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(54) Title: SYNTHETIC IL-10 ANALOGUES (57) Abstract The invention relates to use of a substance or polypeptide according to the formula: X ₁ -X ₂ -X ₃ -Thr-X ₄ -Lys-X ₅ -Arg-X ₆ (SEQ ID NO:22), wherein X ₁ is Ala or Gly, X ₂ is Tyr or Phe, X ₃ , X ₄ and X ₅ are independently selected from the group consisting of Met, Ile, Leu and Val; and X ₆ is selected from the group consisting of Asp, Gln and Glu, optionally at least one of X ₁ , X ₂ , X ₃ , X ₄ , X ₅ and X ₆ is independently substituted with non-natural or unusual amino acids and/or the peptide is cyclized and/or the peptide is stabilized and/or the amino terminal amino acid residue is acylated and/or the carboxy terminal amino acid residue is amidated, and peptidomimetics modelled on the basis of the above formula for the preparation of a pharmaceutical composition for the reduction of TNF α production. In particular, the invention relates to analogues of peptides of the above formula wherein at least one of X ₁ , X ₂ , X ₃ , X ₄ , X ₅ and X ₆ is independently substituted with non-natural or unusual amino acids and/or the peptide is cyclized and/or the peptide is stabilized and/or the amino terminal amino acid residue is acylated and/or the carboxy terminal amino acid residue is amidated, and peptidomimetics modelled on the basis of the above formula.		

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SYNTHETIC IL-10 ANALOGUES

FIELD OF INVENTION

The present invention relates to the pharmaceutical use of a substance which is an interleukin 10 (IL-10) agonist, in particular the use of a substance of the invention for the manufacture of a pharmaceutical composition for prevention and/or treatment of diseases, the pathogenesis of which is related to the decreased production and/or function of immunoinhibitory mediators, especially cytokines, and/or is related to an increased production and/or function of certain immuno-inflammatory mediators, especially cytokines. In particular, the invention relates to the use of a substance of the invention for the manufacture of a pharmaceutical composition for the reduction of TNF α -production, for prevention and/or treatment of auto-immune diseases (diabetes mellitus, type I; inflammatory diseases of the gastro-intestinal tract; rheumatoid arthritis), arthritis urica (gout), allergy of the skin; allergic reactions in the skin, lungs and respiratory tract (including asthma bronchiale); tissue damage as a result of hypoxia/ischemia (infarction; reperfusion); atherosclerosis; psoriasis; granulomatous disease; chronic myeloid leukaemia; acute myeloid leukaemia; cancer; graft vs. host reaction and conditions related to transplant rejection; fibrosis of the lung; fibrosis of the liver; chronic non-infectious inflammation of the lung; glomerulonephritis; pre-term labour; periodontitis; inflammatory reactions due to virus infections, osteoporosis, septic shock and/or for the manufacture of an anti-conceptive agent.

BACKGROUND OF THE INVENTION

Research from the last two decades has shown that the initiation, regulation and ending of inflammatory reactions as well as the regulation of growth and differentiation within the mammalian organisms is under tight control by a special group of signal polypeptides generally called cytokines. Cytokines are polypeptides which can be produced by most nu-

cleated cells and which transmit regulatory signals between cells, thus forming a communication network between identical or different cell types of the organism. The cytokines are extremely potent mediators and active at concentrations down to 10^{-15} M. Cytokines are also key factors for the development of cellular immune reactions, which in turn form the basis for the clinical manifestations of inflammation due to infection, allergy, trauma, graft vs. host reactions and auto-immune diseases. The allergic and auto-immune diseases are explained by abnormalities in the immune system, especially in the T lymphocyte-mediated immunity, but generally these diseases are of unknown etiology. In vitro studies, animal experiments and clinical experimental studies have shown that cytokines play important pathophysiological roles for the inflammatory reactions related to auto-immune diseases, allergy, ischemia, reperfusion injury, trauma, infections, and are important for the development of cancer, atherosclerosis, pregnancy and fetal development, bone homeostasis. Cytokines may be involved in other immunoinflammatory and proliferative diseases as will be described in further detail in the following.

The said diseases are usually chronic and the treatment is palliative, i.e. most of the drugs prescribed in connection with the said diseases are directed at the allaying of symptoms and usually have no curative effect. Other treatments are so-called substitution therapies which involve life-long supplying to the patient of substances, e.g. hormones, needed due to a reduced/insufficient internal production of said substance. Said treatments are often unsatisfactory, imply unwanted and often serious side-effects, and merely delay rather than prevent the progression of the disease. Thus, improved methods of treatments and improved pharmaceutical compositions are highly needed.

Interleukin-10 (IL-10) is a recently described natural endogenous immunosuppressive cytokine, identified in both the murine and human organism. Murine interleukin 10 (mIL-10) was

originally described as a cytokine synthesis inhibitory factor released from TH₂ helper T-cell clones, but it also carries proliferative effects upon various subsets of lymphocytes, including an enhancing effect upon cloning efficacy of CD4⁻, 8⁺ murine splenic T cells (4). Human interleukin 10 (hIL-10) has recently been sequenced and revealed to have high homology with mIL-10 at DNA sequence level as well as on amino acid level. Furthermore, swine interleukin 10 has recently been sequenced and revealed to have high homology with human IL-10 at DNA sequence level as well as on amino acid level (88), see also Figure 2. Also, hIL-10 has high homology with an open reading frame in the Epstein-Barr virus genome, BCRF1, and viral IL-10 does show some activity similar to hIL-10, cf. Fig. 1 (5).

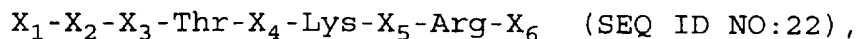
Human IL-10 is produced by activated T cell clones and immortalized B cells, and in addition to its cytokine synthesis inhibitory factor (CSIF) activity, inhibiting the production of several pro-inflammatory cytokines and colony-stimulating factors, it also induces the production of a natural interleukin-1 receptor antagonist protein/peptide (IRAP) by mononuclear cells, thereby indirectly inhibiting IL-1 activity. IL-10 also downregulates its own production by monocytes and inhibits the expression of class II MHC expression (12). Further, hIL-10 reduces antigen-specific proliferation of human T cells and CD4⁺ T cell clones, when using monocytes as antigen-presenting cells. In vivo experiments in mice indicate that the outcome of Leishmania infection is dependent upon the cytokine profile from responding CD4⁺ T lymphocytes (13). In C57BL/6 mice resistant to Leishmania infection, CD4⁺ T cells from draining lymph nodes show up-regulation of IFN- γ and IL-2 cytokines, whereas the sensitive BALB/c mice in their draining lymph nodes have CD4⁺ responding T cells releasing IL-4 and IL-10, which could be demonstrated to correlate with disease progression (13). Thus, IL-10 may exert potent regulatory effects on immunological responses both *in vitro* and *in vivo*. Further IL-10 strongly affects chemokine biology since human interleukin 10 is a specific chemotactic

factor towards CD8+ T cells, while IL-10 suppresses the ability of CD4+, but not CD8+, T cells to migrate in response to the T cell chemotactic cytokine, IL-8 (14). IL-10 also inhibits the chemotactic effect of other chemokines MCP-1/MCAF and RANTES (75). Since IL-10 is a deactivator of monocyte/macrophage functions and an inhibitor of Th1 activity, drugs with full or partial IL-10-like activity may possess therapeutic effect in diseases characterized by imbalance in cytokine production and/or activities.

It has previously been proposed to prepare pharmaceutical compositions comprising hIL-10 or vIL-10, and the use of hIL-10 or vIL-10 for the manufacture of a pharmaceutical composition for the treatment of various conditions such as septic or toxic shock, rheumatoid arthritis, graft-vs-host disease, tissue rejection, diabetes mellitus, autoimmune disorders, leukaemia and cancer has been disclosed in e.g. WO93/02693 and WO94/04180.

SUMMARY OF THE INVENTION

The present invention relates to the use of a substance or polypeptide according to the formula



wherein

X_1 is Ala or Gly,

X_2 is Tyr or Phe,

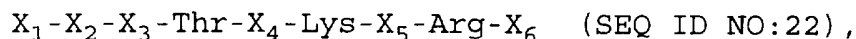
X_3 , X_4 and X_5 are independently selected from the group consisting of Met, Ile, Leu and Val; and

X_6 is selected from the group consisting of Asp, Gln and Glu,

optionally at least one of X_1 , X_2 , X_3 , X_4 , X_5 and X_6 is independently substituted with non-natural or unusual amino acids and/or the peptide is cyclized and/or the peptide is stabilized and/or the amino terminal amino acid residue is acylated

acylated and/or the carboxy terminal amino acid residue is amidated, and peptidomimetics modelled on the basis of the above formula for the preparation of a pharmaceutical composition for the reduction of TNF α production.

- 5 Further, the invention relates to analogues of the peptides having the formula



wherein

X_1 is Ala or Gly,

10 X_2 is Tyr or Phe,

X_3 , X_4 and X_5 are independently selected from the group consisting of Met, Ile, Leu and Val; and

X_6 is selected from the group consisting of Asp, Gln and Glu

15 wherein at least one of X_1 , X_2 , X_3 , X_4 , X_5 and X_6 is independently substituted with non-natural or unusual amino acids and/or the peptide is cyclized and/or the peptide is stabilized and/or the amino terminal amino acid residue is acylated and/or the carboxy terminal amino acid residue is amidated, and peptidomimetics modelled on the basis of the above formula, said analogues having at least one of the following properties:

- a) induces inhibition of spontaneous IL-8 production by human monocytes,
- b) induces inhibition of IL-1 β induced IL-8 production by 25 human peripheral blood mononuclear cells (PBMC),
- c) induces production of interleukin-1 receptor antagonist protein (IRAP) by human monocytes,
- d) induces chemotactic migration of CD8+ human T lymphocytes *in vitro*,
- 30 e) desensitizes human CD8+ T cells resulting in an unresponsiveness towards rhIL-10,

- f) suppresses the chemotactic response of CD4+ T human lymphocytes towards IL-8,
- g) suppresses the chemotactic response of human monocytes towards MCAF/MCP-1,
- 5 h) does not inhibit class II MHC molecule expression on human monocytes, in contrast to human IL-10.
- i) induces the production of IL-4 by cultured normal human CD4+ T cells,
- j) reduces the TNF α production in human mixed leukocyte
10 reaction.

It is contemplated (as described in detail in the following description of immunological mechanisms) that the action mechanism is via interference with the action of mediators of the immune system, in particular cytokines such as monokines,
15 lymphokines, chemokines and monokine-receptor antagonists, i.e. that the substance of the invention interferes with/- suppresses the production and/or action of certain cytokines and thus inhibits pathological processes leading to tissue damage, and that the substance of the invention induces the
20 production of natural monokine-receptor antagonists thus interfering with/suppressing the action of certain cytokines such as TNF α and thereby inhibiting pathological processes which lead to tissue damage.

An important embodiment of the present invention thus relates
25 to a pharmaceutical composition comprising, as the active ingredient, a substance of the invention.

In a further aspect, the present invention relates to the use of a substance of the invention for the manufacture of a pharmaceutical composition for substantially inhibiting a
30 biological effect related to a cytokine, i.e. the use of a substance of the invention as an IL-1 receptor antagonist protein/peptide, lymphokine, monokine, interleukin, interferon, chemokine or colony-stimulating factor. Another aspect relates to the use of a substance of the invention for the
35 manufacture of a pharmaceutical composition for the prophy-

laxis or treatment of a condition related to the disturbance of a cytokine system, i.e. the IL-1 receptor antagonist protein/peptide, lymphokine, monokine, interleukin, interferon, chemokine or colony-stimulating factor system. In another aspect, the invention also relates to a method of treating a condition in a human related to a disturbance in a cytokine system which method comprises administering to the subject an effective amount of a substance of the invention.

The cellular immune system takes part in the development of such disorders as infectious, inflammatory and neoplastic diseases. Immunocompetent cells and their products may play important roles in the initiation, progression and possible chronic nature of development of inflammatory conditions. These disorders are often without known etiology and includes common diseases such as diabetes mellitus, rheumatoid arthritis, inflammatory diseases of the gastro-intestinal tract and of the skin. Apart from these examples, cell-mediated immunity or pro-inflammatory mediators, however, contribute to many other inflammatory and proliferative diseases (see Table 2).

20

TABLE 2

Some diseases where macrophages/T-lymphocyte-mediated immune reactions are considered pathogenetically important

Skin diseases:

25

Psoriasis

Atopic dermatitis

Contact dermatitis

Cutaneous T cell lymphoma (CTCL)

Sezary syndrome

30

Pemphigus vulgaris

Bullous pemphigoid

Erythema nodosum

Scleroderma

Auto-immune (including rheumatic) diseases:

Uveitis

Bechet's disease

Sarcoidosis Boeck

5 Sjögren's syndrome

Rheumatoid arthritis

Juvenile arthritis

Reiter's syndrome

Gout

10 Osteoarthritis

Systemic lupus erythematosus

Polymyositis

Myocarditis

Primary biliary cirrhosis

15 Crohn's disease

Ulcerative colitis

Multiple sclerosis and other demyelinating diseases

Aplastic anaemia

Idiopathic thrombocytopenic purpura

20 Multiple myeloma and B cell lymphoma

Simmons' panhypopituitarism

Graves' disease and Graves' ophthalmopathy

Subacute thyreoditis and Hashimoto's disease

Addison's disease

25 Insulin-dependent diabetes mellitus (type 1)

Other diseases

Various clinical syndromes with vasculitis (e.g. polyarteritis nodosa, Wegener's granulomatosis, Giant cell arteritis
Fever, malaise

30 Anorexia (e.g. in acute and chronic inflammatory and infectious diseases)

Disseminated intravascular coagulation (DIC)

Arteriosclerosis (atherosclerosis)

Shock (e.g. in gram-negative sepsis)

35 Cachexia (e.g. in cancer, chronic infectious and chronic inflammatory diseases)

Transplant rejection and graft vs. host disease

Cytokines:

T-lymphocytes orchestrate the induction and regulation of cell-mediated immune reactions, and cytokine products (lymphokines) of the T cells initiate and control the immune response (1,2). The lymphocyte-activating mediators (lymphokines) produced by antigen-presenting cells belong to a group of polypeptides called cytokines. Cytokines are transmitters of cell-to-cell communication in both physiological and pathophysiological conditions, and may also function as hormones providing signals between the immune system and other tissues and organs. Cytokines may also be produced by cells outside the immune system and it is generally believed that all nucleated cells are capable of producing one or several cytokines. Thus, e.g. keratinocytes and fibroblasts are potent producers of cytokines and in this system cytokines may function as autocrine or paracrine hormones independent of the immune system (3).

Interleukin-10:

Murine interleukin 10 (mIL-10) was originally described as a cytokine synthesis inhibitory factor (CSIF) released from TH₂ helper T-cell clones but it has also proliferative effects upon various subsets of lymphocytes, including an enhancing effect upon cloning efficacy of CD4⁺, 8⁺ murine splenic T cells (4). Human interleukin 10 (hIL-10) has recently been described (5) and has high homology with an open reading frame in the Epstein-Barr virus genome, BCRF1, and viral IL-10 does show some activity similar to hIL-10. In the following, the biochemical, biological, physiological and possible pathophysiological roles of IL-10 will be summarized.

IL-10 structure:

The primary structures of mouse (mIL-10) and human IL-10 (hIL-10) revealed a high degree of nucleotide sequence homology (>80%) throughout their entire length (4, 5). The only

significant difference is the insertion of a human Alu repetitive sequence element in the 3'-untranslated region of the hIL-10 cDNA clone. The mIL-10 and hIL-10 cDNAs encode very similar open reading frames (ORF) of 178 amino acids, including hydrophobic leader sequences and corresponding to 73% amino acid homology. mIL-10, which is active on murine cells, does not cross-react significantly on human cells. hIL-10 is an 18 kDa polypeptide which lacks detectable carbohydrate, but mIL-10 is N-glycosylated at a site near its N-terminus which is missing from hIL-10. Both mIL-10 and recombinant hIL-10 (rhIL-10) are expressed as non-covalent homodimers. The extent to which mIL-10 or hIL-10 monomers are biologically active is not yet certain. mIL-10 and hIL-10 with polypeptide "tags" of at least 8 amino acids at the N-terminus and 21 amino acids at the C-terminus showed no detectable loss of activity according to a publication from Moore et al. who were the first to sequence human IL-10 (6). Tagging C and N terminal ends of the whole IL-10 does not necessarily cause functional changes, since tagging is occasional or incidental. The high number of possible amino acid substitutions as described in further detail in the following might also explain while tagging failed to show missing functions. Recombinant mIL-10 and hIL-10 have been expressed in: CDS7 cells, mouse myeloma cells, chinese hamster ovary cells, a baculovirus expression system, and *E. coli*. The biological activities of these rIL-10 proteins are so far indistinguishable (6).

The mIL-10 gene comprises five exons arrayed over approximately 5.1 kb of DNA. The genomic clone itself encodes an expressible mIL-10 protein. The mIL-10 and hIL-10 genes are on mouse and human chromosome 1 (6, 7).

mIL-10 and hIL-10 exhibit strong DNA and amino acid sequence homology to an open reading frame in the Epstein-Barr virus genome, BCRF1, and the homology is confined to the mature protein coding sequence and is not detected in the signal sequence or 5'- and 3'-untranslated sequences (5). Of the

three sequences, mIL-10 and hIL-10 are the more closely related pair at the DNA sequence level (81%), while the DNA sequences encoding the mature hIL-10 and BCRF1 proteins have 71% homology. The homology between hIL-10 and B CRF1 is 84% on the amino acid level. It is hypothesized that the mIL-10 and hIL-10 genes are evolved from a common ancestor, while BCRF1 represents an ancestral processed, captured cellular cytokine gene and that the BCRF1 protein has been constrained to resemble hIL-10. BCRF1 is expressed during the lytic cycle of the EBV. The BCRF1 ORF encodes a 17 kDa secreted polypeptide which, like hIL-10, contains little or no glycosylation. BCRF1 displays some activities of IL-10 and has been called viral IL-10 (vIL-10), although its activity has been found to be 10% of hIL-10.

