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(54) Title: TREATING AND DIAGNOSING MACROPHAGE-MEDIATED DISEASES USING FC RECEPTOR LIGANDS

(57) Abstract

The invention provides methods and compositions for selectively targeting macrophages in a localized area. The compositions of the invention include an Fc receptor binding agent, and a toxic or a detectable agent. Methods for depleting or inhibiting the activity of macrophages using the compositions of the invention are disclosed. The compositions of the invention can be used therapeutically and diagnostically.

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TREATING AND DIAGNOSING MACROPHAGE-MEDIATED DISEASES USING FC RECEPTOR LIGANDS

Background of the Invention

In normal human skin, two compartmental layers can be distinguished. The upper layer, the epidermis, consists of keratinocytes, Langerhans' cells and T cells. The lower layer, the dermis, consists of fibroblasts, endothelial cells, dendritic cells, T cells, mast cells and macrophages.

The skin serves as an important boundary between the internal milieu and the environment. It primarily prevents contact with potentially harmful antigens. In case of antigen/pathogen penetration, an inflammatory response is induced *in vivo* to eliminate the antigen. This response leads to a dermal infiltrate, the composition of which depends on the type of response induced, but consists predominantly of T cells, polymorphonuclear cells, and monocytes (Williams, I.R., and Kupper, T.S. (1996.) *Life Sci.* 58: 1485-1507; Stingl, G. (1993) *Recent Results Cancer Res.* 128: 45-57). In addition, allergen nonspecific stimuli like tissue injury and ultraviolet light can also trigger an inflammatory response. In general, mechanisms underlying the allergen non-specific response are also employed during the effector phase of the allergen-specific response.

Macrophages are bone-marrow derived cells with great heterogeneity and versatility. These cells can produce a wide range of mediators and exert a multitude of biological functions (Ganz, T. (1993) *New Horiz.* 1: 23-27). Their phenotype and function is largely determined by local environment, whereas macrophage-derived mediators can thereupon influence their microenvironment. This microenvironment leads to regionally different subsets of macrophages and even locally, different macrophage subsets can be present (Gordon, S. (1995) *Bioessays* 17: 977-986). These cells are potent effector cells producing reactive oxygen products and proteolytic enzymes, which can directly damage tissue (Laskin, D.L., and Pendino, K.J. (1995) *Annu Rev Pharmacol. Toxicol.* 35:655-677). Under normal conditions, macrophages regulate proliferation of extracellular matrix-forming cells like fibroblasts in skin (Gonzalez-Ramos, A. *et al.* (1996) *J. Invest. Dermatol.* 106: 305-311). In addition,

macrophages can exert important immunoregulatory functions and in this way play a crucial role in controlling and directing immune responses (Gordon, S. (1995) *Bioessays* 17:9 77-986; Thepen, T. *et al.* (1994) *Ann. N. Y. Acad. Sci.* 725:200-206). These cells can serve as antigen presenting cells, but also directly inhibit antigen presentation by dendritic cells (Holt, P.G. *et al.* (1993) *J. Exp. Med.* 177:397-407). Proliferation, phenotype and thus function of T cells, and thereby the type of immune response induced, can be influenced by macrophages.

Skin macrophages have been shown to play an important role in the regulation of cell growth of different non-hematopoietic cells (such as fibroblasts and keratinocytes), as well as in the functioning of T cells and dendritic cells. Under "steady state" conditions, the number of skin macrophages is relatively low. However, under various pathological conditions (for example, in active lesions), the number of macrophages is significantly increased. Tissue macrophages and infiltrating monocytes have been associated with modified fibroblast and keratinocyte function in inflammatory lesions, as well as aberrant functioning of T cells and/or dendritic cells.

Ultraviolet light exposure has been shown to induce a population of macrophages in the skin that, in contrast to Langerhans' cells, are capable of activating autoreactive T cells. Deregulated macrophage function has been directly correlated with abnormal cutaneous immune responsiveness in various diseases, including cutaneous T cell lymphoma (mycosis fungoides), psoriasis, atopic dermatitis, and cutaneous lupus erythematosus (Cooper, K.D. *et al.* (1993) *J. Invest. Dermatol.* 101: 155-163; Gonzalez-Ramos, A. *et al.* (1996) *J. Invest. Dermatol.* 106: 305-311). These cells can also activate resident and inflammatory macrophages, resulting in a "vicious circle" which maintains the cutaneous inflammation. In addition to the regulation of cell function, macrophages are potent producers of toxic compounds such as oxygen radicals and proteolytic enzymes. These toxic compounds have been shown to cause direct tissue damage.

Summary of the Invention

The present invention provides methods and compositions for selectively targeting cytotoxic compounds via Fc receptors to monocyte-derived phagocytic cells (i.e., macrophages). The invention can thus be used to selectively reduce the number or

activity of a population of macrophages within a localized area, such as the skin, joints or lungs.

In one embodiment, the invention provides a macrophage-binding compound which contains at least a first portion which binds to an Fc receptor present on a macrophage, and at least a second portion which kills or inhibits the function of the macrophage. The portion which binds to the Fc receptor can include any molecule capable of Fc receptor binding, such as an antibody, a peptide (e.g., peptide mimetic)or a chemical compound. In one embodiment, the Fc receptor binding portion is an antibody or antibody fragment (e.g., an Fab, Fab', $F(ab')_2$, Fv, or a single chain Fv). In a preferred embodiment, the anti-Fc receptor antibody or antibody fragment is "humanized" (e.g., has at least a complementarity determining region (CDR) or a portion thereof derived from a non-human antibody (e.g., murine) with the remaining portion(s) are human in origin). In another preferred embodiment, the anti-Fc receptor antibody or antibody fragment is a human monoclonal antibody (e.g., an antibody produced in a mouse genetically-engineered to express a completely human antibody). Also included among these embodiments are compounds (e.g. peptides or chemical species) which "mimic" the binding of such anti-Fc receptor antibodies (Jenks et al. *J. Natl. Cancer Inst.* (1992) 84(2):79; Saragovi et al. *Science* (1991) 253:792; Hinds et al. *J. Med. Chem.* (1991) 34:1777-1789; Fassina *Immunomethods* (1994) 5:121-129). In another embodiment, the Fc receptor binding portion of the macrophage-binding compound is a cyanin composition, such as the fluorescent dye Cy5.18.OSu (referred to herein as "Cy5"), which binds with high affinity and specificity to the FcγRI receptor present on macrophage cells. The cyanin compositions can include at least two moieties: a cyanin succinimidyl ester and a phycobilisome protein, e.g., PE.

The Fc receptor recognized by the macrophage-binding compounds of the invention can be an IgG receptor, e.g., an Fc-gamma receptor (FcγR), such as FcγRI (CD64), FcγRII(CD32), and FcγRIII (CD16), or an IgA receptor, e.g., an FcαR (e.g., Fc αRI, CD89). The Fc receptor is preferably located on the surface of a macrophage, e.g., a skin macrophage, so that it is capable of being recognized and bound by the compound. In a preferred embodiment, the anti-Fc receptor binding portion of the

macrophage-binding compound binds to an Fc receptor at a site which is distinct from that bound by endogenous immunoglobulins (e.g., IgGs or IgAs). Therefore, the binding of the macrophage-binding compounds to the Fc receptor is not blocked by physiological levels of immunoglobulins.

A preferred Fc receptor on a macrophage for targeting is the high affinity Fcγ receptor, FcγRI. Thus, in one embodiment, the anti-Fc receptor binding portion of the macrophage-binding compounds of the invention comprise an anti-FcγRI antibody, or a fragment thereof. Exemplary anti-FcγRI antibodies include mAb 22, mAb 32, mAb 44, mAb 62 and mAb 197. In preferred embodiments, a humanized form of such anti-FcγRI receptor antibodies are used, such as humanized monoclonal antibody 22 (H22), or a fragment thereof.

The portion of the macrophage-binding compound which kills or modulates (e.g., reduces) the activity of a macrophage (the anti-macrophage agent) can be selected from suitable cytotoxins or drugs. For example, the anti-macrophage agent can be Gelonin, Saporin, Onconase, Exotoxin A, Ricin A, dichloromethylene diphosphonate (CL2MDP), or derivatives thereof. In one embodiment, the anti-macrophage agent is directly linked to the anti-Fc receptor binding portion of the macrophage-binding compound. In another embodiment of the invention, the anti-macrophage agent is indirectly linked to the anti-Fc receptor binding portion. For example, the anti-macrophage agent can be encapsulated within a liposome which is linked to the anti-Fc receptor binding portion.

Macrophage-binding compounds of the invention can be used in a variety of therapeutic and diagnostic methods. In one embodiment, these compounds are used to diagnose a disease characterized by abnormal numbers or function of macrophages. The method involves contacting or administering to a test area, or a cultured sample, the macrophage-binding compound under conditions that allow for binding of the compound to macrophages present in the sample. Binding of the compound can then be detected as an indication of the presence (e.g., number) and/or function of macrophages in the sample. For example, a statistically significant elevated level of Fc receptor protein specifically detected, indicating an increase in the number of macrophages, can

be indicative of a disease. The test area or sample can be from, e.g., the skin (e.g., human skin) or other tissue containing macrophage cells.

In another embodiment, the macrophage-binding compounds are used to treat a disease involving proliferation and/or abnormal functioning of macrophages. Upon contacting macrophage-binding compounds with an area needing treatment, the compounds bind to macrophages via their Fc receptors and kill or reduce the activity of these cells. Accordingly, a broad variety of diseases involving macrophages (e.g., macrophage proliferation and/or abnormal functioning) can be treated, prevented or diagnosed using the compounds of the invention. Such diseases can be of intrinsic origin, (e.g. autoimmune disease), or extrinsic origin, (e.g.: contact hypersensitivity, Polymorphic Light Eruption (PLE), and irritants reactions). Skin disease can furthermore be a manifestation of a more systemic disease like atopic dermatitis (AD) in the case of atopy, and systemic lupus erythematosus. A non-limiting list of the diseases that can be treated with the compositions and methods of the present invention include autoimmune diseases, respiratory diseases, infectious diseases, dermatological diseases and inflammatory conditions. Specific examples of such diseases include, but are not limited to, psoriasis, atopic dermatitis, multiple sclerosis, scleroderma, cutaneous lupus erythematosis, rheumatoid arthritis, Human Immunodeficiency Virus (HIV) infections, Chronic Polymorphic Light Dermatosis (CPLD), Chronic Obstructive Pulmonary Diseases (COPD), e.g., allergic asthma and Sarcoidosis, Wegener's Granulomatosis, and inflammatory conditions, such as skin lesions (e.g., open wounds or burn wounds). Additionally, the methods and compositions of the invention can be used *in vitro* to diagnose such diseases, or for research purposes (e.g., to study the pathological role of macrophages in such diseases).

When used *in vivo* for therapeutic purposes, macrophage binding compounds of the invention can be locally administered (e.g., topically, intradermally, subcutaneously or by inhalation as an aerosol) to a selected area in an amount effective to deplete, or reduce the activity of macrophages within the area of administration. In certain embodiments, the macrophage binding compound can include a photosensitizing agent which is inactive when administered (e.g., systemically, topically, intramuscularly), but is activated by exposure to light (e.g., visible or UV light). Similarly, the macrophage

binding compounds can include an Fc binding agent linked to a therapeutic (or diagnostic reagent) via a photocleavable linkage, which upon light exposure releases the reagent. These compounds allow for controlled killing or inactivation of macrophages only within selected tissues exposed to light.

The present invention further provides compositions, e.g., a pharmaceutical compositions, containing macrophage-binding compounds along with an acceptable carrier or diluent, for use in the methods described above.

Other features and advantages of the invention will be apparent from the following figures, detailed description, examples and claims.

Brief Description of the Drawings

Figure 1 is a bar graph depicting the percentage of [^{3}H]-Thymidine incorporation of cultured U937 or IIA1.6 cells grown in the presence or absence of varying concentrations of a CD64-immunotoxin (H22-Ricin A, H22-R, or 197-Ricin A, 197-R) as compared with that of medium control (±SEM). U937 cells were cultured either with (black bars) or without (gray bars) IFNγ in the presence of the indicated concentrations of H22-R (panel A) or 197-R (panel B). In the lower panels C and D, IIA1.6 cells, either transfected with hFcγRI (black bars) or non-transfected (gray bars), were incubated with varying concentrations of H22-R (panel C) or 197-R (panel D).

Figure 2 is a scan of propidium iodide fluorescence of U937 cells as these cells undergo apoptosis after incubation with varying concentrations of H22-R. Nuclear fragmentation was analyzed with propidium iodide staining and subdiploid nuclei are indicated by bars. Numbers above bars specify percentage of subdiploid, hence apoptotic nuclei. Con= Control.

Figures 3A and B are graphs showing the effect of a single intradermal injection of an immunotoxin on inflammatory cells in skin with respect to time. Data points represent mean number of cells per mm^2 (±SEM) and data points represent the average of >3 experiments. Depicted are the kinetics of hFcγRI-expressing cells (filled square, Figure 3*A*), macrophages (blank square, Figure 3A), T cells (filled square, Figure 3*B*), and dendritic cells (blank square, Figure 3*B*).

Figures 4A-4B are graphs showing a decrease in local skin temperature upon intradermal injection of an immunotoxin. Figure 4A depicts local skin temperature readings (±SEM) of SLS treated hFcγRI transgenic mice after a single injection with IT (●)(n=6) or vehicle control (O) (n=6). Figure 4B shows temperature course (± SEM) of SLS treated hFcγRI-transgenic mice, injected with either IT (O) (n=6), or vehicle control (●) (n=6). Local skin temperature was monitored daily, and upon increase, animals were re-injected at the same site (days marked with *).

Detailed Description of the Invention

Abnormal macrophage function, including aberrant proliferation and/or activity, has been implicated in a variety of disorders, such as dermatological diseases, autoimmune diseases, infectious diseases and inflammatory conditions. To date, methods of localized ablation of macrophages using cytotoxic agents, e.g., immunotoxins, have had limited efficacy. The present invention provides methods and compositions for diagnosing, treating and preventing such disorders by selectively depleting and/or inhibiting the activity of macrophages within a localized area. Cells are depleted (e.g., killed) and/or inhibited (e.g., activity reduced) by targeting a toxic agent to them via their Fc receptors. For example, studies described herein demonstrate the use of a macrophage-binding compound consisting of an anti-Fc receptor binding portion, e.g., a humanized antibody against a human FcγRI receptor, conjugated to a toxin, e.g., Ricin A, to selectively eliminate macrophages *in vivo* in transgenic mice expressing human FcγRI. As used herein, the terms "macrophage" and "monocyte-derived phagocytic cell" shall be used interchangeably.

Accordingly, in one embodiment, the invention provides a macrophage-binding compound comprising an agent which binds to an Fc receptor present on a macrophage and an agent which kills or inhibits the activity of the macrophage which is bound. Suitable components for binding Fc receptors include, for example, proteins (e.g., anti-FcR antibodies and peptide or chemical mimetics thereof, or FcR receptor ligands) and chemical moieties (e.g., dyes and synthetic FcR ligands). Such Fc receptor binding agents can be monospecific, bispecific or multispecific in that they contain one, two, or

more than two binding regions, respectively. For example, the agent can bind to two or more different regions of an Fc receptor, or to an Fc receptor and a different component of the same or another cell. In all cases, the agent contains at least one portion which binds to an Fc receptor.

In one embodiment, the Fc receptor binding agent is an antibody, or an antibody fragment, including, e.g., an Fab, Fab', $F(ab')_2$, Fv, or a single chain Fv. The antibody may also be a light chain or heavy chain dimer, or any minimal fragment thereof such as a Fv, or a single chain construct as described in Ladner et al. U.S. Patent No. 4,946,778, issued August 7, 1990, the contents of which is expressly incorporated by reference.

In another embodiment, the Fc receptor binding agent is an antibody mimetic (e.g. peptide or chemical compound)(Jenks et al. *J. Natl. Cancer Inst.* (1992) 84(2):79; Saragovi et al. *Science* (1991) 253:792; Hinds et al. *J. Med. Chem.* (1991) 34:1777-1789; Fassina *Immunomethods* (1994) 5:121-129).

In another embodiment, the Fc binding component is a bispecific or a multispecific molecule. The term "bispecific molecule" is intended to include any compound, e.g., a chemical moiety or a protein, peptide, or protein or peptide complex, which has two different binding specificities which bind to, or interact with (a) an Fc receptor on the surface of a macrophage, and (b) a second, different target antigen. The term "multispecific molecule" or "heterospecific molecule" is intended to include any compound, e.g., a chemical moiety, a protein, peptide, or protein or peptide complex, which has more than two different binding specificities which bind to, or interact with (a) an Fc receptor on the surface of a macrophage, (b) two or more different target antigens. Accordingly, Fc receptor binding agents which can be used in macrophage-binding compounds of the invention include bispecific, trispecific, tetraspecific, and other multispecific molecules which are directed to Fc receptors on macrophages.

For example, the agent can be a heteroantibody comprising two or more antibodies, antibody binding fragments (e.g., Fab), or derivatives thereof, linked together which have different specificities. These different specificities can include two or more different binding specificities on an Fc receptor. Alternatively, they can include a binding specificity on an Fc receptor, and at least one other different binding specificity

on the same cell (i.e., a macrophage) or on a different target cell (e.g., another immune cell or a pathogen).

In such embodiments where the Fc binding agent is a bispecific or multispecific molecule, the agent can function to physically bring together a cytotoxic effector cell to a target macrophage, such that more efficient, targeted elimination of the macrophage can be achieved. As used herein, the term "effector cell" refers to an immune cell which is involved in the effector phase of an immune response, as opposed to the cognitive and activation phases of an immune response. Exemplary immune cells include a cell of a myeloid or lymphoid origin, e.g., lymphocytes (e.g., B cells and T cells including cytolytic T cells (CTLs)), killer cells, natural killer cells, eosinophils, neutrophils, polymorphonuclear cells, granulocytes, mast cells, and basophils. Like macrophages, effector cells express specific Fc receptors and carry out specific immune functions. In preferred embodiments, an effector cell is capable of inducing antibody-dependent cellular toxicity (ADCC), e.g., a neutrophil capable of inducing ADCC. For example, neutrophils, eosinophils, and lymphocytes which express FcαR are involved in specific killing of target cells and presenting antigens to other components of the immune system, or binding to cells that present antigens. In other embodiments, an effector cell can phagocytose a target antigen or cell (e.g., a macrophage), or microorganism, or can lyse a target cell, e.g., a macrophage. The expression of a particular Fc receptor on an effector cell can be regulated by humoral factors such as cytokines. For example, expression of FcγRI has been found to be up-regulated by interferon gamma (IFN-γ). This enhanced expression increases the cytotoxic activity of FcγRI-bearing cells against targets, e.g., macrophages.

In other embodiments of the invention, the Fc receptor binding agent is a monoclonal antibody or fragment thereof. The terms "monoclonal antibody" or "monoclonal antibody composition" as used herein refer to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope. The monoclonal antibody can be murine, or a human monoclonal antibody (e.g., an antibody produced in a mouse genetically-engineered to express completely human antibodies).

In still other embodiments of the invention, the Fc receptor binding agent is a chimeric antibody or fragment thereof, or a humanized antibody or fragment thereof. A "chimeric antibody" is intended to include an antibody in which the variable regions are from one species of animal and the constant regions are from another species of animal. For example, a chimeric antibody can be an antibody having variable regions which derive from a mouse monoclonal antibody and constant regions which are human. In a preferred embodiment of the invention, the macrophage-binding compound comprises a humanized antibody or binding fragment thereof. The term "humanized antibody" is intended to include antibodies in which the hypervariable regions, also termed, the complementarity-determining regions (CDRs) are from one species of animal and the framework regions and constant regions of the antibody are from a different species animal species. In a humanized antibody of the invention, the CDRs are from a mouse monoclonal antibody and the other regions of the antibody are human. In preferred embodiments, a human antibody is derived from known proteins NEWM and KOL for heavy chain variable regions (VHs) and REI for Ig kappa chain, variable regions (VKs). The term antibody as used herein is intended to include chimeric and humanized antibodies, binding fragments of antibodies or modified versions of such.

The terms "fragment" or "binding fragment" of an antibody or protein capable of binding to an antigen is intended to include a fragment of the antibody or protein which is sufficient for binding to the antigen. Binding of a binding fragment of an antibody to an antigen can be with the same affinity or a different affinity, e.g., lower or higher affinity, as binding of the whole antibody to the antigen. Examples of binding fragments encompassed within the term antibody include: an Fab fragment consisting of the V_L, V_H, C_L and C_{H1} domains; an Fd fragment consisting of the V_H and C_{H1} domains; an Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody; a dAb fragment (Ward et al., 1989 *Nature* 341:544-546) consisting of a V_H domain; an isolated complementarity determining region (CDR); and an $F(ab')_2$ fragment, a bivalent fragment comprising two Fab' fragments linked by a disulfide bridge at the hinge region. A binding fragment, e.g., a binding fragment of an antibody, can be an active or functional binding fragment. Accordingly, an active or functional binding fragment is intended to include binding fragments which are capable of

triggering at least one activity or function triggered by the full length molecule. For example, an active binding fragment of monoclonal antibody M22 or H22 is a fragment of the antibody that is capable of binding to the FcγR and triggering a receptor-mediated effector cell activity, e.g., production of superoxide anion. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

The terms "an agent which binds to" or "binding specificity" is used interchangeably herein with the terms "antigen binding site, "antigen binding region" and "binding determinant of an antibody." These terms are intended to include the region of a molecule, e.g., an antibody, that are involved in the binding to an antigen. The antigen binding site of an antibody comprises, but is not limited to, the amino acids of the antibody which contact the antigen. The antigen binding region can be the variable region of an antibody. The antigen binding region of an antibody can also be the hypervariable regions of an antibody. The antigen binding region of an antibody can also be the amino acid residues in the hypervariable region of an antibody which contact the antigen and/or which provide proper tertiary structure of the antigen binding region. Various methods are available for determining which amino acid residues of a variable region or hyper variable region of an antibody contact the antigen and/or are important in having a correctly folded antigen binding region. For example, mutagenesis analyses can be performed. In particular, it is possible to substitute one or more amino acids for other amino acids in a recombinantly produced antibody and to perform *in vitro* binding studies to determine the extent to which the binding affinity of the modified antibody for the antigen has changed compared to the non modified antibody. If binding has decreased due to substitution of an amino acid for another, the amino acid is most likely important in binding of the antibody to the antigen. Other methods for determining which amino acids of a variable region of an antibody are involved in binding of the antibody to an antigen are based on crystallographic analyses, e.g., X-ray crystallography.

