:Fc alone or in combination with IFNγ-1b may be administered together with other treatments presently used for treating fibrotic lung disease. Such additional treatments include glucocorticoids, azathioprine, cyclophosphamide, penicillamine, colchisicine, supplemental oxygen and so forth. Patients with fibrotic lung disease, such as IPF, often present with nonproductive cough, progressive dyspnea, and show a restrictive ventilatory pattern in pulmonary function tests. Chest radiographs reveal fibrotic accumulations in the patient's lungs. When treating fibrotic lung disease in accord with the disclosed methods, sufficiency of treatment may be detected by observing a decrease in the patient's coughing (when cough is present), or by using standard lung function tests to detect improvements in total lung capacity, vital capacity, residual lung volume or by administering a arterial blood gas determination measuring desaturation under exercising conditions, and showing that the patient's lung function has improved according to one or more of these measures. In addition, patient improvement may be determined through chest radiography results showing that the progression of fibrosis in the patient's lungs has become arrested or reduced.

In addition, TNF inhibitors (including soluble TNFRs or antibodies against TNFα or TNFR) are useful for treating organ fibrosis when administered in combination with relaxin, a hormone that down-regulates collagen production thus inhibiting fibrosis, or when given in combination with agents that block the fibrogenic activity of TGF-β. Combination therapies using TNFR:Fc and recombinant human relaxin are useful, for example, for treating systemic sclerosis or fibrotic lung diseases, including cystic fibrosis, idiopathic pulmonary fibrosis, radiation-induced pulmonary fibrosis and bleomycin-induced pulmonary fibrosis.

Other embodiments provide methods for using the disclosed TNFα inhibitors, compositions or combination therapies to treat a variety of rheumatic disorders. These include: adult and juvenile rheumatoid arthritis; systemic lupus erythematosus; gout; osteoarthritis; polymyalgia rheumatica; seronegative spondylarthropathies, including ankylosing spondylitis; and Reiter's disease. The subject TNFα inhibitors, compositions and combination therapies are used also to treat psoriatic arthritis and chronic Lyme arthritis. Also treatable with these compounds, compositions and combination therapies are Still's disease and uveitis associated with rheumatoid arthritis. In addition, the compounds, compositions and combination therapies of the invention are used in treating disorders resulting in inflammation of the voluntary muscle, including dermatomyositis and polymyositis. Moreover, the compounds, compositions ant combinations disclosed herein are useful for treating sporadic inclusion body myositis, as TNFα may play a significant role in the progression of this muscle disease. In addition, the compounds, compositions and combinations disclosed herein are used to treat multicentric reticulohistiocytosis, a disease in which joint destruction and papular nodules of the face and hands are associated with excess production of proinflammatory cytokines by multinucleated giant cells.

For purposes of this invention, patients are defined as having psoriatic arthrisis (PsA) if they have one or more swollen joints or one or more painful or tender joints, and also manifest at least one psoriatic lesion of the skin or nails. The psoriatic lesions may appear before or after the onset of swollen or tender joints. It is understood that prior to treatment, manifestations of PsA may have persisted over time, e.g., for several months or years, and may involve several joints. According to one classification system (reviewed in Alonso et al., 1991), PsA patients can be categorized based on their arthritic symptoms into five clinical subgroups: 1) DIP; 2) mutilans arthritis; 3) symmetrical polyarthritis; 4) oligoarticular arthritis; and 5) ankylosing spondylitis-like. The disclosed therapies, compounds and compositions are suitable for treating all five forms of PsA.

The TNFα inhibitors, compositions and combination therapies of the invention may be used to inhibit hypertrophic scarring, a phenomenon believed to result in part from excessive TNFα secretion. TNF inhibitors may be administered alone or concurrently with other agents that inhibit hypertrophic scarring, such as inhibitors of TGF-α.

Cervicogenic headache is a common form of headache arising from dysfunction in the neck area, and which is associated with elevated levels of TNFα, which are believed to mediate an inflammatory condition that contributes to the patient's discomfort (Martelletti, *Clin Exp Rheumatol* 18(2 Suppl 19):S33-8 (March-April, 2000)). Cervicogenic head-

ache may be treated by administering an inhibitor of TNFα as disclosed herein, thereby reducing the inflammatory response and associated headache pain.

The TNFα inhibitors, compositions and combination therapies of the invention are useful for treating primary amyloidosis. In addition, the secondary amyloidosis that is characteristic of various conditions also are treatable with TNFα inhibitors such as TNFR:Fc, and the compositions and combination therapies described herein. Such conditions include: Alzheimer's disease, secondary reactive amyloidosis; Down's syndrome; and dialysis-associated amyloidosis. Also treatable with the compounds, compositions and combination therapies of the invention are inherited periodic fever syndromes, including familial Mediterranean fever, hyperimmunoglobulin D and periodic fever syndrome and TNF-receptor associated periodic syndromes (TRAPS).

Disorders associated with transplantation also are treatable with the disclosed TNFα inhibitors, compositions or combination therapies, such as graft-versus-host disease, and complications resulting from solid organ transplantation, including transplantion of heart, liver, lung, skin, kidney or other organs. TNFR:Fc may be administered, for example, to prevent or inhibit the development of bronchiolitis obliterans after lung transplantation. Patients undergoing autologous hematopoietic stem cell transplantation in the form of peripheral blood stem cell transplantation may develop "engraftment syndrome," or "ES," which is an adverse and generally self-limited response that occurs about the time of hematopoietic engraftment and which can result in pulmonary deterioration. ES may be treated with inhibitors of either IL-8 or TNFα (such as TNFR:Fc), or with a combination of inhibitors against both of these cytokines.

Ocular disorders also are treatable with the disclosed TNFα inhibitors, compositions or combination therapies, including rhegmatogenous retinal detachment, and inflammatory eye disease, and inflammatory eye disease associated with smoking and macular degeneration.

TNFα inhibitors such as TNFR:Fc and the disclosed compositions and combination therapies also are useful for treating disorders that affect the female reproductive system. Examples include, but are not limited to, multiple implant failure/infertility; fetal loss syndrome or IV embryo loss (spontaneous abortion); preeclamptic pregnancies or eclampsia; and endometriosis.

In addition, the disclosed TNFα inhibitors, compositions and combination therapies are useful for treating obesity, including treatment to bring about a decrease in leptin formation. Also, the compounds, compositions and combination therapies of the invention are used to treat sciatica, symptoms of aging, severe drug reactions (for example, Il-2 toxicity or bleomycin-induced pneumopathy and fibrosis), or to suppress the inflammatory response prior, during or after the transfusion of allogeneic red blood cells in cardiac or other surgery, or in treating a traumatic injury to a limb or joint, such as traumatic knee injury. Various other medical disorders treatable with the disclosed TNFα inhibitors, compositions and combination therapies include; multiple sclerosis; Behcet's syndrome; Sjogren's syndrome; autoimmune hemolytic anemia; beta thalassemia; amyotrophic lateral sclerosis (Lou Gehrig's Disease); Parkinson's disease; and tenosynovitis of unknown cause, as well as various autoimmune disorders or diseases associated with hereditary deficiencies.

The disclosed TNFα inhibitors, compositions and combination therapies furthermore are useful for treating acute polyneuropathy; anorexia nervosa; Bell's palsy; chronic fatigue syndrome; transmissible dementia, including Creutzfeld-Jacob disease; demyelinating neuropathy; Guillain-Barre syndrome; vertebral disc disease; Gulf war syndrome; myasthenia gravis; silent cerebral ischemia; sleep disorders, including narcolepsy and sleep apnea; chronic neuronal degeneration; and stroke, including cerebral ischemic diseases.

Disorders involving the skin or mucous membranes also are treatable using the disclosed TNFα inhibitors, compositions or combination therapies. Such disorders include acantholytic diseases, including Darier's disease, keratosis follicularis and pemphigus vulgaris. Also treatable with the subject TNFα inhibitors, compositions and combination therapies are acne; acne rosacea; alopecia greata; aphthous stomatitis; bullous pemphigoid; burns; eczema; erythema, including erythema multiforme and erythema multiforme bullosum (Stevens-Johnson syndrome); inflammatory skin disease; lichen planus; linear IgA bullous disease (chronic bullous dermatosis of childhood); loss of skin elasticity; mucosal surface ulcers; neutrophilic dermatitis (Sweet's syndrome); pityriasis rubra pilaris; psoriasis; pyoderma gangrenosum; and toxic epidermal necrolysis.

Patients are defined as having ordinary psoriasis if they lack the more serious symptoms of PsA (e.g., distal interphalangeal joint DIP involvement, enthesopathy, spondylitis and dactylitis) but have one of the following: 1) inflamed swollen skin lesions covered with silvery white scale (plaque psoriasis or psoriasis vulgaris); 2) small red dots appearing on the trunk, arms or legs (guttate psoriasis); 3) smooth inflamed lesions without scaling in the flexural surfaces of the skin (inverse psoriasis); 4) widespread reddening and exfoliation of fine scales, with or without itching and swelling (erythrodermic psoriasis); 5) blister-like lesions (pustular psoriasis); 6) elevated inflamed scalp lesions covered by silvery white scales (scalp psoriasis); 7) pitted fingernails, with or without yellowish discoloration, crumbling nails, or inflammation and detachment of the nail from the nail bed (nail psoriasis).

Ordinary psoriasis may be treated by administering to a human patient compositions containing a therapeutically effective amount of a TNFα inhibitor such as a soluble TNF receptor or an antibody against TNFα.

In one preferred embodiment, the therapeutic agent is a soluble TNF receptor, and preferably is a TNFR-Ig. In a preferred embodiment, the TNFR-Ig is TNFR:Fc, which may be administered in the form of a pharmaceutically acceptable composition as described herein. Psoriasis may be treated by administering TNFR:Fc one or more times per week by subcutaneous injection, although other routes of administration may be used if desired. In one exemplary regimen for treating adult human patients, 25 mg of TNFR:Fc is administered by subcutaneous injection two times per week or three times per week for one or more weeks, and preferably for four or more weeks. Alternatively, a dose of 5-12 mg/m^2 or a flat dose of 50 mg is injected subcutaneously one time or two times per week for one or more weeks. In other embodiments, psoriasis is treated with TNFR:Fc in a sustained-release form, such as TNFR:Fc that is encapsulated in a biocompatible polymer, TNFR:Fc that is admixed with a biocompatible polymer (such as topically applied hydrogels), and TNFR:Fc that is encased in a semi-permeable implant.

Various other medicaments used to treat ordinary psoriasis may also be administered concurrently with compositions comprising TNFα inhibitors, such as TNFR:Fc. Such medicaments include: NSAIDs; DMARDs; analgesics; topical steroids; systemic steroids (e.g., prednisone); cytokines; antagonists of inflammatory cytokines; antibodies against T cell surface proteins; anthralin; coal tar; vitamin D3 and its analogs; topical retinoids; oral retinoids; salicylic acid; and hydroxyurea. Suitable analgesics for such combinations

include: acetaminophen, codeine, propoxyphene napsylate, oxycodone hydrochloride, hydrocodone bitartrate and tramadol. DMARDs suitable for such combinations include: azathioprine, cyclophosphamide, cyclosporine, hydroxychloroquine sulfate, methotrexate, leflunomide, minocycline, penicillamine, sulfasalazine, oral gold, gold sodium thiomalate and aurothioglucose. In addition, the TNFR:Fc or other TNFR mimic may be administered in combination with antimalarials or colchicine. NSAIDs suitable for the subject combination treatments of psoriasis include: salicylic acid (aspirin) and salicylate derivatives; ibuprofen; indomethacin; celecoxib; rofecoxib; ketorolac; nambumetone; piroxicam; naproxen; oxaprozin; sulindac; ketoprofen; diclofenac; and other COX-1 and COX-2 inhibitors, propionic acid derivatives, acetic acid derivatives, fumaric acid derivatives, carboxylic acid derivatives, butyric acid derivatives, oxicams, pyrazoles and pyrazolones, including newly developed anti-inflammatories.

If an antagonist against an inflammatory cytokine is administered concurrently with TNFR:Fc to treat psoriasis, suitable targets for such antagonists include TGFβ, Il-6 and Il-8.

In addition, TNFR:Fc may be used to treat psoriasis in combination with topical steroids, systemic steroids, antagonists of inflammatory cytokines, antibodies against T cell surface proteins, anthralin, coal tar, vitamin D3 and its analogs (including 1,25-dihydroxy vitamin D3 and calcipotriene), topical retinoids, oral retinoids (including but not limited to etretinate, acitretin and isotretinoin), topical salicylic acid, methotrexate, cyclosporine, hydroxyurea and sulfasalazine. In addition, TNFR:Fc may be administered to treat psoriasis in combination with one or more of the following compounds; minocycline; misoprostol; oral collagen; 6-mercaptopurine; nitrogen mustard; gabapentin; bromocriptine; somatostatin; peptide T; anti-CD4 monoclonal antibody; fumaric acid; polyunsaturated ethyl ester lipids; zinc; and other drugs that may be used to treat psoriasis. TNFR:Fc may also be used to treat psoriasis in combination with the use of various oils, including fish oils, nut oils and vegetable oils; aloe vera; jojoba; Dead Sea salts; capsaicin; milk thistle; witch hazel; moisturizers; and Epsom salts. In addition, psoriasis may be treated with compositions containing TNFR:Fc in combination with the following therapies: plasmapheresis; phototherapy with ultraviolet light B; psoralen combined with ultraviolet light A (PUVA); and sunbathing.