IL-10 activity on cytokine production:

hIL-10 inhibits the production of a number of cytokines including interferon- γ (IFN- γ), Tumor Necrosis Factor- α (TNF- α), Granulocyte Macrophage Colony Stimulating Factor (GM-CSF), Granulocyte-CSF (G-CSF), IL-1 α , IL-1 β , IL-2, IL-6, IL-8 and Monocyte Chemotactic polypeptide-1 (MCP-1/MCAF) by monocytes/macrophages and/or T lymphocytes (4, 5). IL-10 also inhibits the ability of monocytes to migrate as a response to the chemokine MCP-1/MCAF (75). Further, hIL-10 induces the production of an endogenous, natural interleukin-1 receptor antagonist (IRAP) (6), which inhibits IL-1 α and IL-1 β by competing with receptor binding. Since IL-8 is strongly inducible by IL-1 α and by IL-1 β , IL-10 exerts part of its inhibitory effect on IL-8 production by stimulating the production of the IL-1-receptor antagonist IRAP. This last mechanism is of considerable importance for the present invention as described and exemplified in the following. IRAP has anti-inflammatory activities (9), and its therapeutic effect in rheumatoid arthritis has been suggested (10). Also, IRAP proved to be effective in the treatment of sepsis syndrome and a dose-dependent, 28-day survival benefit was associated with IRAP treatment ($p = 0.015$) in a study by Fisher

et al. (11). IRAP may exert parts of its anti-inflammatory effects by inhibiting chemokine-production such as the production of IL-8.

IL-10 and antigen expression:

- 5 IL-10 inhibits the expression of class II MHC expression on human monocytes (8). Constitutive and IL-4 or IFN- γ induced expression of HLA-DR/DP and DQ was inhibited by hIL-10 (12). In addition, monocytes pre-incubated with IL-10 are refractory to subsequent induction of class II MHC expression by
- 10 IL-4 or IFN- γ . IL-10 inhibits class II expression by human monocytes following activation by LPS (12, 76). BALB/c mice given 1 to 10 mg of IL-10 concomitantly with a lethal dose of LPS were protected from death (6).

- IL-10 inhibits nitrogen intermediates and superoxide anions.
- 15 IL-10 also inhibits reactive nitrogen intermediate (NO) as well as reactive oxygen intermediates (H₂O₂) by macrophages following activation by IFN- γ (13).

IL-10 and T cell activity:

- 20 IL-10 has also modulatory effects on T cell functions/activity. Thus, hIL-10 is a potent chemotactic factor to CD8+ T lymphocytes, while hIL-10 does not show chemotaxis towards CD4+ T cells (14). Additionally, IL-10 suppresses the capacity of CD4+ T cells to respond to the chemotactic signals of
- 25 the β -chemokine RANTES as well as the α -chemokine IL-8. hIL-10 also directly inhibits the proliferation of human peripheral blood T cells and CD4+ T cell clones (14).

IL-10 and B lymphocytes:

hIL-10 co-stimulates B-lymphocyte proliferation induced by cross-linking surface Ig with immobilized anti-IgM antibody, and this effect is enhanced when B-cells are stimulated by cross-linking of their CD40 antigen with anti-CD40 antibody and mouse L cells expressing human Fc γ RII/CD32 (15). The effect of IL-10 on proliferation and differentiation of activated human B cells suggests that this cytokine may account for much of the superior ability of T cells expressing hIL-10 to provide help for B cell responses.

IL-10 as a homeostatic factor for the immune system:

The physiological consequences of the functions of IL-10, mentioned above, are believed to be a certain degree of homeostasis on the immune system. Thus, IL-10 clearly inhibits helper T cells functions and probably stimulates T cells with suppressor functions. Therefore, like IL-4, IL-10 is believed to regulate the balance between Th1 and Th2 cytokine profiles of T cells. Especially, it is believed that IL-10 inhibits the differentiation of Th1 cells. Since Th1 cells are characterized by the production of cytokines (IFN- γ and IL-2) which favour cell-mediated immune responses while Th2 cells produce cytokines (IL-4, IL-5 and IL-10) which favour a humoral response and suppress a cell-mediated immune response, IL-10 is likely to suppress a T cell-mediated immune reaction such as delayed type hypersensitivity reactions, while it favours humoral responses.

As a consequence of the features of IL-10 mentioned above, the activity of IL-10 has been described as an inhibitor of macrophage and Th1 cytokine synthesis. Therefore it has been studied whether lack of IL-10 production and/or activity may play a role in diseases where an enhanced cell-mediated immunoreactivity is believed to play a role for the disease, such as in auto-immune diseases and inflammation. Anti-IL-10 antibody-treated mice show a stronger inflammatory response

to monokine-induced inflammation and are significantly more susceptible to death induced by LPS-induced septic shock, a monokine-mediated inflammatory reaction (16). Also, IL-10 knock-out mice spontaneously develop inflammatory reactions of the gut similar to that of colitis ulcerosa (17). Additionally, it has been investigated whether IL-10 plays a role in different parasite, mycobacterial or viral infections, and IL-10 has been shown to play a pathophysiological role in the immune-paresis related to the infection with *Schistosoma* *mansoni* (18). Also a role in *Mycobacterium leprae* infections has been suggested. Recently, it has been found that AIDS patients with a poor prognosis have a higher level of IL-10 in plasma, and it has been suggested that this contributes to the immune paresis which is known from AIDS (19).

15 *Therapeutic considerations:*

These *in vivo* results/data strongly suggest a homeostatic role of IL-10 in controlling cell-mediated and monokine-amplified immune inflammation and indicate the wide-ranged therapeutical applications of IL-10 or a drug with IL-10-like activity in the treatment of diseases which are characterized by a decreased/insufficient production and/or activity of IL-10. Since the substance of the present invention exemplified by IT9302 exerts IL-10-like activity by

- 1) inducing IRAP production by human monocytes,
- 25 2) inhibiting spontaneous IL-8 production by human monocytes,
- 3) inhibiting IL-1 β -stimulated IL-8 production by peripheral blood mononuclear cells (PBMC),
- 4) stimulating chemotactic migration of CD8+, but not CD4+, human T lymphocytes,
- 30 5) desensitizing human CD8+ T cells towards rhIL-10-induced chemotactic migration,
- 6) inhibiting IL-8-mediated human CD4+ T cell chemotaxis, and

- 7) inhibiting MCP-1/MCAF-mediated human monocyte chemo-taxis,
- 8) inducing the production of IL-4 by cultured normal human CD4+ T cells,
- 5 9) reducing the TNF α production in human mixed leucocyte reaction,

this polypeptide and analogues thereof may thus possess the same therapeutic possibilities as hIL-10. Table 3 lists some diseases where an immune-modulator like IL-10 or an immune-
 10 modulator with IL-10-like activity may have therapeutic importance:

TABLE 3

Some diseases where an immune-modulator with IL-10-like activity, due to its induction of IRAP production and/or
 15 inhibition of cytokine-production and/or activity may have therapeutic importance (ref. 20-74)

	Pre-term labour caused by infection or other conditions
	Rheumatoid arthritis
20	Lyme's arthritis
	Gout
	Sepsis syndrome
	Hyperthermia
	Ulcerative colitis or enterocolitis
25	Osteoporosis
	Cytomegalovirus
	Periodontal diseases
	Glomerulonephritis
	Chronic, non-infectious inflammation of the lung (e.g. sar-
30	coidosis and smoker's lung)
	Granuloma formation
	Fibrosis of the liver
	Fibrosis of the lung
	Transplant rejection
35	Graft vs. host disease
	Chronic myeloid leukaemia
	Acute myeloid leukaemia

- Other neoplastic diseases
 - Asthma bronchiale
 - Diabetes mellitus, type I (insulin dependent)
 - Arteriosclerosis/atherosclerosis
 - 5 Psoriasis
 - Chronic B lymphocyte leukaemia
 - Common variable immunodeficiency
 - Side-effects using other biological response modifiers
 - Disseminated intravascular coagulation
 - 10 Systemic sclerosis
 - Encephalomyelitis
 - Lung inflammation
 - Hyper IgE syndrome
 - Enterocolitis
 - 15 Cancer metastasis and growth
 - Adoptive immune therapy
 - Acquired respiratory distress syndrome
 - Sepsis
 - Reperfusion syndrome
 - 20 Postsurgical inflammation
 - Organ transplantation
 - Alopecia
-

DETAILED DESCRIPTION OF THE INVENTION

- 25 *Development of an IL-10-homologous nonapeptide with IL-10-like activity:*

Partial sequences of hIL-10 having a length of 9 amino acids was chosen according to the principle that the sequences should possess high homology between vIL-10 and hIL-10, but
30 as low homology to mIL-10 as possible. This strategy was based on the fact that vIL-10 cross-reacts partly with hIL-10, while mIL-10 does not cross-react with hIL-10 (see above). Thus, sequences of the hIL-10 responsible for certain activities in the human organism may be located at domains

where there is high homology between hIL-10 and vIL-10 but low in relation to mIL-10.

The signal polypeptide of hIL-10 is supposed to consist of the first 18 amino acids. The mature protein starts from amino acid No. 19 (No. 1 in the functional protein, which is a serine), and the total protein is containing 160 amino acids. By looking at the human and viral IL-10 COOH-terminal sequences and in particular position 157 and 159, containing lysine and arginine residues, this domain was found to be favourable for being partly responsible for receptor binding. (Figs. 1 and 2).

After screening several candidates, obtained by chemical synthesis, for IL-10-like activity, it was found that a synthetic nonapeptide, IT9302, possessed some immuno-suppressive activities which mimic those of hIL-10 as described in further detail in the following examples. IT9302 corresponds to a nonapeptide sequence from the C-terminal end of hIL-10 with the following amino acid sequence:

NH₂-Ala-Tyr-Met-Thr-Met-Lys-Ile-Arg-Asn-COOH (SEQ ID NO:1)

IT9302 has 100% homology with a segment of hIL-10 corresponding to amino acids Nos. 152 to 160 at the C-terminal end. Furthermore, this polypeptide sequence has 6 amino acids in common with the virus vIL-10 in the corresponding area, see Figs. 1 and 2, which means that 6 of 9 or 66% of the amino acids are homologous as defined herein, and 4 amino acids in common with mIL-10, i.e. 4 of 9 or 44%. This nonapeptide is the first sequence obtained or derived from hIL-10 and examined which has the special properties demonstrated in the examples. Until contemplated by the present inventors, no one has pointed to this sequence as a functional domain.

The present invention in particular relates to the use of a substance or polypeptide according to the formula

X_1 - X_2 - X_3 -Thr- X_4 -Lys- X_5 -Arg- X_6 (SEQ ID NO:22),

wherein

X_1 is Ala or Gly,

X_2 is Tyr or Phe,

5 X_3 , X_4 and X_5 are independently selected from the group consisting of Met, Ile, Leu and Val; and

X_6 is selected from the group consisting of Asp, Gln and Glu,

optionally at least one of X_1 , X_2 , X_3 , X_4 , X_5 and X_6 is independently substituted with non-natural or unusual amino acids
10 and/or the peptide is cyclized and/or the peptide is stabilized and/or the amino terminal amino acid residue is acylated and/or the carboxy terminal amino acid residue is amidated, and peptidomimetics modelled on the basis of the above formula for the preparation of a pharmaceutical composition for
15 the reduction of TNF α production. Within the scope of the present invention is the use of any of the substances contemplated or specifically listed in the following for the preparation of a pharmaceutical composition for the reduction of TNF α production.

20 Important embodiments of the present invention are substances according to the above formula wherein at least one of X_1 , X_2 , X_3 , X_4 , X_5 and X_6 is independently substituted with non-natural or unusual amino acids and/or the peptide is cyclized and/or the peptide is stabilized and/or the amino terminal
25 amino acid residue is acylated and/or the carboxy terminal amino acid residue is amidated, and peptidomimetics modelled on the basis of the above formula which exhibits hIL-10 agonist activity as defined above. Within the scope of the present invention are such modifications with respect to any
30 of the substances contemplated or specifically listed in the following.

Within the scope of the present invention are further substances wherein one or more of the residues Thr, Lys or Arg

in the above formula is substituted with non-natural or unusual amino acids as proposed above. All polypeptide sequences in the present specification and claims, are, also when not explicitly stated, written from the N-terminal to the C-terminal end in the conventional format.

The present invention thus relates to an IT9302 analogue which exhibits hIL-10 agonist activity, i.e. a substance other than human interleukin 10 and IT9302 which has one or more of the following properties:

- 10 a) induces inhibition of spontaneous IL-8 production by human monocytes,
 - b) induces inhibition of IL-1 β induced IL-8 production by human peripheral blood mononuclear cells (PBMC),
 - c) induces production of interleukin-1 receptor antagonistic protein (IRAP) by human monocytes,
 - 15 d) induces chemotactic migration of CD8+ human T lymphocytes *in vitro*,
 - e) desensitizes human CD8+ T cells resulting in an unresponsiveness towards rhIL-10,
 - 20 f) suppresses the chemotactic response of CD4+ human T lymphocytes towards IL-8,
 - g) suppresses the chemotactic response of human monocytes towards MCAF/MCP-1,
 - h) does not inhibit class II MHC molecule expression on human monocytes, in contrast to human IL-10,
 - 25 i) induces the production of IL-4 by cultured normal human CD4+ T cells,
 - j) reduces the TNF α production in human mixed leukocyte reaction.
- 30 Among these activities, h) to j), in particular the inhibition of TNF α , are considered the most important.

The nonapeptide IT9302 is very potent to induce different functions and is very stable, and it is presumed that it cannot be incorrectly coupled to receptors. A nonapeptide has

been chosen because generally a 9 amino acid polypeptide sequence is unique for a protein. However, it is to be noted that the 6 amino acids at the very end of hIL-10 seem to be the most important ones. Within the scope of the present invention is thus a substance or polypeptide comprising a subsequence of the amino acid sequence Ala-Tyr-Met-Thr-Met-Lys-Ile-Arg-Asn (SEQ ID NO:1).

It is considered likely that some amino acid substitutions will not have adverse effects on the hIL-10 agonist activity as defined herein although it is preferred that the threonine, the lysine and the arginine are present and with one amino acid placed inbetween.

In its broadest aspect, the present invention thus relates to a polypeptide having the formula

Thr-X₄-Lys-X₅-Arg-X₆ (SEQ ID NO:19),

wherein

X₄ and X₅ are independently selected from the group consisting of Met, Ile, Leu and Val; and

X₆ is selected from the group consisting of Asp, Gln and Glu.

In a further aspect, the invention relates to a polypeptide having the formula

X₃-Thr-X₄-Lys-X₅-Arg-X₆ (SEQ ID NO:20),

wherein

X₃, X₄ and X₅ are independently selected from the group consisting of Met, Ile, Leu and Val; and

X₆ is selected from the group consisting of Asp, Gln and Glu.

In yet a further aspect, the invention relates to polypeptides having the formula

X_2 - X_3 -Thr- X_4 -Lys- X_5 -Arg- X_6 (SEQ ID NO:21),

wherein

- X_2 is Tyr or Phe,
 X_3 , X_4 and X_5 are independently selected from the group
 5 consisting of Met, Ile, Leu and Val; and
 X_6 is selected from the group consisting of Asp, Gln and Glu.

Preferred embodiments of the invention are polypeptides
 having the formula

X_1 - X_2 - X_3 -Thr- X_4 -Lys- X_5 -Arg- X_6 (SEQ ID NO:22),

10 wherein

- X_1 is Ala or Gly,
 X_2 is Tyr or Phe,
 X_3 , X_4 and X_5 are independently selected from the group con-
 sisting of Met, Ile, Leu and Val; and
 15 X_6 is selected from the group consisting of Asp, Gln and Glu.