The term "an antibody which binds specifically to an antigen" is intended to include an antibody which binds to the specific antigen with significantly higher affinity than binding to any other antigen, i.e., it is intended to define the specificity of an antibody as defined in the art. The terms "an antibody recognizing an antigen" and " an antibody specific for an antigen" are used interchangeably herein with the term "an antibody which binds specifically to an antigen".

PRODUCTION OF ANTI-Fc RECEPTOR BINDING AGENTS

I. Production of Anti-Fc Receptor Antibodies

Anti-Fc receptor antibodies for use in macrophage-binding compounds of the invention include antibodies developed using any of a variety of known techniques, provided that the antibody is capable of binding to an Fc receptor on a macrophage. Preferred antibodies are practical for clinical use (e.g., can be administered to humans). Particularly preferred antibodies are non-immunogenic when administered to humans (e.g., are human antibodies produced in transgenic animals), or are modified to reduce immunogenicity when administered to humans (e.g., are humanized).

In one embodiment, the anti-Fc receptor antibody is a monoclonal antibody, e.g., a murine or human monoclonal antibody, which binds to a type IgG receptor or a type IgA receptor, preferably at a site which is not blocked (i.e., bound) by human immunoglobulin G (IgG) or immunoglobulin A (IgA). As used herein, the term "IgG receptor" refers to any of the eight Fcγ receptor genes located on chromosome 1. These genes encode a total of twelve transmembrane or soluble receptor isoforms which are grouped into three Fcγ receptor classes: FcγRI (CD64), FcγRII(CD32), and FcγRIII (CD16). In one preferred embodiment, the Fcγ receptor is a human high affinity FcγRI. The human FcγRI is a 72 kDa molecule, which shows high affinity for monomeric IgG (10^8 - $10^9 M^{-1}$). The production and characterization of these preferred monoclonal antibodies are described by Fanger et al. in PCT application WO 88/00052 and in U.S. Patent No. 4,954,617, the teachings of which are fully incorporated by reference herein. These antibodies bind to an epitope of FcγRI, FcγRII or FcγRIII at a site which is distinct from the Fcγ binding site of the receptor and, thus, their binding is not blocked

substantially by physiological levels of IgG. Specific anti-FcγRI antibodies useful in this invention are mAb 22, mAb 32, mAb 44, mAb 62 and mAb 197. The hybridoma producing mAb 32 is available from the American Type Culture Collection, ATCC Accession No. HB9469. Anti-FcγRI mAb 22, $F(ab')_2$ fragments of mAb 22, and can be obtained from Medarex, Inc. (Annandale, N.J.). The hybridoma producing MAb 22 is available from the ATCC on July 9, 1996 and has been assigned ATCC Accession No. HB-12147. In other embodiments, the anti-Fcγ receptor antibody is a humanized form of monoclonal antibody 22 (H22). The production and characterization of the H22 antibody is described in Graziano, R.F. et al. (1995) *J. Immunol* 155 (10): 4996-5002 and PCT/US93/10384. The H22 antibody producing cell line was deposited at the American Type Culture Collection on November 4, 1992 under the designation HA022CL1 and has the accession no. CRL 11177.

In other embodiments, the anti-FcR antibody is specific for an IgA receptor. The term "IgA receptor" is intended to include the gene product of one α-gene (FcαR) located on chromosome 19. This gene is known to encode several alternatively spliced transmembrane isoforms of 55 to 110 kDa. FcαR (CD89) is constitutively expressed on monocytes/macrophages, eosinophilic and neutrophilic granulocytes, but not on non-effector cell populations. FcαR has medium affinity ($\approx 5 \times 10^7\ M^{-1}$) for both IgA1 and IgA2, which is increased upon exposure to cytokines such as G-CSF or GM-CSF (Morton, H.C. *et al.* (1996) *Critical Reviews in Immunology* 16:423-440). Exemplary anti-Fcα receptor monoclonal antibodies include My 43, A77, A62, A59, and A3 (Monteiro et al. (1992) *J. Immunol.* 148:1764; Shen et al. (1989) *J. Immunol.* 143: 4117). Preferred anti-FcαR antibodies are capable of binding to an FcαR without being inhibited by IgA. The antibody A77 has been produced by immunizing mice with acrylamide gel slices containing FcαR that was IgA affinity purified from human cell lysates. Monoclonal antibodies were screened according to three characteristics: staining of U937 cells at a higher density after PMA activation, selective reactivity with blood monocytes and granulocytes, and their ability to immunoprecipitate molecules of approximately 55 to 75 kDa from neutrophils and activated U937 cells.

Monoclonal anti-Fc receptor antibodies used in the compounds of the invention can be produced by a variety of techniques, including conventional monoclonal antibody methodology, e.g., the standard somatic cell hybridization technique of Kohler and Milstein, (1975) *Nature* 256: 495. Although somatic cell hybridization procedures are preferred, in principle, other techniques for producing monoclonal antibody can be employed e.g., viral or oncogenic transformation of B lymphocytes.

A preferred animal system for preparing hybridomas is the murine system. Hybridoma production in the mouse is a well-established procedure. Immunization protocols and techniques for isolation of immunized splenocytes for fusion are known in the art. Fusion partners (e.g., murine myeloma cells) and fusion procedures are also known.

Human monoclonal antibodies (mAbs) directed against human proteins can be generated using transgenic mice carrying the complete human immune system rather than the mouse system. Splenocytes from these transgenic mice immunized with the antigen of interest are used to produce hybridomas that secrete human mAbs with specific affinities for epitopes from a human protein (see, e.g., Wood et al. International Application WO 91/00906, Kucherlapati et al. PCT publication WO 91/10741; Lonberg *et al.* International Application WO 92/03918; Kay *et al.* International Application 92/03917; Lonberg, N. *et al.* 1994 *Nature* 368:856-859; Green, L.L. *et al.* 1994 *Nature Genet.* 7:13-21; Morrison, S.L. *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 81:6851-6855; Bruggeman *et al.* (1993) *Year Immunol* 7:33-40; Tuaillon *et al.* (1993) *PNAS* 90:3720-3724; Bruggeman *et al.* (1991) *Eur. J. Immunol.* 21:1323-1326).

In an illustrative embodiment, mice (HuMab mice) which produce a fully human antibody response after immunization can be generated by inactivating the genes coding for mouse antibodies. This can be achieved by generating a 'double-knockout mouse' in which the endogenous immunoglobulin heavy chain and the κ-light chain genes are disrupted by targeted deletion of the exons coding for the constant regions (Cμ and JCκ). Separate transgenes can be constructed which contain both the human immunoglobulin heavy chain genes and the human κ light chain genes. In humans, these genes encompass about 1-2 megabases each, a size which is too large to isolate

intact. The essential regions can be assembled in condensed form in so-called 'miniloci'. The heavy chain minilocus contains 2-6 V_h gene segments, 15 D_h and 6 J_h gene segments, and the Sμ and Cμ and Sγl and Cγl gene segments. The κ-light chain minilocus contains 1-17 Vκ-gene segments, 5 Jκ and the Cκ gene segments (Lonberg, N. *et al.* (1994) *Nature* 368: 856-859; Tuaillon, N. *et al.* (1993) *Proc. Natl. Acad. Sci. USA* 90: 3720-3724). These miniloci can be subsequently incorporated into the genome of the 'double-knockout' mice. Several consecutive versions of these double-knockout/double transgenic HuMab mice can be generated, which incorporate increasing amounts of the human heavy- and light-chain loci. For example, HuMab mice have been generated which incorporate a 100 kb heavy chain transgene containing six V segments, and a 200 kb κ light chain transgene containing 17 Vκ-segments. These HuMab mice can be immunized using conventional immunization protocols, and have been shown to efficiently generate high-affinity human IgG1 antibodies against a broad panel of antigens (Fishwild, D.M. *et al.* (1996) *Nature Biotech* 14: 845-851; Lonberg, N. and D. Huszar (1995) *Int. Rev. Immunol.* 13: 65-93). The antibodies generated following these protocols have been shown to have excellent biological activity, and long serum half-lifes.

Chimeric mouse-human monoclonal antibodies (i.e., chimeric antibodies) can be produced by recombinant DNA techniques known in the art. For example, a gene encoding the Fc constant region of a murine (or other species) monoclonal antibody molecule is digested with restriction enzymes to remove the region encoding the murine Fc, and the equivalent portion of a gene encoding a human Fc constant region is substituted. (see Robinson et al., International Patent Publication PCT/US86/02269; Akira, et al., European Patent Application 184,187; Taniguchi, M., European Patent Application 171,496; Morrison et al., European Patent Application 173,494; Neuberger et al., International Application WO 86/01533; Cabilly et al. U.S. Patent No. 4,816,567; Cabilly et al., European Patent Application 125,023; Better et al. (1988 *Science* 240:1041-1043); Liu et al. (1987) *PNAS* 84:3439-3443; Liu et al., 1987, *J. Immunol.* 139:3521-3526; Sun et al. (1987) *PNAS* 84:214-218; Nishimura et al., 1987, *Canc. Res.*

47:999-1005; Wood et al. (1985) *Nature* 314:446-449; and Shaw et al., 1988, *J. Natl Cancer Inst.* 80:1553-1559).

The chimeric antibody can be further humanized by replacing sequences of the Fv variable region which are not directly involved in antigen binding with equivalent sequences from human Fv variable regions. General reviews of humanized chimeric antibodies are provided by Morrison, S. L., 1985, *Science* 229:1202-1207 and by Oi et al., 1986, *BioTechniques* 4:214. Those methods include isolating, manipulating, and expressing the nucleic acid sequences that encode all or part of immunoglobulin Fv variable regions from at least one of a heavy or light chain. Sources of such nucleic acid are well known to those skilled in the art and, for example, may be obtained from 7E3, an anti-$GPII_bIII_a$ antibody producing hybridoma. The recombinant DNA encoding the chimeric antibody, or fragment thereof, can then be cloned into an appropriate expression vector. Suitable humanized antibodies can alternatively be produced by CDR substitution U.S. Patent 5,225,539; Jones et al. 1986 *Nature* 321:552-525; Verhoeyan et al. 1988 *Science* 239:1534; and Beidler et al. 1988 *J. Immunol.* 141:4053-4060.

All of the CDRs of a particular human antibody may be replaced with at least a portion of a non-human CDR or only some of the CDRs may be replaced with non-human CDRs. It is only necessary to replace the number of CDRs required for binding of the humanized antibody to the Fc receptor.

An antibody can be humanized by any method, which is capable of replacing at least a portion of a CDR of a human antibody with a CDR derived from a non-human antibody. Winter describes a method which may be used to prepare the humanized antibodies of the present invention (UK Patent Application GB 2188638A, filed on March 26, 1987), the contents of which is expressly incorporated by reference. The human CDRs may be replaced with non-human CDRs using oligonucleotide site-directed mutagenesis as described in International Application WO 94/10332 entitled, *Humanized Antibodies to Fc Receptors for Immunoglobulin G on Human Mononuclear Phagocytes.*

Also within the scope of the invention are chimeric and humanized antibodies in which specific amino acids have been substituted, deleted or added. In particular,

preferred humanized antibodies have amino acid substitutions in the framework region, such as to improve binding to the antigen. For example, in a humanized antibody having mouse CDRs, amino acids located in the human framework region can be replaced with the amino acids located at the corresponding positions in the mouse antibody. Such substitutions are known to improve binding of humanized antibodies to the antigen in some instances. Antibodies in which amino acids have been added, deleted, or subsituted are referred to herein as modified antibodies or altered antibodies.

The term modified antibody is also intended to include antibodies, such as monoclonal antibodies, chimeric antibodies, and humanized antibodies which have been modified by, e.g., deleting, adding, or substituting portions of the antibody. For example, an antibody can be modified by deleting the constant region and replacing it with a constant region meant to increase half-life, e.g., serum half-life, stability or affinity of the antibody. Any modification is within the scope of the invention so long as the macrophage-binding compound has at least one antigen binding region specific for an FcR and triggers at least one effector function.

Monoclonal antibodies can also be generated by other methods known to those skilled in the art of recombinant DNA technology. An alternative method, referred to as the "combinatorial antibody display" method, has been developed to identify and isolate antibody fragments having a particular antigen specificity, and can be utilized to produce monoclonal antibodies (for descriptions of combinatorial antibody display (see e.g., Sastry *et al.* (1989) *PNAS* 86:5728; Huse *et al.* (1989) *Science* 246:1275; and Orlandi *et al.* (1989) *PNAS* 86:3833). After immunizing an animal with an immunogen as described above, the antibody repertoire of the resulting B-cell pool is cloned. Methods are generally known for obtaining the DNA sequence of the variable regions of a diverse population of immunoglobulin molecules by using a mixture of oligomer primers and PCR. For instance, mixed oligonucleotide primers corresponding to the 5' leader (signal peptide) sequences and/or framework 1 (FR1) sequences, as well as primer to a conserved 3' constant region primer can be used for PCR amplification of the heavy and light chain variable regions from a number of murine antibodies (Larrick et al.,1991, *Biotechniques* 11:152-156). A similar strategy can also been used to amplify human

heavy and light chain variable regions from human antibodies (Larrick et al., 1991, *Methods: Companion to Methods in Enzymology* 2:106-110).

In an illustrative embodiment, RNA is isolated from B lymphocytes, for example, peripheral blood cells, bone marrow, or spleen preparations, using standard protocols (e.g., U.S. Patent No. 4,683,202; Orlandi, et al. *PNAS* (1989) 86:3833-3837; Sastry et al., *PNAS* (1989) 86:5728-5732; and Huse et al. (1989) *Science* 246:1275-1281.) First-strand cDNA is synthesized using primers specific for the constant region of the heavy chain(s) and each of the κ and λ light chains, as well as primers for the signal sequence. Using variable region PCR primers, the variable regions of both heavy and light chains are amplified, each alone or in combination, and ligated into appropriate vectors for further manipulation in generating the display packages. Oligonucleotide primers useful in amplification protocols may be unique or degenerate or incorporate inosine at degenerate positions. Restriction endonuclease recognition sequences may also be incorporated into the primers to allow for the cloning of the amplified fragment into a vector in a predetermined reading frame for expression.

The V-gene library cloned from the immunization-derived antibody repertoire can be expressed by a population of display packages, preferably derived from filamentous phage, to form an antibody display library. Ideally, the display package comprises a system that allows the sampling of very large variegated antibody display libraries, rapid sorting after each affinity separation round, and easy isolation of the antibody gene from purified display packages. In addition to commercially available kits for generating phage display libraries (e.g., the Pharmacia *Recombinant Phage Antibody System*, catalog no. 27-9400-01; and the Stratagene *SurfZAP*™ phage display kit, catalog no. 240612), examples of methods and reagents particularly amenable for use in generating a variegated antibody display library can be found in, for example, Ladner et al. U.S. Patent No. 5,223,409; Kang et al. International Publication No. WO 92/18619; Dower et al. International Publication No. WO 91/17271; Winter et al. International Publication WO 92/20791; Markland et al. International Publication No. WO 92/15679; Breitling et al. International Publication WO 93/01288; McCafferty et al. International Publication No. WO 92/01047; Garrard et al. International Publication No.

WO 92/09690; Ladner et al. International Publication No. WO 90/02809; Fuchs et al. (1991) *Bio/Technology* 9:1370-1372; Hay et al. (1992) *Hum Antibod Hybridomas* 3:81-85; Huse et al. (1989) *Science* 246:1275-1281; Griffths et al. (1993) *EMBO J* 12:725-734; Hawkins et al. (1992) *J Mol Biol* 226:889-896: Clackson et al. (1991) *Nature* 352:624-628; Gram et al. (1992) *PNAS* 89:3576-3580; Garrad et al. (1991) *Bio/Technology* 9:1373-1377; Hoogenboom et al. (1991) *Nuc Acid Res* 19:4133-4137; and Barbas et al. (1991) *PNAS* 88:7978-7982.

In certain embodiments, the V region domains of heavy and light chains can be expressed on the same polypeptide, joined by a flexible linker to form a single-chain Fv fragment, and the scFV gene subsequently cloned into the desired expression vector or phage genome. As generally described in McCafferty et al., *Nature* (1990) 348:552-554, complete V_H and V_L domains of an antibody, joined by a flexible $(Gly_4\text{-}Ser)_3$ linker can be used to produce a single chain antibody which can render the display package separable based on antigen affinity. Isolated scFV antibodies immunoreactive with the antigen can subsequently be formulated into a pharmaceutical preparation for use in the subject method.

Once displayed on the surface of a display package (e.g., filamentous phage), the antibody library is screened with the FcγR, or peptide fragment thereof, to identify and isolate packages that express an antibody having specificity for the FcγR. Nucleic acid encoding the selected antibody can be recovered from the display package (e.g., from the phage genome) and subcloned into other expression vectors by standard recombinant DNA techniques.

Anti-Fc receptor binding agents, and/or other binding agents within macrophage-binding compounds of the invention with high affinities for a target antigen (e.g., surface protein) can be made according to methods known to those in the art, e.g, methods involving screening of libraries (Ladner, R.C., *et al.*, U.S. Patent 5,233,409; Ladner, R.C., *et al.*, U.S. Patent 5,403,484). Further, the methods of these libraries can be used in screens to obtain binding determinants that are mimetics of the structural determinants of antibodies. In particular, the Fv binding surface of a particular antibody molecule interacts with its epitope according to principles of protein-protein

interactions, hence sequence data for V_H and V_L (the latter of which may be of the κ or λ chain type) is the basis for protein engineering techniques known to those with skill in the art. Details of the protein surface that comprises the binding determinants can be obtained from antibody sequence information, by a modeling procedure using previously determined three-dimensional structures from other antibodies obtained from NMR studies or crytallographic data. See for example Bajorath, J. and S. Sheriff, 1996, *Proteins: Struct., Funct., and Genet. 24 (2)*, 152-157; Webster, D.M. and A. R. Rees, 1995. "Molecular modeling of antibody-combining sites,"in S. Paul, Ed., *Methods in Molecular Biol. 51*, Antibody Engineering Protocols, Humana Press, Totowa, NJ, pp 17-49; and Johnson, G., Wu, T.T. and E.A. Kabat, 1995, "Seqhunt: A program to screen aligned nucleotide and amino acid sequences," in *Methods in Molecular Biol.51*, *op. cit.*, pp 1-15.

In one embodiment, the anti-Fc receptor binding agent includes an antigen binding site that is derived from an antibody and which is grafted onto a non-antibody molecule. For example, an antigen binding region can be grafted onto a peptide or protein. In one embodiment, one portion of the antigen binding region, e.g., the portion similar to the antigen binding region from the light chain of an antibody, is grafted onto one protein or peptide and the other portion of the antigen binding region, e.g., the portion similar to the antigen binding region from the heavy chain of an antibody, is grafted onto another protein or peptide. In a preferred embodiment of the invention, the two proteins or peptides having each a portion of the antigen binding region are linked, e.g., by chemical linkage, recombinantly, or by non covalent interaction, such as to produce a protein having an antigen binding site specific for an FcR for human Igs, which triggers at least one Fc receptor-mediated effector cell function.

An antigen binding region can also be obtained by screening various types of combinatorial libraries with a desired binding activity, and to identify the active species, by methods that have been described. For example, phage display techniques (Marks et al. (1992) *J Biol Chem* 267:16007-16010) can be used to identify proteins binding FcγRs. Phage display libraries have been described above. For example, a variegated peptide library can be expressed by a population of display packages to form a peptide

display library. Ideally, the display package comprises a system that allows the sampling of very large variegated peptide display libraries, rapid sorting after each affinity separation round, and easy isolation of the peptide-encoding gene from purified display packages. Peptide display libraries can be in, e.g., prokaryotic organisms and viruses, which can be amplified quickly, are relatively easy to manipulate, and which allows the creation of large number of clones. Preferred display packages include, for example, vegetative bacterial cells, bacterial spores, and most preferably, bacterial viruses (especially DNA viruses). However, the present invention also contemplates the use of eukaryotic cells, including yeast and their spores, as potential display packages. Phage display libraries are described above.

Other techniques include affinity chromatography with an appropriate "receptor", e.g., FcγRI or FcαR, to isolate binding agents, followed by identification of the isolated binding agents or ligands by conventional techniques (e.g., mass spectrometry and NMR). Preferably, the soluble receptor is conjugated to a label (e.g., fluorophores, colorimetric enzymes, radioisotopes, or luminescent compounds) that can be detected to indicate ligand binding. Alternatively, immobilized compounds can be selectively released and allowed to diffuse through a membrane to interact with a receptor.

Combinatorial libraries of compounds can also be synthesized with "tags" to encode the identity of each member of the library (see e.g., W.C. Still *et al.*, International Application WO 94/08051). In general, this method features the use of inert but readily detectable tags, that are attached to the solid support or to the compounds. When an active compound is detected, the identity of the compound is determined by identification of the unique accompanying tag. This tagging method permits the synthesis of large libraries of compounds which can be identified at very low levels among to total set of all compounds in the library.