For determining the sufficiency of treatment when treating ordinary psoriasis in accord with the invention, the TNFR:Fc (or other TNFα inhibitor) is administered in an amount and for a time sufficient to induce an improvement in an indicator such as psoriasis area and severity index (PASI) or an improvement in Target Lesion Assessment score, which is an index for assessing the severity of individual skin lesions. In one embodiment, the treatment is regarded as sufficient when the patient exhibits an at least 50% improvement in his or her PASI score, and in another embodiment, when the patient exhibits an at least 75% improvement in PASI score. The sufficiency of treatment for psoriasis may also be determined by evaluating individual psoriatic lesions for improvement in severity (Psoriasis Target Lesion Assessment Score), and continuing treatment until an improvement is noted according to this scoring system. This scoring system involves determining for an individual lesion whether improvement has occurred in plaque elevation, amount and degree of scaling or degree of erythema, and target lesion response to treatment, each of which is separately scored. Psoriasis Target Lesion Assessment Score is determined by adding together the separate scores for all four of the aforementioned indicia.

In addition to human patients, inhibitors of TNFα are useful in the treatment of autoimmune and inflammatory conditions in non-human animals, such as pets (dogs, cats, birds, primates, etc.), domestic farm animals (horses cattle, sheep, pigs, birds, etc.), or any animal that suffers from a TNFα-mediated inflammatory or arthritic condition. In such instances, an appropriate dose may be determined according to the animal's body weight. For example, a dose of 0.2-1 mg/kg may be used. Alternatively, the dose is determined according to the animal's surface area, an exemplary dose ranging from 0.1-20 mg/m^2, or more preferably, from 5-12 mg/m^2. For small animals, such as dogs or cats, a suitable dose is 0.4 mg/kg. In a preferred embodiment, TNFR:Fc (preferably constructed from genes derived from the same species as the patient), or another soluble TNFR mimic, is administered by injection or other suitable route one or more times per week until the animal's condition is improved, or it may be administered indefinitely.

EXAMPLE

Evaluation of TNFR:Fc in Patients with Psoriatic Arthritis

Sixty patients with active psoriatic arthritis (PsA) were enrolled in a Phase II double-blind, randomized, placebo controlled study to determine whether the subcutaneous biweekly administration of etanercept (recombinant TNFR:Fc) was safe in this patient population and whether efficacy could be documented for both the arthritic and psoriatic aspects of this disease.

In this study, a flat dose of 25 mg of TNFR:Fc was injected subcutaneously two times a week. After 12 weeks, patients who completed the study were eligible for continuation into a 24 week open-label extension of the study, with assessments made at weeks 16, 36 and 30 days post-study. All patients participating in the study extension received etanercept, including those patients who had received placebo during the blinded portion of the study.

In order to qualify for enrollment, subjects had to have at least one of the following forms of PsA: 1) DIP involvement; 2) polyarticular arthritis, absence of rheumatoid nodules and presence of psoriasis; 3) arthritis mutilans; 4) asymmetric peripheral arthritis; or 5) ankylosing spondylitis-like PsA. Subjects furthermore had to exhibit three or more swollen joints and three or more tender or painful joints at the time of enrollment, and to have exhibited an inadequate response to NSAID therapy. Subjects who were on other medications, including methotrexate, NSAIDs or oral corticosteroids were permitted to continue these other treatments at the same dose so long as the investigator considered these other treatments to inadequately control the patient's disease. Methotrexate was concurrently taken by 47% of the etanercept group, and 47% of the placebo group, NSAIDs were concurrently taken by 67% of the etanercept and 77% of the placebos and oral corticosteroids by 40% of the etanercept and 20% of the placebo patients. Pain medications, including acetaminophen, codeine, propoxyphene napsylate, oxycodone hydrochloride, hydrocodone bitartrate and tramadol, also were permitted during the study, as well as the use of topical tar compounds.

To qualify as having PsA, patients had to have experienced at least one psoriatic lesion of the skin or nails. Patients were evaluated at baseline (day 1 of treatment) as follows: 1) complete joint assessment; 2) psoriasis assessment; 3) duration of morning stiffness; 4) health assessment (quality of life) questionnaire, visual analog scale (HAQ/VAS); 5) patient global

assessment; 6) erythrocyte sedimentation rate (ESR, Westergren); 7) C-reactive protein (CRP); and 8) urinalysis. At weeks 4 and 8, patients were evaluated as follows: 1) complete joint assessment; 2) psoriasis assessment; 3) duration of morning stiffness; 4) HAQ/VAS; 5) patient global assessment. At the end of 12 weeks, subjects were evaluated as follows: 1) complete joint assessment; 2) psoriasis assessment; 3) focused physical exam; 4) duration of morning stiffness; 5) HAQ/VAS; 6) patient global assessment; 6) hematology profile; 7) chemistry profile; 8) ESR; 9) CRP; 10) urinalysis; 11) serum tested for antibody to TNFR:Fc. Only those patients whose psoriasis was stable and covered ≧3% of body area were evaluated for psoriasis response during this trial, although patients whose psoriasis was inactive or covered less area were permitted to enroll.

A primary endpoint for clinical improvement or worsening of PsA was the Psoriatic Arthritis Response score, which is a composite score based on the following four measures: 1) patient self-assessment; 2) physician assessment; 3) joint pain or tenderness; 4) joint swelling. Both self- and physician assessments, i.e., overall assessment of disease status, were measured according to a five point Likert scale, in which a patient was considered as "improved" if his or her score decreased by one category, or as "worse" if his or her score increased by one category. Joint pain or tenderness was measured on a 5-point scale, wherein 1=none and 5=severe (withdrawal on examination). Joint swelling was evaluated on a 4-point scale in which 1=none; 2=mild (detectable synovial thickening without loss of bony contour); 3=moderate (loss of distinctness of bony contours); and 4=severe (bulging synovial proliferation with cystic characteristics). For this last measure, a decrease in swelling of ≧30% was scored as an "improvement," and an increase in swelling of ≧30% was scored as a "worsening." Patients were classified as "improved" under the Psoriatic Arthritis Response scoring system if they exhibited an improvement in at least two of the four measures described above, provided that one of the improved areas was joint pain or joint tenderness, and where there was no worsening in any of the four measures.

In addition, a secondary endpoint used for assessing psoriatic arthritis was a modified version of the American College of Rheumatology Preliminary Definition of Improvement in Rheumatoid Arthritis (modified ACR 20 response) (Felson et al., 1995). To qualify as "improved" according to this measure, a patient must have exhibited ≧20% improvement in both tender joint count (78 joints assessed) and swollen joint count (76 joints assessed), and also must have shown an improvement in three of the following five: 1) subject pain assessment; 2) subject global assessment; 3) physician global assessment; 4) subject self-assessed disability; 5) acute-phase reactant (Westergreen erythrocyte sedimentation rate or C-reactive protein level). The joint count was done by scoring several different aspects of tenderness, such as pressure and joint manipulation on physical examination, wherein each joint was scored as "tender" or "nontender." Similarly, each joint is scored after physical examination as "swollen" or "not swollen." The subject's pain assessment was based on a horizontal visual analog scale (usually 10 cm) or Likert scale. The subject's and physician's global assessments of the subject's current disease status was based on an anchored horizontal visual analog scale (usually 10 cm), or Likert scale response. The subject's self-assessment of disability was based on any of the following measures, all of which have been validated in RA trials: Arthritis Impact Measurement Scale (AIMS); Health Assessment Questionnaire; the Quality (or Index) of Well Being Scale; the McMaster Health Inventory Questionnaire (MHIQ); and the McMaster-Toronto Arthritis patient preference questionnaire (MACTAR).

A primary endpoint used to assess the psoriatic aspects of PsA was the standard psoriasis area and severity index (PASI) (Fredriksson and Petersson, *Dermatologica* 157:238-244, 1978). For this study, a positive treatment response was defined as an at least 50% or an at least 75% improvement in a patient's PASI score. For assessing area and severity, the body is divided into four regions: head (10%); trunk (30%); upper extremities (20%); and lower extremities (40%). Each quadrant also was scored for the severity of erythema (E), infiltration (I) and desquamation (D), using a four point scale, in which 0=no symptoms present; 1=slight symptoms; 2=moderate symptoms; 3=striking symptoms; 4=exceptionally striking symptoms. Using a 6-point scale, each region was scored also for the percent of total area that was involved in the psoriatic manifestations of the disease, wherein 0=no involvement; 1=<10% involvement; 2=10-<30% involvement; 3=30-<50% involvement; 4=50-<70% involvement; 5=70-<90% involvement; 6=90-100% involvement. PASI scores were calculated according to the formula given below, in which E=severity score for erythrema, I=severity score for infiltration, D=severity score for desquamation and A=total area involved. In this formula, the letters "h," "t," "u" and "l" represent, respectively, the scores in each of the four body regions, i.e., head, trunk, upper extremities and lower extremities. The PASI score varies in steps of 0.1 units from 0.0 (no psoriatic lesions at all) to 72.0 (complete erythroderma of the severest possible degree).

$$PASI=0.1(Eh+Ih+Dh)Ah+0.3(Et+It+Dt)At+0.2(Eu+Iu+Du)Au+0.4(El+Il+Dl)Al$$

A secondary endpoint used for the psoriatic aspect of psoriatic arthritis was the Target Lesion Assessment Score. This score was determined for a single target lesion that was selected to be monitored throughout the trial. This measurement is a composite of four different evaluations: 1) plaque evaluation; 2) scaling; 3) erythrema; and 4) target lesion response to treatment. The following scale was used for the plaque elevation: 0=none (no evidence of plaque above normal skin level); 1=mild (slight but definite elevation above normal skin level); 2=moderate (moderate elevation with rounded or sloped edges to plaque); 3=severe (hard, marked elevation with sharp edges to plaque); 4=very severe (very marked elevation with very hard sharp edges to plaque). For the scaling assessment: 0=none (no scaling on the lesion); 1=mild (mainly fine scales, with some of the lesion at least partially covered); 2=moderate (somewhat coarser scales, most of the lesion at least partially covered); 3=severe (coarse, thick scales, virtually all the lesion covered, rough surface); 4=very severe (very coarse thick scales, all the lesions covered, very rough surface). For the erythema evaluation: 0=none (no erythema); 1=mild (light red coloration); 2=moderate (red coloration); 3=severe (very red coloration); 4=very severe (extreme red coloration). For target lesion response to treatment score: 0=completely cleared; 1=almost cleared (~90% improvement); 2=marked response (~75% improvement); 3=moderate response (~50% improvement); 4=slight response (~25% improvement); 5=condition unchanged; 6=condition worsened. The patient's Target Lesion Assessment Score was determined by summing the plaque, scaling, erythema and target lesion response scores for the monitored lesion. If the monitored lesion worsened, the percentage change from baseline was recorded as a negative number.

Treatment and placebo groups were compared in accord with the measurements described above, as well as for demo-

graphic and background characteristics; premature discontinuation rate; pain medication requirements; toxicities; serious adverse events; side effects reported by patients; number of weeks on drug until subjects met criteria for improvement, and response according to PsA subtype. Results were analyzed using standard statistical methods.

Dosing Regimen

Recombinant human TNFR:Fc (etanercept) from Immunex Corporation was used in this study. The gene fragments encoding the etanercept polypeptides were expressed in a Chinese hamster ovary (CHO) expression vector.

TNFR:Fc was supplied as a sterile lyophilized powder containing 10 mg or 25 mg TNFR:Fc; 40 mg mannitol, USP; 10 mg sucrose, NF; and 1.2 mg tromethamine (TRIS), USP per vial. Patients received either a dose of 25 mg of etanercept or a placebo. Vials of etanercept or identically-appearing placebo were reconstituted by aseptic injection of 1.0 mL Bacteriostatic Water for Injection, USP, (containing 0.9% benzyl alcohol), and was not filtered during preparation or prior to administration. If storage was required, the reconstituted solutions were stored at 2-8° C. (36-46° F.) in the original vial or in a plastic syringe for a period of no longer than 28 days. Dose was not changed during the study. Study drug was given twice weekly at approximately the same time of day.

Results

Study drug was well tolerated in all patients, and adverse events were consistent with this population and were equally distributed among both treatment groups. As illustrated in Tables 1-4, etanercept induced a significant improvement as compared with the placebo group in Psoriatic Arthritis Response (Table 1), ACR20 (Table 2), ACR50 (Table 3), PASI score, 50% improvement (Table 4), PASI score, 75% improvement (Table 5) and improvement in Target Lesion Assessment Score (Table 6). The fractions shown in Tables 1-5 represent numbers of patients. For example, the first entry in Table 1, which is "4/30," indicates that 4 of 30 patients in the placebo group scored as "improved" according to the Psoriatic Arthritis Response measurements. The tables include P-values for the differences between the two study groups, the groups being labeled as "PLACEBO" and "TNFR:Fc." All of the tables include data calculated after the first four weeks of the open label extension portion of the study ("EXTENSION"), during which all of the patients in both study groups received etanercept.