Examples of specific polypeptides which are presumed to have
 hIL-10 agonist activity as defined above are as follows:

1. NH_2 -Ala-Tyr-Met-Thr-Ile-Lys-Met-Arg-Asn-COOH (SEQ ID NO:2)
2. NH_2 -Ala-Phe-Met-Thr-Leu-Lys-Leu-Arg-Asn-COOH (SEQ ID NO:3)
- 20 3. NH_2 -Ala-Tyr-Met-Thr-Met-Lys-Val-Arg-Glu-COOH (SEQ ID NO:4)
4. NH_2 -Gly-Tyr-Met-Thr-Met-Lys-Ile-Arg-Asp-COOH (SEQ ID NO:5)
5. NH_2 -Ala-Phe-Met-Thr-Met-Lys-Ile-Arg-Asp-COOH (SEQ ID NO:6)
6. NH_2 -Ala-Tyr-Ile-Thr-Met-Lys-Ile-Arg-Asp-COOH (SEQ ID NO:7)
7. NH_2 -Ala-Tyr-Leu-Thr-Met-Lys-Ile-Arg-Asp-COOH (SEQ ID NO:8)
- 25 8. NH_2 -Ala-Tyr-Val-Thr-Met-Lys-Ile-Arg-Asp-COOH (SEQ ID NO:9)
9. NH_2 -Ala-Tyr-Met-Thr-Ile-Lys-Ile-Arg-Asp-COOH (SEQ ID NO:10)
10. NH_2 -Ala-Tyr-Met-Thr-Leu-Lys-Ile-Arg-Asp-COOH (SEQ ID NO:11)
11. NH_2 -Ala-Tyr-Met-Thr-Val-Lys-Ile-Arg-Asp-COOH (SEQ ID NO:12)
12. NH_2 -Ala-Tyr-Met-Thr-Met-Lys-Ile-Arg-Asp-COOH (SEQ ID NO:13)
- 30 13. NH_2 -Ala-Tyr-Met-Thr-Met-Lys-Met-Arg-Asp-COOH (SEQ ID NO:14)
14. NH_2 -Ala-Tyr-Met-Thr-Met-Lys-Val-Arg-Asp-COOH (SEQ ID NO:15)

15. NH₂-Ala-Tyr-Met-Thr-Met-Lys-Ile-Arg-Gln-COOH (SEQ ID NO:16)
16. NH₂-Ala-Tyr-Met-Thr-Met-Lys-Ile-Arg-Glu-COOH (SEQ ID NO:17)

For comparison, another nonapeptide called IT9301 with 100% homology to a sequence of hIL-10 was synthesized. This polypeptide, IT9301, corresponds to a nonapeptide sequence starting at the N-terminal end of hIL-10, (amino acids nos. 19 to 27, i.e. the first 9 amino acids of the mature protein) and has the following amino acid sequence:

NH₂-Ser-Pro-Gly-Gln-Gly-Thr-Gln-Ser-Glu-COOH (SEQ ID NO:18)

- 10 This polypeptide showed no IL-10-like activity using the techniques described below and was only chosen because it is at the other end of hIL-10 and mostly served as a control for negative results. It does not have the characteristics of the C-terminal sequence with regard to sequence homology to vIL-10, and favourable amino acids for receptor binding. IT9301 was tested in the IL-1 β induced peripheral blood mononuclear cell system for IL-8 production, but did not induce any inhibition at all of the IL-8 production from the cells.

- Any of the contemplated peptides of the invention can have an amino terminal amino acid residue which is acylated such as acetylated or benzoylated. Also, any of the contemplated peptides can have a carboxy terminal amino acid residue which is amidated.

- The present invention further contemplates analogues of peptides formed by other conservative amino acid substitutions than the specific substitutions proposed above, substitutions of unusual or non-natural amino acids, stabilization of peptides, cyclization of peptides, and peptidomimetics modelled on the identified IL-10 agonist peptides.

- 30 The principle behind conservative amino acid substitution is that certain amino acid pairs have compatible side chains such that, when one is substituted for the other, there will be only minimal changes in the tertiary structure and the

binding affinity of the peptide. Rules for conservative substitution are explained in (78).

Analogues of synthetic peptides can also be made by substituting individual residues with non-natural or unusual amino acids. Sequences of bioactive peptides are originally derived from proteins which are made up of the naturally occurring twenty L-amino acid residues. However, the process of chemical synthesis used to construct synthetic peptides allows for the substitution of alternate residues including D-amino acids, β -amino acids, N-substituted amino acids, infrequently occurring natural amino acids, or non-natural synthetic amino acid analogues (93) and (99). Non-limiting examples of amino acids which may be useful in the present invention are:

	Aad	2-Aminoadipic acid
15	bAad	3-Aminoadipic acid
	bAla	beta-Alanine, beta-Aminopropionic acid
	Abu	2-Aminobutyric acid
	4Abu	4-Aminobutyric acid, piperidinic acid
	Acp	6-Aminocaproic acid
20	Ahe	2-Aminoheptanoic acid
	Aib	2-Aminoisobutyric acid
	bAib	3-Aminoisobutyric acid
	Apm	2-Aminopimelic acid
	Dbu	2,4-Diaminobutyric acid
25	Des	Desmosine
	Dpm	2,2'-Diaminopimelic acid
	Dpr	2,3-Diaminopropionic acid
	EtGly	N-Ethylglycine
	EtAsn	N-Ethylasparagine
30	Hyl	Hydroxylysine
	aHyl	allo-Hydroxylysine
	3Hyp	3-Hydroxyproline
	4Hyp	4-Hydroxyproline
	Ide	Isodesmosine
35	aIle	allo-Isoleucine
	MeGly	N-Methylglycine, sarcosine

	MeIle	N-Methylisoleucine
	MeLys	6-N-Methyllysine
	MeVal	N-Methylvaline
	Nva	Norvaline
5	Nle	Norleucine
	Orn	Ornithine

The above described alternate residues can be used (a) to replace chemically reactive residues and improve the stability of the synthetic peptide towards e.g. enzymatic and proteolytic degradation, (b) to provide analytic labels useful in the detection of the synthetic peptide, and (c) to modulate the bioactivity of the synthetic peptide by increasing or decreasing the binding affinity of the peptide for the IL-10 receptor, e.g. by introduction of conformational constraints which reduce the rotational freedom for specific chemical bonds.

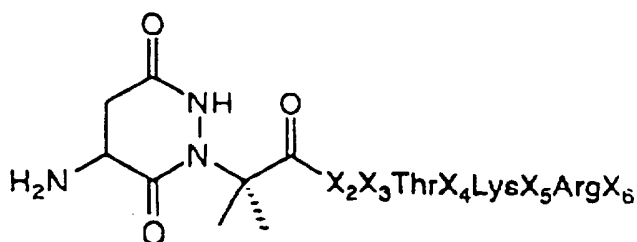
Analogues of synthetic linear peptides can be made by chemically converting the structures to cyclic forms. Cyclization of linear peptides can modulate bioactivity by increasing or decreasing the affinity of binding of the peptide by the target protein (94). Linear peptides are very flexible and tend to adopt many different conformations in solution. Cyclization acts to constrain the number of available conformations and thus favour the more active or inactive structures of the peptide. The immunogenicity of synthetic peptides has been correlated with the experimentally observed conformational preferences in solution (95). Differences in immunogenicity may be indicative of differences in binding affinity of specific antibodies for cyclic peptides.

Cyclization of linear peptides is accomplished either by forming a peptide bond between the free N-terminal and C-terminal ends (homodetic cyclopeptides) or by forming a new covalent bond between amino acid backbone and/or side chain groups (heterodetic cyclopeptides) (93). The latter cyclizations use alternate chemical strategies to form covalent

bonds, e.g. disulfides, -lactones (both also present in natural peptides), ethers, or thioethers. Linear peptides of more than five residues can be cyclized relatively easily. The propensity of the peptide to form a beta-turn conformation in the central four residues facilitates the formation of both homo- and heterodetic cyclopeptides. The presence of proline or glycine residues at the N- or C-terminal ends also facilitates the formation of cyclopeptides, especially from linear peptides shorter than six residues in length.

- Peptidomimetics technology is the design of molecular mimics of peptides. The ability to successfully design such molecules depends on the understanding of the properties of the linear peptide sequence and the conformation in which it is presented to the IL-10 receptor. The synthesis of mimetics can provide compounds exhibiting greater biological activity, improved solubility, and stability (96).

As an example, the following peptidomimetic has been derived on the basis of the α -helical templates in C-terminal peptide mimetics of cytokines disclosed in US 5,446,128 (97) in combination with the knowledge that the C-terminal of IL-10 exists as an α -helix (98).



By this attachment of a small molecule at the N-terminal of the peptide, an α -helical structure of the synthetic peptide is stabilized, and the peptide is made more resistant to proteolytic degradation. Other peptidomimetics may be derived on the basis of the disclosure in US 5,446,128. Such substances wherein substitution has taken place at other residues than X₁ and/or substitution has taken place with other molecules

than the N-terminal molecule shown in the formula above are within the scope of the present invention.

The present invention thus relates to analogues of the peptides having the formula

5 X₁-X₂-X₃-Thr-X₄-Lys-X₅-Arg-X₆ (SEQ ID NO:22),

wherein

X₁ is Ala or Gly,

X₂ is Tyr or Phe,

X₃, X₄ and X₅ are independently selected from the group consisting of Met, Ile, Leu and Val; and

X₆ is selected from the group consisting of Asp, Gln and Glu

wherein one or more amino acids are substituted with non-natural or unusual amino acids and/or the peptide is cyclized and/or the peptide is stabilized and/or the amino terminal amino acid residue is acylated and/or the carboxy terminal amino acid residue is amidated, and peptidomimetics modelled on the basis of the above formula, said analogues having at least one of the following properties:

- a) induces inhibition of spontaneous IL-8 production by human monocytes,
- b) induces inhibition of IL-1 β induced IL-8 production by human peripheral blood mononuclear cells (PBMC),
- c) induces production of interleukin-1 receptor antagonistic protein (IRAP) by human monocytes,
- d) induces chemotactic migration of CD8+ human T lymphocytes *in vitro*,
- e) desensitizes human CD8+ T cells resulting in an unresponsiveness towards rhIL-10,
- f) suppresses the chemotactic response of CD4+ T human lymphocytes towards IL-8,
- g) suppresses the chemotactic response of human monocytes towards MCAF/MCP-1,

- h) does not inhibit class II MHC molecule expression on human monocytes, in contrast to human IL-10.
- i) induces the production of IL-4 by cultured normal human CD4+ T cells,
- 5 j) reduces the TNF α production in human mixed leukocyte reaction.

In accordance with the present invention, the term "an analogue of the peptide" comprises any pharmaceutically active and acceptable compound derived on the basis of the above
10 formula and exhibiting relevant biological actions similar to those of IT9302, including derivatives of such analogues, especially pharmaceutically acceptable salts, esters and solvates thereof.

The following definitions are employed in the present text:

- 15 "Cytokine" is a general term for a proteinaceous mediator released primarily but not exclusively by a cell population of the immune system as a response to a specific stimulating agent, e.g. a specific antigen or an alloantigen; or a non-specific, polyclonal activator, e.g. an endotoxin or other
20 cell wall components of a gram-negative bacterium.

"Lymphokine" is a general term for a proteinaceous mediator released by sensitized lymphocytes as a response to a stimulating agent, e.g. a specific antigen or an alloantigen; or by a lymphocyte challenged by a polyclonal activator, e.g. an
25 endotoxin or other cell wall components of a gram-negative bacterium.

"Interleukin" is a general term for a proteinaceous mediator released primarily but not exclusively by a macrophage, T, B, or NK cell as a response to a stimulating agent, e.g. a specific
30 antigen or an alloantigen; or by a lymphocyte challenged by a polyclonal activator, e.g. an endotoxin or other cell wall components of a gram-negative bacterium.

"Monokine" is a general term for a proteinaceous mediator released primarily but not exclusively by a mononuclear phagocyte (e.g. a monocyte or a macrophage or a Kupffer cell (liver) or a Langerhans' cell (skin) as a response to any stimulating agent.

"Chemokine" is a general term for a proteinaceous chemotactic and/or leukocyte-activating mediator released primarily but not exclusively by a cell population of the immune system as a response to a specific stimulating agent, e.g. a specific antigen or an alloantigen; or a non-specific, polyclonal activator, e.g. an endotoxin or other cell wall components of a gram-negative bacterium and belonging to a particular gene family either the chemokine- α gene family or the chemokine- β gene family.

"Interferon" is a general term for a proteinaceous antiviral and/or monocyte-activating mediator released primarily but not exclusively by a cell population of the immune system as a response to a virus or an interferon inducer such as a polynucleotide; in particular cells of the immune system as a response to a specific stimulating agent, e.g. a specific antigen or an alloantigen; or a non-specific, polyclonal activator, e.g. an endotoxin or other cell wall components of a gram-negative bacterium.

"Colony-stimulating factor" is a general term for a proteinaceous, haematopoietic colony-stimulating mediator released primarily but not exclusively by a cell population of the immune system as a response to a specific stimulating agent, e.g. a specific antigen or an alloantigen; or a non-specific, polyclonal activator, e.g. an endotoxin or other cell wall components of a gram-negative bacterium.

"Polypeptide" as used herein means both short peptides with a length of at least two amino acid residues and at most 10 amino acid residues, oligopeptides (11-100 amino acid residues), and longer peptides (the usual interpretation of

"polypeptide", i.e. more than 100 amino acid residues in length) as well as proteins (the functional entity comprising at least one peptide, oligopeptide, or polypeptide which may be chemically modified by being glycosylated, by being lipi-
5 dated, or by comprising prosthetic groups). The definition of polypeptides also comprises native forms of peptides/proteins in humans as well as recombinant proteins or peptides in any type of expression vectors transforming any kind of host, and also chemically synthesized peptides.

10 One embodiment of the present invention relates to a polypeptide comprising a subsequence of the sequence of human IL-10 and which has a degree of homology to vIL-10 which is 66% and a degree of homology to mIL-10 which is 44%, in particular SEQ ID NO:1. By the term "homology" is meant the identity in
15 sequences of amino acids in segments of two or more amino acids when matched with respect to identity and position of the amino acids of the polypeptides.

Homology as the term is used herein is thus a measure of similarity between two amino acid (or nucleotide) sequences.

20 Homology is expressed as the fraction or percentage of matching amino acids (or bases) after two sequences (possibly of unequal length) have been aligned. The term alignment is used in the sense defined in (76). Roughly, two sequences are aligned by maximizing the number of matching bases (or amino
25 acids) between the two sequences with the insertion of a minimal number of "blank" or "null" bases into either sequence to bring about the maximum overlap.

The term "homologous" is thus used here inter alia to illustrate the degree of identity between the amino acid sequence
30 of a given polypeptide and the amino acid sequence of IT9302. The amino acid sequence to be compared with the amino acid sequence of IT9302 may be deduced from a nucleotide sequence such as a DNA or RNA sequence, e.g. obtained by hybridization as defined in the following, or may be obtained by conventio-
35 nal amino acid sequencing methods. The degree of homology is

preferably determined on the amino acid sequence of a mature polypeptide, i.e. without taking any leader sequence into consideration. Generally, only coding regions are used when comparing nucleotide sequences in order to determine their internal homology.

Although it is contemplated that a substantial degree of homology to hIL-10 and vIL-10 is beneficial, it is not unlikely that subsequences of the subsequence of hIL-10 which show a lower degree of homology to vIL-10, say, e.g., 50%, 55% or 60%, may also show one or more beneficial hIL-10 agonist activities. Moreover, as discussed above, it is not deemed absolutely necessary that the degree of homology to hIL-10 is 100%. Within the scope of the present invention are also polypeptides having a lower degree of homology to hIL-10, such as 75%, 80%, 85%, 90% or 95%, although 100% is preferred.

Such polypeptides may be considered analogues of the nonapeptide. By the term "an analogue or variant thereof" is thus meant a polypeptide not having exactly the amino acid sequence Ala-Tyr-Met-Thr-Met-Lys-Ile-Arg-Asn (SEQ ID NO:1), but still having "hIL-10 agonist activity" as defined above. Generally, such polypeptides will be polypeptides which vary e.g. to a certain extent in the amino acid composition, or the post-translational modifications e.g. glycosylation or phosphorylation, as compared to IT9302 described in the examples.

The term "analogue" or "variant" is thus used in the present context to indicate a protein or polypeptide having a somewhat different, but still similar, amino acid sequence to the amino acid sequence of IT9302, allowing for minor variations that alter the amino acid sequence, e.g. deletions, site directed mutations, insertions of extra amino acids, or combinations thereof, to generate polypeptide analogues of IT9302.

Site-directed mutagenesis provides one convenient way to effect conservative amino acid substitutions, and the like, in the native protein sequence. Alternatively, analogues may be prepared by the well known methods of liquid or solid
5 phase polypeptide synthesis utilizing the successive coupling of the individual amino acids of the polypeptide sequence, or the polypeptide can be synthesized by the coupling of individual amino acids forming fragments of the polypeptide sequence which are later coupled so as to result in the desired
10 polypeptide.

"Conservative" as used herein means (i) that the alterations are as conformationally neutral as possible, that is, designed to produce minimal changes in the tertiary structure of the mutant polypeptides as compared to the native protein,
15 and (ii) that the alterations are as antigenically neutral as possible, that is designed to produce minimal changes in the antigenic determinants of the mutant polypeptides as compared to the native protein. Conformational neutrality is desirable for preserving biological activity, and antigenic neutrality
20 is desirable for avoiding the triggering of immunogenic responses in patients or animals treated with the substances of the invention. Although it is difficult to select with absolute certainty which alternatives will be conformationally and antigenically neutral, rules exist which can guide
25 those skilled in the art to make alterations that have high probabilities of being conformationally and antigenically neutral, see e.g. (77) and (78). Some of the more important rules include (1) replacement of hydrophobic residues is less likely to produce changes in antigenicity because they are
30 likely to be located in the protein's interior, e.g. Berzofsky (cited above) and Bowie et al. (cited above); (2) replacement of physicochemically similar, i.e. synonymous, residues is less likely to produce conformational changes because the replacing amino acid can play the same structural role as
35 the replaced amino acid; and (3) alteration of evolutionarily conserved sequences is likely to produce deleterious conformational effects because evolutionary conservation suggests

sequences may be functionally important. In addition to such basic rules for selecting mutein sequences, assays are available to confirm the biological activity and conformation of the engineered molecules. Biological assays for the substances of the invention are described more fully in the examples. Changes in conformation can be tested by at least two well known assays: the microcomplement fixation method, e.g. (79) and (80) used widely in evolutionary studies of the tertiary structures of proteins; and affinities to sets of conformation-specific monoclonal antibodies, e.g. (81).

An important embodiment of the present invention thus relates to a polypeptide in which at least one amino acid residue has been substituted with a different amino acid residue and/or in which at least one amino acid residue has been deleted or added so as to result in a polypeptide comprising an amino acid sequence being different from the amino acid sequence or a subsequence of said amino acid sequence as defined in the following, but essentially having hIL-10 agonist activity as defined above.

An interesting embodiment of the invention relates to a polypeptide which is an analogue and/or comprises at least part of the polypeptide of the invention amounting in total from 10 to 100 amino acids, e.g. at least 12 amino acids, at least 15 amino acids, at least 20 amino acids or at least 30 amino acids.