II. Cyanin Compositions

In another embodiment of the invention, the Fc receptor binding agent of the macrophage-binding compound is a chemical moiety, such as a cyanin composition, including but not limited to the fluorescent dye Cy5.18.OSu (referred to as Cy5) and conjugates and derivatives thereof. Cyanin compositions are known to bind with high affinity and specificity to FcγRI receptors. In certain cases, the cyanin compositions can contain two or more moieties, such as a cyanin succinimidyl ester and a phycobilisome protein, e.g., PE. The term "PE-Cy5" as used here designates the specific tandem dye comprised of phycoerythrin and Cy5.18.OSu; the term "PE-Cy5 reagent" designates, for example but not limited to, PE-Cy5 conjugates to antibodies, to genetically engineered binding proteins and peptides (U.S.P.N. 5,233,409 and 5,403,484), to avidin, to biotin, or to other molecular entities. PE-Cy5 conjugates can be used in therapeutic and diagnostic applications.

Cyanin was isolated from cornflower (*Centaurea cyanus*), and is structurally the 3,5-diglucoside of cyanidin, which is 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-1-benzopyrylium chloride and was isolated from banana (Merck Index). Another cyanidin derivative, the 3-rhamnoglucoside isolated from sour cherries, is described as having therapeutic application for night blindness. Anthocyanosides of bilberry (*Vaccinium myrtillus*) fruit are marketed as nutraceutical food supplements, which according to one manufacturer (Amrion, Inc., Boulder, CO), are consumed orally to improve vasodilation, decrease capillary permeability, protect collagen in blood vessels, operate as antioxidants and support control of the inflammatory process, improving general vision, stomach linings, blood-brain barrier and the veins of the legs and colon (*Gen. Engin. News 16* (11), p.27, 1996).

The cyanidin derivative dye Cy5, also designated Cy5.18.OSu, has the chemical structure 5,5'-bis-sulfo-1,1'-(ε-carboxyphenyl)-3,3,3',3'-tetramethylindodicarbocyanin-disuccinimidyl ester (A.S. Waggoner *et al.*, In: Clinical Flow Cytometry, p.185 (Eds) A. Landay *et al.* The New York Academy of Sciences, New York, New York, 1993). Cyanin dye labeling reagents for sulfhydryl groups (Ernst, L.A. *et al.*, 1989, *Cytometry 10*:3) and carboxymethylindocyanin succinimidyl esters (Southwick, P.L. *et al.*, 1990,

Cytometry 11:418) have been described, and compositions claimed in patent applications (USPN 4,981,977 and 5,268,486), the contents of which are hereby incorporated by reference. Structure of Cy5, and its synthesis and spectra for absorption and emission of light are given in Mujumdar, R.B., 1993, *Bioconj. Chem. 4*:105. Cy5 is a sulfoindocyanin succinimidyl ester, which is an amino-reactive cyanin dye that contains a negatively charged sulfonate group on the aromatic nucleus of the indocyanin fluorophore. The Cy5 members of this family are characterized by a 5-carbon, unsaturated polymethine bridge connecting two substituted ring structures. Cy5 can be excited with a 633 nm HeNe laser line or a 647 nm line of a Dr laser. Cy5 and its derivatives are noted for photostability, which is comparable to or better than that of fluorescein. The extinction coefficient (L/mol cm) of 250,000 is very high. Related dyes (Mujumdar *et al.*, *supra*), with similar structures and modes of synthesis are here encompassed within the expression "Cy5" so that this expression encompasses sulfoindocyanin succinimidyl esters of cyanin dye labeling reagents in general, for example, Cy3.29.OSu (known as Cy3) and Cy7.18.OH. The terms Cy5 reagent, Cy5 conjugate and Cy5 derivatives shall mean a conjugate comprising at least a Cy5 moiety and another molecular entity. Additional new derivatives of this basic structure have been described, the sulfobenzindocyanin succinimidyl esters of cyanin reagents (Mujumdar, S.R. *et al.*, 1996, *Bioconj. Chem* 7:356), which share properties of Cy5 and other sulfoindocyanin succinimidyl esters, and are contemplated to bind FcγRI with affinity and specificity.

Use of the Cy5 reagent PE-Cy5, comprised of Cy5 in tandem with PE, to provide three-color fluorescence by excitation with a single 488 nm argon ion laser line is described in Waggoner *et al.*, 1993, *supra*, as are conditions for optimization. Major problems with tandem dyes based on Texas Red are attributed to instability of one moiety, resulting during use in leakage of emission into the spectrum of the other moiety, limiting the ability to use Texas Red dyes emitting light at or near the wavelength of that second moiety. Cy5 and its reagent family of dyes, however, emit light at longer wavelengths than Texas Red, so that analysis of data obtained from using Cy5 with other dyes requires minimal channel compensation in setting detection

windows and in downstream calculations. Considerations for best mode use of Cy5 reagents include the process of synthesis of the Cy5 reagent from the components, since the ratio of number of Cy5 molecules bound per molecule of conjugate affects the relative emission wavelength spectrum of the synthesis product. Thus for PE-Cy5, the efficiency of energy transfer from PE to Cy5 increases as more Cy5 molecules are bound to each PE up to an optimal range, beyond which quenching interactions among excess Cy5 moieties is observed. The optimum ratio is 4 to 8 Cy5 per PE in the PE-Cy5 tandem dye (Waggoner *et al.*, 1993, *supra*). Tandem dyes are light sensitive, and stability during usage is improved if dyes are stored and handled and experiments are performed under dark conditions.

The improved signal size due to extent of fluorescence and absence of background for PE-Cy5, compared to that of previously synthesized tandem dyes, make it a successful analytical tool for cell analysis studies with antibody-dye conjugates. However at least one report of "non-specific" binding of a variety of PE-Cy5 products from different suppliers to myeloid cells has been reported (Stewart SJ, *et al.*, *supra*), attributed to the Cy5 moiety because PE-Texas Red conjugates do not exhibit this property. In contrast, Takizawa *et al.* report binding of PE and its mAb conjugates to low affinity mouse IgG receptors FcγRII and FcγRIII (*J. Immunol. Methods*, 1993, *162*:269).

PRODUCTION OF CYTOTOXIC AGENTS WHICH KILL MACROPHAGES OR WHICH REDUCE THEIR ACTIVITY

I. Cytotoxins

A variety of cytotoxic agents can be targeted to macrophages via compounds of the invention (i.e., by virtue of being linked to an agent which binds to an Fc receptor on a macrophage). As used herein, the terms "cytotoxin" and "cytotoxic agent" includes any compound (e.g., drug) capable of killing or reducing the activity of a macrophage. For example, the compound can be a toxin, such as Gelonin, Saporin, Exotoxin A, Onconase or Ricin A, or a drug, such as dichloromethylene diphosphonate (CL2MDP)

or a derivative thereof. Cytotoxins for use in the invention can additionally include an agent or a moiety which enhances the therapeutic activity of these compounds.

For example, the cytotoxin can include an agent which promotes apoptosis, a mitotic inhibitor, an alkylating agent, an antimetabolite, a nucleic acid intercalating agent, a topoisomerase inhibitor, a macrophage-specific drug, or a radionuclide. The present invention offers the advantage of targeting such cytotoxins to high affinity Fcγ receptors (e.g., using an antibody such as Mab 22, Mab 32, or humanized forms thereof) on macrophages where they, for example, are internalized by the cell. Therefore, these cytotoxins can be more effective in cell killing or modulating cell function than other agents which are not internalized, or that are internalized with slower kinetics.

The cytoxic agent can be a toxic drug or an enzymatically active toxin of bacterial or plant origin, or a biologically active fragment ("A chain") of such a toxin. Exemplary enzymatically active toxins and fragments thereof include diphtheria A chain, nonbinding active fragments of diphtheria toxin, exotoxin A chain (from Pseudomonas aeruginosa), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthinproteins, phytolacca americana proteins (PAPI, PAPII, and PAP-S), momordicacharantia inhibitor, curcin, crotin, saponaria officinalis inhibitor, gelonin, mitogellin, restrictocin, phenomycin and enomycin. Preferred toxins that can be used include Gelonin, Saporin, Exotoxin A, Onconase, Ricin A, diphtheria toxin, and Pseudomonas exotoxin or subunits of these toxins. Studies the preparation, *in vivo* uses and pharmacokinetics of these toxins are described in, for example, Vitetta *et al.* (1987) *Science* 238: 1098-1104; Spitlet, L. *et al.* (1987) *Clin. Chem.* 33(b): 1054; Uhr et al., *Monoclonal Antibodies and Cancer*, Academic Press, Inc., pp. 85-98 (1983).

Conjugates of the compounds of the invention and such toxic agents may be prepared using a variety of bifunctional protein coupling agents as described in detail below in the section entitled "Methods of Making Conjugates of Macrophage-Binding Compounds." Examples of such reagents are SPDP, IT, bifunctional derivatives of imidoesters such as dimethyl adipimidate, HCl, active esters such as disuccinimidyl suberate, aldehydes such as glutaraldehyde, bis-azido compounds such as bis-(p-azidobenzoyl) hexanediamine, bis-diazonium derivatives such as bis-(p-diazoniumbenzoyl)-

ethylenediamine, diisocyanates such as tolulene 2,6-diisocyanate, and bis-active fluorine compounds such as 1,5-difluoro-2,4-dinitrobenzene.

In other embodiments, the cytotoxin is a drug. Exemplary drugs include dichloromethylene diphosphonate (CL2MDP) or other chlodronate derivatives (Bogers *et al.* (1991) *Clin. Exp. Immunol.* 86: 328-333). Alternatively, the cytotoxin can be an agent which promotes apoptosis, a mitotic inhibitor, an alkylating agent, an antimetabolite, a nucleic acid intercalating agent, and a topoisomerase inhibitor. Examples of such agents which can be used in the compounds of the invention include the topoisomerase II inhibitors ellipticine, amsacrine, adriamycin and mitrozantrone, the prokaryotic DNA gyrase inhibitor coumermycin A1 and the DNA binding agents neocarzinostatin and chloroquine (which either intercalate or nick DNA). Methods for delivery of such drugs, e.g., liposome-delivery, are described below.

In certain embodiments, the cytotoxin can comprise a photosensitizing moiety (e.g., a photosensitizing drug). Cytotoxins which constitute such photosensitizing moieties are useful in sensitizing a target, e.g., a macrophage, to destruction upon photoactivation, e.g., by irradiation using visible light. Preferably, the photosensitizing moiety has no direct biological effect prior to photoactivation. Compounds comprising such moieties can be administered to a subject, e.g., topically or by injection. Upon photoactivation by exposing these compounds to a particular wavelength of light, e.g., by visible light exposure, the moiety becomes toxic (either itself or by activating a cytotoxin associated with the moiety) and selectively destroys the macrophages. Without being bound by any particular theory, the mechanism of photoactivation is believed to include transfer of energy from a photosensitizing moiety to endogenous oxygen, thereby converting it to singlet oxygen. The singlet oxygen is thought to be responsible for the cytotoxic effect. Macrophage binding compounds containing photosensitizing moieties are particularly useful for treatment of dermatological diseases.

Exemplary photosensitizing agents that can be used in the present invention include porphyrin related compounds, e.g. hematoporphyrin derivatives (Lipson, R.L. *et al.* (1961), *J. National Cancer Inst.* 26:1-8; Photophrin II compositions (US 4,649,151, Dougherty, T.J. (1983) *Adv. Exp. Med. Bio.* 160: 3-13. Kessel, D. *et al.* (1987)

Photochem. Photobiol. 46: 463-568 and Scourides, P.A. *et al.* (1987) *Cancer Res.* 47: 3439-3445), pyropheophorbide compounds (US 5,459, 159; US 4,996, 312, and US 4,849,207, and EP 220686); chlorophyll and bacteriophyll derivatives (EPA 93111942.4); 9-substituted porphycene derivatives (WO 96/31451); phrobine derivatives (WO 95/08551): as well as chlorins, phthalocyanines and porphins (reviewed in Harvey, I. Pass. (1993) *J. Natl. Canc. Inst.* 85: 443-457). Photoactivated forms of photosensitizing agent which are capable of emitting a fluorescent signal can also be used in diagnostic applications to label macrophage-binding compounds of the invention.

In other embodiments, the macrophage binding compounds of the invention can include an Fc binding agent coupled to a therapeutic or a diagnostic reagent, e.g., toxic agent, via a photocleavable linkage. Preferably, the linkage is mediated by a photoactivable agent, such as a chromophore, which releases the therapeutic or diagnostic reagent upon exposure to light (Goldmacher *et al.* (1992) *Bioconj. Chem.* 3: 104-107). For example, in dermatological applications, light will induce degradation of the linkage, liberating the active toxin locally (e.g., skin). Photoactivatable agents suitable for releasing the bound therapeutic or diagnostic reagent include any agent which can be linked to a functional group (e.g., a phenol) of the therapeutic or diagnostic reagent and which, upon exposure to light, releases the therapeutic or diagnostic reagent in functional form. As an illustration, the photoactivatable agent can be a chromophore. Suitable chromophores are generally selected for absorption of light that is deliverable from common radiation sources (e.g. UV light ranging from 240-370 nm). Examples of chromophores which are photoresponsive to such wavelengths include, but are not limited to, acridines, nitroaromatics and arylsulfonamides.

When using chromophores, the efficiency and wavelength at which the chromophore becomes photoactivated and thus releases or "uncages" the therapeutic reagent will vary depending on the particular functional group(s) attached to the chromophore. For example, when using nitroaromatics, such as derivatives of o-nitrobenzylic compounds, the absorption wavelength can be significantly lengthened by addition of methoxy groups. In one embodiment, nitrobenzyl (NB)

and nitrophenylethyl (NPE) is modified by addition of two methoxy residues into 4,5-dimethoxy-2-nitrobenzyl (DMNB) and 1-(4,5-dimethoxy-2-nitrophenyl)ethyl (DMNPE), respectively, thereby increasing the absorption wavelength range to 340-360 nm (λ_{max} = 355 nm). Radiation to promote photorelease of the therapeutic or diagnostic agent can be provided by a variety of sources including, but not limited to, non-coherent UV light sources and excimer sources. In one embodiment, a KrF excimer laser operating at 248 nanometers can be used. Alternatively, a frequency-quadrupled, solid state, Neodymium-doped YAG laser or the like operating at 266 nm can be used, or an Argon ion laser operating at 257 or 275 nm can be used. The photoactivatable agent can be reacted with the therapeutic agent to create a photoreleasable linkage. When using chromophores as photoactivatable agents, the excitation wavelength may be chosen so as to selectively excite particular chromophores. For example, it is possible to photoreleasably attach two different drugs or to two different chromophores to the substrate, and then independently or sequentially release the two drugs by selecting the excitation wavelength to match the corresponding chromophore. The chromophore and the excitation wavelength may further be selected to avoid undesired photolytic reactions of the drug (e.g., inactivation) or of the surrounding tissue. For example, the photosensitivity of nucleic acids is well known. When the drug is a nucleic acid, excitation energy which may damage the nucleic acid (e.g. wavelengths shorter than 280 nm) should be avoided.

In addition, macrophage-binding compounds of the invention can be labeled (e.g., for diagnostic use) by coupling the compound to radionuclides, such as 131I, 90Y, 105Rh, 47Sc, 67Cu, 212Bi and 211At, as described, e.g., in Goldenberg, D.M. *et al.* (1981) *Cancer Res.* 41: 4354-4360; in EP 0365 997; Carrasquillo et al., *Cancer Treat. Rep.*, 68:317-328 (1984); Zaloberg et al., *J. Natl. Cancer Institute* 72:697-704 (1984); Jones et al., *Int. J. Cancer* 35:715-720 (1985); Lange et al., *Surgery* 98:143-150 (1985); Kaltovich et al., *J. Nucl. Med.* 27:897 (1986); Order et al., *Intl. J. Radiother. Oncl. Biol. Phys.* 8:259-261 (1982); Courtenay-Luck et al. *Lancet* 1:1441-1443 (1983); Ettinger et al., *Cancer Treat. Rep.* 56:289-297 (1982); the disclosures of all of which are

incorporated herein by reference. Such radionuclides can also enhance the cytotoxic effect of the photosensitizing moiety.

In such diagnostic applications, it is desirable to attach a label group to the macrophage-binding compounds to facilitate their detection (e.g.. their binding to macrophages in a sample). Accordingly, in addition to the radionuclides listed above, suitable labeling groups include, for example, a fluorophore, a colorimetric enzyme, a radioisotope, or a luminescent compound. For example, when the labeling group is an enzyme, the enzyme which is linked to the macrophage binding compound will react with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical signal which can be detected, for example, by spectrophotometric, fluorimetric or by visual means. Enzymes which can be used to detectably label the antibody include, but are not limited to, malate dehydrogenase, staphylococcal nuclease, delta-5-steroid isomerase, yeast alcohol dehydrogenase, alpha-glycerophosphate, dehydrogenase, triose phosphate isomerase, horseradish peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase and acetylcholinesterase. The detection can be accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection of binding of macrophage-binding compounds to macrophages can also be accomplished using any of a variety of immunoassays. For example, a radioimmunoassay (RIA) can be used (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). Alternatively, enzyme immunoassays (EIA) can be used (Voller, "The Enzyme Linked Immunosorbent Assay (ELISA)", Diagnostic Horizons 2:1-7, 1978, Microbiological Associates Quarterly Publication, Walkersville, MD; Voller, et al., J. Clin. Pathol. 31:507-520 (1978); Butler, Meth. Enzymol. 73:482-523 (1981); Maggio, (ed.) Enzyme Immunoassay, CRC Press, Boca Raton, FL, 1980; Ishikawa, et al., (eds.) Enzyme

Immunoassay. Kgaku Shoin, Tokyo, 1981). The radioactive isotope can be detected by such means as the use of a γ counter or a scintillation counter or by autoradiography.

It is also possible to label the macrophage-binding compounds with a fluorescent compound. When the fluorescently labeled compound is exposed to light of the proper wavelength. its presence can then be detected. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

The compounds of the present invention can also be labeled using fluorescence emitting metals such as 152Eu, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA). Alternatively, these compounds can be labeled by coupling them to a chemiluminescent compound. The presence of the chemiluminescent-tagged compound is then determined by detecting luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester.

Likewise, a bioluminescent compound may be used to label the macrophage-binding compounds of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in, which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

CONJUGATING ANTI-Fc RECEPTOR BINDING AGENTS TO CYTOTOXINS

Macrophage-binding compounds of the present invention contain, along with other optional components, an agent which binds to an Fc receptor on a macrophage linked to a cytotoxin. Accordingly, to produce such compounds, the anti-Fc receptor binding agent is conjugated (e.g., by covalently crosslinking) to a cytotoxin using a variety of known techniques (see e.g., D. M. Kranz et al. (1981) *Proc. Natl. Acad. Sci.*

USA 78:5807, U.S. Patent 4,474,893), or by recombinantly expressing the anti-Fc receptor binding agent and the cytotoxin together as a fusion molecule.

Suitable agents, such as crosslinking agents, which can be employed for this purpose are well known in the art. The terms "crosslinking agent" and "crosslinker" are intended to include molecules which can function as bridging molecules between two other molecules by way of having two reactive functional groups, one of which reacts to form a covalent bond with the first molecule and the other of which reacts to form a covalent bond with the second molecule, thereby effectively connecting the two molecules together. Preferably, the crosslinker has two reactive functional groups of different functional moieties. Examples of suitable functional groups include amino groups, carboxyl groups, sulfhydryl groups and hydroxy groups. When one functional group of the crosslinker is reacted with a molecule (e.g., an Fc receptor binding agent), the other functional group can be, if necessary, prevented from reacting with that molecule by means of a protecting group which modifies the second functional group of the crosslinker so that it cannot react with the molecule. After the first reaction is completed, the protecting group can be removed, restoring the second functional group, and then the second functional group can be reacted with another molecule (e.g., a toxin).

Macrophage-binding compounds of the present invention can be prepared by conjugating their constituent agents, e.g., the anti-FcR and cytotoxin, using methods known in the art. For example, each agent of the macrophage-binding compound can be generated separately and then conjugated to one another. When the binding specificities are proteins or peptides, a variety of coupling or cross-linking agents can be used for covalent conjugation. Examples of cross-linking agents include protein A, carbodiimide, N-succinimidyl-S-acetyl-thioacetate (SATA), N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), and sulfosuccinimidyl 4-(N-maleimidomethyl) cyclohaxane-1-carboxylate (sulfo-SMCC) (see e.g., Karpovsky et al. (1984) J. Exp. Med. 160:1686; Liu, MA et al. (1985) Proc. Natl. Acad. Sci. USA 82:8648). Other methods include those described by Paulus (Behring Ins. Mitt. (1985) No. 78, 118-132); Brennan et al. (Science (1985) 229:81-83), and Glennie et al. (J. Immunol. (1987) 139:

2367-2375). Preferred conjugating agents are SATA and sulfo-SMCC, both available from Pierce Chemical Co. (Rockford, IL).

In cases where the macrophage-binding molecule contains two antibodies (e.g., a bispecific antibody), these antibodies can be conjugated via sulfhydryl bonding of the C-terminus hinge regions of the two heavy chains. In a particularly preferred embodiment, the hinge region is modified to contain an odd number of sulfhydryl residues, preferably one, prior to conjugation. Alternatively, both agents can be encoded in the same vector and expressed and assembled in the same host cell. This method is particularly useful where the macrophage-binding compound is a mAb x mAb, mAb x Fab, Fab x $F(ab')_2$ or ligand x Fab fusion protein. A macrophage-binding compound of the invention, e.g., a bispecific molecule can be a single chain molecule, such as a single chain bispecific antibody, a single chain bispecific molecule comprising one single chain antibody and a binding determinant, or a single chain bispecific molecule comprising two binding determinants. Macrophage-binding compounds can also be single chain molecules or may comprise at least two single chain molecules. Methods for preparing bi- and multispecific molecules are described for example in U.S. Patent Number 5,260,203; U.S. Patent Number 5,455,030; U.S. Patent Number 4,881,175; U.S. Patent Number 5,132,405; U.S. Patent Number 5,091,513; U.S. Patent Number 5,476,786; U.S. Patent Number 5,013,653; U.S. Patent Number 5,258,498; and U.S. Patent Number 5,482,858.