Table 1 shows the number of patients in each treatment group who scored as "improved" according to the Psoriatic Arthritis Response scoring system described above. By four weeks, there was a highly significant difference between etanercept and placebo groups. Moreover, after being switched to etanercept during the extension, those patients who had received placebo during the blinded portion of the study were seen to exhibit an improvement over baseline (Table 1, Placebo, EXTENSION). These results indicate that etanercept acts rapidly to alleviate many aspects of psoriatic arthritis.

Tables 2 and 3, respectively, illustrate the study results for the ACR20 and ACR50 endpoints. For either measure, a significant difference between etanercept and placebo groups was observed at all three time points during the blinded portion of the study. Given the differences between test and placebo groups after only four weeks of treatment (P=0.000 for ACR20 and P=0.011 for ACR50), these data suggest that notable improvement in ACR scores occurred within the etanercept group very soon after treatment was initiated, possibly after a single dose of etanercept. During the 4 week extension period, during which all of the patients received etanercept, a striking improvement in both ACR20 and ACR50 was seen in those patients who had received placebo during the first 12 weeks (Tables 2 and 3).

TABLE 2

ACR20 Response

	Placebo	TNFR:Fc	P-value
4 weeks	1/30 (3%)	18/30 (60%)	0.000
8 weeks	3/30 (10%)	19/30 (63%)	0.000
12 weeks	4/30 (13%)	22/30 (73%)	0.000
EXTENSION	11/23 (48%)	18/25 (72%)	0.093

TABLE 3

ACR50 Response

	Placebo	TNFR:Fc	P-value
4 weeks	0/30 (0%)	6/30 (20%)	0.011
8 weeks	1/30 (3%)	11/30 (37%)	0.001
12 weeks	1/30 (3%)	15/30 (50%)	0.000
EXTENSION	7/23 (30%)	11/25 (44%)	0.316

The results of the psoriasis evaluations are presented in Tables 4-6. Tables 4 and 5, respectively, present the numbers and percentages of patients in each group who exhibited a 50% or 75% improvement in PASI score, while Table 6 presents Target Lesion Assessment scores, these latter being denoted as percent improvement over baseline. The data in Tables 4-6 clearly indicate that etanercept induced an improvement in psoriasis for a large percentage of the patients who received it. When single lesions were evaluated (Table 6), the improvement in psoriasis was even more apparent than when PASI scores were used (Tables 4 and 5). It is notable also that, for either PASI scores (Tables 4 and 5) or Psoriasis Target Lesion Assessment Score (Table 6), the scores of the placebo group improved after these patients were switched to etanercept during the extension.

Though not shown in Table 6, Target Lesion Assessment Scores for patients who were concurrently receiving methotrexate (14 of the 30 patients in the etanercept group, and 14 patients in the placebo group) were compared with the scores of those patients who did not take methotrexate. Little difference in this index was noted between the patients who received methotrexate and those who did not receive it.

TABLE 1

Psoriatic Arthritis Response

	Placebo	TNFR:Fc	P-value
4 weeks	4/30 (13%)	23/30 (77%)	0.000
8 weeks	7/30 (23%)	25/30 (83%)	0.000
12 weeks	6/30 (20%)	26/30 (87%)	0.000
EXTENSION	17/23 (74%)	21/25 (84%)	0.356

TABLE 4

PASI Score - 50% Improvement

	Placebo	TNFR:Fc	P-value
4 weeks	0/19 (0%)	4/19 (21%)	0.037
8 weeks	1/19 (5%)	7/19 (37%)	0.019
12 weeks	4/19 (21%)	8/19 (42%)	0.165
EXTENSION	6/16 (38%)	6/15 (40%)	0.856

TABLE 5

PASI Response Rate 75% Improvement

	Placebo	TNFR:Fc	P-value
4 weeks	0/19 (0%)	1/19 (5%)	0.264
8 weeks	0/19 (0%)	2/19 (11%)	0.153
12 weeks	0/19 (0%)	4/19 (21%)	0.037
EXTENSION	1/16 (6%)	4/15 (27%)	0.113

TABLE 6

Psoriasis Target Lesion Assessment
(Percent Improvement or Worsening Compared with Baseline)

		Placebo	TNFR:Fc	P-value
4 weeks	Mean (SD)	2.7 (27.6)	21.2 (35.2)	0.120
	Median	0.0	14.3	
	MIN--MAX	-50.0-50.0	-33.3-100.0	
	N	19	19	
8 weeks	Mean (SD)	-7.5 (25.3)	28.5 (34.1)	0.003
	Median	0.0	29.2	
	MIN--MAX	-50.0-20.0	-33.3-100.0	
	N	17	18	
12 weeks	Mean (SD)	9.5 (23.2)	45.7 (31.6)	0.001
	Median	0.0	50.0	
	MIN--MAX	-25.0-50.0	-16.7-100.0	
	N	16	19	
EXTENSION	Mean (SD)	28.9 (41.2)	47.1 (35.8)	0.263
	Median	36.7	50.0	
	MIN--MAX	-100.0-66.7	-33.3-100.0	
	N	16	15	

What is claimed:

1. A method for treating a patient having ordinary psoriasis comprising administering to the patient a therapeutically effective dose of TNFR:Fc.

2. The method of claim **1**, wherein the dose of TNFR:Fc administered is either 50 mg once per week or 25 mg twice per week.

3. The method of claim **1**, wherein the dose of TNFR:Fc administered is 50 mg twice per week.

4. The method of claim **1**, wherein the TNFR:Fc is administered by subcutaneous injection.

5. The method of claim **1**, wherein cyclosporine is administered concurrently.

6. The method of claim **1**, wherein acitretin is administered concurrently.

7. The method of claim **1**, wherein ultraviolet light B phototherapy or psoralen combined with ultraviolet light A (PUVA) phototherapy is administered concurrently.

8. The method of claim **1**, wherein a corticosteroid is administered concurrently.

9. The method of claim **1**, wherein methotrexate is administered concurrently.

10. The method of claim **1**, wherein

(a) a dose of 50 mg of TNFR:Fc is administered two times per week for at least two months and then

(b) TNFR:Fc is administered at a reduced dose or at a reduced frequency.

11. The method of claim **10**, wherein the administration of (b) is at a dose 25 mg of TNFR:Fc twice per week.

12. The method of claim **10**, wherein the administration of (b) is at a dose of 50 mg once per week.

13. The method of claim **10**, wherein the TNFR:Fc is administered by subcutaneous injection.

* * * * *

Exhibit G


US008722631B2

(12) **United States Patent**
Finck

(10) **Patent No.: US 8,722,631 B2**
(45) **Date of Patent: *May 13, 2014**

(54) **SOLUBLE TUMOR NECROSIS FACTOR RECEPTOR TREATMENT OF MEDICAL DISORDERS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **13/773,319**

(22) Filed: **Feb. 21, 2013**

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Related U.S. Application Data

(60) Division of application No. 13/367,071, filed on Feb. 6, 2012, now Pat. No. 8,410,060, which is a division of application No. 13/021,545, filed on Feb. 4, 2011, now Pat. No. 8,119,605, which is a continuation of application No. 12/394,962, filed on Feb. 27, 2009, now Pat. No. 7,915,225, which is a division of application No. 10/853,479, filed on May 25, 2004, now abandoned, which is a division of application No. 09/602,351, filed on Jun. 23, 2000, now abandoned, and a continuation-in-part of application No. 09/378,828, filed on Aug. 13, 1999, now abandoned.

(60) Provisional application No. 60/164,676, filed on Nov. 10, 1999, provisional application No. 60/184,864, filed on Feb. 25, 2000, provisional application No. 60/130,074, filed on Apr. 19, 1999, provisional application No. 60/134,320, filed on May 14, 1999, provisional application No. 60/143,959, filed on Jul. 15, 1999, provisional application No. 60/148,234, filed on Aug. 11, 1999.

(51) **Int. Cl.**

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C07K 14/525	(2006.01)

(52) **U.S. Cl.**

USPC **514/21.2**; 514/18.7; 514/169; 552/588; 607/94; 530/350; 530/866

(58) **Field of Classification Search**

None

See application file for complete search history.

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(57) **ABSTRACT**

The invention pertains to methods and compositions for treating medical disorders characterized by elevated levels or abnormal expression of TNFα by administering a TNFα antagonist, such as recombinant TNFR:Fc.

22 Claims, No Drawings

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SOLUBLE TUMOR NECROSIS FACTOR RECEPTOR TREATMENT OF MEDICAL DISORDERS

This application is a divisional of U.S. application Ser. No. 13/367,071, filed Feb. 6, 2012, now allowed; which is a divisional of U.S. application Ser. No. 13/021,545, filed Feb. 4, 2011, now U.S. Pat. No. 8,119,605; which is a continuation of U.S. application Ser. No. 12/394,962, filed Feb. 27, 2009, now U.S. Pat. No. 7,915,225; which is a divisional of U.S. application Ser. No. 10/853,479, filed May 25, 2004, now abandoned; which is a divisional of U.S. application Ser. No. 09/602,351, filed Jun. 23, 2000, now abandoned, which claims benefit of U.S. Provisional Application Nos. 60/164,676, filed Nov. 10, 1999, now abandoned, and 60/184,864, filed Feb. 25, 2000, now abandoned; and which is a continuation-in-part of U.S. application Ser. No. 09/373,828, filed Aug. 13, 1999, now abandoned, which claims the benefit of U.S. Provisional Application Nos. 60/130,074, filed Apr. 19, 1999, now abandoned, 60/134,320, filed May 14, 1999, now abandoned, 60/143,959, filed Jul. 15, 1999, now abandoned, and 60/148,234, filed Aug. 11, 1999, now abandoned; all of which are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

The invention pertains to methods for treating various medical disorders that are characterized by abnormal or excessive TNFα levels by administering a TNFα antagonist, preferably a soluble TNFα. The TNFα inhibitor may be administered in combination with other biologically active molecules.

BACKGROUND OF THE INVENTION

The pleiotropic cytokine tumor necrosis factor alpha (TNFα) is associated with inflammation and binds to cells through membrane receptor molecules, including two molecules having molecular weights of approximately 55 kDa and 75 kDa (p55 and p75). In addition to binding TNFα, the p55 and p75 TNF receptors mediate the binding to cells of homotrimers of TNFβ, which is another cytokine associated with inflammation and which shares structural similarities with TNFα (e.g., see Cosman, *Blood Cell Biochem* 7:51-77, 1996). TNFβ is also known as lymphotoxin-α (LTα).

It has been proposed that a systemic or localized excess of TNFα contributes to the progression of numerous medical disorders. For example, patients with chronic heart failure have elevated levels of serum TNFα, which have been shown to increase with disease progression (see, for example, Levine et al., *N Eng J Med* 323:236-241, 1990). A variety of other diseases are associated with elevated levels of TNFα (see, for example, Feldman et al., *Transplantation Proceedings* 30:4126-4127, 1998).

Psoriatic arthritis (PsA) is a chronic autoimmune condition that shares some features with both rheumatoid arthritis (RA) and the inflammatory skin disease psoriasis (for review, see Breathnach, In Klippel and Dieppe eds. *Rheumatology*, 2nd Ed., Mosby, 1998, 22.1-22.4). Psoriasis is characterized by epidermal keratinocyte hyperproliferation, accompanied by neutrophil and T cell infiltration, and is associated with elevated levels of inflammatory cytokines, including TNFα, IL-6 and TGFβ (see, for example, Bonifati et al., *Clin Exp Dermatol* 19:383-387, 1994). Psoriasis and PsA are different clinical entities, and are associated with somewhat different MHC haplotypes (Gladman, *Rheum Dis Clin NA.* 18:247-256, 1992; Breathnach, 1998). The overall prognosis for PsA is far worse than for ordinary psoriasis. Nonetheless, treatments used for the psoriatic lesions of PsA generally are similar to those used to treat psoriasis.

Psoriatic skin lesions are present in patients with PsA, although only a minority of psoriasis sufferers actually have PsA. Ordinary psoriasis occasionally is accompanied by joint pain, but does not involve the extreme pain and often deforming degeneration of joints and bone that occurs in PsA patients.

Treatments that sometimes are effective in treating ordinary psoriasis include topical medications (e.g., steroids, coal tar, anthralin, Dead Sea salts, various natural oils, vitamin D3 and its analogs, sunshine, topical retinoids), phototherapy (e.g., ultraviolet light, photochemotherapy (PUVA)), and internal medications (e.g., methotrexate, systemic steroids, oral retinoids, cyclosporine, or a rotating regimen of these three). In addition, it has been proposed that psoriasis could be treated with TNF-derived peptides, quinolinesulfonamides, pyrrolidinone derivatives, catechol diether compounds, isoxazoline compounds, matrix metalloproteinase inhibitors or mercapto alkyl peptidyl compounds, all of which inhibit either TNFα production or its release from cultured cells (see, for example, U.S. Pat. No. 5,691,382, U.S. Pat. No. 5,834,485, U.S. Pat. No. 5,420,154, U.S. Pat. No. 5,563,143, U.S. Pat. No. 5,869,511 and U.S. Pat. No. 5,872,146), as well as with various combination therapies involving TNFα antagonists (for example, see U.S. Pat. No. 5,888,511 or U.S. Pat. No. 5,958,413).