In a preferred embodiment of the invention, the substance or polypeptide is used in substantially pure form. To obtain this, purification of the polypeptide may be required. Examples of the procedures employed for the purification of polypeptides are: (i) immunoprecipitation or affinity chromatography with antibodies, (ii) affinity chromatography with a suitable ligand, (iii) other chromatography procedures such as gel filtration, ion exchange or high performance liquid chromatography or derivatives of any of the above, (iv) electrophoretic procedures like polyacrylamide gel electrophore-

sis, denaturing polyacrylamide gel electrophoresis, agarose gel electrophoresis and isoelectric focusing, (v) any other specific solubilization and/or purification techniques.

Within the scope of the present invention is also a nucleotide sequence encoding a polypeptide as defined above, in particular a nucleotide sequence encoding a polypeptide comprising or being the amino acid sequence SEQ ID NO:1, e.g. a nucleotide sequence comprising the sequence GCC TAC ATG ACA ATG AAG ATA CGA AAC (SEQ ID NO:23) or a nucleotide sequence encoding a polypeptide having a subsequence of said amino acid sequence. Moreover, a modified nucleotide sequence which differs from the nucleotide sequence above in that at least one nucleotide has been deleted, substituted or modified or at least one additional nucleotide has been inserted so as to result in a nucleotide sequence which encodes a polypeptide having hIL-10 agonist activity is within the scope of the invention.

In the present specification and claims, the term "subsequence" designates a sequence which preferably has a size of at least 18 nucleotides, more preferably at least 21 nucleotides, and most preferably at least 24 nucleotides. In a number of embodiments of the invention, the subsequence or analogue of the nucleotide sequence of the invention will comprise at least 27 nucleotides, such as at least 30 nucleotides or at least 45 nucleotides. The polypeptide encoded by the "subsequence" should conform to at least one of the criteria h)-j) above and/or the nucleotide "subsequence" should hybridize with the nucleotide sequence comprising the sequence SEQ ID NO:23 under high stringency conditions.

The term "highly stringent" when used in conjunction with hybridization conditions is as defined in the art that is 5-10°C under the melting point T_m , cf. Sambrook et al, 1989, pages 11.45-11.49.

The term "analogue" with regard to the DNA fragments of the invention includes a nucleotide sequence which encodes a polypeptide identical or substantially identical to the polypeptide encoded by the DNA referred to above. It is well known
5 that the same amino acid may be encoded by various codons, the codon usage being related, *inter alia*, to the preference of the organisms in question expressing the nucleotide sequence. Thus, one or more nucleotides or codons of the DNA fragment of the invention may be exchanged by others which,
10 when expressed, result in a polypeptide identical or substantially identical to the polypeptide encoded by the DNA fragment shown above.

Furthermore, the terms "analogue" and "subsequence" are intended to allow for variations in the sequence such as substitution, insertion (including introns), addition and rearrangement of one or more nucleotides, which variations do not have any substantial adverse effect on the hIL-10 agonist activity of the polypeptide encoded by the DNA fragment or a subsequence thereof. The invention thus also includes a nucleotide sequence encoding a polypeptide having a subsequence
20 of the amino acid sequence SEQ ID NO:1.

The polypeptides of the invention can be produced using recombinant DNA technology. An important embodiment of the present invention relates to an expression system comprising a
25 nucleotide sequence of the invention. Within the scope of the invention is thus also an expression system comprising a nucleotide sequence of the invention, such as a replicable expression vector which carries and is capable of mediating the expression of a nucleotide sequence as defined above.

30 The organism which is used for the production of the polypeptide of the invention may be a higher organism, e.g. an animal, or a lower organism, e.g. a microorganism such as *Escherichia coli*, a yeast, a protozoan, or cell derived from a multicellular organism such as a fungus, an insect cell, a

plant cell, a mammalian cell or a cell line, which carries an expression system as defined above.

Irrespective of the type of organism used, the DNA fragment of the invention is introduced into the organism either directly or by means of a suitable vector. Alternatively, the polypeptides may be produced in the mammalian cell lines by introducing the DNA fragment or an analogue or a subsequence thereof of the invention either directly or by means of an expression vector. The polypeptides of the invention may also be produced by chemical synthesis as discussed above.

The invention furthermore relates to a plasmid vector containing a DNA sequence coding for a polypeptide of the invention or a fusion polypeptide as defined herein. In one particular important embodiment, the DNA fragment or an analogue or subsequence thereof of the invention or a fusion DNA fragment of the invention as defined herein may be carried by a replicable expression vector which is capable of replicating in a host organism or a cell line.

The vector may in particular be a plasmid, phage, cosmid, mini-chromosome or virus. In an interesting embodiment of the invention, the vector may be a vector which, when introduced in a host cell, is integrated in the host cell genome.

The polypeptide produced as described above may be subjected to post-translational or post-synthetic modifications as a result of thermal treatment, chemical treatment (formaldehyde, glutaraldehyde etc.) or enzyme treatment (peptidases, proteinases and protein modification enzymes). The polypeptide may be processed in a different way when produced in an organism as compared to its natural production environment. As an example, glycosylation is often achieved when the polypeptide is expressed by a cell of a higher organism such as yeast or preferably a mammal. Glycosylation is normally found in connection with amino acid residues Asn, Ser, Thr or hydroxylysine.

One embodiment of the invention thus relates to a method of producing a polypeptide as defined above comprising the following steps of:

- 5 (a) inserting a nucleotide sequence as defined above in an expression vector,
- (b) transforming a suitable host organism with the vector produced in step (a),
- (c) culturing the host organism produced in step (b) under suitable conditions for expressing the polypeptide,
10
- (d) harvesting the polypeptide, and
- (e) optionally subjecting the polypeptide to post-translational modification.

15 Within the scope of the present invention is also a pharmaceutical composition comprising a substance or polypeptide of the invention.

A very important aspect of the invention relates to a pharmaceutical composition comprising an analogue of the peptides of the formula

20 $X_1-X_2-X_3\text{-Thr-}X_4\text{-Lys-}X_5\text{-Arg-}X_6$ (SEQ ID NO:22),

wherein

X_1 is Ala or Gly,
 X_2 is Tyr or Phe,
 X_3 , X_4 and X_5 are independently selected from the group consisting of Met, Ile, Leu and Val; and
25 X_6 is selected from the group consisting of Asp, Gln and Glu

wherein at least one of X_1 , X_2 , X_3 , X_4 , X_5 and X_6 is independently substituted with non-natural or unusual amino acids and/or the peptide is cyclized and/or the peptide is stabilized and/or the amino terminal amino acid residue is acylated and/or the carboxy terminal amino acid residue is amidated, and peptidomimetics modelled on the basis of the above formula, said analogues having at least one of the following properties:

- a) induces inhibition of spontaneous IL-8 production by human monocytes,
- b) induces inhibition of IL- 1β induced IL-8 production by human peripheral blood mononuclear cells (PBMC),
- c) induces production of interleukin-1 receptor antagonistic protein (IRAP) by human monocytes,
- d) induces chemotactic migration of CD8+ human T lymphocytes *in vitro*,
- e) desensitizes human CD8+ T cells resulting in an unresponsiveness towards rhIL-10,
- f) suppresses the chemotactic response of CD4+ T human lymphocytes towards IL-8,
- g) suppresses the chemotactic response of human monocytes towards MCAF/MCP-1,
- h) does not inhibit class II MHC molecule expression on human monocytes, in contrast to human IL-10.
- i) induces the production of IL-4 by cultured normal human CD4+ T cells,
- j) reduces the TNF α production in human mixed leukocyte reaction.

and a pharmaceutically acceptable excipient. The composition may comprise e.g. purified synthesized protein or a purified recombinant polypeptide.

The IL-10 agonist used in this invention may be prepared as formulations in pharmaceutically acceptable media, for example, saline, phosphate buffered saline (PBS), Ringer's solution, dextrose/saline, Hank's solution, and glucose. The

compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents, wetting agents, detergents, and the like. Additives
5 may also include additional active ingredients, e.g. bactericidal agents, or stabilizers. The amount administered to the patient will vary depending upon what is being administered, the purpose of the administration, such as prophylaxis or therapy, the state of the host, the manner of administration,
10 nistration, and the like.

The pharmaceutical compositions are typically intended for transdermal or parenteral administration, e.g. intravenously, subcutaneously, or intramuscularly. Orally administrative forms are also desired and can be provided by modifying the
15 composition to bypass the stomach environment. The composition can be used for prophylactic and/or therapeutic treatment. Preferably, the pharmaceutical compositions are administered intravenously. Thus, the invention provides compositions which comprise an IL-10 agonist substance dissolved or
20 suspended in an acceptable carrier, preferably an aqueous carrier. These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered.

The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration.
25 The IL-10 agonist may also be administered with a second biologically active agent, such as a standard chemotherapeutic agent. Such agents include but are not limited to vincristine, daunorubicin, L-asparaginase, mitoxantrone and
30 amsacrine.

In therapeutic applications, the pharmaceutical compositions are administered to a patient in an amount sufficient to produce the desired effect, defined as a "therapeutically effective dose". The therapeutically effective dose of a
35 IL-10 agonist will vary according to, for example, the parti-

cular use for which the treatment is made, the manner of administration, the health and condition of the patient, and the judgment of the prescribing physician. For example, the dose for continuous infusion will typically be in the range
5 of about 500 ng to about 800 μ g per day for a 70 kg patient, preferably between about 10 μ g and about 300 μ g. The dose will typically be between 700 ng/kg/day and 16 μ g/kg/day.

The concentration of IL-10 agonist in the pharmaceutical formulations can vary widely, i.e. from about 10 μ g to about
10 5 mg/ml, preferably between about 100 μ g and about 2 mg/ml. The concentration will usually be selected primarily by fluid volumes, viscosities, etc., in accordance with the particular mode of administration selected. Thus, a typical pharmaceutical composition for intravenous infusion could be made up
15 to contain 250 ml of dextrose/saline solution and 2.5 mg of IL-10 agonist.

For solid compositions, conventional non-toxic solid carriers may be used which include, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharin, talcum, cellulose, glucose, sucrose, magnesium carbonate, and the like. For oral administration, a pharmaceuti-
20 cally acceptable non-toxic composition is formed by incorporating normally employed excipients, such as those carriers previously listed, and generally 10-95% of active ingredient, that is, an IL-10 agonist substance, preferably 25-75%.
25

For aerosol administration, the IL-10 agonist is preferably supplied in finely divided form along with a surfactant and propellant. Typical percentages of IL-10 agonist are 0.01-20% by weight, preferably 1-10%. The surfactant must, of course,
30 be non-toxic, and preferably soluble in the propellant. Representative of such agents are the esters or partial esters of fatty acids containing from 6 to 22 carbon atoms, such as caproic, octanoic, lauric, palmitic, stearic, linoleic, linolenic, olesteric and oleic acids with an aliphatic
35 polyhydric alcohol or its cyclic anhydride such as, for

example, ethylene glycol, glycerol, erythritol, arbitol, mannitol, sorbitol, the hexitol anhydrides derived from sorbitol, and the polyoxyethylene and polyoxypropylene derivatives of these esters. Mixed esters, such as mixed or
5 natural glycerides may be employed.

The surfactant may constitute 0.1-20% by weight of the composition, preferably 0.25-5%. The balance of the composition is ordinarily propellant. Liquified propellants are typically
10 gases at ambient conditions, and are condensed under pressure. Among suitable liquified propellants are the lower alkanes containing up to 5 carbons, such as butane and propane; and preferably fluorinated or fluorochlorinated alkanes. Mixtures of the above may also be employed. In producing the aerosol, a container equipped with a suitable valve is filled
15 with the appropriate propellant, containing the finely divided polypeptide(s) and surfactant. The ingredients are thus maintained at an elevated pressure until released by action of the valve.

To enhance the serum half-life, the IL-10 agonist may be encapsulated, introduced into the lumen of liposomes, prepared
20 as a colloid, or other conventional techniques may be employed which provide an extended lifetime of the polypeptides. Thus, in certain embodiments, the IL-10 agonist may be encapsulated in a liposome. A variety of methods are available for preparing liposomes, as described in, e.g., (83),
25 (84), (85) and (86).

Use of the substance according to the present invention

In accordance with the present invention, it has as described above been found that IT9302 and analogues and variants
30 thereof are useful for preventing effects of cytokines known to be pathogenetically involved in the previously described pathological conditions.

Therefore, the potentials of therapy by using the polypeptide of the invention or analogues or derivatives thereof is contemplated and should be investigated in all diseases where a therapeutic effect of hIL-10 and/or IRAP is expected (see
5 above, Table 3).

Very important embodiments of the invention relate to use of a substance or polypeptide of the invention for the treatment or prophylaxis of one or more of the diseases mentioned in Table 3, to the use of a substance or polypeptide according
10 to the invention for the manufacture of a pharmaceutical composition for the treatment or prophylaxis of one or more of the diseases mentioned in Table 3, as well as to a method of treating and/or preventing one or more of the diseases mentioned in Table 3, the method comprising administering to a
15 patient in need thereof a therapeutically or prophylactically effective amount of a substance or polypeptide according to the invention.

One important aspect of the invention is thus the use of an analogue of IT9302 or a functional derivative thereof for the
20 manufacture of a pharmaceutical composition for substantially inhibiting a biological effect in a human related to a cytokine, such as a lymphokine, interleukin, monokine, chemokine, interferon, colony-stimulating factor, prostaglandin and/or leukotriene, for the prophylaxis or treatment of a condition
25 related to a disturbance of a cytokine system such as the lymphokine, interleukin, monokine, chemokine, interferon or colony-stimulating factor system and/or a disturbance in the prostaglandin and/or leukotriene system. As used herein, the term "pharmaceutical composition" comprises any composition
30 suitable for human use as described in detail above.

The invention particularly relates to the use of an analogue of IT9302 or a functional derivative thereof

- for substantially *inhibiting* a biological effect in a human related to a cytokine for the prophylaxis or treatment of a

condition related to a disturbance of a cytokine system;
and/or

- 5 - for substantially *inhibiting* a biological effect in a human
related to a lymphokine for the prophylaxis or treatment of
a condition related to a disturbance of a lymphokine sys-
tem; and/or
- 10 - for substantially *inhibiting* a biological effect in a human
related to a interleukin for the prophylaxis or treatment
of a condition related to a disturbance of a interleukin
system; and/or
- 15 - for substantially *inhibiting* a biological effect in a human
related to a monokine for the prophylaxis or treatment of a
condition related to a disturbance of a monokine system;
and/or
- 20 - for substantially *inhibiting* a biological effect in a human
related to a chemokine for the prophylaxis or treatment of
a condition related to a disturbance of a chemokine system;
and/or
- 25 - for substantially *inhibiting* a biological effect in a human
related to an interferon for the prophylaxis or treatment
of a condition related to a disturbance of an interferon
system; and/or
- 30 - for substantially *inhibiting* a biological effect in a human
related to a colony-stimulating factor for the prophylaxis
or treatment of a condition related to a disturbance of a
colony-stimulating factor system; and/or

- for substantially *inhibiting* a biological effect in a human related to a prostaglandin for the prophylaxis or treatment of a condition related to a disturbance of a prostaglandin system; and/or
- 5 - for substantially *inhibiting* a biological effect in a human related to a leukotriene for the prophylaxis or treatment of a condition related to a disturbance of a leukotriene system.

LEGENDS TO FIGURES

- 10 Fig. 1 shows a comparison of the predicted amino acid sequences of mIL-10, hIL-10, and BCRFI. Amino acid sequence identities are indicated by vertical lines.

Fig. 2 shows the COOH terminal polypeptide sequences of IL-10 including 9 amino acids and comparing porcine, human, BCRFI
15 and mouse proteins.

Fig. 3 is a diagram showing that IT9302 inhibits spontaneous IL-8 production by purified cultured human monocytes.

Fig. 4 is a diagram showing that IT9302 inhibits IL-1 induced (1 ng/ml) IL-8 production by human peripheral blood mono-
20 nuclear cells.

Fig. 5 illustrates IRAP production by IT9302-stimulated human monocytes.

Fig. 6 illustrates IRAP production by IL-10-stimulated human monocytes.

25 Fig. 7 illustrates the chemotactic activity of IT9302 on CD8+ T cells

Fig. 8 illustrates the desensitization of DC8+ T cells by IT9302, resulting in unresponsiveness of CD8+ T cells towards IL-10 (10 ng/ml) induced chemotaxis.

Fig. 9 illustrates the suppression of IL-8 activity by
5 IT9302.

Fig. 10 is a diagram showing that IT9302 inhibits MCAF/MCP-1 induced monocyte chemotaxis.

Fig. 11 shows IL-4 production in CD4+ T cell cytosolic fractions by ECL - Western Blotting.

10 Fig. 12 shows TNF- α production in human mixed lymphocyte culture cytosolic fractions by ECL - Western Blotting. TNF- α Western Blotting was carried out as the IL-4 described in Materials and Methods, but using a rabbit anti-human TNF- α antibody (Pepro Tech. Inc., London, England) and a horse-
15 radish peroxidase-labelled secondary antibody (Cat.no. P 217, Dako, Denmark).

Fig. 13 shows that LPS induced shock and leukopenia are modulated by IT9302, shown by total leukocyte counting.

Fig. 14 shows that injection of murine IT9302 into rabbits
20 before induced pancreatitis prevented leukopenia.