Once produced in accordance with the guidelines above, macrophage-binding compounds can be tested for binding to macrophages using known techniques, such as enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), or Western Blot Assay. Each of these assays generally detects the presence of protein-antibody complexes of particular interest by employing a labeled reagent (e.g., an antibody) specific for the complex of interest. For example, the FcR-antibody complexes can be detected using e.g., an enzyme-linked antibody or antibody fragment which recognizes and specifically binds to the antibody-FcR complexes. Alternatively, the complexes can be detected using any of a variety of other immunoassays. For example, the antibody can be radioactively labeled and used in a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is

incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a γ counter or a scintillation counter or by autoradiography.

PHARMACEUTICAL COMPOSITIONS AND ADMINISTRATION ROUTES

Macrophage-binding compounds of the invention are preferably present in a composition along with a carrier or diluent. For *in vivo* administration to a subject (e.g., to treat or diagnose a disorder), the compounds are preferably present along with a pharmaceutically acceptable carrier or diluent. As described in detail below, pharmaceutical compositions of the present invention may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets, boluses, powders, granules, pastes; (2) parenteral administration, for example, by subcutaneous, intramuscular or intravenous injection as, for example, a sterile solution or suspension; (3) topical application, for example, as a cream, ointment or spray applied to the skin; (4) intravaginally or intrarectally, for example, as a pessary, cream or foam; or (5) aerosol, for example, as an aqueous aerosol, liposomal preparation or solid particles containing the compound.

Pharmaceutical compositions of the invention also can be administered in a combination therapy, i.e., combined with other agents. For example, the combination therapy can include a composition of the present invention with at least one other anti-macrophage agent, or other conventional therapy. Exemplary anti-macrophage agents include chlodronate compounds, e.g., dichloromethylene diphosphonate (CL2MDP).

The phrase "pharmaceutically-acceptable carrier" as used herein means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject chemical from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch

and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

A "pharmaceutically acceptable salt" refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired toxicological effects (see *e.g.*, Berge, S.M., *et al.* (1977) *J. Pharm. Sci.* 66:1-19). Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanoic acids, hydroxy alkanoic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chloroprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

A composition of the present invention can be administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results.

The term "administration," is intended to include any route of introducing into a subject a macrophage-binding compound of the invention which allows the compound to perform its intended function (i.e., macrophage reduction and/or inhibition). Examples of routes of administration which can be used include injection (subcutaneous, intravenous, parenterally, intraperitoneally, intrathecal, etc.), oral, inhalation, rectal and

transdermal. The pharmaceutical preparations are of course given by forms suitable for each administration route. For example, these preparations are administered in tablets or capsule form, by injection, inhalation, eye lotion, ointment, suppository, etc.; administration by injection, infusion or inhalation; topical by lotion or ointment; and rectal by suppositories. The injection can be bolus or can be continuous infusion. Depending on the route of administration, the macrophage-binding compound can be coated with or disposed in a selected material to protect it from natural conditions which may detrimentally effect its ability to perform its intended function. The macrophage-binding compound can be administered alone, or in conjunction with either another agent as described above or with a pharmaceutically acceptable carrier, or both. The macrophage-binding compound can be administered prior to the administration of the other agent, simultaneously with the agent, or after the administration of the agent. Furthermore, the compound can also be administered in a proform or inactive form (e.g., a macrophage-binding compound which includes a light-sensitive toxin) which is converted into its active metabolite, or more active metabolite *in vivo*, e.g., upon light exposure.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous; subcuticular, intraarticulare, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

The phrases "systemic administration," "administered systemically," "peripheral administration" and "administered peripherally" as used herein mean the administration of a macrophage-binding compound, such that it enters the subject's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

In general, macrophage-binding compounds of the invention are administered locally to treat or diagnose disorders characterized by an abnormal number and/or function of macrophages within a particular area or region of the body (e.g., skin, lungs, joints, or muscle/nerve tissue). For dermatological applications, the compounds are

preferably delivered or administered topically or by transdermal patches. Topical administration is preferred in treatment of skin lesions, including lesions of the scalp, lesions of the cornea (keratitis), and lesions of mucous membranes where such direct application is practical. Shampoo formulations are sometimes advantageous for treating scalp lesions such as seborrheic dermatitis and psoriasis of the scalp. Mouthwash and oral paste formulations can be advantageous for mucous membrane lesions, such as oral lesions and leukoplakia. A preferred way to practice the invention is to apply the macrophage-binding compound, in a cream or oil based carrier, directly to the lesion, e.g., the psoriatic lesion. Typically, the concentration of macrophage-binding compound in a cream or oil is 1-2%. In addition, intra-dermal administration is an alternative for dermal lesions such as those of psoriasis and wounds. Alternatively, an aerosol can be used topically. Oral administration is a preferred alternative for treatment of skin lesions and other lesions discussed above where direct topical application is not as practical, and it is a preferred route for other applications.

Additionally, the compositions can be delivered parenterally, especially for treatment of arthritis, such as psoriatic arthritis or rheumatoid arthritis, and for direct injection of skin lesions. Parenteral therapy is typically intra-dermal, intra-articular, intramuscular or intravenous. Intra-articular injection is a preferred alternative in the case of treating one or only a few (such as 2-6) joints. Additionally, the therapeutic compounds are injected directly into lesions (intra-lesion administration) in appropriate cases. As an alternative in the treatment of arthritis, the compounds of the invention can be administered systemically.

For the treatment of respiratory diseases, compositions of the invention can be administered by nasal aerosol or inhalation. Such compositions can be prepared as solutions in saline, employing benzyl alcohol or other suitable preservatives, absorption promoters to enhance bioavailability, fluorocarbons, and/or other conventional a solubilizing or dispersing agents.

In certain embodiments, compositions including the compounds can be administered systemically or locoregionally. For example, compositions of macrophage-binding compounds which include a light-sensitive moiety, e.g., a toxin or a linker, can be administered in such manner. Furthermore, some autoimmune conditions

such as multiple sclerosis are preferentially treated by either of locoregional or systemic administration of the compositions of the invention.

Powders and sprays can contain, in addition to compounds of the invention, carriers such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Ordinarily, an aqueous aerosol is made by formulating an aqueous solution or suspension of the agent together with conventional pharmaceutically acceptable carriers and stabilizers. The carriers and stabilizers vary with the requirements of the particular compound, but typically include nonionic surfactants (Tweens, Pluronics, or polyethylene glycol), innocuous proteins like serum albumin, sorbitan esters, oleic acid, lecithin, amino acids such as glycine, buffers, salts, sugars or sugar alcohols. Aerosols generally are prepared from isotonic solutions.

Regardless of the route of administration selected, the macrophage-binding compound, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present invention, are formulated into pharmaceutically-acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels and time course of administration of the active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. See, *e.g.*, *Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

To administer a compound of the invention by certain routes of administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan *et al.*, (1984) *J. Neuroimmunol.* 7:27). Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

Sterile solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by sterilization microfiltration. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those

enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying (lyophilization) that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It is especially advantageous to formulate compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

For the therapeutic compositions, formulations of the present invention include those suitable for topical, dermal or epidermal administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the subject being treated, and the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage

form will generally be that amount of the composition which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 0.01 per cent to about ninety-nine percent of active ingredient, preferably from about 0.1 per cent to about 70 per cent, most preferably from about 1 per cent to about 30 per cent.

Dosage forms for the topical or transdermal administration of compositions of this invention include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants which may be required.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, *supra*, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

When the compounds of the present invention are administered as pharmaceuticals, to humans and animals, they can be given alone or as a pharmaceutical composition containing, for example, 0.01 to 99.5% (more preferably, 0.1 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of this invention may be varied so as to obtain an amount of the active ingredient which

is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, a suitable daily dose of a compositions of the invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. It is preferred that administration be local, e.g., topical, subcutaneous, intradermal, preferably administered proximal to the site of the target. If desired, the effective daily dose of a therapeutic compositions may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms. While it is possible for a compound of the present invention to be administered alone, it is preferable to administer the compound as a pharmaceutical formulation (composition).

Therapeutic compositions can be administered with medical devices known in the art. For example, in a preferred embodiment, a therapeutic composition of the invention can be administered with a needleless hypodermic injection device, such as the devices disclosed in U.S. Patent Nos. 5,399,163, 5,383,851, 5,312,335, 5,064,413, 4,941,880, 4,790,824, or 4,596,556. Examples of well-known implants and modules useful in the present invention include: U.S. Patent No. 4,487,603, which discloses an

implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Patent No. 4,486,194, which discloses a therapeutic device for administering medicants through the skin; U.S. Patent No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Patent No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Patent No. 4,439,196, which discloses an osmotic drug delivery system having multi-chamber compartments; and U.S. Patent No. 4,475,196, which discloses an osmotic drug delivery system. These patents are incorporated herein by reference. Many other such implants, delivery systems, and modules are known to those skilled in the art.

In certain embodiments, the compounds of the invention can be formulated to ensure proper distribution *in vivo*. In one embodiment, the macrophage-binding molecules can be encapsulated into liposomes. For methods of manufacturing liposomes, see, e.g., U.S. Patents 4,522,811; 5,374,548; and 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery (see, e.g., V.V. Ranade (1989) *J. Clin. Pharmacol.* 29:685). For example, certain embodiments, it is preferable to use single chain antibodies against an Fc receptor (scFv), for example, H22 scFv, to target the compounds of the invention to Fc-bearing macrophages. Protocols for preparing liposome encapsulated scFv fragments are described in de Kruif, J. *et al.* (1996) *FEBS* 399: 232-236. For example, lipid-modified H22 scFv can be coupled to liposomes composed of egg phosphatidylcholine (EPC), egg phosphatidylglycerol (EPG), cholesterol and, optionally, rhodamine-phospatidylethanolamine (rhodamine) as a fluorescent bilayer marker, at a molar ratio of 10:1:5:0.01, by diluting mixed micelles containing n-octyl β-D-glucoside, lipid and lipid modified scFv to a level far below the critical micelle concentration of the detergent. Incorporation of scFv molecules in the liposomes can be verified by SDS-PAGE.

A "therapeutically effective dosage" is that dosage which reduces the number of macrophages within a selected treatment area relative to an untreated control, or which inhibits activity of macrophages within a selected area so that, for example, they no

longer proliferate or contribute to inflammatory responses within the area. As a consequence, the symptoms of the macrophage-mediated disease are improved. The ability of compounds of the invention to kill or inhibit a population of macrophages can be evaluated in an animal model system, such as a transgenic animal expressing a human Fc receptor as described in the Examples herein. Alternatively, these functions can be evaluated in *in vitro* assays known to the skilled practitioner. A therapeutically effective amount of a therapeutic compound can decrease the macrophage cell population or activity, or otherwise ameliorate symptoms in a subject. One of ordinary skill in the art would be able to determine such amounts based on such factors as the subject's size, the severity of the subject's symptoms, and the particular composition or route of administration selected.

The composition must be sterile and fluid to the extent that the composition is deliverable by syringe. In addition to water, the carrier can be an isotonic buffered saline solution, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. Proper fluidity can be maintained, for example, by use of coating such as lecithin, by maintenance of required particle size in the case of dispersion and by use of surfactants. In many cases, it is preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol or sorbitol, and sodium chloride in the composition. Long-term absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate or gelatin.

USES AND METHODS OF THE INVENTION

Macrophage-binding compounds of the present invention have several diagnostic, therapeutic and research utilities. They can be administered to cells *in vitro* (in culture), *ex vivo*, or *in vivo* (in a subject), to treat, diagnose or study a variety of disorders.

In one embodiment, a method of depleting (e.g., reducing the number) or inhibiting the activity of macrophages in a selected treatment or diagnostic area is provided. The method involves contacting the selected area with the macrophage-binding compound in an amount sufficient to achieve the aforementioned result. As used

herein, the terms "selected area" or "local area" collectively refer to any selected sample of tissue or cells (either *in vitro* or *in vivo*) which contain, or may contain, macrophages which contribute to a disorder, such as a localized area of the human body (skin, lungs, joints, etc.) or a tissue culture sample. The contacting can occur *in vitro* (e.g., cells in culture) or *in vivo* (e.g., by administering the compounds of the invention to a subject).

As used herein, the term "subject" is intended to include human and non-human animals. Preferred human animals include a human patient having a disorder characterized by aberrant activity of a macrophage cell, e.g., a skin macrophage cell. The term "activity" is intended to include all biological functions of a macrophage cell, including proliferation, differentiation, survival, growth factor or cytokine secretion, among others. The term "non-human animals" of the invention includes all vertebrates, e.g., mammals and non-mammals, such as non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, etc.

Macrophage-binding compounds of the invention can be initially tested *in vitro*. For example, the activity of these molecules killing and/or modulating, e.g., reducing, macrophage activity can be assayed in macrophage-derived cell lines, cultured differentiated blood monocytes, and primary culture systems. Protocols for assaying *in vitro* activity of macrophage-binding compounds can be found, for example, in *Immunopharmacology of Macrophages and Other Antigen-presenting Cells* (ISBN 0-12-137800-4, 1994, Academic Press Limited). For example, primary skin macrophage cultures can be established from skin cells derived from healthy and dermatologic subjects. Macrophage activity, e.g., cell proliferation or cytokine secretion, can be assayed at specific time intervals after the addition of a range of concentrations of the compounds of the present invention. In one embodiment, 'punch biopsies' obtained from healthy and dermatologic subjects can be used. Punch biopsies can be cultured either submerged, or with the epidermal side surfaced in culture medium, to which the compounds of the invention can be added. Following culture with the macrophage-binding compounds of the invention, the effect(s) of these compounds in macrophage activity can be assayed immunohistochemically or by ELISA, RIA or EIA.

Protocols for detecting changes in cell proliferation, e.g., thymidine or BrdU incorporation assays, are known in the art. Preferred macrophage-binding compounds of the invention decrease or eliminate macrophage activity. Protocols for detecting changes in cytokine concentration can be detected via a variety of immunoassays, such as enzyme-linked immunoassay (ELISA), enzyme immunoassay (EIA) or radioimmunoassay (RIA) which are known in the art (see e.g., Keler, T. *et al.* (1997) *Cancer Research* 57: 4008-14). Exemplary cytokines that can be assayed include: granulocyte/macrophage colony stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), interleukins 1 -12 (IL-1 to IL-12), and TNF-α. The concentration of a cytokine can be measured using an EIA by detecting the interaction of the cytokine with an antibody, which is in turn conjugated to an enzyme. The activity of the enzyme is detected by the reaction with an appropriate substrate, preferably a chromogenic substrate, in such a manner as to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorimetric or by visual means (Voller, "The Enzyme Linked Immunosorbent Assay (ELISA)." Diagnostic Horizons 2:1-7, 1978, Microbiological Associates Quarterly Publication, Walkersville, MD; Voller, et al., J. Clin. Pathol. 31:507-520 (1978); Butler, Meth. Enzymol. 73:482-523 (1981); Maggio, (ed.) Enzyme Immunoassay, CRC Press, Boca Raton, FL, 1980; Ishikawa, et al., (eds.) Enzyme Immunoassay, Kgaku Shoin, Tokyo, 1981). Enzymes which can be used to detectably label the antibody are described above. The detection can be accomplished by colorimetric methods which employ a chromogenic substrate for the enzyme. Detection may also be accomplished by visual comparison of the extent of enzymatic reaction of a substrate in comparison with similarly prepared standards.

Detection of a cytokine may also be accomplished using a radioimmunoassay (RIA) (see, for example, Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, which is incorporated by reference herein). The radioactive isotope can be detected by such means as the use of a γ counter or a scintillation counter or by autoradiography.

It is also possible to label the anti-cytokine antibody with a fluorescent compound. When the fluorescently labeled antibody is exposed to light of the proper wave length, its presence can then be detected. Among the most commonly used fluorescent labeling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine. The antibody can also be detectably labeled using fluorescence emitting metals such as 152Eu, or others of the lanthanide series. These metals can be attached to the antibody using such metal chelating groups as diethylenetriaminepentacetic acid (DTPA) or ethylenediaminetetraacetic acid (EDTA). The antibody also can be detectably labeled by coupling it to a chemiluminescent compound. The presence of the chemiluminescent-tagged antibody is then determined by detecting luminescence that arises during the course of a chemical reaction. Examples of particularly useful chemiluminescent labeling compounds are luminol, isoluminol, theromatic acridinium ester, imidazole, acridinium salt and oxalate ester. Likewise, a bioluminescent compound may be used to label the antibody. Bioluminescence is a type of chemiluminescence found in biological systems in, which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Important bioluminescent compounds for purposes of labeling are luciferin, luciferase and aequorin.

Macrophage-binding compounds also can be tested *in vivo*. For example, these compounds can be tested using mice expressing human Fc receptors as described in the Examples herein. In one embodiment, macrophage-binding compounds can be injected intradermally into these transgenic mice. Vehicle-injected controls can be processed in parallel. Chronic cutaneous inflammation can be induced experimentally in these mice by repeated topical application of 5% sodium lauryl sulfate. The effects of these compounds can be monitored immunohistochemically, e.g., macroscopically or clinically, at various time intervals after injection.

The macrophage-binding compounds of the invention can be used in the treatment of disorders characterized by aberrant macrophage activity or numbers. The term "aberrant" refers to a macrophage density within a selected site which is different

(e.g., higher) than that found in the same area in normal, healthy patients. The term "aberrant" also includes abnormal macrophage activity, such as abnormally high cell proliferation or cytokine secretion. Accordingly, in one embodiment, the invention provides a method of treating or prophylactively preventing disorders characterized by aberrant numbers or activity of macrophages in a selected area, comprising administering to a subject, generally in the local area needing treatment, a pharmaceutical composition containing one or more macrophage-binding compounds.

Macrophage-binding compounds are generally used as targeting agents to deliver cytotoxins (e.g., drugs) to Fc receptor-bearing macrophages. In one embodiment of the invention, the cytotoxin is encapsulated within a liposome which itself is targeted to Fc receptor-bearing macrophages. Thus, the macrophage-binding compound comprises an anti-Fc receptor binding portion linked to a liposome containing a cytotoxin. In a preferred embodiment, the anti-Fc receptor binding portion is a single chain antibody directed against an Fc receptor (scFv), such as H22 scFv. The anti-FcR scFv is linked or inserted into the lipid bilayers of the liposome in a manner which allows the scFv still to recognize and bind to Fc receptors outside the liposome. This can be done using known protocols, such as those described by de Kruif, J. *et al.* (1996) *FEBS* 399: 232-236. The end result is an FcR targeted cytotoxin which is delivered to cells in the form of a liposome.

As used herein, a "therapeutically effective amount" of a macrophage-binding compound refers to an amount of a compound which is effective, upon single or multiple dose administration to the subject, at inhibiting the growth of the cells, or an improvement in the clinical symptoms in the absence of such treatment.

As used herein, "a prophylactically effective amount" of a compound refers to an amount of a macrophage-binding compound which is effective, upon single- or multiple-dose administration to the patient, in preventing or delaying the occurrence of the onset or recurrence of a macrophage-mediated disease state.

The terms "induce", "inhibit", "potentiate", "elevate", "increase", "decrease" or the like, e.g., which denote quantitative differences between two states, refer to at least statistically significant differences between the two states. For example, "an amount

effective to inhibit growth of the macrophage cells" means that the rate of growth of the cells will at least statistically significantly different from the untreated cells.

Macrophage-binding compounds of the invention can be used to treat a variety of macrophage-mediated diseases. These diseases are not necessarily characterized solely by aberrant macropage numbers and/or activity, but they each involve undesired macrophage activity which is harmful to patients. In one embodiment, the compounds are used to treat autoimmune diseases including, for example, diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis), multiple sclerosis, encephalomyelitis, diabetes, myasthenia gravis, systemic lupus erythematosis, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjögren's Syndrome, including keratoconjunctivitis sicca secondary to Sjögren's Syndrome, alopecia areata, allergic responses due to arthropod bite reactions, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Crohn's disease, Graves ophthalmopathy, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis). Downmodulation of immune activity will also be desirable in cases of allergy such as, atopic allergy.

Exemplary of preferred autoimmune/dermatological disorders for which the subject method may be used as part of a treatment regimen include: psoriasis, atopic dermatitis, multiple sclerosis, scleroderma and cutaneous lupus erythematosis. For example, the methods and compositions of the invention can be used to treat atopic dermatitis (AD). Without being bound by theory, it is believed that during the acute phase of cutaneous inflammation in AD, the phenotype of local T cells switches from an initial Th2 type to a Thl type in the chronic phase. At this timepoint, an increase in IL-12 production in the lesion is found, together with a strong influx of activated

inflammatory macrophage. Macrophages are potent producers of IL-12 which induces T cells to produce IFN-γ, which in turn is a potent macrophage activator (Thepen, T. *et al.* (1996) *J Allergy Clin. Immunol.* 97: 828-837; Grewe, M. *et al.* (1998) *Immunol. Today* 19:359361). Such positive feedback potentially creates a vicious circle, which by itself may be capable of maintaining local inflammation without the necessity of external stimuli. Other such mechanisms, resulting in a continual allergen non-specific response, resulting from dysregulation of macrophage are plausible, considering the regulatory potential of macrophages. The selective, localized elimination of inflammatory macrophages by targeting an Fc receptor, e.g., FcγRI, described in the Examples below makes the compositions of the invention useful for reducing or eliminating the positive feedback loop created upon macrophage secretion, and thus treating diseases such as AD.