Conflicting results have been reported regarding the role of TNFα in psoriasis. Some investigators have proposed that overproduction of TNFα contributes to the pathology of psoriasis (e.g., Pigatto et al., *J Invest Dermatol* 94:372-376, 1990; Sagawa et al., *Dermatol* 187:81-83, 1993; Ameglio et al., *Dermatol* 189:359-363, 1994). One group reported some improvement after treatment with pentoxifylline, a drug that can inhibit the release of TNFα, but which exerts many of its physiological effects by inhibiting cyclic AMP phosphodiesterase (Omulecki et al., *J Am Acad Dermatol* 34:714-715, 1996; Centola et al., *J Androl* 16:136-142, 1995; Elferinck et al., *Biochem Pharmacol* 54:475-480, 1997). However, other reports have cast doubt on the hypothesis that overproduction of TNFα exacerbates psoriasis. For example, some investigators have reported that treatment with TNFα itself actually can mitigate psoriasis (see, e.g., Takematsu et al., *Br J Dermatol* 124:209-210, 1991; Creaven et al., *J Am Acad Dermatol* 24:735-737, 1991).

In addition to psoriatic lesions, PsA is characterized by distal interphalangeal joint (DIP) involvement, enthesopathy, nail lesions, spondylitis and dactylitis. The histopathogenesis of PsA and the more well-studied rheumatoid arthritis share certain features. In both RA and in active PsA, patients exhibit increased levels of HLA-DR$^+$ T cells and MHC class II antigens in their synovial membranes and synovial fluid, as well as increased expression of the cytokine TNFα. In addition, both diseases are associated with prominent synovial vascular changes.

The discovery of rheumatoid factor in the serum of RA patients provided an important tool for differentiating PsA from RA, but the realization that RA and PsA are distinct diseases was based primarily on their many clinical differences (e.g., Helliwell and Wright, In Klippel and Dieppe eds. *Rheumatology*, 2nd Ed., Mosby, 1998, 21.1-21.8). Studies have shown that levels of TNFα, Il-1β, Il-8 as well as TNFα receptors in synovial fluids were higher in PsA patients than in osteoarthritis patients, though they were lower than in RA patients (Partsch et al., *J Rheumatol* 24:518-523, 1997; Partsch et al., *J Rheumatol* 25:105-110, 1998; Partsch et al.,

Ann Rheum Dis 57:691-693, 1998). PsA is distinguished from RA also by radiographic appearance, a notably higher degree of synovial membrane vascularity as well as differences in the levels of various cytokines in the synovial fluids (Ritchlin et al., *J Rheumatol* 25:1544-52, 1998; Veale et al., *Arth Rheum* 36:893-900, 1993). Veale et al. noted differences in synovial membrane adhesion molecules and numbers of macrophages when they compared RA and PsA patients, as well as observing a minimal degree of hyperplasia and hypertrophy of synoviocytes in PsA as compared with RA patients. Because of such differences, coupled with the association of PsA but not RA with class I MHC antigens, Ritchlin et al. have suggested that PsA must be triggered by different mechanisms than those underlying RA. Veale et al. suggested for similar reasons that different cytokines were likely to be interacting in the synovium of PsA and RA patients.

Most of the drugs used for treating the arthritic aspects of PsA are similar to those used in RA (Salvarini et al., *Curr Opin Rheumatol* 10:229-305, 1998), for example the non-steroidal antiinflammatories (NSAIDs), which may be used alone or in combination with the disease-modifying anti-rheumatic drugs, or "DMARDs." However, one group found that long-term administration of the DMARD methotrexate failed to slow the progression of joint damage in PsA patients (Abu-Shakra et al., *J Rheumatol* 22:241-45, 1995), and another group reported very little improvement in PsA patients who had received methotrexate (Willkens et al., *Arthr Rheum* 27:376-381, 1984). Similarly, Clegg et al. found only a slight improvement over placebo in PsA patients treated with sulfasalazine, another drug classified as a DMARD (Clegg et al., *Arthritis Rheum* 39: 2013-20, 1996). Some studies have indicated that the immunosuppressor cyclosporine is effective in treating PsA (reviewed in Salvarini et al., 1998), though this drug has severe side effects. In addition, others have proposed that PsA could be treated with truncated TNFα receptors or with a combination of methotrexate and antibodies against TNFα (WO 98/01555; WO 98/0537).

A recent meta-analysis of a number of PsA treatment studies concluded that PsA and RA differed not only in their response to treatment with specific drugs, but in the relative magnitudes of improvement in the placebo arms of the studies (Jones et al., *Br J Rheumatol* 36:95-99, 1997). As an example, PsA patients responded better to gold salt therapy than did RA patients, though the gold did not affect the psoriatic skin lesions (Dorwart et al., *Arthritis Rheum* 21:515-513, 1978).

It has been suggested that the suppression of TNFα might be beneficial in patients suffering from various disorders characterized by abnormal or excessive TNFα expression. However, although progress has been made in devising effective treatment for such diseases, improved medicaments and methods of treatment are needed.

SUMMARY OF THE INVENTION

Provided herein are methods for treating a number of medical disorders characterized by abnormal TNFα expression by repeatedly administering an antagonist of TNFα, such as a soluble TNFα receptor, for a period of time sufficient to induce a sustained improvement in the patient's condition.

DETAILED DESCRIPTION OF THE INVENTION

This invention provides compounds, compositions and methods for treating a mammalian patient, including a human patient, who is suffering from a medical disorder that is characterized by abnormal or elevated expression of TNFα. For purposes of this disclosure, the terms "illness," "disease," "medical condition," "abnormal condition" and the like are used interchangeably with the term "medical disorder."

The subject methods involve administering to the patient a soluble TNFα antagonist that is capable of reducing the effective amount of endogenous biologically active TNFα, such as by reducing the amount of TNFα produced, or by preventing the binding of TNFα to its cell surface receptor (TNFR). Antagonists capable of inhibiting this binding include receptor-binding peptide fragments of TNFα, antibodies directed against TNFα, and recombinant proteins comprising all or portions of receptors for TNFα or modified variants thereof, including genetically-modified muteins, multimeric forms and sustained-release formulations. Other compounds suitable for treating the diseases described herein include thalidomide and pentoxifylline.

Preferred embodiments of the invention utilize soluble TNFRs as the TNFα antagonist. Soluble forms of TNFRs may include monomers, fusion proteins (also called "chimeric proteins), dimers, trimers or higher order multimers. In certain embodiments of the invention, the soluble TNFR derivative is one that mimics the 75 kDa TNFR or the 55 kDa TNFR and that binds to TNFα in the patient's body. The soluble TNFR mimics of the present invention may be derived from TNFRs p55 or p75 or fragments thereof. TNFRs other than p55 and p75 also are useful for deriving soluble compounds for treating the various medical disorders described herein, such for example the TNFR described in WO 99/04001. Soluble TNFR molecules used to construct TNFR mimics include, for example, analogs or fragments of native TNFRs having at least 20 amino acids, that lack the transmembrane region of the native TNFR, and that are capable of binding TNFα. Antagonists derived from TNFRs compete for TNFα with the receptors on the cell surface, thus inhibiting TNFα from binding to cells, thereby preventing it from manifesting its biological activities. Binding of soluble TNFRs to TNFα or LTα can be assayed using ELISA or any other convenient assay. This invention provides for the use of soluble TNFα receptors in the manufacture of medicaments for the treatment of numerous diseases.

The soluble TNFR polypeptides or fragments of the invention may be fused with a second polypeptide to form a chimeric protein. The second polypeptide may promote the spontaneous formation by the chimeric protein of a dimer, trimer or higher order muimer that is capable of binding a TNFα and/or LTα molecule and preventing it from binding to cell-bound receptors. Chimeric proteins used as antagonists include, for example, molecules derived from an antibody molecule and a TNFR. Such molecules are referred to herein as TNFR-Ig fusion proteins. A preferred TNFR-Ig fusion protein suitable for treating diseases in humans and other mammals is recombinant TNFR:Fc, a term which as used hercin refers to "etanercept," which is a dimer of two molecules of the extracellular portion of the p75 TNFα receptor, each molecule consisting of a 235 amino acid TNFR-derived polypeptide that is fused to a 232 amino acid Fc portion of human IgG_1. Etanercept is currently sold by Immunex Corporation under the trade name ENBREL.® Because the p75 receptor protein that it incorporates binds not only to TNFα, but also to the inflammatory cytokine LTα, etanercept can act as a competitive inhibitor not only of TNFα, but also of LTα. This is in contrast to antibodies directed against TNFα, which cannot inhibit LTα. Also encompassed by the invention are treatments using a compound that comprises the extracellular portion of the 55 kDa TNFR fused to the Fc portion of IgG, as well as compositions and combinations containing such a molecule. Encompassed also are therapeutic methods involv-

ing the administration of TNFR-Ig proteins derived the extracellular regions of TNFα receptor molecules other than the p55 and p75 TNFRs, such as for example the TNFR described in WO 99/04001.

In one preferred embodiment of the invention, sustained-release forms of soluble TNFRs are used, including sustained-release forms of TNFR:Fc. Sustained-release forms suitable for use in the disclosed methods include, but are not limited to, TNFRs that are encapsulated in a slowly-dissolving biocompatible polymer (such as the alginate microparticles described in U.S. Pat. No. 6,036,978), admixed with such a polymer (including topically applied hydrogels), and or encased in a biocompatible semi-permeable implant. In addition, the soluble TNFR may be conjugated with polyethylene glycol (pegylated) to prolong its serum half-life or to enhance protein delivery.

In accord with this invention, medical disorders characterized by abnormal or excess expression of TNFα are administered a therapeutically effective amount of a TNFα inhibitor. The TNFα inhibitor may be a TNFα-binding soluble TNFα receptor, preferably TNFR:Fc. As used herein, the phrase "administering a therapeutically effective amount" of a therapeutic agent means that the patient is treated with the agent in an amount and for a time sufficient to induce a sustained improvement in at least one indicator that reflects the severity of the disorder. An improvement is considered "sustained" if the patient exhibits the improvement on at least two occasions separated by one or more weeks. The degree of improvement is determined based on signs or symptoms, and determinations may also employ questionnaires that are administered to the patient, such as quality-of-life questionnaires.

Various indicators that reflect the extent of the patient's illness may be assessed for determining whether the amount and time of the treatment is sufficient. The baseline value for the chosen indicator or indicators is established by examination of the patient prior to administration of the first dose of the etanercept or other TNFα inhibitor. Preferably, the baseline examination is done within about 60 days of administering the first dose. If the TNFα antagonist is being administered to treat acute symptoms, such as for example to treat a traumatic knee injury, the first dose is administered as soon as practically possible after the injury has occurred.

Improvement is induced by administering TNFR:Fc or other TNFα antagonist until the patient manifests an improvement over baseline for the chosen indicator or indicators. In treating chronic conditions, this degree of improvement is obtained by repeatedly administering this medicament over a period of at least a month or more, e.g., for one, two, or three months or longer, or indefinitely. A period of one to six weeks, or even a single dose, often is sufficient for treating acute conditions. For injuries or acute conditions, a single dose may be sufficient.

Although the extent of the patient's illness after treatment may appear improved according to one or more indicators, treatment may be continued indefinitely at the same level or at a reduced dose or frequency. Once treatment has been reduced or discontinued, it later may be resumed at the original level if symptoms should reappear.

Any efficacious route of administration may be used to therapeutically administer TNFR:Fc or other TNFα antagonists. If injected, TNFR:Fc can be administered, for example, via intra-articular, intravenous, intramuscular, intralesional, intraperitoneal or subcutaneous routes by bolus injection or by continuous infusion. Other suitable means of administration include sustained release from implants, aerosol inhalation, eyedrops, oral preparations, including pills, syrups, lozenges or chewing gum, and topical preparations such as lotions, gels, sprays, ointments or other suitable techniques. Alternatively, proteinaceous TNFα inhibitors, such as a soluble TNFR, may be administered by implanting cultured cells that express the protein, for example, by implanting cells that express TNFR:Fc. In one embodiment, the patient's own cells are induced to produce TNFR:Fc by transfection in vivo or ex vivo with a DNA that encodes TNFR:Fc. This DNA can be introduced into the patient's cells, for example, by injecting naked DNA or liposome-encapsulated DNA that encodes TNFR:Fc, or by other means of transfection. When TNFR:Fc is administered in combination with one or more other biologically active compounds, these may be administered by the same or by different routes, and may be administered simultaneously, separately or sequentially.