EXAMPLES

Materials and Methods*Cytokines and chemoattractants*

Recombinant hIL-10 was obtained from Pepro Tech Inc., NJ.
5 (Cat.No. 200 10). Recombinant hIL-1 β and recombinant hIL-8
were a kind gift from Dainippon Pharmaceutical Company,
Osaka, Japan. The culture medium was RPMI 1640 GIBCO, LPS-
free according to the Limulus Amoebocyte Lysate assay (Sigma
E-TOXATE Kit Cat.No. 210-A1). rhMCAF/MCP-1 was a kind gift
10 from professor Kouji Matsushima, Kanazawa, Japan.

*Leukocyte chemotaxis assay**T cell chemotaxis*

CD4+ and CD8+ T lymphocyte subsets characterized by expres-
sing either CD4 or CD8 antigens were purified from hepari-
15 nized blood of normal donors. Thus, peripheral blood mononu-
clear cells (PBMC) were purified from the heparinized blood
by diluting 100 ml of the blood with Hanks balanced salt
solution (HBSS) 1:1 and then separated by layering the cells
on Lymphopac™ (Nycomed Pharma, Oslo, Norway) followed by gra-
20 dient centrifugation at 2000 rpm for 20 minutes. The mononu-
clear cells were washed 3 times in HBSS and the cell pellet
was diluted in 4 ml of HBSS containing 1% fetal calf serum
and sorted at 4°C by using Dynabeads coated with monoclonal
antibody towards CD4 or CD8 antigen (Dynabeads M-450 CD4
25 Cat.No. 111.16, Dynabeads M-450 CD8 Cat.No. 111.08,
DETACHaBEAD Cat.No. 125.04). The bead:cell ratio was 10:1 and
the incubation time 1 hour. The beads were detached by adding
polyclonal anti-mouse antibody according to the manufactu-
rer's instructions.

30 The chemotaxis assay was a 48-well micro chamber technique
(Neuroprobe, Rockville, MD) as previously described (74; see

ref. 3 and ref. 14). Chemoattractants were diluted in RPMI 1640 (GIBCO Cat.No. 61870-010) with 1% sterile filtered fetal calf serum and placed in the lower 25 μ l chamber. In the case of determining T cell chemotaxis, T cells (5×10^6 /ml) were
5 suspended in medium and 50 μ l was placed in the upper chamber separated from the lower chamber by a 5 μ m pore-size polycarbonate, polyvinylpyrrolidone-free filter (Nucleopore Corp., Pleasanton, CA) coated with type IV collagen (Sigma Cat.No. C 0543). Cells were allowed to migrate for 2 hours at
10 37°C at 5% CO₂. The filters were then carefully removed, fixed in 70% methanol, and stained for 5 minutes in Coomassie's Brilliant Blue. Cells attached to the lower surface of the filter were counted by measuring their area using a video
15 camera on the microscope connected to a computer system for digital analysis and supported by software for objective determination of chemotactic migration. Approximately 5% of the T cells will migrate spontaneously corresponding to between 12,000 and 13,000 cells; this may vary from day to
20 day, but very little in the same day's experiments. As has been described earlier (ref. 3 and ref. 14), it was therefore chosen to report the results as a ratio between number of cells migrating in the sample and in the negative control, which reflects spontaneous migration. This ratio is referred
25 to as the chemotactic index (CI). All samples were analyzed in triplicates and cell migration in each well was measured in three fields before the median value of area was estimated. In some experiments the chemotaxis membrane was not coated with collagen, and in the present assay system migrating cells will therefore drop to the bottom of the lower
30 well of the chemotaxis chamber.

In one experiment, the chemotactic activity of IT9302 on CD8+ T cells was performed by testing serial dilutions of IT9302 added to the lower chamber and evaluating chemotaxis as described above.

35 In a second experiment, the ability of IT9302 to desensitize the migration of CD8+ T cells as a response to rHIL-10

(10 ng/ml) was studied by adding IT9302 to the target cells 30 minutes before chemotaxis. IT9302 was added in serial concentrations and the chemotactic response of rhIL-10 was evaluated as described above.

- 5 In a third experiment, the ability of IT9302 to suppress the chemotactic response of CD4+ T cells towards rhIL-8 (10 ng/ml) was studied by adding IT9302 to the target cells 30 minutes before performing chemotaxis. IT9302 was added in serial concentrations and the chemotactic response of rhIL-8
10 was evaluated as described above.

Monocyte chemotaxis

- Monocyte chemotaxis was measured using the same Boyden chamber equipment as described for T cells above. The chemo-attractant MCAF/MCP-1 was diluted in RPMI 1640 medium with
15 0.5% BSA and added to the lower chamber at a concentration of 10 ng/ml. Monocytes, purified by the standard plastic adherence technique, from normal human PBMC, obtained as described above were suspended in RPMI 1640 medium with 0.5% BSA and then incubated for 30 minutes in the presence of IT9302
20 at different concentrations. Subsequently, the cells were added to the upper chemotaxis chambers at a concentration of 10^6 cells/ml. The upper and lower chambers were separated by an 8 μ m pore size polyvinyl pyrrolidone-free polycarbonate filter (Nucleopore, Pleasanton, CA). The chamber was incubated
25 at 37°C for 90 minutes. The membranes containing migrating cells were treated as described above and a chemotactic index calculated according to the technique described above.

Production of IL-8 by normal human peripheral mononuclear cells (PBMC)

- 30 PBMC was purified from heparinized blood of normal human donors. Following gradient centrifugation with Lymphoprep™ (Nycomed Pharma, Oslo, Norway), the mononuclear cells were

diluted to 2×10^6 cells/ml in LPS-free RPMI 1640 medium (Gibco Cat.No. 6187-010) containing 1% sterile filtered heat-inactivated fetal calf serum and penicillin (10,000 IE/ml), streptomycin (10 mg/ml) and gentamycin (2.5 mg/ml). Cells
5 were cultured in 24 wells Nunc Micro Plates (Nunc, Denmark) and in the presence of different concentration of IT9302 (0, 1 μ g, 100 ng, 10 ng, 1 ng, 0.1 ng, 0.01 ng/ml) for 24 hours. Following 24 hours of incubation, another dose of IT9302 was added once more, and 1 hour later r-hIL-1 β (1 ng/ml) was
10 added to the cell cultures. Supernatants were collected after a total of 48 hours of incubation, and the concentration of the secreted IL-8 was measured by IL-8 ELISA by using an IL-8 ELISA Kit (Dainippon Pharmaceutical Co. Ltd, Osaka, Japan). Briefly, standards and cell supernatants were incubated for
15 one hour and in duplicates at 20°C on a micro-plate shaker. Then after washing, a second antibody was added for one hour, followed by one hour of incubation with peroxidase-labelled goat anti-rabbit IgG. After washing, the reaction was developed with O-phenylenediamine. Thirty minutes later the reac-
20 tion was stopped with 1.6 N sulphuric acid. Optical density (OD) was measured in an ELISA reader at 490 nm. IL-8 concentration was calculated by a calibration curve of absorbance of unknown vs concentrations of IL-8 standards.

Determination of IRAP concentration

25 PBMC was purified as described above. PBMC was cultured in RPMI 1640, 10% sterile heat-inactivated fetal calf serum (including penicillin 10,000 IE/ml, streptomycin 10 mg/ml, gentamycin 2.5 mg/ml) and the cell concentration was 5×10^6 cells/ml. The monocytes were then purified by standard plastic adherence technique. Monocytes were then cultured in RPMI
30 1640 with 2% FCS (2.5×10^6 cells/ml) and with different dilutions of rhIL-10 or IT9302. The cells were stimulated for 24 hours and the supernatants were collected for IRAP determination. IRAP ELISA was carried out by using Human IL-1ra
35 Quantikin Immunoassay Kit from R&D Systems Europe Ltd. (Cat.No. DRA 00, Abingdon, Oxon, UK).

Determination of class II MHC antigen expression on monocytes

PBMC was purified as described above and monocytes isolated by plastic adherence technique. Monocytes were then cultured in RPMI 1640 with 2% FCS including penicillin 10,000 IE/ml, streptomycin 10 mg/ml, gentamycin 2.5 mg/ml at a cell concentration of 3×10^6 /ml. Cells were stimulated for 40 hours in microwells (Nunc, Denmark) with 100 ng/ml rhIL-10, or IT9302 1 μ g/ml, 100 ng/ml, 10 ng/ml. At the end of stimulation, the supernatants were removed and the cells were detached from the plastic by freezing at -20°C for 20 minutes. The cells were collected in 1 ml of cold HBSS with 1% FCS and sorted at 20°C by using 50 μ l/ml Dynabeads M 450 coated with monoclonal antibody for HLA class II β chain (Cat. No. 210.03). After 20 minutes of incubation, the cells were washed 3 times with cold HBSS with 1% FCS and collected on a magnetic separation device. The cells were diluted in the above buffer and stained with methylene blue, and the rosetting cells (cells carrying Dynabeads with HLA class II mAb) were counted.

*Determination of IL-4 production by CD4+ T lymphocytes*20 *Cell cultures*

CD4+ T lymphocytes were purified from heparinized normal human blood. Following Lymphoprep™ (Nycomed Pharma, Oslo, Norway) gradient centrifugation, the mononuclear cells were further sorted at 4°C using Dynabeads (DynaL AS, Norway) coated with monoclonal anti-CD4 antibodies. The beads were detached by adding polyclonal anti-mouse antibody (DynaL AS, Norway). The purity of the positively selected cells were higher than 99% as judged by FACS analysis. When examining the *de novo* production of IL-4 by IL-8-stimulated T cells, T cells were cultures, 5×10^6 cells/ml in LPS-free RPMI 1640 (Gibco Cat.no. 61870-010) containing 1% sterile-filtered, heat-inactivated fetal calf serum (FCS), penicillin (10,000 IU/ml), streptomycin (10 mg/ml) and gentamycin (2.5 mg/ml).

T cells were stimulated for 3 days using rIL-8 (100 ng/ml), rIL-10 (100 ng/ml), IT9302 (10 ng/ml) and IFN- γ (10 ng/ml). Recombinant human IL-8 (rh IL-8) was a kind gift from Dainippon Pharmaceuticals Co. Ltd., Osaka, Japan), and IFN- γ was purchased from Boehringer Ingelheim Am Rhein, Germany. To obtain specific inhibition of IL-8 stimulation, a neutralizing monoclonal anti-IL-8 antibody (WS.4) was used (a kind gift from Dr. K. Matsushima, Japan). Recombinant IL-10 was purchased from Pepro Tech. Inc. (London, England).

10 *Preparation of cell material and culture supernatant for gel electrophoresis*

Cultured T cells and culture media were separated by centrifugation at 2000 rpm for 5 minutes. The supernatants were freeze-dried and then dissolved in 100 μ l of lysis buffer. The cells were resuspended directly in 100 μ l of gel lysis buffer (9). The material was kept at -80°C until further examination.

ECL-Western blotting of CD4+ T cell derived proteins

Cells or freeze-dried cell culture supernatants were used for IL-4 protein content determination. Proteins from one-dimensional 15% SDS-PAGE gels were transferred by blotting onto Hybond-ECL nitrocellulose membranes (Amersham RPN 2020D, UK) and blocked with 5% bovine serum albumin (Sigma) in Tris buffer saline pH 7.8 containing 0.1% Tween-20. The blots were then incubated with a polyclonal goat anti-human IL-4 antibody (R&D Systems, UK) followed by a horseradish peroxidase-labelled secondary antibody (Cat.no. RPN 2106 ECL, Amersham, UK), and the immunostaining was detected by exposing a film (Kodak X-OMAT-S, USA) for 90 seconds.

30 *Specificity of IT9302 amino acid sequence*

Searching for possible homology of the IT9302 sequence with other known proteins was carried out by searching on the EMBO

protein database kindly assisted by Dr. Henrik Leffers, Institute for Medical Biochemistry, University of Aarhus, Denmark.

Amino acid sequence specificity of IT9302

- 5 According to information from the EMBO protein database (Heidelberg) performed on 10 June 1994, IT9302 has 100% homology with a sequence of the human IL-10 and 75% with a sequence of an Epstein-Barr virus derived protein identical with vIL-10. Other virus proteins as phage T7 and tomato
10 yellow leaf curl virus revealed 75% and 85% identity, respectively.

Results

- The conception of the invention took place in October and November 1992, when a nonapeptide designated IT9302 was de-
15 signed according to the strategy outlined above, and the chemical synthesis of the first prototype (IT9302) was decided and ordered on 27 November 1992. It was expected that this nonapeptide should exert immunomodulatory activities of which some would mimic IL-10-like activity, and the following con-
20 trol experiments (examples) were planned during this period as well. The chemical synthesis of IT9302 was carried out by using an automatic polypeptide synthesizer performed by Carlbiotech Ltd. A/S, Denmark as a payed order by the inven-
25 tors. Afterwards the protein was purified on HPLC and the purity confirmed to exceed 95% by HPLC. This confirmed that the synthesized product was identical with the IT9302 designed by the inventors in October and November 1992.

EXAMPLE 1

IT9302 induced inhibition of spontaneous IL-8 production by human monocytes

The test was performed as described in "Production of IL-8 by
5 normal human peripheral mononuclear cells (PBMC)". Monocytes
were purified by plastic adherence technique and 3.0×10^6
cells/ml were stimulated for 40 hours. As shown in Fig. 3,
IT9302 inhibited the production of IL-8 by monocytes, and at
0.1 ng/ml of IT9302 the IL-8 production was suppressed to 35%
10 of the spontaneous production *in vitro*. The viability of
cells always exceeded 80% after 1 day in culture and the
addition of IT9302 did not in this or in the following exam-
ples affect viability at any concentration of IT9302 between
0.1 and 1000 ng/ml (IT9302 MW: 1,127 dalton, rhIL-10 predict-
15 ed MW: 18,400 dalton).

EXAMPLE 2

IT9302 induced inhibition of IL-1 β induced IL-8 production by human peripheral blood mononuclear cells (PBMC)

The test was performed as described in "Production of IL-8 by
20 normal human peripheral blood mononuclear cells (PBMC)". As
shown in Fig. 4, IT9302, in a dose dependent manner, inhi-
bited the IL-1 β induced production of IL-8 by human periphe-
ral blood mononuclear cells *in vitro*. The suppression of IL-8
production plateaued at IT9302 concentrations between 0.01
25 and 100 ng/ml.

EXAMPLE 3

*IT9302 induced production of interleukin-1 receptor antago-
nist protein (IRAP) by human monocytes*

The test was performed as described in "Determination of IRAP
30 concentration". As shown in Fig. 5, IT9302 dose-dependently

induced the production of IRAP by human monocytes. The production drastically increased when using concentrations of IT9302 above 10 ng/ml. Fig. 6 shows the induction of IRAP by rhIL-10 and since hIL-10 is approximately 20 times larger than IT9302, 10 ng/ml of IT9302 equals 200 ng/ml of IL-10 in molarity. Therefore the potencies of IT9302 and rhIL-10 are comparable and approximately equal with respect to the induction of IRAP at lower concentrations. At IT9302 concentrations exceeding 10 ng/ml, the induction of IRAP drastically increased and reached levels of approximately 700 ng/ml.

EXAMPLE 4

The chemotactic effect of IT9302 on human CD8+ T lymphocytes

The experiment was carried out as described in "Leukocyte chemotaxis assay". As shown in Fig. 7, IT9302 induced the chemotactic migration of CD8+ human T lymphocytes *in vitro*, while there was no effect on CD4+ T cells (data not shown). Again, the potency of IT9302 shown in this experiment is comparable with that of rhIL-10 shown previously (ref. 14).

EXAMPLE 5

IT9302 desensitizes human CD8+ T cells resulting in an unresponsiveness towards rhIL-10

The experiment was carried out as described in "Leukocyte chemotaxis assay". IT9302 was added to a suspension of CD8+ T cells 30 minutes before these cells were tested towards their chemotactic response to rhIL-10. As shown in Fig. 8, the pre-incubation of cells with IT9302 results in a suppressed responsiveness of the CD8+ T cells towards hrIL-10. This indicates that IT9302 may affect the binding of rhIL-10 to the IL-10 receptor.

EXAMPLE 6

IT9302 suppresses the chemotactic response of CD4+ T lymphocytes towards IL-8

The experiment was carried out as described in "Leukocyte chemotaxis assay". As shown in Fig. 9, IT9302, in a dose-dependent manner and added to a suspension of human CD4+ T lymphocytes, inhibits the response of the CD4+ T cells towards IL-8.

EXAMPLE 7

IT9302 suppresses the chemotactic response of human monocytes towards MCAF/MCP-1

The experiment was carried out as described in "Monocyte chemotaxis". As shown in Fig. 10, IT9302, in a dose-dependent manner and added to a suspension of human monocytes, inhibits the chemotactic response of the monocytes towards MCAF/MCP-1.

EXAMPLE 8

IT9302 does not inhibit class II MHC molecule expression on human monocytes

The experiment was carried out as described in Materials and Methods and show that rhIL-10 inhibits class II MHC expression but IT9302 does not, as shown below in Table 4.