Additional examples of diseases that can be treated via therapeutic methods of the invention include infectious diseases, e.g., HIV infections, respiratory conditions, e.g., Chronic Polymorphic Light Dermatosis (CPLD), Chronic Obstructive Pulmonary Diseases (COPD), for example, allergic asthma and Sarcoidosis, and inflammatory reactions such as those observed in open wounds or burn wounds.

In other embodiments, the compositions and methods of the present invention can be used in cosmetic applications. For example, the macrophage-binding compounds can be applied locally (e.g., topically) to the skin to delay and/or prevent the aging process of the skin.

The therapeutic methods of the present invention can be performed in conjunction with other techniques for removal of macrophage cells. For example, therapy using macrophage-binding compounds of the invention can be used in conjunction with surgery, chemotherapy or radio-therapy.

Macrophage-binding compounds of the invention can also be used to modulate FcγR levels on effector cells, such as by capping and elimination of receptors on the cell surface. Mixtures of anti-Fc receptors can also be used for this purpose.

The present invention further provides a kit comprising one or more dosages of a macrophage-binding compound and instructions for use.

In other embodiments, combinations of macrophage-binding compounds of the invention can be used to selectively kill or reduce the activity of macrophages, e.g., a combination of a first compound having at least one antigen binding region specific for an FcR and a toxin, and a second compound having an antigen binding region to a different epitope of the FcR receptor or a different Fc receptor, e.g., an Fcα receptor. In certain embodiments, a second macrophage-binding compounds of the invention can be used in conjunction with the first. For example, this second macrophage-binding compound can have at least one antigen binding region specific for an IgA receptor, e.g., Fcα receptor, IgE receptor, e.g., Fcε receptor, an Fcδ receptor and/or an Fcμ receptor.

Prior to administering macrophage-binding compounds to a subject, the subject can be pre-treated with an agent that modulates, e.g., enhances or inhibits, the expression or activity of Fcγ receptors, by for example, treating the subject with a cytokine. Preferred cytokines for administration during treatment with the macrophage-binding compound include of granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), and tumor necrosis factor (TNF).

Macrophage-binding compounds of the invention can also be used diagnostically *in vitro and in vivo* to detect and/or measure macrophage populations by measuring levels of Fc receptor binding. For example, as shown in the Examples provided herein, abundant expression of FcγRI is detected in the dermis of both acute and chronic cutaneous inflammation in humans. Therefore, the macrophage-binding compounds described herein can be used to diagnose such inflammatory conditions. For such uses, the compound can be linked to a molecule that can be detected. The detectable label can be, for example, a radioisotope, a fluorescent compound, an enzyme, or an enzyme co-factor. Accordingly, in another embodiment, the invention provides a method of diagnosing *in vitro* or *in vivo* disorders characterized by aberrant numbers of macrophages (e.g., macrophage proliferation) and/or Fc receptor expression (e.g., increased number of cells expressing an Fc receptor and/or increased Fc receptor expression in a given cell). By measuring the level of binding of the compounds of the invention in a given test sample or within a localized area, the presence of macrophages

within the area or sample can be deduced, provided that the anti-Fc receptor component of the compound is specific for macrophage Fc receptors. This can be done by (i) obtaining a body sample, such as a body fluid, tissue (e.g., a skin sample) or biopsy from a patient; (ii) contacting the body sample with a macrophage-binding compound of the invention or a fragment thereof: (iii) determining the level of binding of said macrophage-binding compound to the body sample; (iv) comparing the amount of molecule bound to the body sample to a control sample, e.g., a biological sample from a healthy subject, or to a predetermined base level, so that a binding greater than the control level is indicative of the presence of a macrophage disease, e.g., skin disease. Preferably, the level of Fc receptor expression is detected primarily on the macrophage cell population relative to other Fc receptor-expressing cells. Protocols for *in vivo* and *in vitro* diagnostic assays are provided in PCT/US88/01941, EP 0 365 997 and US 4,954,617.

The following invention is further illustrated by the following examples, which should not be construed as further limiting. The contents of all references, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference.

EXAMPLES

Materials and Methods

The following methodologies were used in the studies described below. The terms macrophage-binding compounds, CD64 immunotoxins (CD64 IT), or immunotoxins (IT) are used interchangeably herein.

Monoclonal Antibodies

The examples below describe the use of an anti-CD64 (anti-FcR) antibody corresponding to a humanized form of monoclonal antibody 22 (H22), described in U.S.P.N. 5,635,600, which is incorporated by reference. The production and characterization of the H22 antibody is described in Graziano, R.F. et al. (1995) *J. Immunol* 155 (10): 4996-5002 and PCT/US93/10384. The H22 antibody producing cell

line was deposited at the American Type Culture Collection on November 4, 1992 under the designation HA022CL1 and has the ATCC accession number CRL 11,177.

Other specific anti-CD64 antibodies which can be used in the methods and compositions of the invention are murine antibodies mAb 32.2, mAb 44, mAb 62 and mAb 197. The hybridoma producing mAb 32.2 is available from the American Type Culture Collection. ATCC accession number HB9469. The preparation of mAb 197-Ricin A conjugates is described in the Examples below.

The anti-FcR mAbs were purified from each respective hybridoma supernatant by protein A affinity chromatography (Bio-Rad, Richmond, CA).

Immunohistochemical Staining

A: CD64 staining

Biopsies were cut into 6μm sections on a freezing microtome and mounted on coated slides. After drying overnight, the sections were fixed for 10 minutes with dry acetone and air dried. Slides were incubated with FITC conjugated 10.1 (Serotec 1 :40) in PBS 2% normal mouse serum (NMS) for 45 min. Slides were washed three times for 5 minutes with PBS, 0.05% Tween, after which alkaline phosphatase (AP) conjugated sheep anti FITC (Boehringer Mannheim, 1 :400) in PBS (1% Human AB serum, 1% NMS for 30 min). After washing twice in PBS/Tween and once in Tris-HCl (0.1M, pH 8.5), AP activity was demonstrated using naphtol AS-BI phosphate (sodium salt, 50 mg/100 ml; Sigma) as substrate and new fuchsin (10 mg/100 ml; Merck, Whitehouse Station, N.J.) as chromogen dissolved in 0.1M TrisHCl, pH 8.5, resulting in pink/red staining. Endogenous AP activity was inhibited by addition of levamisole (35 mg/100ml, Sigma) to the reaction mixture. Slides were lightly counterstained with hematoxylin.

B: Markers

Sections were fixed in dry acetone with H_2O_2 (30%, 100μl/100ml) for 7 min. Slides were incubated with primary rat antibodies in optimal dilution for 45 min in PBS 2% NMS. The following antibodies were used to stain macrophages: MOMA-2 (Kraal,

G. *et al.* (1987) *Scand. J. Immunol.* 26: 653-661); dendritic cells: NLDC145 (Kraal, G. *et al.* (1986) *J. Exp. Med. 163*: 981 -997); T cells: KT3 (Tomonari, K. (1988) *Immunogenetics* 28:455-458). After washing three times (5 minutes in PBS, 0.05% Tween 20), incubation with peroxidase labeled rabbit anti rat conjugate (DAKO, 1:200), in PBS (1% Human AB serum, 1% NMS) followed for 30 minutes. After rinsing twice with PBS and once with NaAc (0.1M, pH 5.0), PO activity was revealed using H_2O_2 as substrate and DAB (Sigma) as chromogen, resulting in brown staining.

Animal Studies

Induction of cutaneous inflammation, Immunotoxin injections, and Biopsies. In the experiments described herein, transgenic FVB/N mice expressing human FcγRI were used (Heijnen, I.A., *et al.* (1996) *J. Clin. Invest.* 97:331 338). Nontransgenic littermates served as controls. To induce chronic cutaneous inflammation, an area of 1.5 by 1.0 cm on both flanks of the mice was shaved and the irritant Sodium Lauryl Sulfate (SLS) (5% in saline) was applied epicutaneous daily for ten consecutive days.

Animals were anaesthetized with 20 µl of a 4:3 mixture of Aescoket (Aesculaap, Gent, Belgium) and Rompun (Bayer, Leverkussen, Germany), intramuscularly injected. Two adjacent intradermal injections, (10µl each, $2x10^{-8}$ M, referring to the Ricin-A moiety, in saline) were administered. For control purposes, identical saline injections were administered contralaterally.

Animals were anaesthetized as described above and 3 mm punch biopsies were taken, snap frozen in liquid nitrogen and stored at -70°C prior to use. The skin was closed with one suture.

Punch biopsies (3mm) were taken under local anesthesia (1% lidocaine) from lesional AD skin (n=3), 24h APT (n=3), 48h SLS (n=2), and 72h WB challenged PLE skin. Biopsies were snap frozen in liquid nitrogen and stored at -70 °C prior to use.

EXAMPLE I: PREPARATION OF CD64 IMMUNOTOXINS

The CD64 monoclonal antibodies 197 (Guyre, P.M., *et al.* 1989. *J. Immunol.* 143: 1650-1655) and H22 (Graziano, R.F., *et al.* 1995. *J. Immunol.* 155:4996-5002)

were conjugated to de-glycosylated Ricin A (30 KDa, Sigma) using using a suitable linker (such as the heterobifunctional cleavable crosslinker N-succinimidyl 3-(2-pirydyldithio) proprionate (SPDP) (Pierce) under GLP conditions according to the manufacturers' instruction. Briefly, SPDP was conjugated to the CD64 mAb, e.g., H22, then the molar ratio of mAb-PDP was determined. After determining the molar ratio of mAb-PDP, Ricin A was added. Free PDP groups and free Ricin A chains were inactivated and the mixture was purified by size exclusion chromatography. The purity of H22-Ricin A conjugates was further checked by SDS-PAGE. H22-Ricin A conjugates were sterilized using an 0.2 μm filter. All preparation steps were performed under Good Manufacturing Practice conditions.

EXAMPLE II: EFFECTIVE CELL KILLING OF MACROPHAGES USING CD64 IMMUNOTOXINS

Constitutive expression of FcγRI is primarily restricted to cells of the myeloid lineage, and is strongly upregulated under proinflammatory and inflammatory conditions (Velde, A.A., *et al.* (1992) *J. Immunol.* 149:4048-4052; Schiff, D.E., *et al.* (1997) *Blood* 90:3187-3194). The ability of FcγRI to rapidly and efficiently mediate endocytosis makes this receptor an effective target for activated inflammatory macrophages (Heijnen, I.A *et al.* (1996) *J. Clin. Invest.* 97:331 338). Several immunotoxins against hFcγRI were prepared as described in Example I using conjugates of the toxin Ricin-A and CD64 antibodies.

To establish the efficiency of these conjugates in inducing macrophage killing, the cultured human promonocytic cell line U937, either unstimulated, or stimulated with IFN-γ, was examined in the presence or absence of the compositions of the present invention (Figs. 1, A and B). Culture conditions and stimulation of U937 cells with cytokines is described in Guyre, P.M., *et al.* (1983) *J. Clin. Invest.* 72:393-397. Briefly, U937 cells were cultured in the presence of 300 U/ml IFNγ for 24 hours to upregulate Fc γRI expression. FcγRI levels were monitored by flow cytometry. In addition, IIA1.6 cells, either non-transfected or transfected with FcγRIa cDNA were tested. IIA1.6 cells are derived from the murine A20 B cell lymphoma and were recently shown to belong to

a distinct subset of CD5+ B cell/macrophage cells (van Vugt, M.J., *et al.* 1998. *Clin. Exp. Immunol.* 113:415-422).

The cytotoxic efficacy of the CD64 immunotoxin (IT) was assessed by measuring the inhibition of [^{3}H]Thymidine incorporation in a concentration-dependent fashion (Post, J *et al. Leuk. Res.* 19:241 -247). Briefly, cells were seeded at $5x10^4$ cells/well in a 96 wells round bottom plate and incubated with CD64 IT for 72 hours in concentrations ranging from 10^{-12} to 10^{-7} M referring to the ricin moiety. Cells were pulsed for 4 h with [^{3}H]-Thymidine (1μCi) and subsequently harvested on glasswool filters and counted on a beta plate scanner. All incubations were performed in culture medium supplemented with 2 % human AB serum to block the Fc-binding site of FcγRI, thereby allowing binding of the IT by its antigen recognition site only. Cell numbers seeded were chosen such, that [^{3}H]-Thymidine incorporation was a linear function of the number of cells. Background values of [^{3}H]Thymidine incorporation were obtained by incubation with 0.1 mM cycloheximide.

Results were expressed as percentage [^{3}H]Thymidine incorporation compared to mock-treated cells. In Figures 1A-1B, the bar graphs represent the percentage of [^{3}H]-Thymidine incorporation as compared with that of medium control (±SEM). The dose dependent decrease in [^{3}H]-Thymidine incorporation as a function of increasing concentrations of H22-R or 197-R shows the cytotoxicity of the immunotoxins on the stimulated U937 cells. For panels 1C-1D, the bar graphs represent the percentage of [^{3}H]-Thymidine incorporation as compared with that of medium control (±SEM). The dose dependent decrease in [^{3}H]-Thymidine incorporation with resepect to increasing concentrations of H22-R or 197-R shows the cytotoxicity of the immunotoxins on hFcγ RI-transfected IIA1.6 cells. This demonstrates the specificity of both IT for hFcγ RI-expressing cells.

The two immunotoxins tested were potent inducers of cell killing. However, H22 Ricin-A (H22-R) was on the whole more effective than 197 Ricin-A (197-R) in inducing cell killing, especially on unstimulated cells. Incubation with Ricin-A alone at 10^{-8} and 10^{-9} M had no significant effect (88.9 ± 14.2 and 100.4 ± 13.5 percent, respectively). Furthermore, no significant effect of either IT was found on the

non-transfected IIA1.6 cells, in contrast to the effective killing of hFcγRI-transfected IIA1.6 cells detected using either of these ITs (Figure 1, panels C and D).

These results demonstrate both the efficacy and specificity of CD64 IT in killing hFcγRI-expressing cells *in vitro*. On the basis of these experiments, H22-R was used at a concentration of 2×10^{-8} M in the *in vivo* experiments described below.

EXAMPLE III: INDUCTION OF APOPTOSIS BY CD64-IMMUNOTOXINS

To establish whether the cytotoxic effect of H22 Ricin-A was due to apoptosis induction, propidium iodide staining in hypotonic buffer was performed. In this assay segmented apoptotic nuclei are recognized by subdiploid DNA content. To conduct these experiments, nuclear fragmentation was detected using propidium iodide staining as described in Nicoletti, I., *et al.* (1991) *J. Immunol. Methods* 139:271-279. In short, cells were incubated with IT and harvested at different timepoints. Cells were fixed with ethanol at -20°C, incubated with extraction buffer (0.05M Na_2HPO_4; 0.0025M citric acid; 0.1% Triton X- 100; 20μg/ml propidium iodide). Propidium iodide fluorescence was analyzed using a Fluorescent Activated Cell Sorter (FACScan) flow cytometer (Beckton and Dickinson, San Jose, CA).

As shown in Figure 2, apoptotic nuclei were detected in IT-treated cultures relative to control. In this experiment, U937 cells were stimulated with IFNγ and incubated for 6h with different concentrations of H22-R. Apoptotic nuclei were detected as early as 2 hours after IT exposure, and was still evident after 16 hours of treatment. This finding shows that the cytotoxic effect of H22 Ricin-A IT results from the induction of apoptosis. Apoptosis-mediated cell killing limits the potential damaging effects by depletion of hFcγRI-expressing cells *in vivo*. In addition, the long lasting cell killing induced by H22-R (even after 16 hours) suggests the practicability of H22-R as IT to deplete hFcγRI-expressing cells *in vivo*.

EXAMPLE IV: DETECTION OF FcγRI-EXPRESSING CELLS IN CHRONIC CUTANEOUS INFLAMMATION IN HUMANS

The staining ability of another CD64 monoclonal antibody, 10.1(Dougherty, G.J *et al.* 1987. *Eur. J. Immunol.* 17:1453-1459), was tested after pre-incubation of sections with H22 antibodies and in the presence of varying concentrations of H22 antibody. Since the 10.1 and H22 recognize different epitopes on hFcγRI, no significant change in staining intensity, or pattern was detected upon simultaneous incubation. Based on these results, the 10.1 antibody was used in all experiments involving immuno-histochemical evaluation of collected tissues.

To examine the presence of FcγRI-expressing cells in chronic cutaneous inflammation in humans, biopsies from chronically inflamed skin from patients with atopic dermatitis (AD) were collected. The diagnosis of AD was made according to the criteria of Hanifin and Raijka (Hanifin, J.M., and Rajka, G. (1980) *Acta Derm. Venereol. (Stockholm)* 92:44-47). Atopy Patch Test (APT) was performed as described in Langeveld-Wildschut, E.G., *et al.* (1995) *J. Allergy Clin. Immunol.* 96:66-73. In short, skin was tape stripped ten times and the allergen Dermatophagoides pteronyssinus (Haarlem's Allergenen Laboratorium, Haarlem, The Netherlands; 80,µl, 10,000 AU/ml) was applied using Leucotests (Beiersdorf, Hamburg, Germany) on clinically normal skin of the back of patients diagnosed with AD. On analogous skin, Sodium Lauryl Sulfate (SLS, Sigma, 0.1% in saline) was applied in a similar fashion. Polymorphic Light Eruption (PLE) was diagnosed on the basis of a polymorphic clinical picture, with presence of papules and vesicles, severe itching and clinical response after WA and / or WB irradiation. Previously unexposed skin was irradiated with 6 minimal erythema dose, using a Philips TL12 UVB source.

Sections from human skin were immunohistochemically stained using FcγRI antibodies. FcγRI-expressing cells were detected resulting as pink/red staining and counterstained with hematoxiline. In normal unaffected skin, few cells expressed FcγRI. These cells were located primarily in dermis. In contrast, abundant expression of FcγRI in dermis was observed in chronically lesioned skin, for example, atopic dermatitis skin. The stained cells were localized both in infiltrates and scattered through dermis. No

significant staining in epidermis was observed. Next to these, biopsies from acute phase models, such as 24hr after atopic patch test (APT), 72hr after polymorphic light eruption skin (PLE), and 48hr after treatment with Sodium Lauryl Sulfate (SLS), were collected. These biopsies gave similar results, however, the number of FcγRI-expressing cells was somewhat higher than in the chronically affected tissues. The very presence of large numbers of FcγRI-expressing cells in both acute and chronic phase is indicitive of a role for these cells in the inflammatory cutaneous response.

EXAMPLE V: ESTABLISHMENT OF A MURINE MODEL FOR CHRONIC CUTANEOUS INFLAMMATION

To determine whether elimination of inflammatory macrophages from skin is feasible and has a beneficial effect on cutaneous inflammation, the H22-R was tested in experimental animals. Induction of chronic cutaneous inflammation was studied using shaved skin of hFcγRI-transgenic mice and their nontransgenic littermates after repeated topical application of SLS. The expression pattern, gene regulation and function of hFcγ RI in these mice mirrors that in humans (Heijnen, I.A., *et al.* (1996) J. *Clin. Invest.* 97:331 338). Several protocols were tested and daily application of 5% SLS for ten days proved adequate as described in the section entitled Materials and Methods.

Low numbers of T cells, dendritic cells, and macrophages were detected in normal, untreated skin (5±4; 7±4 and 15±3 per mm^2 respectively). In addition, few hFc γRI-expressing cells were detected in normal, untreated skin (5±2 per mm^2), and the distribution resembled that of normal unaffected human skin. Treatment with SLS resulted in thickening of epidermis and a vast dermal infiltrate consisting of T cells, dendritic cells, and macrophages (Figure 3A). For these experiments, a single intradermal injection of IT was administered into chronically inflamed skin and at different intervals punch biopsies were taken and stained immunohistochemically. The number of cells expressing hFcγRI also increased dramatically (Figure 3A) (75±11 per mm^2) and like in chronically affected human skin, these cells were primarily distributed in the dermis . There was no significant difference in cellular composition between the hFcγRI-transgenic and non-transgenic mice. In the latter however, no significant cells

staining for hFcγRI were observed. No detectable presence of either hFcγRI-expressing cells or macrophages was observed after injection with H22-R only.

The similarities with respect to cellular composition and hFcγRI expression between chronically inflamed human skin and the SLS induced inflammation in hFcγ RI-transgenic mice make this a suitable model to study the role of hFcγRI expressing cells during chronic cutaneous inflammation. This model in combination with the H22-R IT was used in the Examples set forth below.

EXAMPLE VI: EFFECTIVE DEPLETION OF FcγRI-EXPRESSING MACROPHAGES IN VIVO

To determine whether H22-R was as effective in killing hFcγRI-expressing cells *in vivo* as it proved to be *in vitro*, H22-R was injected intradermally in mice treated with SLS. Chronic cutaneous inflammation was induced in the human FcγRI-expressing transgenic mice by repeated topical application of an irritant, 5% sodium lauryl sulfate as described in the section entitled Materials and Methods, *supra*. Two adjacent 10 μl intradermal injections of 2×10^{-8} M (3 μg of H22 and 0.6 μg of Ricin A) were administered once to SLS treated skin of hFcγRI-transgenic and nontransgenic mice. Identical vehicle control injections were administered contralaterally. SLS application was continued while at different timepoints skin samples, draining lymph nodes, liver, and spleen were collected for immuno-histochemical analysis.

The localized nature of the intradermal injections was examined by detecting uptake of carbon particle by macrophages. A cross-section of murine skin after intradermal injection of carbon particles revealedthe presence of carbon particles primarily in the dermis, but not below cutaneous musculature. This distribution demonstrates the localized nature of the intradermal injections.

A representative immunohistochemical cross-section of skin of human FcγRI-expressing transgenic mouse after repeated topical applications of sodium lauryl sulfate, and intradermal injection with vehicle control or Ricin A-H22 revealed the thickening of the epidermis and large number of infiltrating cells in the dermis 24 hours after treatment. This pattern of staining indicates chronic inflammation induced by the

irritant. The majority of the infiltrating cells detected were FcγRI-positive macrophages (stained in pink). In contrast, the staining of Fcγ receptor-expressing infiltrated cells was significantly reduced 24 hours after injection of the immunotoxin Ricin A-H22.