TNFR:Fc or other soluble TNFRs preferably are administered in the form of a physiologically acceptable composition comprising purified recombinant protein in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers are nontoxic to recipients at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the TNFα antagonist with buffers, antioxidants such as ascorbic acid, low molecular weight polypeptides (such as those having fewer than 10 amino acids), proteins, amino acids, carbohydrates such as glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents. In accordance with appropriate industry standards, preservatives may also be added, such as benzyl alcohol. TNFR:Fc preferably is formulated as a lyophilizate using appropriate excipient solutions (e.g., sucrose) as diluents. Appropriate dosages can be determined in standard dosing trials, and may vary according to the chosen route of administration. The amount and frequency of administration will depend, of course, on such factors as the nature and severity of the indication being treated, the desired response, the age and condition of the patient, and so forth.

In one embodiment of the invention, TNFR:Fc is administered one time per week to treat the various medical disorders disclosed herein, in another embodiment is administered at least two times per week, and in another embodiment is administered at least three times per week. An adult patient is a person who is 18 years of age or older. If injected, the effective amount of TNFR:Fc per adult dose ranges from 1-20 mg/m^2, and preferably is about 5-12 mg/m^2. Alternatively, a flat dose may be administered, whose amount may range from 5-100 mg/dose. Exemplary dose ranges for a flat dose to be administered by subcutaneous injection are 5-25 mg/dose, 25-50 mg/dose and 50-100 mg/dose. In one embodiment of the invention, the various indications described below are treated by administering a preparation acceptable for injection containing TNFR:Fc at 25 mg/dose, or alternatively, containing 50 mg per dose. The 25 mg or 50 mg dose may be administered repeatedly, particularly for chronic conditions. If a route of administration other than injection is used, the dose is appropriately adjusted in accord with standard medical practices. In many instances, an improvement in a patient's condition will be obtained by injecting a dose of about 25 mg of TNFR:Fc one to three times per week over a period of at least three weeks, or a dose of 50 mg of TNFR:Fc one or two times per week for at least three weeks, though treatment for longer periods may be necessary to induce the desired degree of improvement. For incurable chronic conditions, the regimen may be continued indefinitely, with adjustments being made to dose and frequency if such are deemed necessary by the patient's physician.

For pediatric patients (age 4-17), a suitable regimen involves the subcutaneous injection of 0.4 mg/kg, up to a maximum dose of 25 mg of TNFR:Fc, administered by subcutaneous injection one or more times per week.

The invention further includes the administration of TNFR:Fc concurrently with one or more other drugs that are administered to the same patient in combination with the TNFR:Fc, each drug being administered according to a regimen suitable for that medicament. "Concurrent administration" encompasses simultaneous or sequential treatment with the components of the combination, as well as regimens in which the drugs are alternated, or wherein one component is administered long-term and the other(s) are administered intermittently. Components may be administered in the same or in separate compositions, and by the same or different routes of administration. Examples of drugs to be administered concurrently include but are not limited to antivirals, antibiotics, analgesics, corticosteroids, antagonists of inflammatory cytokines, DMARDs and non-steroidal anti-inflammatories. DMARDs that can be administered in combination with the subject TNFα inhibitors such as TNFR:Fc include azathioprine, cyclophosphamide, cyclosporine, hydroxychloroquine sulfate, methotrexate, leflunomide, minocycline, penicillamine, sulfasalazine and gold compounds such as oral gold, gold sodium thiomalate and aurothioglucose. Additionally, TNFR:Fc may be combined with a second TNFα antagonist, including an antibody against TNFα or TNFR, a TNFα-derived peptide that acts as a competitive inhibitor of TNFα (such as those described in U.S. Pat. No. 5,795,859), a TNFR-IgG fusion protein other than etanercept, such as one containing the extracellular portion of the p55 TNFα receptor, a soluble TNFR other than an IgG fusion protein, or other molecules that reduce endogenous TNFα levels, such as inhibitors of the TNFα converting enzyme (see e.g., U.S. Pat. No. 5,594,106). In further embodiments of this invention, TNFR:Fc is administered in combination with pentoxifylline or thalidomide.

If an antibody against TNFα is used as the TNFα inhibitor, a preferred dose range is 0.1 to 20 mg/kg, and more preferably is 1-10 mg/kg. Another preferred dose range for anti-TNFα antibody is 0.75 to 7.5 mg/kg of body weight. Humanized antibodies are preferred, that is, antibodies in which only the antigen-binding portion of the antibody molecule is derived from a non-human source. such antibodies may be injected or administered intravenously.

In one preferred embodiment of the invention, the various medical disorders disclosed herein as being treatable with inhibitors such as TNFR:Fc are treated in combination with another cytokine or cytokine inhibitor. For example, TNFR:Fc may be administered in a composition that also contains a compound that inhibits the interaction of other inflammatory cytokines with their receptors. Examples of cytokine inhibitors used in combination with TNFR:Fc include, for example, antagonists of TGFβ, Il-6 or Il-8. TNFα inhibitors such as TNFR:Fc also may be administered in combination with the cytokines GM-CSF, IL-2 and inhibitors of protein kinase A type 1 to enhance T cell proliferation in HIV-infected patients who are receiving anti-retroviral therapy. Other combinations for treating the hereindescribed diseases include TNFR:Fc administered concurrently with compounds that block the binding of RANK and RANK-ligand, such as antagonistic antibodies against RANK or RANK-ligand, soluble forms of RANK-ligand that do not trigger RANK, osteoprotegerin or soluble forms of RANK, including RANK:Fc. Soluble forms of RANK suitable for these combinations are described, for example, in U.S. Pat. No. 6,017,729. The concurrent administration of TNFR:Fc and RANK:Fc or TNFR:Fc and osteoprotegerin is useful for preventing bone destruction in various settings including but not limited to various rheumatic disorders, osteoporosis, multiple myeloma or other malignancies that cause bone degeneration, or anti-tumor therapy aimed at preventing metastasis to bone, or bone destruction associated with prosthesis wear debris or with periodontitis.

The present invention also relates to the use of the disclosed TNFα inhibitors, such as TNFR:Fc, in the manufacture of a medicament for the prevention or therapeutic treatment of each medical disorder disclosed herein.

The disclosed TNFα inhibitors, compositions and combination therapies described herein are useful in medicines for treating bacterial, viral or protozoal infections, and complications resulting therefrom. One such disease is *Mycoplasma pneumonia*. In addition, provided herein is the use of TNFR:Fc to treat AIDS and related conditions, such as AIDS dementia complex, AIDS associated wasting, lipidistrophy due to antiretroviral therapy; and Kaposi's sarcoma. Provided herein is the use of TNFR:Fc for treating protozoal diseases, including malaria and schistosomiasis. Additionally provided is the use of TNFR:Fc to treat erythema nodosum leprosum; bacterial or viral meningitis; tuberculosis, including pulmonary tuberculosis; and pneumonitis secondary to a bacterial or viral infection. Provided also herein is the use of TNFR:Fc to prepare medicaments for treating louse-borne relapsing fevers, such as that caused by *Borrelia recurrentis*. TNFR:Fc can also be used to prepare a medicament for treating conditions caused by Herpes viruses, such as herpetic stromal keratitis, corneal lesions, and virus-induced corneal disorders. In addition, TNFR:Fc can be used in treating human papillomavirus infections. TNFR:Fc is used also to prepare medicaments to treat influenza.

Cardiovascular disorders are treatable with the disclosed TNFα inhibitors, pharmaceutical compositions or combination therapies, including aortic aneurisms; arteritis; vascular occlusion, including cerebral artery occlusion; complications of coronary by-pass surgery; ischemia/reperfusion injury; heart disease, including atherosclerotic heart disease, myocarditis, including chronic autoimmune myocarditis and viral myocarditis; heart failure, including chronic heart failure (CHF), cachexia of heart failure; myocardial infarction; restenosis after heart surgery; silent myocardial ischemia; post-implantation complications of left ventricular assist devices; Raynaud's phenomena; thrombophlebitis; vasculitis, including Kawasaki's vasculitis; giant cell arteritis, Wegener's granulomatosis; and Schoenlein-Henoch purpura.

TNFα and IL-8 have been implicated as chemotactic factors in atherosclerotic abdominal aortic aneurism (Szekanecz et al., *Pathobiol* 62:134-139 (1994)). Abdominal aortic aneurism may be treated in human patients by administering a soluble TNFR, such as TNFR:Fc, which may be administered in combination with an inhibitor of IL-8, such treatment having the effect of reducing the pathological neovascularization associated with this condition.

A combination of a TNFα inhibitor and one or more other anti-angiogenesis factors may be used to treat solid tumors, thereby reducing the vascularization that nourishes the tumor tissue. Suitable anti-angiogenic factors for such combination therapies include IL-8 inhibitors, angiostatin, endostatin, kringle 5, inhibitors of vascular endothelial growth factor (such as antibodies against vascular endothelial growth factor), angiopoietin-2 or other antagonists of angiopoietin-1, antagonists of platelet-activating factor and antagonists of basic fibroblast growth factor

In addition, the subject TNFα inhibitors, compositions and combination therapies are used to treat chronic pain condi-

tions, such as chronic pelvic pain, including chronic prostatitis/pelvic pain syndrome. As a further example, TNFR:Fc and the compositions and combination therapies of the invention are used to treat post-herpetic pain.

Provided also are methods for using TNFα inhibitors, compositions or combination therapies to treat various disorders of the endocrine system. For example, the TNFα inhibitors are used to treat juvenile onset diabetes (includes autoimmune and insulin-dependent types of diabetes) and also to treat maturity onset diabetes (includes non-insulin dependent and obesity-mediated diabetes). In addition, the subject compounds, compositions and combination therapies are used to treat secondary conditions associated with diabetes, such as diabetic retinopathy, kidney transplant rejection in diabetic patients, obesity-mediated insulin resistance, and renal failure, which itself may be associated with proteinurea and hypertension. Other endocrine disorders also are treatable with these compounds, compositions or combination therapies, including polycystic ovarian disease, X-linked adrenoleukodystrophy, hypothyroidism and thyroiditis, including Hashimoto's thyroiditis (i.e., autoimmune thyroiditis).

Conditions of the gastrointestinal system also are treatable with TNFα inhibitors, compositions or combination therapies, including coeliac disease. In addition, the compounds, compositions and combination therapies of the invention are used to treat Crohn's disease; ulcerative colitis; idiopathic gastroparesis; pancreatitis, including chronic pancreatitis and lung injury associated with acute pancreatitis; and ulcers, including gastric and duodenal ulcers.

Included also are methods for using the subject TNFα inhibitors, compositions or combination therapies for treating disorders of the genitourinary system, such as glomerulonephritis, including autoimmune glomerulonephritis, glomerulonephritis due to exposure to toxins or glomerulonephritis secondary to infections with haemolytic streptococci or other infectious agents. Also treatable with the compounds, compositions and combination therapies of the invention are uremic syndrome and its clinical complications (for example, renal failure, anemia, and hypertrophic cardiomyopathy), including uremic syndrome associated with exposure to environmental toxins, drugs or other causes. Further conditions treatable with the compounds, compositions and combination therapies of the invention are complications of hemodialysis; prostate conditions, including benign prostatic hypertrophy, nonbacterial prostatitis and chronic prostatitis; and complications of hemodialysis.

Also provided herein are methods for using TNFα inhibitors, compositions or combination therapies to treat various hematologic and oncologic disorders. For example, TNFR:Fc is used to treat various forms of cancer, including acute myelogenous leukemia, Epstein-Barr virus-positive nasopharyngeal carcinoma, glioma, colon, stomach, prostate, renal cell, cervical and ovarian cancers, lung cancer (SCLC and NSCLC), including cancer-associated cachexia, fatigue, asthenia, paraneoplastic syndrome of cachexia and hypercalcemia. Additional diseases treatable with the subject TNFα inhibitors, compositions or combination therapies are solid tumors, including sarcoma, osteosarcoma, and carcinoma, such as adenocarcinoma (for example, breast cancer) and squamous cell carcinoma. In addition, the subject compounds, compositions or combination therapies are useful for treating leukemia, including acute myelogenous leukemia, chronic or acute lymphoblastic leukemia and hairy cell leukemia. Other malignancies with invasive metastatic potential can be treated with the subject compounds, compositions and combination therapies, including multiple myeloma. In addition, the disclosed TNFα inhibitors, compositions and combination therapies can be used to treat anemias and hematologic disorders, including anemia of chronic disease, aplastic anemia, including Fanconi's aplastic anemia; idiopathic thrombocytopenic purpura (ITP); myelodysplastic syndromes (including refractory anemia, refractory anemia with ringed sideroblasts, refractory anemia with excess blasts, refractory anemia with excess blasts in transformation); myelofibrosis/myeloid metaplasia; and sickle cell vasoocclusive crisis.

Various lymphoproliferative disorders also are treatable with the disclosed TNFα inhibitors, compositions or combination therapies. These include, but are not limited to autoimmune lymphoproliferative syndrome (ALPS), chronic lymphoblastic leukemia, hairy cell leukemia, chronic lymphatic leukemia, peripheral T-cell lymphoma, small lymphocytic lymphoma, mantle cell lymphoma, follicular lymphoma, Burkitt's lymphoma. Epstein-Barr virus-positive T cell lymphoma, histiocytic lymphoma, Hodgkin's disease, diffuse aggressive lymphoma, acute lymphatic leukemias, T gamma lymphoproliferative disease, cutaneous B cell lymphoma, cutaneous T cell lymphoma (i.e., mycosis fungoides) and Sézary syndrome.