TABLE 4

Stimulation	Number of rosetting monocytes
0	$12.0 \pm 1.0 \times 10^4$
5 100 ng/ml rhIL-10	$4.6 \pm 1.4 \times 10^4$
1 μ g/ml IT9302	$11.0 \pm 3.0 \times 10^4$
100 ng/ml IT9302	$14.4 \pm 2.4 \times 10^4$
10 ng/ml IT9302	$11.2 \pm 3.2 \times 10^4$

10 Discussion related to the experiments

The present data demonstrate a dose-dependent inhibitory effect of the synthetic nonapeptide, IT9302, on processes which reflect pro-inflammatory activity, including IL-8 production and monocyte and/or T cell migration. Thus, IT9302

15 was able to suppress the spontaneous production of IL-8 by human monocytes cultured overnight. This could be explained by a direct inhibitory effect on IL-8 mRNA production and/or subsequent protein production and or release. Another mechanism could be explained by the fact that monocytes cultured

20 *in vitro* will express and produce IL-1, which then in turn induces the production of IL-8. This is supported by the fact that it has been demonstrated that IT9302 potently induces the production of IRAP from monocytes. IT9302 may therefore also inhibit spontaneous IL-8 production by interfering with

25 the activity of IL-1. The observed IRAP induction by IT9302 appears to induce a biologically active IRAP, since IT9302 added to the cultures counteracts IL-1-induced IL-8 production, but only when added at least 16 hours before adding IL-1 to the cultures. This could mean that IT9302 inhibits

30 IL-1-induced IL-8 production by inducing the production of IRAP, which then in turn blocks the activity of IL-1 through its receptor. If IT9302 directly inhibited IL-8 production, it would have been expected that addition of IT9302 to the cultures 1 hour before adding IL-1 should inhibit IL-8 pro-

35 duction, which was not the case (data not shown). Therefore,

the observed inhibition of IL-8 production of IT9302 is likely to be due to an induction of IRAP production rather than a direct inhibition of IL-8 production. These functions of IT9302 can also be found for hIL-10 indicating that IT9302 possesses IL-10-like activities. IT9302 also mimics IL-10 activity by suppressing the ability of CD4+ T cells to migrate as a response to IL-8. Since IL-8 is related to many different states of inflammation and since CD4+ T cells appear early in the infiltrate of T cell-mediated immune inflammation such as allergy of the skin, this feature may prove to have significant therapeutic value for the control of T cell-mediated immune inflammation.

The demonstrated CD8+ T cell chemotactic activity of IT9302 is also parallel to that of IL-10, and IT9302 may thus activate T cell populations with suppressor activity contributing to the ending of T cell-mediated immune inflammation. Therefore IT9302 according to the examples which are demonstrated above, possesses therapeutic value in diseases where IL-10 and/or IRAP may also have therapeutic value. Additionally, IT9302 may have therapeutic value in diseases where IL-8 and/or MCAF and/or IL-1 are believed to have pathogenetic roles.

EXAMPLE 9

IT9302 and IL-10 induce the production of IL-4 in CD4+ T lymphocytes

Background

Like IL-10, IL-4 is a product of CD4+ T cells of T_H2 type. It was observed that recombinant human IL-10 induces the production of IL-4 by cultured human CD4+ T cells. This means that IL-10, in addition to its own immunosuppressive functions, also induces the production of another immunosuppressive cytokine, IL-4. It was therefore tested whether IT9302 also induces the production of IL-4 by CD4+ T cells.

Thus, CD4+ T cells, purified as described in "Methods for T cell chemotaxis", and cultured as described in the section "Determination of IL-4 production by CD4+ T lymphocytes", were stimulated for 3 days with IT9302 (10 ng/ml) or IL-10 (100 ng/ml). Cytosolic fractions were collected and analyzed for their IL-4 content using Western blotting (Fig. 11) and a goat anti-human IL-4 polyclonal antibody.

As demonstrated in Fig. 11, it was observed that IL-10 as well as IT9302 induce the production of IL-4 by cultured normal human CD4+ T cells.

EXAMPLE 10

IT9302 inhibits the production of TNF- α during a mixed leukocyte reaction (MLR)

It has been demonstrated that the mixed leukocyte reaction is partly dependent on the production of TNF- α during the reaction. It has been shown that IT9302 does not significantly reduce the MLR, but it was found that there is a significant reduction in the production of TNF- α during the MLR.

Thus, MLR was performed by purifying human leukocytes and then culturing 1 million cells/ml from allogeneic donors for 4 days. Before establishing the cultures, one group of cells was irradiated for 2 minutes using beta irradiation. Cytosolic protein fractions were purified as described in the section "Determination of IL-4 production by CD4+ T lymphocytes", and Western blotting was performed using a rabbit anti-human TNF- α antibody.

As demonstrated in Fig. 12, a significant reduction in the production of TNF- α was observed during a human mixed leukocyte reaction.

EXAMPLE 11

The modulation of the LPS induced shock and leukopenia in swine using IT9302

Since it was found that IT9302 modulates cytokine production, including TNF- α and IL-8, and supported by the published sequence of porcine IL-10 (Blanchon et al., 1995) (see Fig. 2 for homology to the COOH-terminal peptide), it was tested whether IT9302 was able to modulate the course of LPS induced leukopenia in swine (Fig. 13).

10 In a preliminary experiment, it was tested how the intravenous injection of IT9302 0.1 mg/kg modulated the effect of intravenous injection of 2 μ g/kg LPS in swine (N = 3). IT9302 was injected 30 minutes before injection of LPS, and blood samples were drawn as described in Fig. 13. Total leukocyte
15 count as well as differential cell count were determined, and the total number of neutrophilic leukocytes was calculated on the basis of these results.

As demonstrated, it was observed that injection of LPS caused a transient leukopenia. Injection of IT9302, however, prevented leukopenia as demonstrated in the figure.
20

EXAMPLE 12

Antibody against the synthetic polypeptide IT9302

Synthetic polypeptide IT9302 was purchased from Kem-En-Tec A/S, Copenhagen, Denmark, with higher purity than 95%. This
25 polypeptide was also conjugated to Keyhole Limpet Hemocyanin (KLH) by the producer. The soluble IT9302/KLH is more immunogenic than the polypeptide alone, and it may also be used as control protein for ELISA or Western Blotting.

Immunization of rabbits

250 μ g of IT9302 coupled to KLH, and as an emulsion with complete Freund's adjuvant, is used for intradermal or subcutaneous injections. The injections are repeated 4 times with
5 two-week intervals and the rabbits are bled 8 and 12 days thereafter. Later booster injections of 250 μ g of IT9302/KLH in incomplete Freund's adjuvant are administered with one-month intervals using subcutaneous injections. The formation
10 of IT9302 antibody is tested by dot-blot immunoassay or Western Blotting.

EXAMPLE 13

The effect of murine IT9302 homologous to human IT9302 on TNF- α release in plasma after LPS administration in mice

It has been demonstrated in animals that recombinant murine
15 IL-10 can protect mice from lethal endotoxemia (89) and in addition, it has been demonstrated that IL-10 administered in humans has inhibitory effects on T-cells and suppresses production of proinflammatory cytokines (90). The aim of the study was to test whether IT9302 can suppress production of
20 TNF- α in mice exposed to endotoxin-induced shock after administration of LPS.

Eight week old BALB/C mice were used (obtained from Center for Research Animals, Bomholtgaard, Bomholtvej 10, DK-8680 Ry, Denmark). LPS from *E. coli* was from Sigma (batch 3444114)
25 and murine IT9302 was from Schafer-N, Lersø Parkallé 42, DK-2100 Copenhagen Ø, Denmark.

Mice were injected intraperitoneally with 100 μ g of LPS in 1 ml of 0.9 M NaCl. 60 minutes prior to LPS injection mice were given murine IT9302 equivalent to 0.1 μ g, 1 μ g and 10 μ g
30 of human IL-10. Groups of 5 animals were anaesthetized (Imobilon, Pherrovet, Malmö, Sweden) after 1, 2, 3 and 4 hours, respectively, after which a maximum of whole blood was drawn

through cardiac puncture. The blood was centrifuged in the cold and sera were collected and stored at -70°C for subsequent analysis of $\text{TNF-}\alpha$ content by ELISA ($\text{TNF-}\alpha$ Mouse Immuno Assay Diagnostic kits, Genzyme, Cambridge, MA, USA).

- 5 As demonstrated, it was observed that $10\text{ }\mu\text{g}$ of murine IT9302 clearly suppressed $\text{TNF-}\alpha$ in mouse sera compared to the control group not treated with mIT9302 (see Table 5).

TABLE 5

m-TNF- α ng/ml + SEM in serum of mice

10	Murine IT9302 equivalent to hIL-10 added 60 minutes before LPS				
15	0	0.1 μg	1 μg	10 μg	
1 h	2.48 $\pm$ 0.16	1.92 $\pm$ 0.48	2.64 $\pm$ 0.38	1.84 $\pm$ 0.16	
2 hs	1.43 $\pm$ 0.17	1.68 $\pm$ 0.27	1.16 $\pm$ 0.21	0.62 $\pm$ 0.14	
3 hs	0.48 $\pm$ 0.04	0.05 $\pm$ 0.02	0.18 $\pm$ 0.05	0.0	
4 hs	0.09 $\pm$ 0.06	0.06 $\pm$ 0.02	0.20 $\pm$ 0.04	0.05 $\pm$ 0.01	
20					

EXAMPLE 14

The modulation of acute pancreatitis induced by bile acid using murine IT9302

- Rodents, mice and rabbits are often used in animal models, and recently Genzyme Diagnostics has advertized with Cross-reactivity Kits for Cytokine Research Products. Especially, it was possible to measure rabbit $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ by mouse Immunoassay Kits. As we were working on a rabbit model, studying the pathophysiological role of IL-8 in experimental acute pancreatitis, we investigated the effect of the murine IT9302 on induced leukopenia (Fig. 14). Our hypothesis was that IL-10 could be quite identical in mice and rabbits and

might have effects on IL-8-induced leukocyte invasion in the pancreas during acute pancreatitis.

Experimental model

New Zealand white rabbits (*Oryctolagus cuniculus*) weighing
5 1.7-4.0 kg were fasted for 18-24 hours. The operative procedures according to (91) and (92) were followed, and acute pancreatitis was induced in rabbits by 5% bile acid given into the pancreatic duct. Murine IT9302 (100 μ g/kg) was given
10 through a central vein 30 minutes before the bile and subcutaneously in a dose of 100 μ g/kg rabbit immediately afterwards. Plasma samples were collected according to Fig. 14.

As demonstrated, it was observed that injection of murine IT9302 into rabbits before induced pancreatitis prevented leukopenia as demonstrated in the Figure.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(B) STREET: Pilegårdsvej 233
(C) CITY: Hasselager, Kolt
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(F) POSTAL CODE (ZIP): 8361

(ii) TITLE OF INVENTION: Immunomodulators

(iii) NUMBER OF SEQUENCES: 23

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Ala Tyr Met Thr Met Lys Ile Arg Asn
1 5

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Ala Tyr Met Thr Ile Lys Met Arg Asn
1 5

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Ala Phe Met Thr Leu Lys Leu Arg Asn
1 5

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ala Tyr Met Thr Met Lys Val Arg Glu
1 5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Gly Tyr Met Thr Met Lys Ile Arg Asp
1 5

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Ala Phe Met Thr Met Lys Ile Arg Asp
1 5

(2) INFORMATION FOR SEQ ID NO: 7:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Ala Tyr Ile Thr Met Lys Ile Arg Asp
1 5

(2) INFORMATION FOR SEQ ID NO: 8:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Ala Tyr Leu Thr Met Lys Ile Arg Asp
1 5

(2) INFORMATION FOR SEQ ID NO: 9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 9 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Ala Tyr Val Thr Met Lys Ile Arg Asp
1 5

(2) INFORMATION FOR SEQ ID NO: 10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 9 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Ala Tyr Met Thr Ile Lys Ile Arg Asp
1 5

(2) INFORMATION FOR SEQ ID NO: 11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 9 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

Ala Tyr Met Thr Leu Lys Ile Arg Asp
1 5

(2) INFORMATION FOR SEQ ID NO: 12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 9 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS:

(D) TOPOLOGY: linear -

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Ala Tyr Met Thr Val Lys Ile Arg Asp
1 5

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

Ala Tyr Met Thr Met Lys Ile Arg Asp
1 5

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

Ala Tyr Met Thr Met Lys Met Arg Asp
1 5

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

Ala Tyr Met Thr Met Lys Val Arg Asp
1 5

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

Ala Tyr Met Thr Met Lys Ile Arg Gln
1 5

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

Ala Tyr Met Thr Met Lys Ile Arg Glu
1 5

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

Ser Pro Gly Gln Gly Thr Gln Ser Glu
1 5

(2) INFORMATION FOR SEQ ID NO: 19:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

Thr Xaa Lys Xaa Arg Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 20:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

Xaa Thr Xaa Lys Xaa Arg Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 21:

- (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

Xaa Xaa Thr Xaa Lys Xaa Arg Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 22:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 9 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

Xaa Xaa Xaa Thr Xaa Lys Xaa Arg Xaa
1 5

(2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

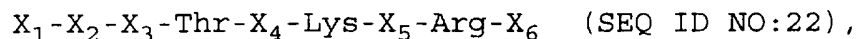
(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GCCTACATGA CAATGAAGAT ACGAAAC

CLAIMS

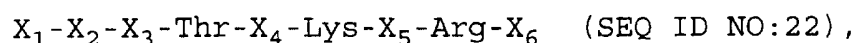
1. Use of a substance or polypeptide according to the formula



wherein

- 5 X_1 is Ala or Gly,
 X_2 is Tyr or Phe,
 X_3 , X_4 and X_5 are independently selected from the group consisting of Met, Ile, Leu and Val; and
 X_6 is selected from the group consisting of Asp, Gln and Glu,
- 10 optionally at least one of X_1 , X_2 , X_3 , X_4 , X_5 and X_6 is independently substituted with non-natural or unusual amino acids and/or the peptide is cyclized and/or the peptide is stabilized and/or the amino terminal amino acid residue is acylated and/or the carboxy terminal amino acid residue is amidated,
- 15 and peptidomimetics modelled on the basis of the above formula for the preparation of a pharmaceutical composition for the reduction of TNF α production.

2. Analogues of the peptides having the formula



20 wherein

- X_1 is Ala or Gly,
 X_2 is Tyr or Phe,
 X_3 , X_4 and X_5 are independently selected from the group consisting of Met, Ile, Leu and Val; and
- 25 X_6 is selected from the group consisting of Asp, Gln and Glu

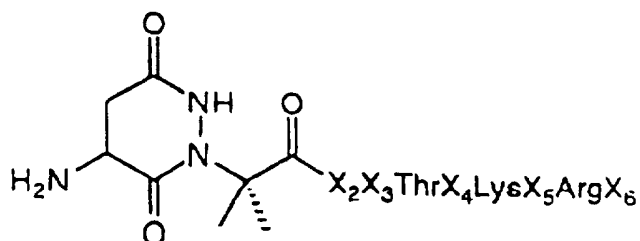
wherein at least one of X_1 , X_2 , X_3 , X_4 , X_5 and X_6 is independently substituted with non-natural or unusual amino acids and/or the peptide is cyclized and/or the peptide is stabilized

zed and/or the amino terminal amino acid residue is acylated and/or the carboxy terminal amino acid residue is amidated, and peptidomimetics modelled on the basis of the above formula, said analogues having at least one of the following

5 properties:

- a) induces inhibition of spontaneous IL-8 production by human monocytes,
- b) induces inhibition of IL-1 β induced IL-8 production by human peripheral blood mononuclear cells (PBMC),
- 10 c) induces production of interleukin-1 receptor antagonistic protein (IRAP) by human monocytes,
- d) induces chemotactic migration of CD8+ human T lymphocytes *in vitro*,
- e) desensitizes human CD8+ T cells resulting in an unresponsiveness towards rhIL-10,
- 15 f) suppresses the chemotactic response of CD4+ T human lymphocytes towards IL-8,
- g) suppresses the chemotactic response of human monocytes towards MCAF/MCP-1,
- 20 h) does not inhibit class II MHC molecule expression on human monocytes, in contrast to human IL-10.
- i) induces the production of IL-4 by cultured normal human CD4+ T cells,
- j) reduces the TNF α production in human mixed leukocyte
- 25 reaction.

3.



4. A pharmaceutical composition comprising a substance or polypeptide according to claim 2 or 3.

5. Use of a substance or polypeptide according to claim 2 or 3 for the treatment or prophylaxis of one or more of the diseases mentioned in Table 3.

5 6. Use of a substance or polypeptide according to claim 2 or 3 for the manufacture of a pharmaceutical composition for the treatment or prophylaxis of one or more of the diseases mentioned in Table 3.

10 7. A method of treating and/or preventing one or more of the diseases mentioned in Table 3, the method comprising administering to a patient in need thereof a therapeutically or prophylactically effective amount of a substance or polypeptide according to claim 2 or 3.

[illegible]

Comparison of the predicted amino acid sequences of mL-10, hIL-10, and BCRF1. Amino acid sequence identities are indicated by vertical lines.

Fig. 1

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COOH TERMINAL PEPTIDE SEQUENCES OF IL-10 INCLUDING
9 AMINO ACIDS OF PORCINE, HUMAN and VIRUS PROTEINS.