The disappearance of hFcγRI-expressing cells from the skin was detected within 24 hours of exposure to IT (Figure 3A). Despite continued SLS application, the depletion was complete till approximately 96 h after which repopulation occurred. Repopulation was complete only at 120 h (Figure 3A). In draining lymph nodes, liver, and spleen, no significant changes in hFcγRI expression were observed. This observation emphasizes the fact that the effect remains restricted to the site of injection. In the vehicle control injected site and in the non-transgenic mice no significant changes were observed. The rapid and nearly complete disappearance of hFcγRI-expressing cells and their protracted absence from skin showed the practicability of the H22-R IT to eliminate hFcγRI-expressing cells in chronic cutaneous inflammation *in vivo*.

EXAMPLE VII: EFFECT OF DEPLETION OF hFcγRI-EXPRESSING CELLS ON LOCAL CUTANEOUS INFLAMMATION

Simultaneously with the reduction in hFcγRI-expressing cells, the abundance of MOMA-2-expressing macrophages was also diminished. This finding shows that injection of H22-R results in efficient depletion of inflammatory macrophages from affected skin (Figure 3A). In contrast, no significant change in macrophage populations occurred in non-transgenic mice. This selective depletion confirms the specificity of H22-R in targeting and eliminating macrophages from skin.

To further assess the localized nature of the macrophage depletion, hematopoietic tissues such as lymph nodes, spleen, and liver were examined. No significant cell depletion by the immunotoxin was observed in other hematopoietic tissues. Identical treatment of non-transgenic littermates resulted in undetectable changes in any of the cell populations examined. These results indicate that the macrophage depletion was specific for human Fcγ RI-bearing cells and remained limited to the site of injection.

The specificity of the procedure in eliminating macrophages locally is further demonstrated by the disappearance, as early as within 24 hours, of macrophages, while no significant depletion was observed in dendritic cells, T cell populations, or Langerhans' cells during the timepoints examined. The H22-R injections had no direct effect on the numbers of T cells and dendritic cells (Figure 3B). However, after the disappearance of hFcγRI-expressing macrophages, T cell and dendritic cell numbers started to decrease in the skin. The reduction of T cell and dendritic cell numbers is indicative of resolving local inflammation and thus a beneficial effect of deletion of inflammatory macrophages on local inflammation, even in the continued presence of the inflammatory stimulus (Figure 3B).

These findings demonstrate the efficiency and specificity of the CD64 IT in depleting inflammatory macrophages from skin at the histological level. The subsequent disappearance of other inflammatory cells points to a deleterious role of macrophages in chronic cutaneous inflammation.

EXAMPLE VIII: LOCAL MACROPHAGE DEPLETION RESULTS IN CLINICAL IMPROVEMENT OF THE SKIN

To determiner whether local macrophage depletion resulted in clinical improvement of the skin two parameters were measured: local skin temperature and erythema. Erythema is primarily due to increased capillary dilatation, and directly related to this increased skin temperature.

To detect changes in skin temperature induced by SLS application and IT injection, animals were immobilized by mild ether sedation and local temperature was measured using a skinprobe (Ellab A-H1, Denmark). To elucidate capillary dilatation and vascular leakage as parameter for inflammation, animals were sedated with ether and intravenously injected with a 1% Evans blue solution. After 15 minutes animals were sacrificed and skin was removed for assessment.

Using a small skin probe, local changes in skin temperature were measured in IT-treated and control animals. A rise in temperature after SLS treatment was detected confirming the induction of local inflammation. Figure 4A is a bar graph depicting the

effect of intradermal injection of H22-R on local skin temperature as a function of time. A drop in temperature reaching levels comparable to untreated, unaffected skin. This decrease in temperature in IT-treated animals was detected typically lasting 96 hours. After that time, the temperature increased again, reaching levels comparable to that prior to IT injection. These changes in temperature are indicative of the resolution of inflammation. Neither the vehicle control nor the nontransgenic mice showed a similar decrease in temperature. Moreover, a close temporal correlation between the disappearance of the macrophages and the decrease in local skin temperature was observed. Conversely, upon reappearance of the macrophages, an increase in temperature was detected. This findings are highly suggestive of a critical role of macrophages in local inflammation.

In mice, redness of the skin is difficult to assess due to the thinness of murine skin. To facilitate visualization of local capillary dilatation, Evans blue was intravenously injected into these animals. For these experiments, chronic cutaneous inflammation was induced in hFcγRI-transgenic mice (n=9) or non-transgenic mice (n=9) by epicutaneous application of SLS and IT or vehicle control was administered intradermally. Evans blue was injected intravenously at 24h and 30 min later animals were killed and skin from the middle section was removed. Using this technique, the presence of an inflammatory response after SLS treatment was detected. No significant effect of the IT in capillary dilation was detected in non-transgenic mice or the vehicle control. At the H22-R-injected side of the latter however, the injection site itself was devoid of blue staining showing resolution of local inflammation. Moreover, the overall intensity of the blue staining was less at the H22-R-injected side.

EXAMPLE IX: PROLONGED SUPPRESSION OF INFLAMMATION IN VIVO UPON REPEATED INJECTIONS WITH CD64-IT

To establish whether the IT could be employed for a prolonged time, skin temperature was measured daily and upon increase the animals were again injected at the same site. During the experiment, SLS application was continued. Figure 4B shows that inflammation could be controlled for at least 18 days in hFcγRI-transgenic mice

injected with H22-R only. Vehicle control and non-transgenic mice did not show a significant decrease in temperature at any of the timepoints tested. Repeated injections with the IT demonstrated that it was possible to suppress inflammation for a prolonged period. This finding demonstrates the applicability of prolonged IT treatment in chronic cutaneous inflammation in patients. Taken together, these experiments show a beneficial effect of local macrophages elimination on the chronic cutaneous inflammation induced by SLS application.

In sum, the experiments described in the Examples herein show that activated macrophages can be eliminated selectively and efficiently eliminated using the methods and composition of the present invention, without significantly affecting other cutaneous or hematopoietic cell populations. Moreover, the effects of the immunotoxin remains primarily localized to the area of delivery, thus reducing negative systemic effect on other FcγR-expressing cells. The reduction in inflammation upon macrophage elimination underscores the importance of inflammatory macrophages as an agent in inducing and maintaining cutaneous inflammation. A reduction in the inflammation is detected at a histological level, as well as by decreases in clinical parameters such as local skin temperature and redness of the skin. Moreover, repeated application resulted in suppression of inflammation for prolonged periods. Prolonged effectivenes suggests the potential use of the methods and composition of the present invention in managing local cutaneous inflammation in patients suffering from chronic cutaneous diseases. This approach described herein may have wider applications since inflammatory macrophages are likely to play a key role in chronicity of other types of chronic inflammation, such as rheumatoid arthritis.

Staining for CD64 in human skin showed numerous FcγRI-expressing cells during both acute and chronic cutaneous inflammation. This observation indicates that targeting macrophages through FcγRI can indeed provide a new therapeutic approach for cutaneous inflammatory disease in humans. In fact, the effective reduction in SLS induced chronic inflammation in hFcγRI-transgenic mice showed herein supports potential therapeutic uses of these immunotoxins.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

We claim:

1. A method of selectively reducing the number or activity of macrophages, comprising contacting the macrophages with a macrophage-binding compound comprising (a) an agent which binds to an Fc receptor; and (b) an agent which kills or reduces the activity of the macrophages.

2. A method of treating or preventing a disease in a subject characterized by aberrant activity or number of macrophages within a selected area of the subject, comprising locally administering to the area a macrophage-binding compound comprising (a) an agent which binds to an Fc receptor; and (b) an agent which kills or reduces the activity of the macrophages.

3. The method of either of claims 1 or 2, wherein the portion which binds to an Fc receptor binds at a site which is not bound by an endogenous immunoglobulin.

4. The method of either of claims 1 or 2, wherein the Fc receptor is an Fcγ receptor (FcγR) or an Fcα receptor (FcαR).

5. The method of claim 4, wherein the Fcγ receptor is selected from the group consisting of FcγRI, FcγRII and FcγRIII.

6. The method of claim 5, wherein the Fcγ receptor is a human FcγRI.

7. The method of claim 4, wherein the Fc receptor is a human FcαR.

8. The method of either of claims 1 or 2, wherein the macrophage-binding compound comprises an anti-Fc receptor antibody conjugated to a toxin.

9. The method of claim 8, wherein the anti-Fc receptor antibody is an anti-Fcγ receptor antibody or a fragment thereof.

10. The method of claim 9, wherein the anti-Fcγ receptor antibody is a monoclonal antibody selected from the group consisting of mab 22, 32 and 197, or a fragment thereof.

11. The method of claim 9, wherein the anti-Fcγ receptor antibody is a humanized antibody H22 produced by the cell line having ATCC accession number CRL 1117 or a fragment thereof.

12. The method of claim 8, wherein the toxin is selected from the group consisting of Gelonin, Saporin, Exotoxin A, Onconase and Ricin A.

13. The method of claim 1, wherein the agent which kills or reduces the activity of the macrophages is encapsulated within a liposome.

14. The method of claim 13, wherein the agent which kills or reduces the activity of a macrophage is dichoromethylene diphosphonate (CL2MDP) or derivatives thereof.

15. The method of claim 13, wherein the agent which binds to an Fc receptor is a single chain antibody.

16. The method of claim 13, wherein the agent which binds to an Fc receptor is an anti-Fcγ receptor antibody or a fragment thereof.

17. The method of claim 13, wherein the agent which binds to an Fc receptor is a single chain anti-Fcγ receptor antibody or a fragment thereof.

18. The method of claim 1, wherein the contacting step occurs in culture.

19. The method of either of claims 1 or 2, wherein the macrophage-binding compound is administered topically, intradermally or subcutaneously in a pharmaceutically acceptable carrier.

20. The method of claim 2, wherein the disease is characterized by enhanced proliferation and/or growth factor secretion of the macrophage.

21. The method of claim 2, wherein the disease is selected from the group consisting of psoriasis, atopic dermatitis, scleroderma, cutaneous lupus erythematosis, Human Immunodeficiency Virus infection, multiple sclerosis, rheumatoid arthritis, Chronic Polymorphic Light Dermatosis, Chronic Obstructive Pulmonary Diseases, and Wegener's Granulomatosis.

22. A method of diagnosing a disease in a subject characterized by aberrant numbers or activity of macrophages, comprising:

contacting a biological sample from the subject with a macrophage-binding compound comprising an agent which binds to an Fc receptor; and

detecting the level of Fc receptor binding as an indication of the amount of Fc receptor protein in the sample,

wherein elevated expression of the Fc receptor protein, or an increase in the number of macrophages expressing the Fc receptor protein, is indicative of a macrophage-mediated disease.

23. The method of claim 22, wherein the macrophage-binding compound further comprises a detectable label.

24. The method of claim 22, wherein the Fc receptor protein expression is detected by autoradiographic, colorimetric, luminescent or fluorescent detection.

25. The method of claim 22 wherein the disease is selected from the group consisting of psoriasis, atopic dermatitis, multiple sclerosis, scleroderma, cutaneous lupus erythematosis, Human Immunodeficiency Virus infection, Chronic Polymorphic Light Dermatosis, Chronic Obstructive Pulmonary Diseases, and Wegener's Granulomatosis.

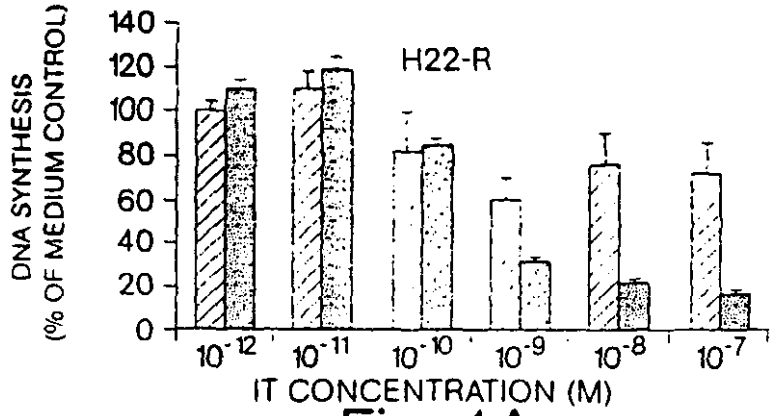

Fig. 1A

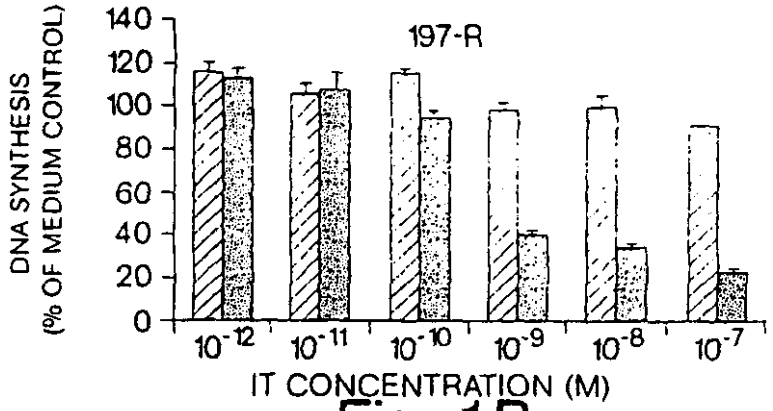

Fig. 1B

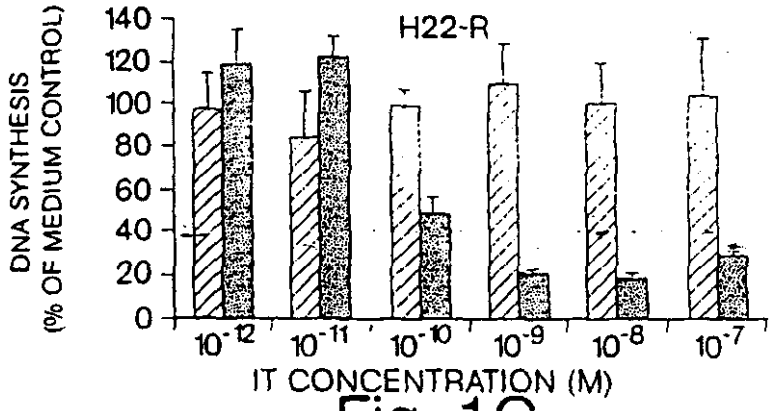

Fig. 1C

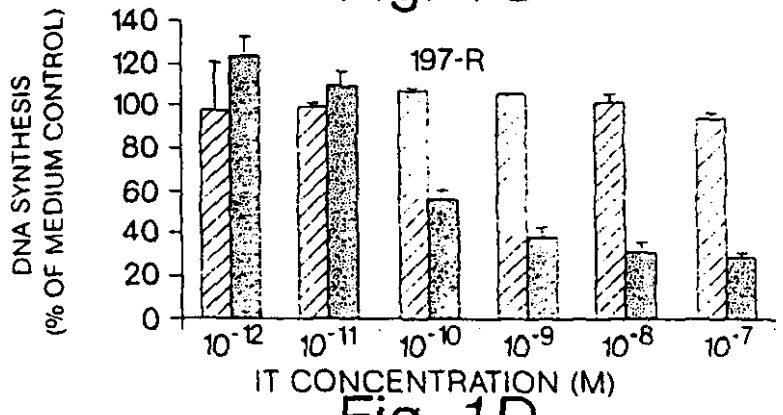

Fig. 1D

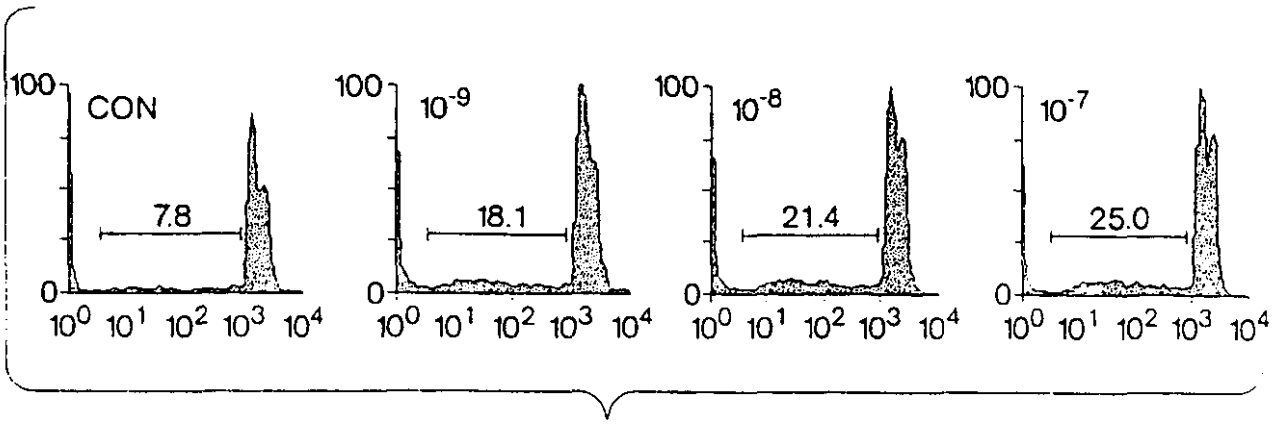
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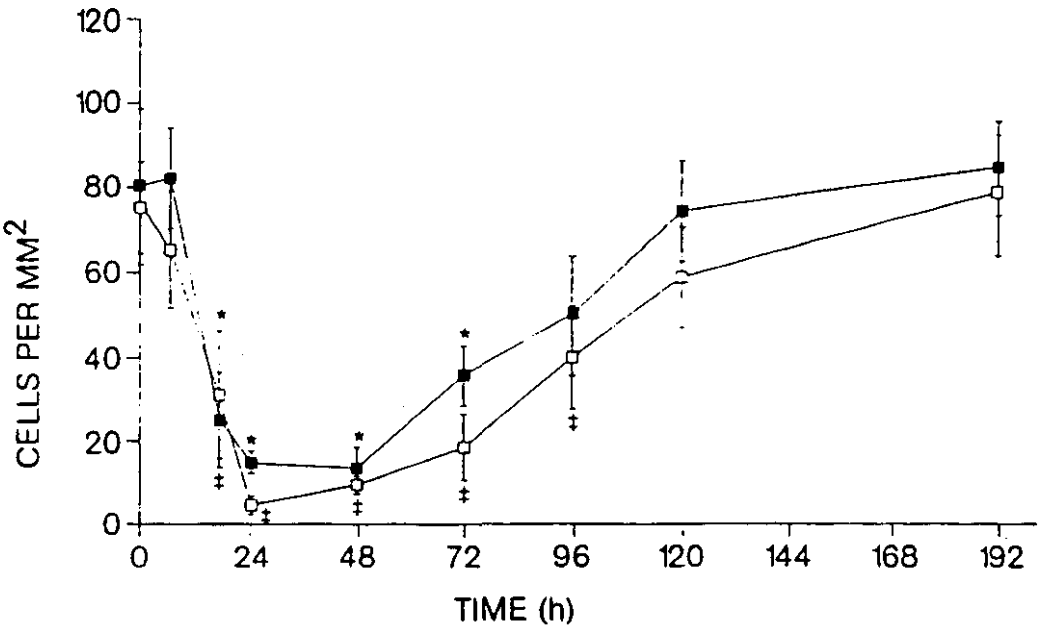
Fig. 2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80
60
40
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24
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96
120
144
168
192
TIME (h)