In addition, the subject TNFα inhibitors, compositions and combination therapies are used to treat hereditary conditions such as Gaucher's disease, Huntington's disease, linear IgA disease, and muscular dystrophy.

Other conditions treatable by the disclosed TNFα inhibitors, compositions and combination therapies include those resulting from injuries to the head or spinal cord, and including subdural hematoma due to trauma to the head.

The disclosed TNFα inhibitors, compositions and combination therapies are further used to treat conditions of the liver such as hepatitis, including acute alcoholic hepatitis, acute drug-induced or viral hepatitis, hepatitis A, B and C, sclerosing cholangitis and inflammation of the liver due to unknown causes.

In addition, the disclosed TNFα inhibitors, compositions and combination therapies are used to treat various disorders that involve hearing loss and that are associated with abnormal TNFα expression. One of these is inner ear or cochlear nerve-associated hearing loss that is thought to result from an autoimmune process, i.e., autoimmune hearing loss. This condition currently is treated with steroids, methotrexate and/or cyclophosphamide, which may be administered concurrently with the TNFR:Fc or other TNFα inhibitor. Also treatable with the disclosed TNFα inhibitors, compositions and combination therapies is cholesteatoma, a middle ear disorder often associated with hearing loss.

In addition, the subject invention provides TNFα inhibitors, compositions and combination therapies for the treatment of non-arthritic medical conditions of the bones and joints. This encompasses osteoclast disorders that lead to bone loss, such as but not limited to osteoporosis, including post-menopausal osteoporosis, periodontitis resulting in tooth loosening or loss, and prosthesis loosening after joint replacement (generally associated with an inflammatory response to wear debris). This latter condition also is called "orthopedic implant osteolysis." Another condition treatable by administering TNFRα inhibitors, such as TNFR:Fc, is temporal mandibular joint dysfunction (TMJ).

A number of pulmonary disorders also can be treated with the disclosed TNFα inhibitors, compositions and combination therapies. One such condition is adult respiratory distress syndrome (ARDS), which is associated with elevated TNFα, and may be triggered by a variety of causes, including exposure to toxic chemicals, pancreatitis, trauma or other causes. The disclosed compounds, compositions and combination

therapies of the invention also are useful for treating bronchopulmonary dysplasia (BPD); lymphangioleiomyomatosis; and chronic fibrotic lung disease of preterm infants. In addition. the compounds, compositions and combination therapies of the invention are used to treat occupational lung diseases, including asbestosis, coal worker's pneumoconiosis, silicosis or similar conditions associated with long-term exposure to fine particles. In other aspects of the invention, the disclosed compounds, compositions and combination therapies are used to treat pulmonary disorders, including chronic obstructive pulmonary disease (COPD) associated with chronic bronchitis or emphysema; fibrotic lung diseases, such as cystic fibrosis, idiopathic pulmonary fibrosis and radiation-induced pulmonary fibrosis; pulmonary sarcoidosis; and allergies, including allergic rhinitis, contact dermatitis, atopic dermatitis and asthma.

Cystic fibrosis is an inherited condition characterized primarily by the accumulation of thick mucus, predisposing the patient to chronic lung infections and obstruction of the pancreas, which results in malabsorption of nutrients and malnutrition. TNFR:Fc may be administered to treat cystic fibrosis. If desired, treatment with TNFR:Fc may be administered concurrently with corticosteroids, mucus-thinning agents such as inhaled recombinant deoxyribonuclease 1 (such as PULMOZYME®; Genentech, Inc.) or inhaled tobramycin (TOBI®; Pathogenesis, Inc.). TNFR:Fc also may be administered concurrently with corrective gene therapy, drugs that stimulate cystic fibrosis cells to secrete chloride or other yet-to-be-discovered treatments. Sufficiency of treatment may be assessed, for example, by observing a decrease in the number of pathogenic organisms in sputum or lung lavage (such as *Haemophilus influenzae, Stapholococcus aureus*, and *Pseudomonas aeruginosa*), by monitoring the patient for weight gain, by detecting an increase in lung capacity or by any other convenient means.

TNFR:Fc or TNFR:Fc combined with the cytokine IFNγ-1b (such as ACTIMMUNE®; InterMune Pharmaceuticals) may be used for treating cystic fibrosis or fibrotic lung diseases, such as idiopathic pulmonary fibrosis, radiation-induced pulmonary fibrosis and bleomycin-induced pulmonary fibrosis. In addition, this combination is useful for treating other diseases characterized by organ fibrosis, including systemic sclerosis (also called "scleroderma"), which often involves fibrosis of the liver. For treating cystic fibrosis, TNFR:Fc and IFNγ-1b may be combined with PULMOZYME® or TOBI® or other treatments for cystic fibrosis.

TNFR:Fc alone or in combination with IFNγ-1b may be administered together with other treatments presently used for treating fibrotic lung disease. Such additional treatments include glucocorticoids, azathioprine, cyclophosphamide, penicillamine, colchisicine, supplemental oxygen and so forth. Patients with fibrotic lung disease, such as IPF, often present with nonproductive cough, progressive dyspnea, and show a restrictive ventilatory pattern in pulmonary function tests. Chest radiographs reveal fibrotic accumulations in the patient's lungs. When treating fibrotic lung disease in accord with the disclosed methods, sufficiency of treatment may be detected by observing a decrease in the patient's coughing (when cough is present), or by using standard lung function tests to detect improvements in total lung capacity, vital capacity, residual lung volume or by administering a arterial blood gas determination measuring desaturation under exercising conditions, and showing that the patient's lung function has improved according to one or more of these measures. In addition, patient improvement may be determined through chest radiography results showing that the progression of fibrosis in the patient's lungs has become arrested or reduced.

In addition, TNF inhibitors (including soluble TNFRs or antibodies against TNFα or TNFR) are useful for treating organ fibrosis when administered in combination with relaxin, a hormone that down-regulates collagen production thus inhibiting fibrosis, or when given in combination with agents that block the fibrogenic activity of TGF-β. Combination therapies using TNFR:Fc and recombinant human relaxin are useful, for example, for treating systemic sclerosis or fibrotic lung diseases, including cystic fibrosis, idiopathic pulmonary fibrosis, radiation-induced pulmonary fibrosis and bleomycin-induced pulmonary fibrosis.

Other embodiments provide methods for using the disclosed TNFα inhibitors, compositions or combination therapies to treat a variety of rheumatic disorders. These include: adult and juvenile rheumatoid arthritis; systemic lupus erythematosus; gout; osteoarthritis; polymyalgia rheumatica; seronegative spondylarthropathies, including ankylosing spondylitis; and Reiter's disease. The subject TNFα inhibitors, compositions and combination therapies are used also to treat psoriatic arthritis and chronic Lyme arthritis. Also treatable with these compounds, compositions and combination therapies are Still's disease and uveitis associated with rheumatoid arthritis. In addition, the compounds, compositions and combination therapies of the invention are used in treating disorders resulting in inflammation of the voluntary muscle, including dermatomyositis and polymyositis. Moreover, the compounds, compositions ant combinations disclosed herein are useful for treating sporadic inclusion body myositis, as TNFα may play a significant role in the progression of this muscle disease. In addition, the compounds, compositions and combinations disclosed herein are used to treat multicentric reticulohistiocytosis, a disease in which joint destruction and papular nodules of the face and hands are associated with excess production of proinflammatory cytokines by multinucleated giant cells.

For purposes of this invention, patients are defined as having psoriatic arthrisis (PsA) if they have one or more swollen joints or one or more painful or tender joints, and also manifest at least one psoriatic lesion of the skin or nails. The psoriatic lesions may appear before or after the onset of swollen or tender joints. It is understood that prior to treatment, manifestations of PsA may have persisted over time, e.g., for several months or years, and may involve several joints. According to one classification system (reviewed in Alonso et al., 1991), PsA patients can be categorized based on their arthritic symptoms into five clinical subgroups: 1) DIP; 2) mutilans arthritis; 3) symmetrical polyarthritis; 4) oligoarticular arthritis; and 5) ankylosing spondylitis-like. The disclosed therapies, compounds and compositions are suitable for treating all five forms of PsA.

The TNFα inhibitors, compositions and combination therapies of the invention may be used to inhibit hypertrophic scarring, a phenomenon believed to result in part from excessive TNFα secretion. TNF inhibitors may be administered alone or concurrently with other agents that inhibit hypertrophic scarring, such as inhibitors of TGF-α.

Cervicogenic headache is a common form of headache arising from dysfunction in the neck area, and which is associated with elevated levels of TNFα, which are believed to mediate an inflammatory condition that contributes to the patient's discomfort (Martelletti, *Clin Exp Rheumatol* 18(2 Suppl 19):S33-8 (March-April, 2000)). Cervicogenic head-

ache may be treated by administering an inhibitor of TNFα as disclosed herein, thereby reducing the inflammatory response and associated headache pain.

The TNFα inhibitors, compositions and combination therapies of the invention are useful for treating primary amyloidosis. In addition, the secondary amyloidosis that is characteristic of various conditions also are treatable with TNFα inhibitors such as TNFR:Fc, and the compositions and combination therapies described herein. Such conditions include: Alzheimer's disease, secondary reactive amyloidosis; Down's syndrome; and dialysis-associated amyloidosis. Also treatable with the compounds, compositions and combination therapies of the invention are inherited periodic fever syndromes, including familial Mediterranean fever, hyperimmunoglobulin D and periodic fever syndrome and TNF-receptor associated periodic syndromes (TRAPS).

Disorders associated with transplantation also are treatable with the disclosed TNFα inhibitors, compositions or combination therapies, such as graft-versus-host disease, and complications resulting from solid organ transplantation, including transplantion of heart, liver, lung, skin, kidney or other organs. TNFR:Fc may be administered, for example, to prevent or inhibit the development of bronchiolitis obliterans after lung transplantation. Patients undergoing autologous hematopoietic stem cell transplantation in the form of peripheral blood stem cell transplantation may develop "engraftment syndrome," or "ES," which is an adverse and generally self-limited response that occurs about the time of hematopoietic engraftment and which can result in pulmonary deterioration. ES may be treated with inhibitors of either IL-8 or TNFα (such as TNFR:Fc), or with a combination of inhibitors against both of these cytokines.

Ocular disorders also are treatable with the disclosed TNFα inhibitors, compositions or combination therapies, including rhegmatogenous retinal detachment, and inflammatory eye disease, and inflammatory eye disease associated with smoking and macular degeneration.

TNFα inhibitors such as TNFR:Fc and the disclosed compositions and combination therapies also are useful for treating disorders that affect the female reproductive system. Examples include, but are not limited to, multiple implant failure/infertility; fetal loss syndrome or IV embryo loss (spontaneous abortion); preeclamptic pregnancies or eclampsia; and endometriosis.

In addition, the disclosed TNFα inhibitors, compositions and combination therapies are useful for treating obesity, including treatment to bring about a decrease in leptin formation. Also, the compounds, compositions and combination therapies of the invention are used to treat sciatica, symptoms of aging, severe drug reactions (for example, 11-2 toxicity or bleomycin-induced pneumopathy and fibrosis), or to suppress the inflammatory response prior, during or after the transfusion of allogeneic red blood cells in cardiac or other surgery, or in treating a traumatic injury to a limb or joint, such as traumatic knee injury. Various other medical disorders treatable with the disclosed TNFα inhibitors, compositions and combination therapies include; multiple sclerosis; Behcet's syndrome; Sjogren's syndrome; autoimmune hemolytic anemia; beta thalassemia; amyotrophic lateral sclerosis (Lou Gehrig's Disease); Parkinson's disease; and tenosynovitis of unknown cause, as well as various autoimmune disorders or diseases associated with hereditary deficiencies.

The disclosed TNFα inhibitors, compositions and combination therapies furthermore are useful for treating acute polyneuropathy; anorexia nervosa; Bell's palsy; chronic fatigue syndrome; transmissible dementia, including Creutzfeld-Jacob disease; demyelinating neuropathy; Guillain-Barre syndrome; vertebral disc disease; Gulf war syndrome; myasthenia gravis; silent cerebral ischemia; sleep disorders, including narcolepsy and sleep apnea; chronic neuronal degeneration; and stroke, including cerebral ischemic diseases.

Disorders involving the skin or mucous membranes also are treatable using the disclosed TNFα inhibitors, compositions or combination therapies. Such disorders include acantholytic diseases, including Darier's disease, keratosis follicularis and pemphigus vulgaris. Also treatable with the subject TNFα inhibitors, compositions and combination therapies are acne; acne rosacea; alopecia greata; aphthous stomatitis; bullous pemphigoid; burns; eczema; erythema, including erythema multiforme and erythema multiforme bullosum (Stevens-Johnson syndrome); inflammatory skin disease; lichen planus; linear IgA bullous disease (chronic bullous dermatosis of childhood); loss of skin elasticity; mucosal surface ulcers; neutrophilic dermatitis (Sweet's syndrome); pityriasis rubra pilaris; psoriasis; pyoderma gangrenosum; and toxic epidermal necrolysis.