PORCINE IL-10		*		*		*														
PIT	-	A	-	Y	-	M	-	T	-	M	-	K	-	M	-	R	-	K	-	N
HUMAN IL-10						*		*		*										
IT 9302	-	A	-	Y	-	M	-	T	-	M	-	K	-	I	-	R	-	N		
BCRFI IL-10						*		*		*										
VIT	-	A	-	Y	-	M	-	T	-	I	-	K	-	A	-	R	-			

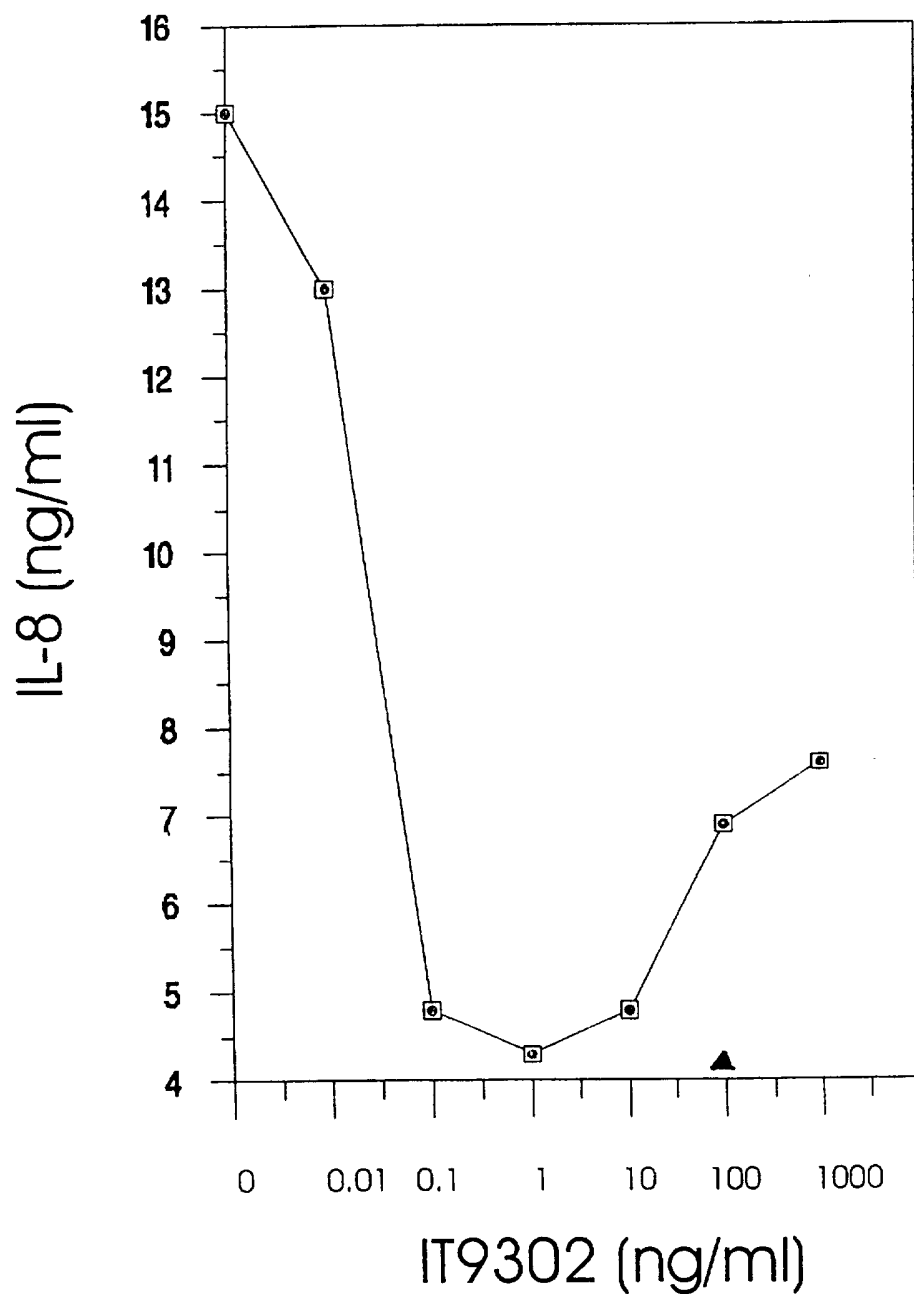
Aminoacids marked with * represent key position for
receptor binding and are evolutionary well conserved.
Compare the IL-10 aminoacid sequence from mouse below.

MOUSE IL-10								*												
MIT	-	A	-	Y	-	M	-	M	-	I	-	K	-	M	-	K	-	S		

This sequence is lacking the homology to the important
amino acids.

Fig. 2

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IT9302 inhibits spontaneous IL-8 production by purified cultured monocytes. (▲) Indicates the level of IL-8 when using rh IL-10 (100 ng/ml).

Fig. 3

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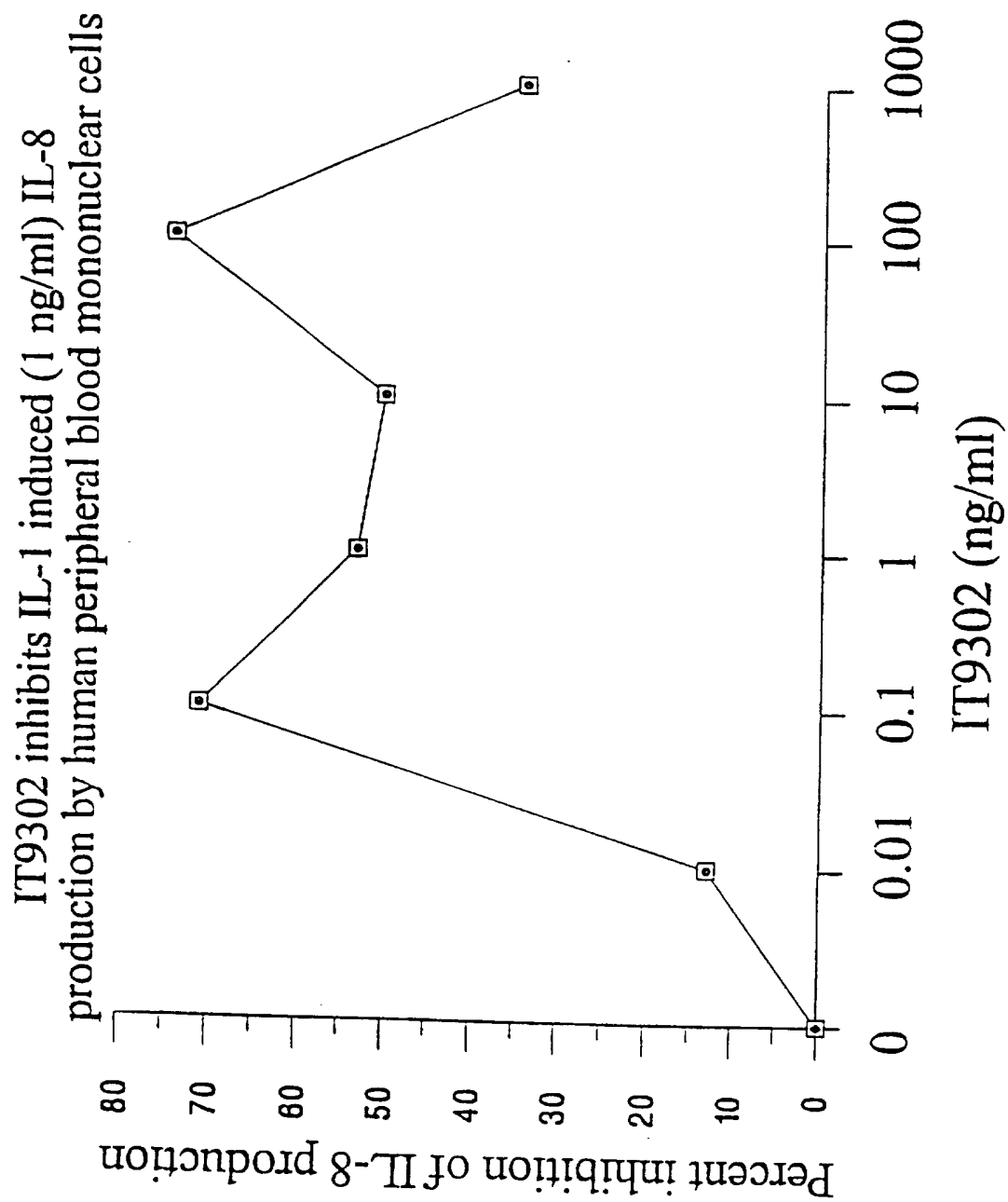


Fig. 4

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IT9302 stimulated human monocytes

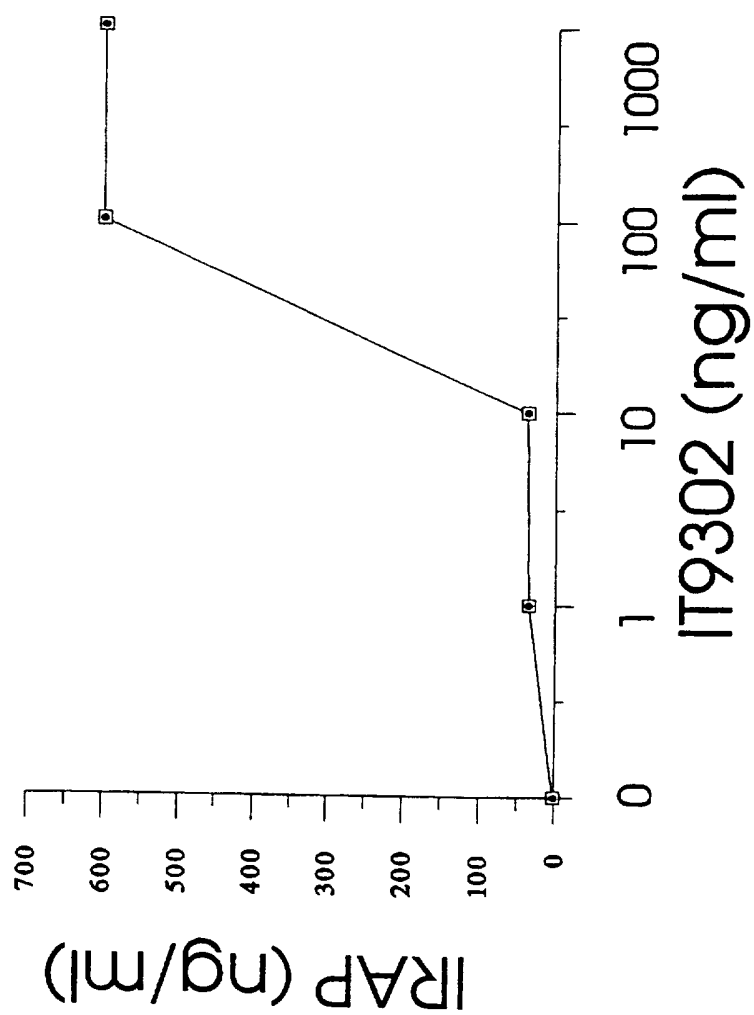
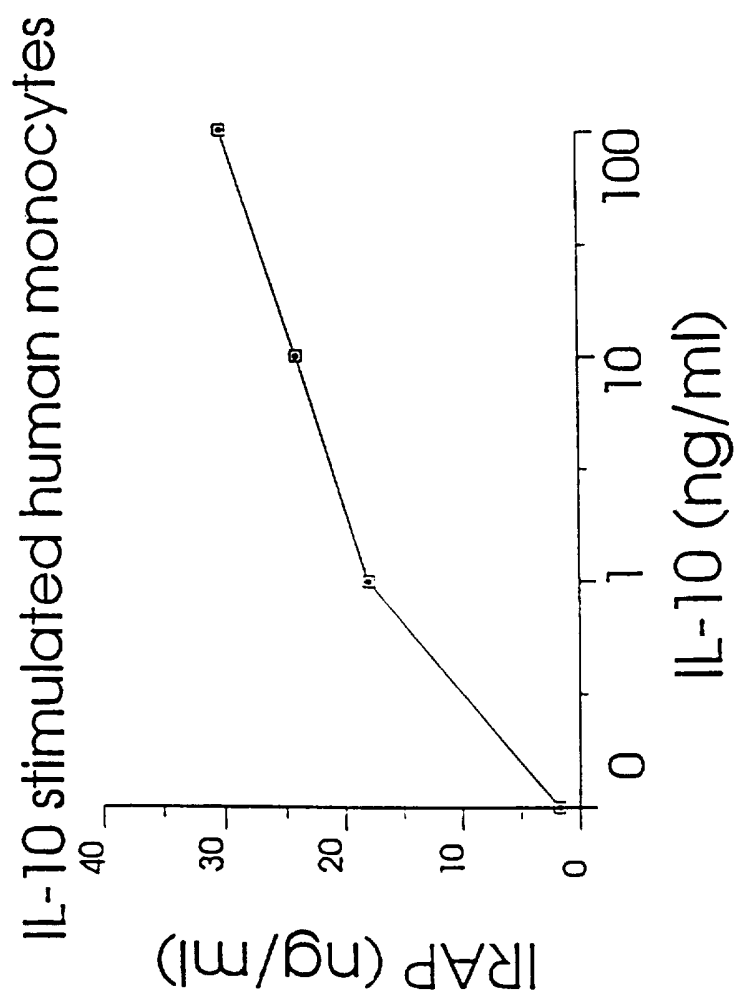
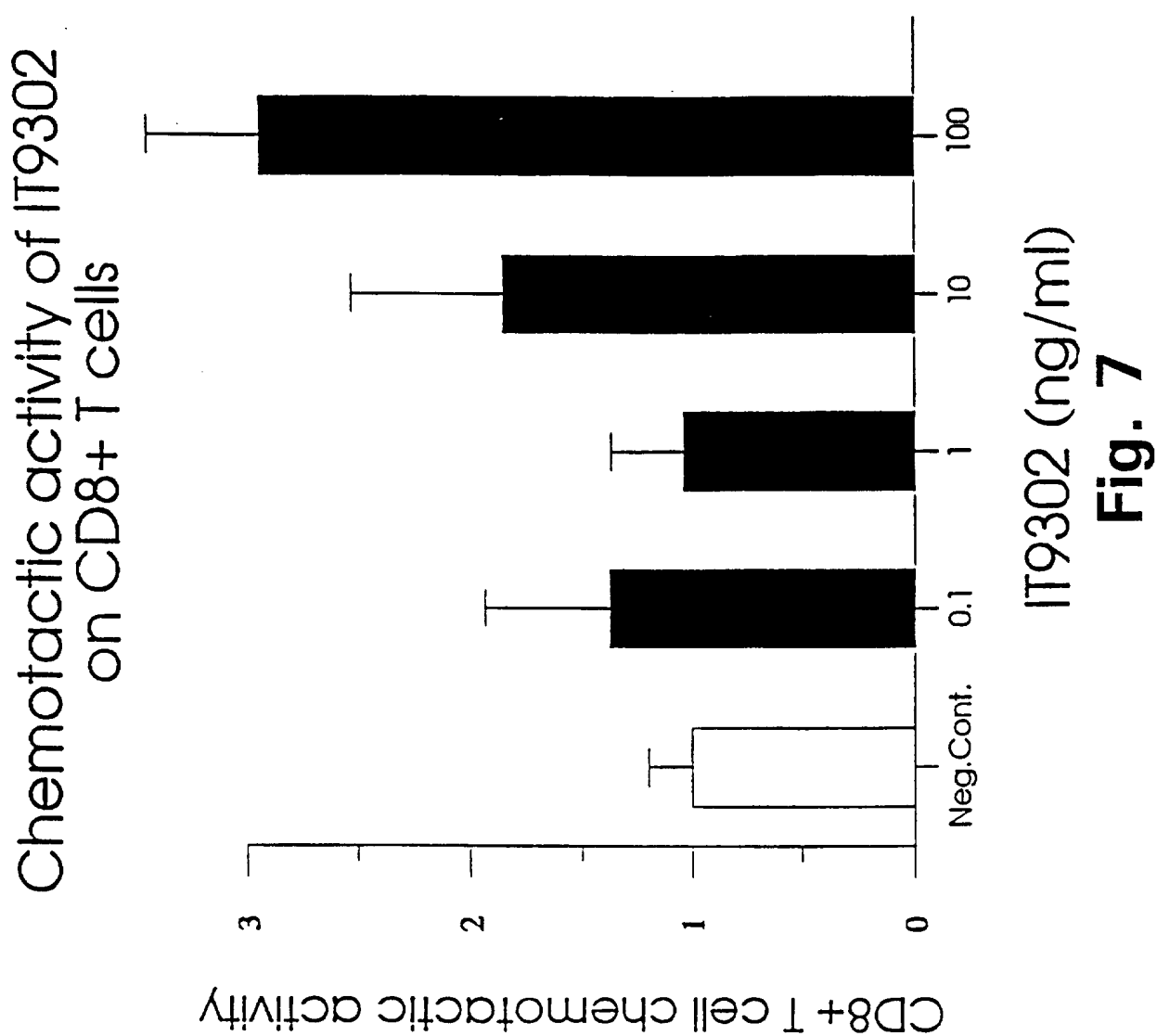


Fig. 5

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**Fig. 6**

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Desensitization of CD8+ T cells by
IT9302, resulting in unresponsiveness
of CD8+ T cells towards IL-10 (10 ng/ml) induced chemotaxis

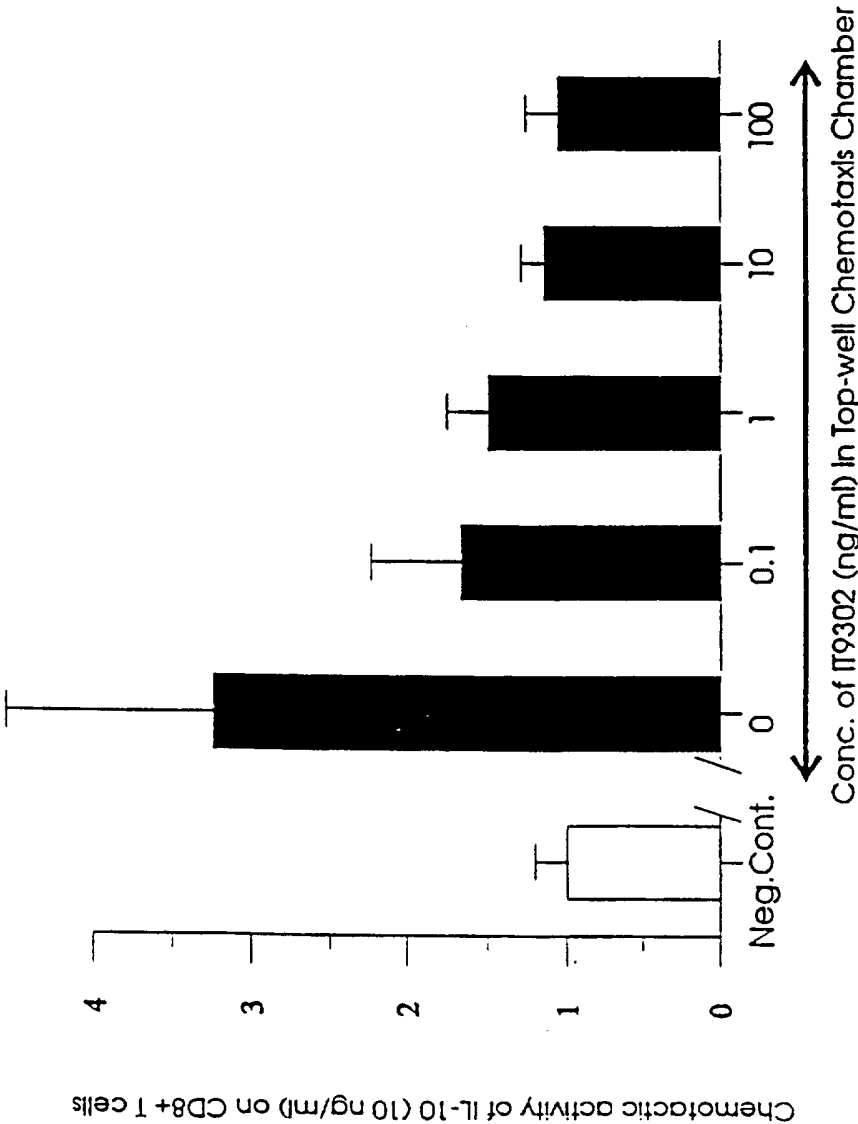


Fig. 8

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Suppression of IL-8 activity by IT9302

Effect of IT9302 on IL-8 (10 ng/ml) mediated chemotaxis.