Fig. 3A

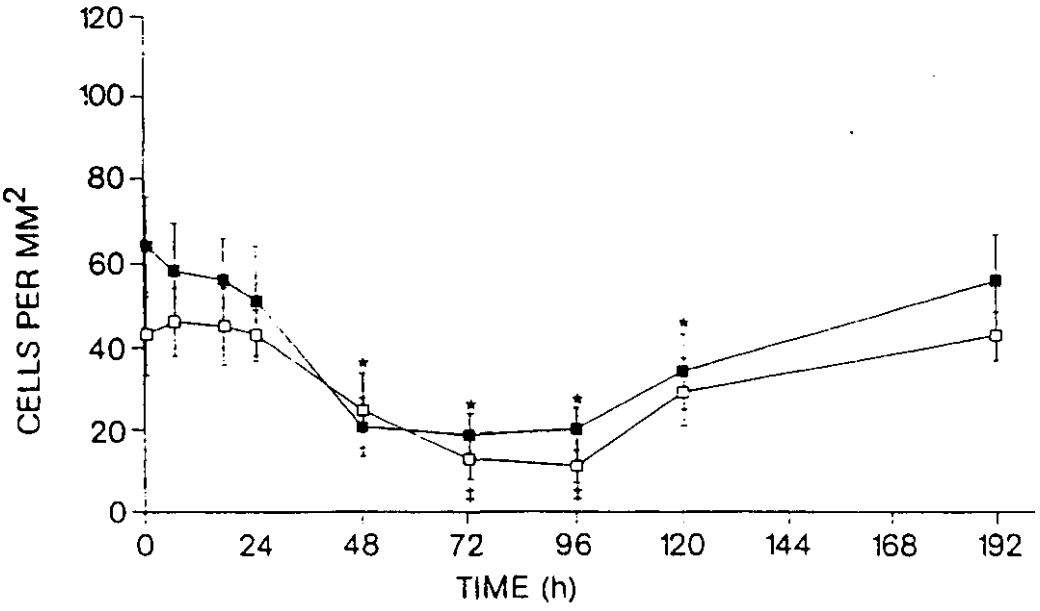
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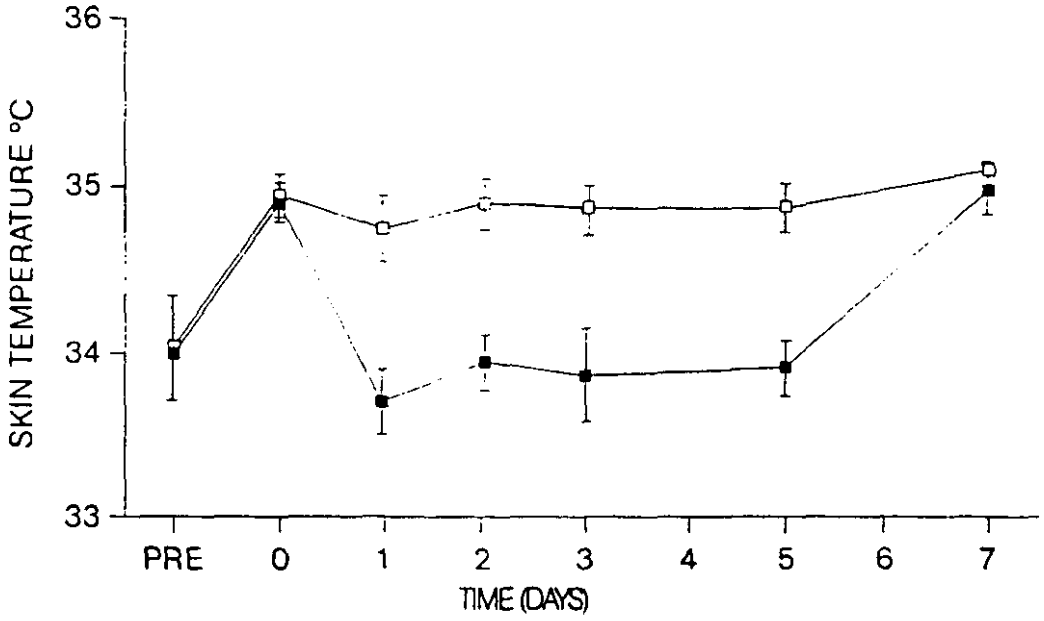
Fig. 3B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35
34
33
PRE
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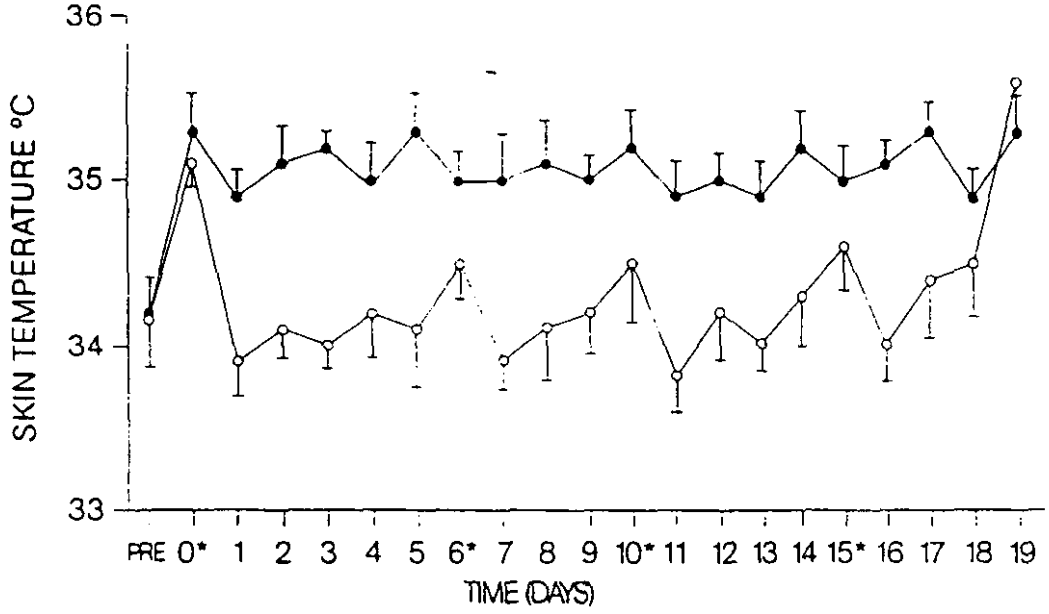
1
2
3
4
5
6
7
TIME (DAYS)

Fig. 4A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35
34
33
PRE 0* 1 2 3 4 5 6* 7 8 9 10* 11 12 13 14 15* 16 17 18 19
TIME (DAYS)

Fig. 4B

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权利要求书2页 说明书50页 附图页数4页

[54]发明名称 用FC受体配基治疗和诊断巨噬细胞介导的疾病

[57]摘要

本发明提供用于在局部区域选择性地靶向巨噬细胞的方法和组 合物。本发明的组合物包含Fc受体结合试剂,以及毒性的或可检测 的试剂。公开了缺失或抑制巨噬细胞活性的方法。本发明的组合物可 用于治疗或诊断目的。

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权 利 要 求 书

1. 有选择地减少巨噬细胞的数量或降低巨噬细胞活性的方法，包括用巨噬细胞结合化合物接触巨噬细胞，该巨噬细胞结合化合物包含（a）结合 Fc 受体的试剂（b）杀伤巨噬细胞或降低巨噬细胞活性的试剂。

2. 治疗或预防患者疾病方法，该疾病的特征是在患者的特定区域内巨噬细胞活性或数量异常，包括在局部区域施用巨噬细胞结合化合物，上述巨噬细胞结合化合物包含（a）结合 Fc 受体的试剂（b）杀伤巨噬细胞或降低巨噬细胞活性的试剂。

3. 根据权利要求 1 或权利要求 2 的方法，其中结合 Fc 受体的组成部分的结合位点不会被内源性免疫球蛋白结合。

4. 根据权利要求 1 或权利要求 2 的方法，其中 Fc 受体是 Fcγ受体（FcγR）或 Fcα受体（FcαR）。

5. 根据权利要求 4 的方法，其中 Fcγ受体选自 FcγRI、FcγRII、FcγRIII。

6. 根据权利要求 5 的方法，其中 Fcγ受体是人 FcγRI。

7. 根据权利要求 4 的方法，其中 Fc 受体是人 FcαR。

8. 权利要求 1 或 2 的方法，其中巨噬细胞结合化合物包含偶联了毒素的抗－Fc 受体抗体。

9. 根据权利要求 8 的方法，其中抗－Fc 受体抗体是抗－Fcγ受体抗体或其片段。

10. 根据权利要求 9 的方法，其中抗－Fcγ受体抗体是单克隆抗体，它选自 mab22、32 和 197 或其片段。

11. 根据权利要求 9 的方法，其中抗－Fcγ受体抗体是人源化的抗体 H22 或其片段，该抗体由 ATCC 登记号为 CRL 1117 的细胞系产生。

12. 根据权利要求 8 的方法，其中毒素选自 Gelonin、皂草素、外毒素 A、癌酶和蓖麻毒蛋白 A。

13. 根据权利要求 1 的方法，其中杀伤巨噬细胞或减少巨噬细胞活性的试剂被包入脂质体中。

14. 根据权利要求 13 的方法，其中杀伤巨噬细胞或减少巨噬细胞活性的试剂是二氯亚甲基二磷酸酯（CL2MDP）或其衍生物。

15. 根据权利要求 13 的方法，其中结合 Fc 受体的试剂是单链抗

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体。

16. 根据权利要求 13 的方法，其中结合 Fc 受体的试剂是抗－Fcγ受体抗体或其片段。

17. 根据权利要求 13 的方法，其中结合 Fc 受体的试剂是单链抗－Fcγ受体抗体或其片段。

18. 根据权利要求 1 的方法，其中接触步骤发生在培养中。

19. 根据权利要求 1 或权利要求 2 的方法，其中巨噬细胞结合化合物是在药用上可接受的载体中被局部、皮内或皮下施用。

20. 根据权利要求 2 的方法，其中疾病的特征是巨噬细胞的增殖和/或生长因子的分泌增强。

21. 根据权利要求 2 的方法，其中疾病选自牛皮癣、遗传性过敏皮炎、硬皮病、皮肤红斑狼疮、人类免疫缺陷病毒感染、多发性硬化症、风湿性关节炎、慢性多形性光照皮肤病、慢性梗阻性肺病和 Wegener's 肉芽肿病。

22. 诊断患者中以巨噬细胞数量或活性异常为特征的疾病的方法，包括用包含结合 Fc 受体试剂的巨噬细胞结合化合物接触源自患者的生物样品；检测 Fc 受体结合的水平，该水平表明样品中 Fc 受体蛋白的数量，其中 Fc 受体蛋白表达水平的升高或表达 Fc 受体蛋白的巨噬细胞数量的增加表明存在巨噬细胞介导的疾病。

23. 根据权利要求 22 的方法，其中巨噬细胞结合化合物进一步包含可探测的标记物。

24. 根据权利要求 22 的方法，其中 Fc 受体蛋白的检测是通过放射性自显影、比色法、发光或荧光检测。

25. 根据权利要求 22 的方法，其中疾病选自牛皮癣、遗传性过敏皮炎、多发性硬化症、硬皮病、皮肤红斑狼疮、人类免疫缺陷病毒感染、慢性多形性光照皮肤病、慢性梗阻性肺病和 Wegener's 肉芽肿病。

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说　明　书

用 FC 受体配基治疗和诊断巨噬细胞介导的疾病

发明背景

在正常人的皮肤中可区分出两个分开的层．上层，表皮层，由角质细胞、郎格罕氏细胞和 T 细胞组成．下层，真皮层，由纤维原细胞、内皮细胞和树突状细胞、T 细胞、肥大细胞和巨噬细胞组成．

皮肤是外环境和内环境之间的重要分界．它主要防止和潜在的有害物质接触．在抗原/病原体侵入时在体内诱导炎症反应以清除抗原．该反应导致真皮渗透，渗透的成分依赖于所诱导的应答的类型，但主要由 T 细胞、多形核细胞和单细胞组成（Williams. I. R., and Kupper. T. S. (1996) Life Sci. 58: 1485-1507; Stingl, G. (1993) Recent Results Cancer Res. 128: 45-47）．此外，非特异性的过敏原刺激如组织损伤和紫外线也能诱发炎症反应．通常，在过敏原特异性反应的效应器阶段也应用了过敏原非特异性反应的机制．

巨噬细胞是来自骨髓的细胞，具有很大的异质性和多样性．这些细胞能产生范围很广泛的介导物质并执行许多生物功能（Ganz, T. (1993) New Horiz. 1: 23-27）．它们的表型和功能很大程度上受局部环境决定，而源自巨噬细胞的介导物质因此能影响它们的微环境．这种微环境导致区域性的不同巨噬细胞子集，出现区域化的不同巨噬细胞亚群（Gordon, S. (1995) Bioessays 17: 977-986）．这些细胞是强有力的效应细胞，它们产生能直接损伤组织的活性氧产物和蛋白水解酶（Laskin, D. L., and Pendino, K. J., (1995) Annu Rev Pharmacol Toxicol. 35: 655-677）．在正常条件下巨噬细胞调节细胞外基质形成细胞如皮肤中的纤维原细胞（Gonzalez-Ramos, A. et al. (1996) J. Invest. Dermal. 106: 305-311）．此外，巨噬细胞能执行重要的免疫调节功能，通过这种方式在控制和导向免疫应答中起着重要的作用（Gordon, S. (1995) Bioessays 17: 977-986; Thepen, T. et al., (1994) Ann. N. Y. Acad. Sci. 725:200-206）．这些细胞可作为抗原递呈细胞，但也通过树突状细胞直接抑制抗原递

呈（Holt, P. G. et al. (1993) J. Exp Med. 177: 397-407）。巨噬细胞可影响T细胞的增殖、表型和功能，从而影响所诱导的免疫应答。

皮肤巨噬细胞在调节不同非造血细胞（例如纤维原细胞和角化细胞）以及T细胞和树枝状的细胞的生长中起了重要的作用。在"稳态"条件下，皮肤巨噬细胞的数量相对较少。然而，在各种病理条件下（例如，活跃的伤口），巨噬细胞的数量显著增加。组织巨噬细胞和渗透单核细胞与发炎伤口中的修饰纤维原细胞和角化细胞的功能以及T细胞和/或树枝状细胞的异常功能是相关的。

和郎格罕氏细胞相反，暴露于紫外线能在皮肤中诱导出一类能激活自活性（autoreactive）T细胞的巨噬细胞群。已经发现巨噬细胞功能失控与各种疾病，包括皮肤T细淋巴瘤（霉菌类）、牛皮癣、遗传性过敏皮肤炎和红斑狼疮中的异常皮肤免疫应答直接相关（Cooper, K. D. et al., (1993) J. Invest. Dermatol. 101: 155-163; Gonzalez-Ramos, A. et al. (1996) J. Invest. Dermatol. 106: 305-311）。这些细胞也能激活常驻的或炎症的巨噬细胞，导致维持着皮肤炎症的"恶性循环"。除了调节细胞功能外巨噬细胞还是产生毒性化合物如氧自由基和蛋白水解酶的有力生产者。已知这些毒性化合物导致直接的组织损伤。

发明概述

本发明提供通过 Fc 受体选择性地将细胞毒化合物靶向到单核细胞来源的噬菌细胞（即巨噬细胞）的组合物和方法。因此本发明被用于在局部区域如皮肤、关节和肺部选择性地减少一群巨噬细胞的数量。

在一个实施方案中，本发明提供了一种结合巨噬细胞的化合物，该化合物至少含有能结合到呈现于巨噬细胞的 Fc 受体的第一部分，和至少含有能杀灭或抑制巨噬细胞功能的第二部分。上述结合到 Fc 受体的部分可以包括任何能结合Fc受体的分子，例如抗体、肽(例如肽模拟物)或化学化合物。在一个实施方案中，Fc 受体结合部分是抗

体或抗体片段（例如 Fab, Fab', F(ab')$_2$, Fv, 或单链 Fv）. 在一个优选的实施方案中，抗-Fc 受体抗体或抗体片段是"人源化的"（例如至少有一个源自非人类抗体(例如鼠)的互补性决定区（CDR）或互补性决定区的一部分，其它部分是人源的）在另一个优选的实施方案中，抗-Fc 受体抗体或抗体片段是人单克隆抗体(例如由能表达完整人抗体的基因工程鼠产生的抗体）. 这些方案中还包含"模仿"这样的抗-Fc 受体抗体的结合的化合物（例如肽或化学物质）（Jenks et al. J. Natl. Cancer Inst. (1992) 84 (2): 79 ; Saragovi et al. Science, (1991) 253: 792; Hinds et al. J. Med, Chem. (1991) 34: 1777-1789; Fassina Immunomethods (1994) 5: 121-129）. 在另一个实施方案中，巨噬细胞结合化合物的 Fc 受体结合部分是花青苷组合物，如荧光染料 Cy5.18. OSu(此处用"Cy5"表示)，它和呈现于巨噬细胞的 FcγRI 受体以很高的亲和性和特异性结合. 花青苷组分可包括至少两个部分：花青苷琥珀酰亚胺（succimidyl）酯和藻胆蛋白体蛋白，例如 PE.

为本发明的巨噬细胞结合化合物所识别的 Fc 受体可以是 IgG 受体，例如 Fc-γ受体（FcγR），如 FcγRI（CD64），FcγRII（CD32）和 FcγRIII（CD16），或 IgA 受体，如 FcαR（例如 FcαRI, CD89）. Fc 受体优选地位于巨噬细胞，例如皮肤巨噬细胞，的表面，以便它能被上述化合物所识别和结合. 在一个优选的实施方案中上述巨噬细胞结合化合物的抗 Fc 受体结合部分和 Fc 受体结合的位点和内源性免疫球蛋白（例如 IgG 或 IgA）所结合的位点截然不同. 因此，上述巨噬细胞结合化合物与 Fc 受体的结合不会被生理水平的免疫球蛋白阻断.

巨噬细胞上用于定位的一种优选的 Fc 受体是高亲和性的 Fcγ受体，FcγRI. 因此在本发明的巨噬细胞结合化合物的抗 Fc 受体结合部分包括一个抗 FcγRI 抗体，或该抗体的一个片段. 抗 FcγRI 抗体的例子包括 mAb22、mAb32、mAb44、mAb62 和 mAb197. 在一个优选实施方案中使用了这样的抗 FcγRI 受体抗体的人源化形式，如人源化单克隆抗体 22（H22）或其片段.

上述巨噬细胞结合化合物的杀死巨噬细胞或调节（即降低）巨噬细胞活性的部分（抗巨噬细胞物质）可选自适当的细胞毒素或药物. 例如抗巨噬细胞物质可以是 Gelonin、皂草素、癌酶（Onconase）、

外毒素 A、蓖麻蛋白 A（Ricin A）、二氯甲烷二膦酸酯（CL2MDP）或它们的衍生物．在一个实施方案中抗巨噬细胞物质是直接连接到巨噬细胞结合化合物的抗 Fc 受体结合部分的．在另一个实施方案中抗巨噬细胞物质是不直接连接到巨噬细胞结合化合物的抗 Fc 受体结合部分的．例如，抗巨噬细胞物质被包到和抗 Fc 受体结合部分连接的脂质体中．

本发明的巨噬细胞结合化合物可被用于多种治疗和诊断方法．在一个实施例中这些化合物被用于诊断一种疾病，该疾病的特征为巨噬细胞的数量或功能不正常．这些方法包括在允许上述化合物和样品中的巨噬细胞结合的条件下，在一个测试区域，或人工培养的样品中接触或施用上述巨噬细胞结合化合物．然后可检测化合物的结合，作为样品中巨噬细胞的存在（数量）和/或功能的指标．例如，特异性检测到的 Fc 受体蛋白水平的统计学意义上的显著升高表明巨噬细胞数量的增加，这可能意味着疾病．测试区域或样品可来自，例如，皮肤（如人类皮肤）或其它含巨噬细胞的组织．

在另一个实施方案中，巨噬细胞结合化合物被用于治疗一种涉及巨噬细胞的增殖和/或异常功能的疾病．当巨噬细胞化合物接触需要治疗的区域时，该化合物通过巨噬细胞的 Fc 受体结合到巨噬细胞上并且杀死这些细胞或降低这些细胞的活性．相应地，利用本发明的化合物可以治疗、预防或诊断广泛的涉及巨噬细胞（例如，巨噬细胞增殖和/或异常功能）的各种疾病．这些疾病可以是内源性的（例如自体免疫疾病），或外源性的（例如接触过敏症，多形性光疹（polymorphic light eruption, PLE），和过敏反应）．在遗传性过敏症和全身性红斑狼疮的情况下皮肤疾病可能是更为全身性的疾病，如遗传性过敏皮炎（AD），的先兆．可以用本发明的组合物和方法治疗非限制性疾病列表包括自体免疫疾病、呼吸道疾病、感染疾病、皮肤疾病和炎症．这些疾病的具体例子包括，但不限于，牛皮癣、遗传性过敏皮炎、多发性硬化、硬皮病、皮肤红斑狼疮、类风湿性关节炎、人类免疫缺陷病毒感染、慢性多形性光照皮肤病（CPLD）、慢性阻塞性肺病（COPD），例如过敏性哮喘和类肉状瘤病、Wegener 氏肉芽肿病和炎症，如皮肤损伤（例如开放的伤口或烧伤）．进而，本发明的方法和组合物可被用于体外诊断这样的疾病，或用于研究目的

（例如研究巨噬细胞在这样的疾病中的角色）.

当用于体内治疗目的时可将优选剂量的本发明的巨噬细胞结合化合物局部施用（例如局部地，皮内地，皮下地或作为雾剂吸入）到选中区域以衰竭或降低施用区域内的巨噬细胞活性. 在某些实施方案中巨噬细胞结合化合物可包括在施用（例如全身施用、局部施用或肌肉内施用）无活性，但暴露于光（如可见或紫外光）时被激活的光敏物质. 类似地，结合化合物可包括通过光敏连接连接到 Fc 结合剂上的治疗（或诊断）试剂，暴露于光时这些试剂被释放. 这些化合物允许可控制地仅仅杀死或失活所选的暴露于光组织中的巨噬细胞.

本发明进而提供用于上述方法的组合物，例如含有巨噬细胞结合化合物以及可接受的自体或稀释液的药用组合物.

在下文的附图、详述、实施例和权利要求中本发明的其它特征和优点将是显而易见的.

附图简述

图 1 是描述和培养基对照（±SEM）相比，[^{3}H]－胸腺嘧啶掺入到培养的 U937 或 IIA1.6 细胞的百分比的柱状图. 上述细胞是在存在或不存在不同浓度的 CD64－免疫毒素（H22 蓖麻毒素 A，H22－R 或 197 蓖麻毒素 A，197R）的条件下生长的. 在图示浓度的 H22－R（图 A）或 197－R 存在的情况下 U937 细胞与 IFNγ（黑条）或不与（灰条）IFNγ一起培养. 在图 C 和 D 中转化了 hFcγRI（黑条）或未转化（灰条）的 IIA1.6 细胞与不同浓度的 H22－R（图 C）或 197－R（图 D）一起培养.

图 2 是 U937 细胞的碘化丙锭荧光扫描，因为这些细胞和不同浓度的 H22－R 保温后导致了细胞调亡. 用碘化丙锭染色分析了核碎片并用柱图表示了亚二倍体核. 柱图上的数字表示亚二倍体的百分比，即调亡核. con=对照.

图 3A 和 B 表明一次皮下注射免疫毒素对皮肤的炎症细胞的效力与时间的关系. 数据点代表每平方毫米（±SEM）中细胞的平均数量并且数据点代表了大于三次实验的平均结果. 所描述的是 hFcγRI－表达细胞（实心方格，图 3A）、巨噬细胞（空方格，图 3A）、T 细胞（实心方格，图 3B）和树枝状细胞（空方格，图 3B）的动力学.