Patients are defined as having ordinary psoriasis if they lack the more serious symptoms of PsA (e.g., distal interphalangeal joint DIP involvement, enthesopathy, spondylitis and dactylitis) but have one of the following: 1) inflamed swollen skin lesions covered with silvery white scale (plaque psoriasis or psoriasis vulgaris); 2) small red dots appearing on the trunk, arms or legs (guttate psoriasis); 3) smooth inflamed lesions without scaling in the flexural surfaces of the skin (inverse psoriasis); 4) widespread reddening and exfoliation of fine scales, with or without itching and swelling (erythrodermic psoriasis); 5) blister-like lesions (pustular psoriasis); 6) elevated inflamed scalp lesions covered by silvery white scales (scalp psoriasis); 7) pitted fingernails, with or without yellowish discoloration, crumbling nails, or inflammation and detachment of the nail from the nail bed (nail psoriasis).

Ordinary psoriasis may be treated by administering to a human patient compositions containing a therapeutically effective amount of a TNFα inhibitor such as a soluble TNF receptor or an antibody against TNFα.

In one preferred embodiment, the therapeutic agent is a soluble TNF receptor, and preferably is a TNFR-Ig. In a preferred embodiment, the TNFR-Ig is TNFR:Fc, which may be administered in the form of a pharmaceutically acceptable composition as described herein. Psoriasis may be treated by administering TNFR:Fc one or more times per week by subcutaneous injection, although other routes of administration may be used if desired. In one exemplary regimen for treating adult human patients, 25 mg of TNFR:Fc is administered by subcutaneous injection two times per week or three times per week for one or more weeks, and preferably for four or more weeks. Alternatively, a dose of 5-12 mg/m^2 or a flat dose of 50 mg is injected subcutaneously one time or two times per week for one or more weeks. In other embodiments, psoriasis is treated with TNFR:Fc in a sustained-release form, such as TNFR:Fc that is encapsulated in a biocompatible polymer, TNFR:Fc that is admixed with a biocompatible polymer (such as topically applied hydrogels), and TNFR:Fc that is encased in a semi-permeable implant.

Various other medicaments used to treat ordinary psoriasis may also be administered concurrently with compositions comprising TNFα inhibitors, such as TNFR:Fc. Such medicaments include: NSAIDs; DMARDs; analgesics; topical steroids; systemic steroids (e.g., prednisone); cytokines; antagonists of inflammatory cytokines; antibodies against T cell surface proteins; anthralin; coal tar; vitamin D3 and its analogs; topical retinoids; oral retinoids; salicylic acid; and hydroxyurea. Suitable analgesics for such combinations

include: acetaminophen, codeine, propoxyphene napsylate, oxycodone hydrochloride, hydrocodone bitartrate and tramadol. DMARDs suitable for such combinations include: azathioprine, cyclophosphamide, cyclosporine, hydroxychloroquine sulfate, methotrexate, leflunomide, minocycline, penicillamine, sulfasalazine, oral gold, gold sodium thiomalate and aurothioglucose. In addition, the TNFR:Fc or other TNFR mimic may be administered in combination with antimalarials or colchicine. NSAIDs suitable for the subject combination treatments of psoriasis include: salicylic acid (aspirin) and salicylate derivatives; ibuprofen; indomethacin; celecoxib; rofecoxib; ketorolac; nambumetone; piroxicam; naproxen; oxaprozin; sulindac; ketoprofen; diclofenac; and other COX-1 and COX-2 inhibitors, propionic acid derivatives, acetic acid derivatives, fumaric acid derivatives, carboxylic acid derivatives, butyric acid derivatives, oxicams, pyrazoles and pyrazolones, including newly developed anti-inflammatories.

If an antagonist against an inflammatory cytokine is administered concurrently with TNFR:Fc to treat psoriasis, suitable targets for such antagonists include TGFβ, Il-6 and Il-8.

In addition, TNFR:Fc may be used to treat psoriasis in combination with topical steroids, systemic steroids, antagonists of inflammatory cytokines, antibodies against T cell surface proteins, anthralin, coal tar, vitamin D3 and its analogs (including 1,25-dihydroxy vitamin D3 and calcipotriene), topical retinoids, oral retinoids (including but not limited to etretinate, acitretin and isotretinoin), topical salicylic acid, methotrexate, cyclosporine, hydroxyurea and sulfasalazine. In addition, TNFR:Fc may be administered to treat psoriasis in combination with one or more of the following compounds; minocycline; misoprostol; oral collagen; 6-mercaptopurine; nitrogen mustard; gabapentin; bromocriptine; somatostatin; peptide T; anti-CD4 monoclonal antibody; fumaric acid; polyunsaturated ethyl ester lipids; zinc; and other drugs that may be used to treat psoriasis. TNFR:Fc may also be used to treat psoriasis in combination with the use of various oils, including fish oils, nut oils and vegetable oils; aloe vera; jojoba; Dead Sea salts; capsaicin; milk thistle; witch hazel; moisturizers; and Epsom salts. In addition, psoriasis may be treated with compositions containing TNFR:Fc in combination with the following therapies: plasmapheresis; phototherapy with ultraviolet light B; psoralen combined with ultraviolet light A (PUVA); and sunbathing.

For determining the sufficiency of treatment when treating ordinary psoriasis in accord with the invention, the TNFR:Fc (or other TNFα inhibitor) is administered in an amount and for a time sufficient to induce an improvement in an indicator such as psoriasis area and severity index (PASI) or an improvement in Target Lesion Assessment score, which is an index for assessing the severity of individual skin lesions. In one embodiment, the treatment is regarded as sufficient when the patient exhibits an at least 50% improvement in his or her PASI score, and in another embodiment, when the patient exhibits an at least 75% improvement in PASI score. The sufficiency of treatment for psoriasis may also be determined by evaluating individual psoriatic lesions for improvement in severity (Psoriasis Target Lesion Assessment Score), and continuing treatment until an improvement is noted according to this scoring system. This scoring system involves determining for an individual lesion whether improvement has occurred in plaque elevation, amount and degree of scaling or degree of erythema, and target lesion response to treatment, each of which is separately scored. Psoriasis Target Lesion Assessment Score is determined by adding together the separate scores for all four of the aforementioned indicia.

In addition to human patients, inhibitors of TNFα are useful in the treatment of autoimmune and inflammatory conditions in non-human animals, such as pets (dogs, cats, birds, primates, etc.), domestic farm animals (horses cattle, sheep, pigs, birds, etc.), or any animal that suffers from a TNFα-mediated inflammatory or arthritic condition. In such instances, an appropriate dose may be determined according to the animal's body weight. For example, a dose of 0.2-1 mg/kg may be used. Alternatively, the dose is determined according to the animal's surface area, an exemplary dose ranging from 0.1-20 mg/m^2, or more preferably, from 5-12 mg/m^2. For small animals, such as dogs or cats, a suitable dose is 0.4 mg/kg. In a preferred embodiment, TNFR:Fc (preferably constructed from genes derived from the same species as the patient), or another soluble TNFR mimic, is administered by injection or other suitable route one or more times per week until the animal's condition is improved, or it may be administered indefinitely.

EXAMPLE

Evaluation of TNFR:Fc in Patients with Psoriatic Arthritis

Sixty patients with active psoriatic arthritis (PsA) were enrolled in a Phase II double-blind, randomized, placebo controlled study to determine whether the subcutaneous biweekly administration of etanercept (recombinant TNFR:Fc) was safe in this patient population and whether efficacy could be documented for both the arthritic and psoriatic aspects of this disease.

In this study, a flat dose of 25 mg of TNFR:Fc was injected subcutaneously two times a week. After 12 weeks, patients who completed the study were eligible for continuation into a 24 week open-label extension of the study, with assessments made at weeks 16, 36 and 30 days post-study. All patients participating in the study extension received etanercept, including those patients who had received placebo during the blinded portion of the study.

In order to qualify for enrollment, subjects had to have at least one of the following forms of PsA: 1) DIP involvement; 2) polyarticular arthritis, absence of rheumatoid nodules and presence of psoriasis; 3) arthritis mutilans; 4) asymmetric peripheral arthritis; or 5) ankylosing spondylitis-like PsA. Subjects furthermore had to exhibit three or more swollen joints and three or more tender or painful joints at the time of enrollment, and to have exhibited an inadequate response to NSAID therapy. Subjects who were on other medications, including methotrexate, NSAIDs or oral corticosteroids were permitted to continue these other treatments at the same dose so long as the investigator considered these other treatments to inadequately control the patient's disease. Methotrexate was concurrently taken by 47% of the etanercept group, and 47% of the placebo group, NSAIDs were concurrently taken by 67% of the etanercept and 77% of the placebos and oral corticosteroids by 40% of the etanercept and 20% of the placebo patients. Pain medications, including acetaminophen, codeine, propoxyphene napsylate, oxycodone hydrochloride, hydrocodone bitartrate and tramadol, also were permitted during the study, as well as the use of topical tar compounds.

To qualify as having PsA, patients had to have experienced at least one psoriatic lesion of the skin or nails. Patients were evaluated at baseline (day 1 of treatment) as follows: 1) complete joint assessment; 2) psoriasis assessment; 3) duration of morning stiffness; 4) health assessment (quality of life) questionnaire, visual analog scale (HAQ/VAS); 5) patient global

assessment; 6) erythrocyte sedimentation rate (ESR, Westergren); 7) C-reactive protein (CRP); and 8) urinalysis. At weeks 4 and 8, patients were evaluated as follows: 1) complete joint assessment; 2) psoriasis assessment; 3) duration of morning stiffness; 4) HAQ/VAS; 5) patient global assessment. At the end of 12 weeks, subjects were evaluated as follows: 1) complete joint assessment; 2) psoriasis assessment; 3) focused physical exam; 4) duration of morning stiffness; 5) HAQ/VAS; 6) patient global assessment; 6) hematology profile; 7) chemistry profile; 8) ESR; 9) CRP; 10) urinalysis; 11) serum tested for antibody to TNFR:Fc. Only those patients whose psoriasis was stable and covered ≥3% of body area were evaluated for psoriasis response during this trial, although patients whose psoriasis was inactive or covered less area were permitted to enroll.

A primary endpoint for clinical improvement or worsening of PsA was the Psoriatic Arthritis Response score, which is a composite score based on the following four measures: 1) patient self-assessment; 2) physician assessment; 3) joint pain or tenderness; 4) joint swelling. Both self- and physician assessments, i.e., overall assessment of disease status, were measured according to a five point Likert scale, in which a patient was considered as "improved" if his or her score decreased by one category, or as "worse" if his or her score increased by one category. Joint pain or tenderness was measured on a 5-point scale, wherein 1=none and 5=severe (withdrawal on examination). Joint swelling was evaluated on a 4-point scale in which 1=none; 2=mild (detectable synovial thickening without loss of bony contour); 3=moderate (loss of distinctness of bony contours); and 4=severe (bulging synovial proliferation with cystic characteristics). For this last measure, a decrease in swelling of ≥30% was scored as an "improvement," and an increase in swelling of 30% was scored as a "worsening." Patients were classified as "improved" under the Psoriatic Arthritis Response scoring system if they exhibited an improvement in at least two of the four measures described above, provided that one of the improved areas was joint pain or joint tenderness, and where there was no worsening in any of the four measures.

In addition, a secondary endpoint used for assessing psoriatic arthritis was a modified version of the American College of Rheumatology Preliminary Definition of Improvement in Rheumatoid Arthritis (modified ACR 20 response) (Felson et al., 1995). To qualify as "improved" according to this measure, a patient must have exhibited ≥20% improvement in both tender joint count (78 joints assessed) and swollen joint count (76 joints assessed), and also must have shown an improvement in three of the following five: 1) subject pain assessment; 2) subject global assessment; 3) physician global assessment; 4) subject self-assessed disability; 5) acute-phase reactant (Westergreen erythrocyte sedimentation rate or C-reactive protein level). The joint count was done by scoring several different aspects of tenderness. such as pressure and joint manipulation on physical examination, wherein each joint was scored as "tender" or "nontender." Similarly, each joint is scored after physical examination as "swollen" or "not swollen." The subject's pain assessment was based on a horizontal visual analog scale (usually 10 cm) or Likert scale. The subject's and physician's global assessments of the subject's current disease status was based on an anchored horizontal visual analog scale (usually 10 cm), or Likert scale response. The subject's self-assessment of disability was based on any of the following measures, all of which have been validated in RA trials: Arthritis Impact Measurement Scale (AIMS); Health Assessment Questionnaire; the Quality (or Index) of Well Being Scale; the McMaster Health Inventory Questionnaire (MHIQ); and the McMaster-Toronto Arthritis patient preference questionnaire (MACTAR).