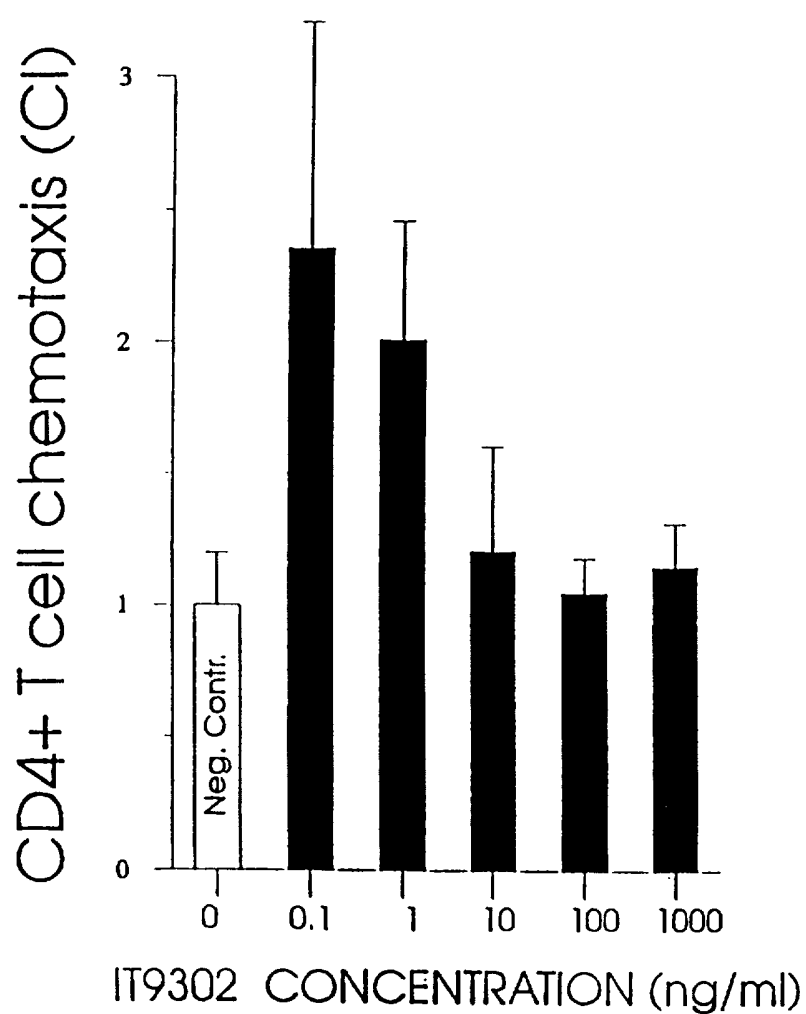


Fig. 9

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IT9302 inhibits MCAF/MCP-1
induced monocyte chemotaxis

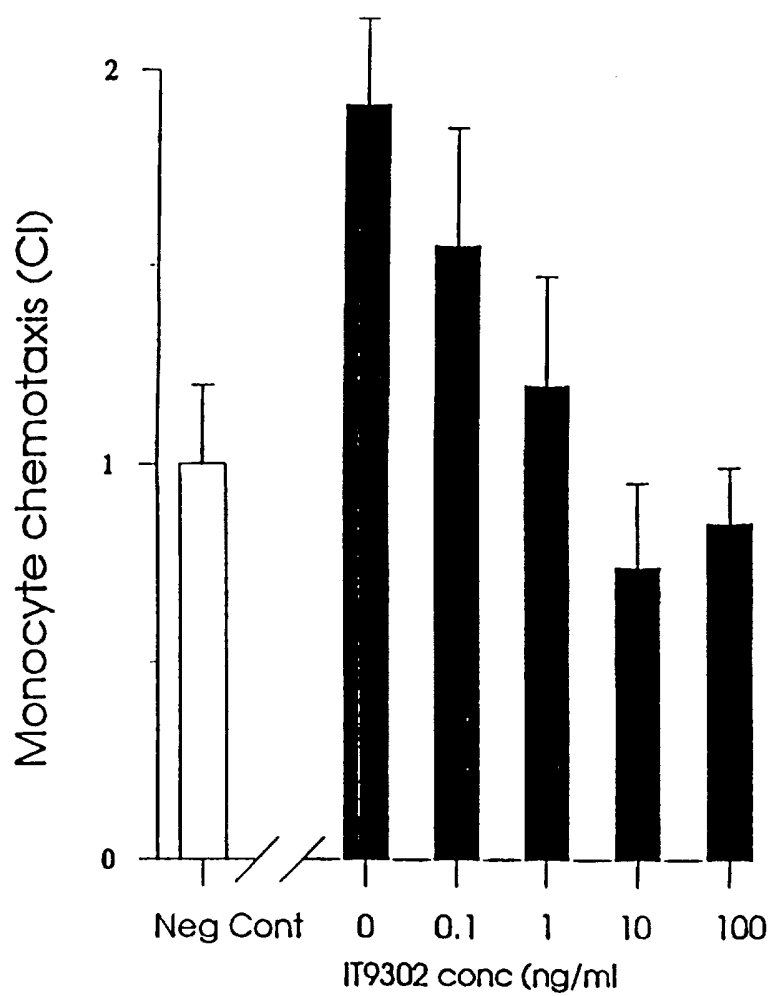
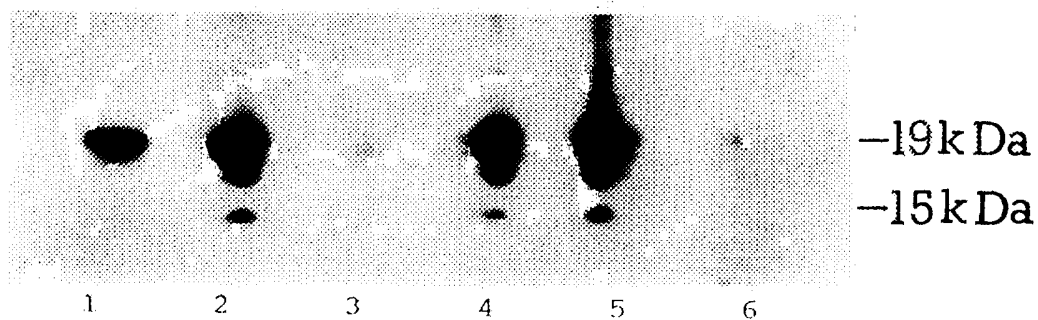


Fig. 10

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ECL-Western Blotting of CD4+ T cell cytosolic proteins using a goat anti-human IL-4 antibody.



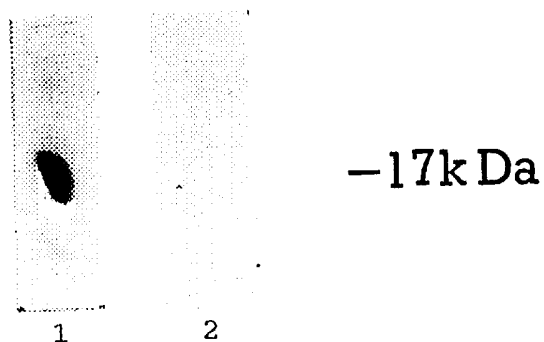
Stimulation for 3 days with:

1. Control
2. mon.anti-IL-8 antibody WS.4 10 μ g/ml,
3. rIL-8 100 ng/ml,
4. rIL-10 100 ng/ml,
5. IT9302 10 ng/ml,
6. rIFN gamma 10 ng/ml.

Fig. 11
SUBSTITUTE SHEET (RULE 26)

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ECL-Western Blotting of Human Mixed lymphocyte culture cytosolic proteins using a rabbit anti-human TNF- α antibody.



Stimulation for 24 hours with:

1. Control
2. IT9302 10 ng/ml

Fig. 12
SUBSTITUTE SHEET (RULE 26)

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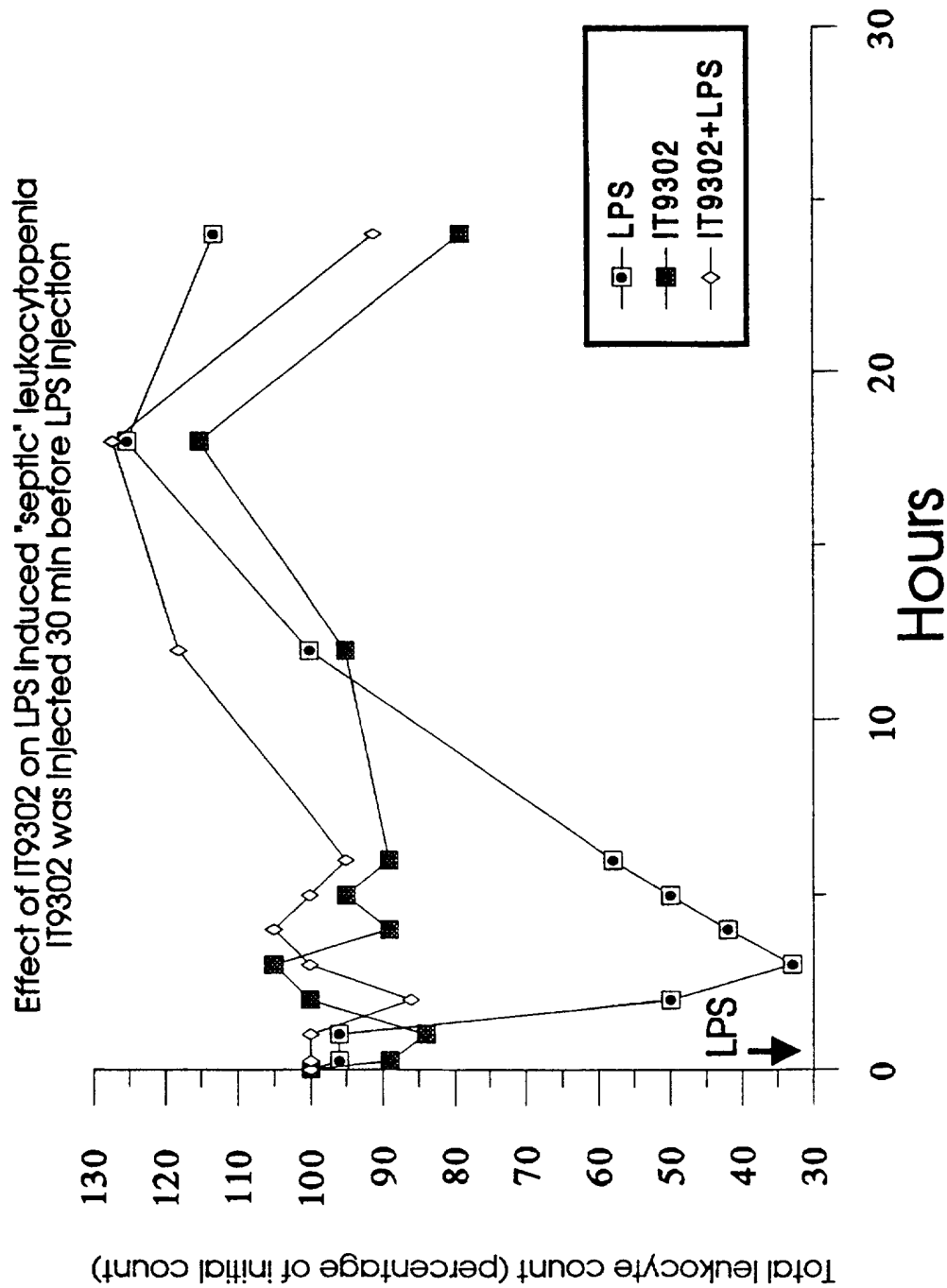
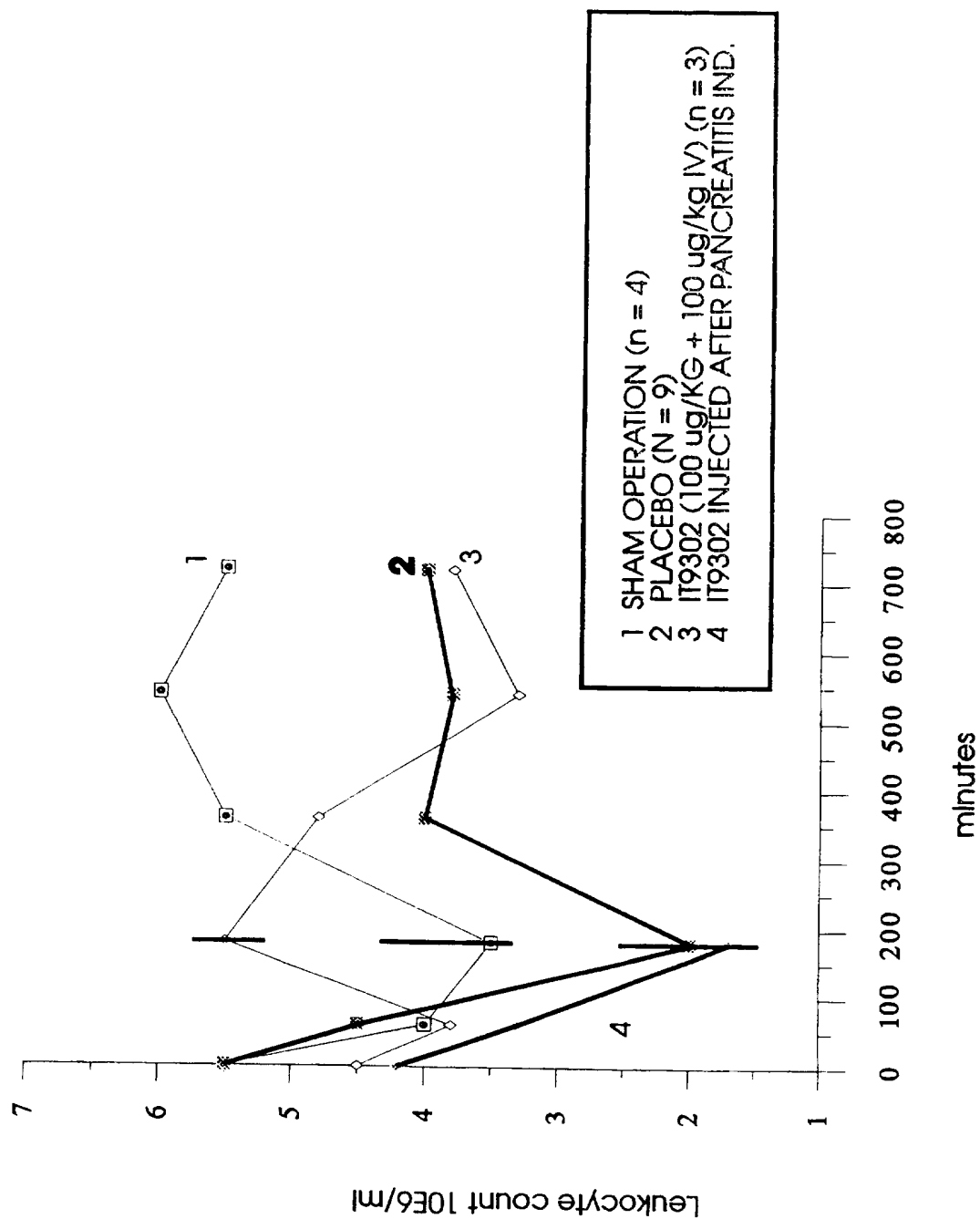


Fig. 13

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Leukocyte count in peripheral blood (rabbits)
after induction of acute pancreatitis (5% bile acid)

**Fig. 14**

INTERNATIONAL SEARCH REPORT

International Application No

PC 1/DK 96/00029

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/54 A61K38/20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 405 980 (SCHERING CORPORATION) 2 January 1991 see the whole document ---	1-7
E	WO,A,96 01318 (NYCOMED DAK A/S) 18 January 1996 see the whole document -----	1



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

Date of the actual completion of the international search

3 September 1996

Date of mailing of the international search report

16. 09. 96

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Authorized officer

Masturzo, P

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK96/00029

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 7
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 7 refers to a method of treatment of the human body, the search was carried out and based on the alleged effects of the compounds.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/DK 96/00029

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