A primary endpoint used to assess the psoriatic aspects of PsA was the standard psoriasis area and severity index (PASI) (Fredriksson and Petersson, *Dermatologica* 157:238-244, 1978). For this study, a positive treatment response was defined as an at least 50% or an at least 75% improvement in a patient's PASI score. For assessing area and severity, the body is divided into four regions: head (10%); trunk (30%); upper extremities (20%); and lower extremities (40%). Each quadrant also was scored for the severity of erythema (E), infiltration (I) and desquamation (D), using a four point scale, in which 0=no symptoms present; 1=slight symptoms; 2=moderate symptoms; 3=striking symptoms; 4=exceptionally striking symptoms. Using a 6-point scale, each region was scored also for the percent of total area that was involved in the psoriatic manifestations of the disease, wherein 0=no involvement; 1=<10% involvement; 2=10-<30% involvement; 3=30-<50% involvement; 4=50-<70% involvement; 5=70-<90% involvement; 6=90-100% involvement. PASI scores were calculated according to the formula given below, in which E=severity score for erythrema, I=severity score for infiltration, D=severity score for desquamation and A=total area involved. In this formula, the letters "h," "t," "u" and "l" represent, respectively, the scores in each of the four body regions, i.e., head, trunk, upper extremities and lower extremities. The PASI score varies in steps of 0.1 units from 0.0 (no psoriatic lesions at all) to 72.0 (complete erythroderma of the severest possible degree).

$$PASI=0.1(Eh+Ih+Dh)Ah+0.3(Et+It+Dt)At+0.2(Eu+Iu+Du)Au+0.4(El+Il+Dl)Al$$

A secondary endpoint used for the psoriatic aspect of psoriatic arthritis was the Target Lesion Assessment Score. This score was determined for a single target lesion that was selected to be monitored throughout the trial. This measurement is a composite of four different evaluations: 1) plaque evaluation; 2) scaling; 3) erythrema; and 4) target lesion response to treatment. The following scale was used for the plaque elevation: 0=none (no evidence of plaque above normal skin level); 1=mild (slight but definite elevation above normal skin level); 2=moderate (moderate elevation with rounded or sloped edges to plaque); 3=severe (hard, marked elevation with sharp edges to plaque); 4=very severe (very marked elevation with very hard sharp edges to plaque). For the scaling assessment: 0=none (no scaling on the lesion); 1=mild (mainly fine scales, with some of the lesion at least partially covered); 2=moderate (somewhat coarser scales, most of the lesion at least partially covered); 3=severe (coarse, thick scales, virtually all the lesion covered, rough surface); 4=very severe (very coarse thick scales, all the lesions covered, very rough surface). For the erythema evaluation: 0=none (no erythema); 1=mild (light red coloration); 2=moderate (red coloration); 3=severe (very red coloration); 4=very severe (extreme red coloration). For target lesion response to treatment score: 0=completely cleared; 1=almost cleared (-90% improvement); 2=marked response (-75% improvement); 3=moderate response (-50% improvement); 4=slight response (-25% improvement); 5=condition unchanged; 6=condition worsened. The patient's Target Lesion Assessment Score was determined by summing the plaque, scaling, erythema and target lesion response scores for the monitored lesion. If the monitored lesion worsened, the percentage change from baseline was recorded as a negative number.

Treatment and placebo groups were compared in accord with the measurements described above, as well as for demo-

graphic and background characteristics; premature discontinuation rate; pain medication requirements; toxicities; serious adverse events; side effects reported by patients; number of weeks on drug until subjects met criteria for improvement, and response according to PsA subtype. Results were analyzed using standard statistical methods.

Dosing Regimen

Recombinant human TNFR:Fc (etanercept) from Immunex Corporation was used in this study. The gene fragments encoding the etanercept polypeptides were expressed in a Chinese hamster ovary (CHO) expression vector.

TNFR:Fc was supplied as a sterile lyophilized powder containing 10 mg or 25 mg TNFR:Fc; 40 mg mannitol, USP; 10 mg sucrose, NF; and 1.2 mg tromethamine (TRIS), USP per vial. Patients received either a dose of 25 mg of etanercept or a placebo. Vials of etanercept or identically-appearing placebo were reconstituted by aseptic injection of 1.0 mL Bacteriostatic Water for Injection, USP, (containing 0.9% benzyl alcohol), and was not filtered during preparation or prior to administration. If storage was required, the reconstituted solutions were stored at 2-8° C. (36-46° F.) in the original vial or in a plastic syringe for a period of no longer than 28 days. Dose was not changed during the study. Study drug was given twice weekly at approximately the same time of day.

Results

Study drug was well tolerated in all patients, and adverse events were consistent with this population and were equally distributed among both treatment groups. As illustrated in Tables 1-4, etanercept induced a significant improvement as compared with the placebo group in Psoriatic Arthritis Response (Table 1), ACR20 (Table 2), ACR50 (Table 3), PASI score, 50% improvement (Table 4), PASI score, 75% improvement (Table 5) and improvement in Target Lesion Assessment Score (Table 6). The fractions shown in Tables 1-5 represent numbers of patients. For example, the first entry in Table 1, which is "4/30," indicates that 4 of 30 patients in the placebo group scored as "improved" according to the Psoriatic Arthritis Response measurements. The tables include P-values for the differences between the two study groups, the groups being labeled as "PLACEBO" and "TNFR:Fc." All of the tables include data calculated after the first four weeks of the open label extension portion of the study ("EXTENSION"), during which all of the patients in both study groups received etanercept.

Table 1 shows the number of patients in each treatment group who scored as "improved" according to the Psoriatic Arthritis Response scoring system described above. By four weeks, there was a highly significant difference between etanercept and placebo groups. Moreover, after being switched to etanercept during the extension, those patients who had received placebo during the blinded portion of the study were seen to exhibit an improvement over baseline (Table 1, Placebo, EXTENSION). These results indicate that etanercept acts rapidly to alleviate many aspects of psoriatic arthritis.

TABLE 1

Psoriatic Arthritis Response

	Placebo	TNFR:Fc	P-value
4 weeks	4/30 (13%)	23/30 (77%)	0.000
8 weeks	7/30 (23%)	25/30 (83%)	0.000
12 weeks	6/30 (20%)	26/30 (87%)	0.000
EXTENSION	17/23 (74%)	21/25 (84%)	0.356

Tables 2 and 3, respectively, illustrate the study results for the ACR20 and ACR50 endpoints. For either measure, a significant difference between etanercept and placebo groups was observed at all three time points during the blinded portion of the study. Given the differences between test and placebo groups after only four weeks of treatment (P=0.000 for ACR20 and P=0.011 for ACR50), these data suggest that notable improvement in ACR scores occurred within the etanercept group very soon after treatment was initiated, possibly after a single dose of etanercept. During the 4 week extension period, during which all of the patients received etanercept, a striking improvement in both ACR20 and ACR50 was seen in those patients who had received placebo during the first 12 weeks (Tables 2 and 3).

TABLE 2

ACR20 Response

	Placebo	TNFR:Fc	P-value
4 weeks	1/30 (3%)	18/30 (60%)	0.000
8 weeks	3/30 (10%)	19/30 (63%)	0.000
12 weeks	4/30 (13%)	22/30 (73%)	0.000
EXTENSION	11/23 (48%)	18/25 (72%)	0.093

TABLE 3

ACR50 Response

	Placebo	TNFR:Fc	P-value
4 weeks	0/30 (0%)	6/30 (20%)	0.011
8 weeks	1/30 (3%)	11/30 (37%)	0.001
12 weeks	1/30 (3%)	15/30 (50%)	0.000
EXTENSION	7/23 (30%)	11/25 (44%)	0.316

The results of the psoriasis evaluations are presented in Tables 4-6. Tables 4 and 5, respectively, present the numbers and percentages of patients in each group who exhibited a 50% or 75% improvement in PASI score, while Table 6 presents Target Lesion Assessment scores, these latter being denoted as percent improvement over baseline. The data in Tables 4-6 clearly indicate that etanercept induced an improvement in psoriasis for a large percentage of the patients who received it. When single lesions were evaluated (Table 6), the improvement in psoriasis was even more apparent than when PASI scores were used (Tables 4 and 5). It is notable also that, for either PASI scores (Tables 4 and 5) or Psoriasis Target Lesion Assessment Score (Table 6), the scores of the placebo group improved after these patients were switched to etanercept during the extension.

Though not shown in Table 6, Target Lesion Assessment Scores for patients who were concurrently receiving methotrexate (14 of the 30 patients in the etanercept group, and 14 patients in the placebo group) were compared with the scores of those patients who did not take methotrexate. Little difference in this index was noted between the patients who received methotrexate and those who did not receive it.

TABLE 4

PASI Score-50% Improvement

	Placebo	TNFR:Fc	P-value
4 weeks	0/19 (0%)	4/19 (21%)	0.037
8 weeks	1/19 (5%)	7/19 (37%)	0.019
12 weeks	4/19 (21%)	8/19 (42%)	0.165
EXTENSION	6/16 (38%)	6/15 (40%)	0.856

TABLE 5

PASI Response Rate 75% Improvement

	Placebo	TNFR:Fc	P-value
4 weeks	0/19 (0%)	1/19 (5%)	0.264
8 weeks	0/19 (0%)	2/19 (11%)	0.153
12 weeks	0/19 (0%)	4/19 (21%)	0.037
EXTENSION	1/16 (6%)	4/15 (27%)	0.113

TABLE 6

Psoriasis Target Lesion Assessment
(Percent Improvement or Worsening Compared with Baseline)

		Placebo	TNFR:Fc	P-value
4 weeks	Mean (SD)	2.7 (27.6)	21.2 (35.2)	0.120
	Median	0.0	14.3	
	MIN-MAX	−50.0 −50.0	−33.3 −100.0	
	N	19	19	
8 weeks	Mean (SD)	−7.5 (25.3)	28.5 (34.1)	0.003
	Median	0.0	29.2	
	MIN-MAX	−50.0 −20.0	−33.3 −100.0	
	N	17	18	
12 weeks	Mean (SD)	9.5 (23.2)	45.7 (31.6)	0.001
	Median	0.0	50.0	
	MIN-MAX	−25.0 −50.0	−16.7 −100.0	
	N	16	19	
EXTENSION	Mean (SD)	28.9 (41.2)	47.1 (35.8)	0.263
	Median	36.7	50.0	
	MIN-MAX	−100.0 −66.7	−33.3 −100.0	
	N	16	15	

What is claimed is:

1. A method of treatment comprising administering a dose of TNFR:Fc to a patient having psoriatic arthritis and/or plaque psoriasis,

wherein the dose is administered one time or two times per week, and

wherein the dose administered is 25-50 mg or 50-100 mg, and

wherein the dose is administered by subcutaneous injection.

2. The method of claim **1**, wherein the dose is administered once per week and is 50-100 mg.

3. The method of claim **1**, wherein the dose is administered twice per week.

4. The method of claim **3**, wherein the dose administered is 25-50 mg.

5. The method of claim **3**, wherein the dose administered is 50-100 mg.

6. The method of claim **1**, wherein the patient has plaque psoriasis.

7. The method of claim **1**, wherein the patient has psoriatic arthritis.

8. A method for treating plaque psoriasis comprising administering TNFR:Fc by subcutaneous injection to a patient having plaque psoriasis once or twice a week at a dose of 25-50 mg or a dose of 50-100 mg.

9. The method of claim **8**, comprising administering the TNFR:Fc once a week at a dose of 50-100 mg.

10. The method of claim **9**, comprising administering the TNFR:Fc once a week at a dose of 50 mg.

11. The method of claim **8**, comprising administering the TNFR:Fc twice a week at a dose of 50-100 mg.

12. The method of claim **11**, comprising administering the TNFR:Fc twice a week at a dose of 50 mg.

13. The method of claim **8**, comprising administering the TNFR:Fc twice a week at a dose of 25-50 mg.

14. The method of claim **13**, comprising administering the TNFR:Fc twice a week at a dose of about 25 mg.

15. The method of claim **8**, comprising administering the TNFR:Fc twice a week at a dose of 50-100 mg for at least 3 weeks and then administering the TNFR:Fc once a week at a dose of 50-100 mg or twice a week at a dose of 25-50 mg.

16. The method of claim **8**, wherein a steroid, vitamin D3 or an analog thereof, cyclosporine, a retinoid, acitretin, ultraviolet light B phototherapy, psoralen plus ultraviolet A (PUVA) phototherapy, fumaric acid, or methotrexate is administered concurrently with the TNFR:Fc.

17. A method for treating psoriatic arthritis comprising administering TNFR:Fc by subcutaneous injection to a patient having psoriatic arthritis, wherein the TNFR:Fc is administered once a week at a dose of 50-100 mg or wherein the TNFR:Fc is administered twice a week at a dose of 25-50 mg.

18. The method of claim **17**, wherein the TNFR:Fc is administered once a week at a dose of 50-100 mg.

19. The method of claim **18**, wherein the TNFR:Fc is administered once a week at a dose of 50 mg.

20. The method of claim **17**, wherein the TNFR:Fc is administered twice a week at a dose of 25-50 mg.

21. The method of claim **20**, wherein the TNFR:Fc is administered twice a week at a dose of 25 mg.

22. The method of claim **17**, wherein methotrexate, cyclosporine, leflunomide, or a nonsteroidal anti-inflammatory drug (NSAID) is administered concurrently with the TNFR:Fc.

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