图 4A－4B 表明皮下注射免疫毒素时局部皮肤温度的降低. 图

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4A 描述了 SLS 处理的 hFcγRI 转基因鼠经过一次注射 IT（●）（n=6）或载体对照（O）（n=6）以后局部皮肤温度读数（±SEM）. 图 4B 表明 SLS 处理的 hFcγRI 转基因鼠注射 IT(●)(n=6)或载体对照(O)(n=6)时的温度过程. 每天监测局部皮肤温度，并且温度升高时就在同样的部位重新注射动物（天数用*标记）.

发明详述

在许多种病症中，例如皮肤疾病、自体免疫疾病、传染疾病和炎症中都表明存在巨噬细胞功能异常，包括增殖和/或活性异常. 目前为止，利用细胞毒素例如免疫毒素局部消除巨噬细胞的方法的功效是有限的. 本发明提供了用于诊断、治疗和预防这些疾病的方法和组合物，该方法和组合物通过在局部区域有选择地减少巨噬细胞和/或抑制巨噬细胞的活性. 通过巨噬细胞的 Fc 受体将毒性物质定位到巨噬细胞上使细胞减少（例如被杀死）和/或被抑制（例如活性降低）. 例如，此处描述的研究演示了巨噬细胞结合化合物的应用，该化合物含有偶联到一种毒素上，例如蓖麻毒蛋白 A，的抗－Fc 受体结合部分，例如抗人 FcγRI 受体的人源抗体，以便在表达人 hFcγRI 的转基因鼠体内有选择地排除巨噬细胞 . 此处所用的术语“巨噬细胞”“源自单核细胞的吞噬细胞”应该是可以互换使用的.

相应地，在一个实施方案中，本发明提供了一种巨噬细胞结合化合物，该化合物包含一种能结合到呈现于巨噬细胞的 Fc 受体上的试剂和一种能杀死所结合的巨噬细胞或抑制该巨噬细胞活性的试剂. 用于结合 Fc 受体的适当组分包括，例如，蛋白（例如抗－FcR 抗体和肽或其化学模拟物，或 FcR 受体配基）和化学部分（例如染料和合成的 FcR 配基）. 这些 Fc 受体结合试剂可以是单特异性、双特异性、或多特异性的，因为它们分别含有一个、两个或两个以上的结合区域. 例如，该试剂可结合到 Fc 受体的两个或更多个不同区域，或结合到 Fc 受体以及同一细胞或另一细胞的不同组分. 在所有的情况下该试剂含有至少一个结合 Fc 受体的部分.

在一个实施方案中 Fc 受体结合试剂是抗体或抗体片段，包括，如，Fab，Fab'，$F(ab)_2$，Fv，或单链 Fv. 上述抗体也可以是轻链或重链二聚体或其任何极小片段，如 Fv 或 1990 年 8 月 7 日发表的 Ladner 等，美国专利第 4，946，778 号中所公布的单链构建物，上述专利的

内容在此引入作为参考.

在另一个实施方案中Fc受体结合试剂是抗体模拟物(如肽或化学化合物)(Jenks et al. J. Natl. Cancer Inst. (1992) 84 (2): 79; Saragovi et al. Science (1991) 253: 792; Hinds et al. J. Med. Chem. (1991) 34: 1777-1789; Fassina Immunomethods (1994) 5: 121-129).

在另一个实施方案中 Fc 结合组分是双特异性或多特异性分子. 术语“双特异性分子”是用于包括任何具有两个不同的结合特异性的化合物，如，化学部分或蛋白、肽或蛋白或肽的复合物，它和（a）巨噬细胞表面上的Fc受体，以及（b）第二个不同的目的抗原结合或相互作用. 术语“多特异性分子”或“杂合特异性分子”是用于包括任何具有两个以上不同的结合特异性的化合物，如，化学部分或蛋白、肽或蛋白或肽的复合物，它和（a）巨噬细胞表面上的 Fc 受体，以及（b）两个或更多个不同的目的抗原结合或相互作用. 相应地，可被用于本发明的巨噬细胞结合化合物的 Fc 受体结合试剂包括针对巨噬细胞 Fc 受体的双特异性、三特异性、四特异性和其它多特异性分子.

例如，该试剂可以是包含连接到一起的具有不同特异性的两个或多个抗体、抗体结合片段（例如 Fab）或其衍生物的杂合抗体. 这些不同的特异性可包括 Fc 受体上的两个或更多个不同的结合特异性. 替代地，它们可包括一个 Fc 受体上的结合特异性和至少一个同一细胞（即巨噬细胞）或不同靶细胞（即其它免疫细胞或病原体）上的其它不同的结合特异性.

在 Fc 结合试剂是双特异性或多特异性分子的实施方案中该试剂可通过在物理上使得细胞毒性效应细胞和靶巨噬细胞靠近而发挥功能，从而更有效地靶向消灭巨噬细胞. 如此处所用，术语“效应细胞”指参与免疫应答的效应阶段的免疫细胞，而不是参与免疫应答的识别阶段或激活阶段. 免疫细胞的例子包括源自骨髓或淋巴的细胞，如淋巴细胞（如B细胞和T细胞，包括细胞毒性T细胞（CTLs））、杀伤细胞、自然杀伤细胞、嗜酸性粒细胞、嗜中性粒细胞、多形核细胞、粒细胞、肥大细胞和嗜碱性粒细胞. 类似巨噬细胞，效应细胞表达特异的 Fc 受体并执行特异的免疫功能. 在优选的实施方案中效应细胞

能诱导抗体依赖的细胞毒性（ADCC），如嗜中性粒细胞能诱导 ADCC. 例如，表达 FcαR 的嗜中性粒细胞、嗜曙红细胞和 T 细胞参与特异性地杀死靶细胞并将抗原递呈给免疫系统的其它成员，或和呈现该抗原的细胞结合. 在另一个实施方案中效应细胞能吞噬靶抗原或细胞（例如巨噬细胞），或微生物或能裂解该靶细胞，如巨噬细胞. 特定的 Fc 受体在效应细胞上的表达可为体液因子如细胞因子所调节. 例如有人发现 FcγRI 的表达为干扰素γ（IFNγ）所上调. 这种增强后的表达能提高带 FcγRI 的细胞针对目标，如巨噬细胞，的细胞毒性.

在本发明的另一个实施方案中 Fc 受体结合试剂是单克隆抗体或其片段. 此处所用的术语“单克隆”或“单克隆抗体组分”指单一分子组分的抗体制备物. 单克隆抗体表现出对特定表位的单一结合特异性和亲和力. 单克隆抗体可以是鼠的或人的单克隆抗体（例如，表达完整人抗体的遗传工程鼠产生的抗体）.

在本发明的另一个实施方案中 Fc 受体结合试剂是嵌合抗体或其片段，或人源抗体或其片段. “嵌合抗体”是用于包括一种抗体，其可变区源自一种动物而其恒定区源自另一种动物. 例如，一种嵌合抗体可以是含有源自小鼠单克隆抗体的可变区和源自人的恒定区的抗体. 在本发明的一个优选实施方案中，巨噬细胞结合化合物含有人源抗体或其结合片段. 术语“人源抗体”是用于包括一种抗体，其高变区，也称为互补决定区（CDRs）源自一种动物，而其框架区和恒定区源自不同不同种的动物. 在本发明的一种人源抗体中 CDRs 是源自小鼠而该抗体的其它区域源自人类. 在优选的实施方案中重链可变区（VHs）源自已知蛋白 NEWM 和 KOL，Ig Kappa 链可变区源自 REI. 此处所用的术语抗体是用于包括嵌合和人源抗体、抗体的结合片段或其修饰形式.

术语抗体或蛋白的能结合抗原的 “片段”或“结合片段”是用来包括抗体或蛋白能结合抗原的片段. 和完整抗体和抗原的结合相比，抗体的结合片段和抗原的结合可以具有相同的亲和性或不同的亲和性，例如，更高或更低的亲和性. 术语抗体中所包含的结合片段的例子包括：由 V_L，V_H，C_L 和 C_{H1} 结构域组成的 Fab 片段；由 V_H 和 C_{H1} 组成的 Fd 片段；由一个抗体的一条臂的 V_H 和 V_L 组成的 Fv 片段；有 V_H 结构域组成的 dAb 片段（Ward et al. 1989 Nature 341: 544-546）；

分离的互补决定区（CDR）；和F（ab）'$_2$片段，一种由两个Fab片段在铰链区通过二硫键连接在一起的片段．结合片段，例如抗体的结合片段，可以是活性或功能性的结合片段．相应地，活性或功能性结合片段用来包括能引发至少一种全长分子所引发的功能或活性的结合片段．例如单克隆抗体M22或H22的活性结合片段是该抗体能和结合FcγR并引发受体介导的效应细胞活性的片段．这些抗体是通过本领域中具有一定技术的人员所已知的传统技术得到的，并通过和完整抗体一样的方法筛选这些片段的用途．

在此所用的“和……结合的试剂”或“结合特异性”与术语“抗原结合位点”、“抗原结合区域”以及“抗体的结合决定簇”是可以互换使用的．这些术语用于包括分子，例如抗体，的参与结合抗原的区域．抗体的抗原结合位点包括，但不限于，抗体和抗原接触的氨基酸．抗原结合区域可以是抗体的可变区．抗原结合区域也可以是抗体的高变区．抗体的抗原结合区域也可以是抗体高变区的与抗原接触和/或为抗原结合区域提供适当三级结构的氨基酸残基．有许多方法可用于决定抗体可变区或高变区的哪些氨基酸残基接触抗原和/或对抗原结合区域的正确折叠是重要的．例如，可进行基因突变分析．特别地，可以在重组抗体中把一个或多个氨基酸替换成其它氨基酸，并且进行体外结合实验以确定和非修饰的抗体相比，修饰抗体抗原结合力的改变程度．如果结合力下降是由于一个氨基酸替换成另一个氨基酸，那么该氨基酸在抗体结合抗原时很可能是重要的．确定抗体可变区的哪些氨基酸残基参与抗体和抗原结合的其它方法是建立在晶体学分析，例如，X-射线晶体学，的基础上的．

术语“特异性结合抗原的抗体”用于包括一种抗体，该抗体和特异性抗原结合的亲和性显著高于和其它抗原的结合，即，它用于按照本领域中的方式定义抗体的特异性．术语“识别抗原的抗体”、“对抗原特异的抗体” 在此处可以与“特异性结合抗原的抗体”互换使用．

抗－Fc受体结合试剂的生产

I．抗－Fc受体抗体的生产

用于本发明巨噬细胞结合化合物的抗－Fc受体抗体包括任何通过各种已知的技术生产的抗体，前提是该抗体能结合到巨噬细胞的

Fc 受体上. 优选的抗体是可用作临床用途的(例如, 可施用于人类). 特别优选的抗体对人类施用时是非免疫原性的(例如, 转基因动物产生的人抗体), 或该抗体被修饰以便当抗体施用于人类时免疫原性减小(例如, 人源化的).

在一个实施方案中, 抗-Fc 受体抗体是单克隆抗体, 例如, 鼠或人的单克隆抗体, 该抗体结合到 IgG 受体或 IgA 受体上, 优选地结合到不会被人类免疫球蛋白 G(IgG)或免疫球蛋白 A(IgA)阻断(例如, 结合)的位点上. 此处所用的术语"IgG 受体"指位于染色体 1 上的任意八个 FcγRI 受体基因. 这些基因编码全部的十二种跨膜或可溶的同工型受体, 这些同工型受体分为三类 FcγR 受体: FcγRI(CD64), FcγRII(CD32)和 FcγRIII(CD16). 在一个优选的实施方案中, Fcγ受体是人高亲和性的 FcγRI. 该人 FcγRI 是 72KD 的分子, 表现出对单体 IgG($10^8-10^9M^{-1}$)的高亲和性. Fanger et al. 在 PCT 申请 WO 88/00052 和在美国专利号, 4, 954, 617, 中, 描述了这些优选的单克隆抗体的生产和特征, 其中的讲授内容在此全文引入作为参考. 这些抗体结合到 FcγRI、FcγRII 或 FcγRIII 表位上与受体 Fcγ结合位点不同的位点上, 因此它们的结合基本上不会被生理学水平的 IgG 所阻断. 本发明中有用的抗-FcγRI 特异性抗体是 mAb22、mAb32、mAb44、mAb62 和 mAb197. 产生 mAb32 的杂交瘤可从美国典型培养物保藏中心获得, ATCC 登记号 HB9469. 抗-FcγRI mAb22, mAb22 的 F(ab')2 片段可以从 Medarex, Inc(Annandale, N. J.)获得. 产生 mAb32 的杂交瘤于 1996 年 7 月从 ATCC 得到, 并且指定的 ATCC 登记号为 HB-12147. 在另一个实施方案中, 抗-Fcγ受体是人源化的单克隆抗体 22(H22). Graziano, R. F. et al. (1995) J. Immunol 155 (10):4996-5002 和 PCT/US93/10384 描述了 H22 抗体的生产和特征. 产生 H22 抗体的细胞系于 1992 年 11 月储存在美国典型培养物保藏中心, 名称为 HA022CL1, 登记号为 CRL 11177.

在其它的实施方案中, 抗-FcR 抗体是对 IgA 受体特异的. 术语"IgA 受体"用于包括位于 19 号染色体上的一个α-基因(FcαR)的基因产物. 已知该基因编码几个 55-110KDa 的可选择剪切的跨膜同工型. FcαR(CD89)在单核细胞/巨噬细胞、嗜酸性粒细胞和嗜碱性粒细胞中组成性地表达, 但不是在非效应细胞群中表达. FcαR 对 IgA1

和 IgA2 有中等的亲和力（$\approx 5\times10^{7}M^{-1}$），暴露于例如 G－CSF 或 FM－CSF 的细胞因子时上述两种抗体增加(Morton, H. C. et al. (1996) Critical Reviews in Immunology 16:423-440)。抗－Fcα受体单克隆抗体的例子包括 My43、A77、A62、A59 和 A3(Aonteiro et al. (1992) J. Immunol. 148:1764;Shen et al. (1989) J. Immunol. 143:4117)。优选的抗－FcαR 抗体能结合到 FcαR 上而不会被 IgA 抑制。通过用含 FcαR 的丙烯酰胺凝胶片免疫小鼠产生了抗体 A77，上述 FcαR 是利用 IgA 亲和性从人类细胞溶解物纯化得到的。单克隆抗体的筛选根据三个特征：被 PMA 激活后对 U937 细胞的染色密度更高，选择性地与血液单核细胞和粒细胞反应，能与嗜中性粒细胞和激活的 U937 细胞的大约 55－75KDa 的分子发生免疫沉淀反应。

用于本发明的化合物的抗－Fc 单克隆抗体可通过多种技术得到，包括传统的单克隆抗体方法，例如，常规体细胞杂交技术，Kohler and Milstein，（1975）Nature 256:495。虽然体细胞杂交方法在是优选的，但是从原理上说也可以应用其它单克隆抗体制备技术，例如，B 淋巴细胞的病毒性或致癌性的转化。

制备杂交瘤的优选动物体系是鼠系。在鼠中生产杂交瘤是一种已经建立得很完善的方法。分离用于融合的免疫脾细胞的免疫方法和技术在本领域中是已知的。融合伙伴（fusion partner）（例如，鼠的骨髓瘤细胞）和融合方法也是已知的。

产生抗人蛋白的人单克隆抗体（mAbs）用的是携带完整的人免疫体系的转基因鼠而不是小鼠系统。源自用感兴趣的抗原免疫的这些转基因鼠的脾细胞被用于产生杂交瘤，该杂交瘤分泌对人蛋白表位具有特异亲和性的人 mAbs。(参见，例如，Wood et al. 国际申请 WO 91/00906，Kucherlapati et al. PCT 发表 WO 91/10741；Lonberg et al. 国际申请 WO 92/03918; Key et al. 国际申请 92/03917; Lonberg, N. et al. 1994 Nature 368:856-859; Green, L. L. et al. 1994 Nature Genet 7:13-21;Morrison, S. L. et al (1994) Proc. Natl. Acad. Sci. USA 81:6851-6855;Bruggeman et al. (1993) Year Immunol. 7:33-40;Tuaillon et al. (1993)PNAS 90:3720-3724;Bruggeman et al. (1991) Eur J. Immunol. 21:1323-1326)。

在一个示例性的实施方案中，免疫后产生完全的人抗体应答的

小鼠（HuMab 鼠）可通过失活编码鼠抗体的基因而得到．这可以通过产生“双重敲除（double knockout）小鼠”而实现；通过定向缺失编码恒定区外显子（Cμ和 JCκ）上述小鼠的内源免疫球蛋白重链和κ－轻链基因被破坏．可以分别构建包含人免疫球蛋白重链基因和人κ－轻链基因的基因．在人类中，这些基因分别包含 1－2 兆碱基，这样的长度太大了，不能完整分离．必需的区域可以被组装成被称为'微基因座(minilocus)'的简化形式．重链微基因座包含 2－6 个 V_h基因片段、15D_h和 6J_h基因片段，以及 S_μ、C_μ、$S_\gamma 1$、$C_\gamma 1$．κ－轻链微基因座包含 1－17Vκ－基因片段、5Jκ和 Cκ基因片段(Lonberg, N. et al. (1994) Nature 368:856-859;Tuaillon, N. et al. (1993) Proc. Natl. Acad. Sci. USA 90:3720-3724)．随后这些微基因座可以插入'双重敲除'小鼠的基因组中．可以产生这些双重敲除/双转基因 HuMab 小鼠的几个连续株，这些小鼠中插入了越来越多的人重链和轻链 loci．例如，已经得到的一种 HuMab 小鼠中插入了一段含六个 V 片段的 100kb 重链转基因和一段含 17 个 Vκ－片段的 200kb 的轻链转基因．这些 HuMab 小鼠可以用传统的方法免疫，并且已经发现这些小鼠能高效地产生针对许多抗原的 IgG1 高亲和性抗体(Fishwild, D. M. et al. (1996) nature Biotech 14: 845-851; Lonberg, N. and D. Huszar (1995) Int. Rev. Immunol. 13: 65-93)．已经发现按照这些方法得到的抗体具有很好的生物学活性已经很长的血清半衰期．

可通过本领域中一种的重组 DNA 技术得到小鼠-人嵌合单克隆抗体（即嵌合抗体）．例如，用限制性内切酶消化编码小鼠单克隆抗体 Fc 恒定区基因以除去编码小鼠 Fc 的区域，并用编码人 Fc 恒定区基因的对等部分替代（参见 Robinson et al., 国际专利 PCT/US86/02269; Akira, et al., 欧洲专利申请 184, 187; tANIGUCHI, m., 欧洲专利申请 171,496; Morrison et al., 欧洲专利申请 173,494; Neuberger et al., 国际专利申请 WO 86/01533; Cabilly et al. 美国专利第 4,816,567 号; Cabilly et al. 欧洲专利申请 125. 023, Better et a;/ (1988 Science 240: 1041-1043); Liu et al. (1987) PNAS 84: 3439-3443; Liu et al. 1987, J Immunol 129: 3521-3526; Sun et al. (1987) PNAS 84: 214-218; Nishimura et al., 1987, Canc. Res. 47: 999-1005; Wood et al. (1985)

Nature 314: 446-449; and Shaw et al., 1988, J. Natl Cancer Inst. 80: 1553-1559).

嵌合抗体可通过用对等的人 Fv 可变区序列取代不直接参与抗原结合的 Fv 可变区的序列而进一步人源化. 人源抗体的总体综述参见 Morrison, S. L., 1985, Science 229: 1202-1207 和 Qi et al., 1986, BioTechniques 4: 214. 这些方法包括编码源自至少重链或轻链之一的免疫球蛋白的所有或部分 Fv 可变区的核酸序列的分离、操作、和表达. 这些核酸的来源对于本领域中技术熟练的人员是众所周知的, 例如, 可来自 7E3, 一种产生抗 $GPII_bIII_a$ 抗体的杂交瘤. 然后编码嵌合抗体或其片段的重组 DNA 可被克隆到适当的表达载体中. 替代地, 人源抗体可通过 CDR 替换产生美国专利 5, 225, 539; Jones et al. 1986 Nature 321: 552-525; Verhoeyan et al. 1988 Science 239: 1534; 和 Beidler et al. 1988 J. Immunol. 141: 4053-4060.

一种特定人类抗体的 CDRs 可被至少一部分非人类 CDR 置换, 或只有一些 CDRs 能被非人类 CDRs 置换. 只需要置换对人源抗体和 Fc 受体的结合必需的 CDRs.

任何能用来自非人类抗体的 CDR 置换至少一部分人类抗体 CDR 的方法都可用于抗体的人源化. Winter 描述了一种可用于制备本发明的人源化抗体的方法(英国专利申请 GB2188638A, 1987 年 3 月提交.) 其内容清楚地在此引入作为参考. 可用题为 Humanized Antibodies to Fc Receptors for Immunoglobulin G on Human Monoculear Phagocytes 的国际专利申请 WO 94/10332 中所描述的寡聚核苷酸定点突变的方法用非人类 CDRs 置换人类 CDRs.

特定氨基酸被替换、缺失或添加的人源化抗体也包括在本发明的范围之内. 特别是, 优选的人源化抗体在框架区域进行了氨基酸替换, 以增进和抗原的结合. 例如, 在 CDRs 为小鼠的人源化抗体中位于人类框架区域的可用小鼠抗体相应区域的氨基酸替换. 已知这种替换在一些情况下能增进人源化抗体和抗原的结合. 氨基酸被添加、缺失或替换的抗体在此被称为修饰抗体或改变的抗体.

术语修饰抗体也用来包括抗体的部分被缺失、添加或替换的抗体, 如单克隆抗体, 嵌合抗体和人源化抗体. 例如, 可通过缺失一种抗体的恒定区并用一种恒定区替换从而修饰该抗体以提高抗体的半

衰期，如血清半衰期，稳定性或亲和性．只要巨噬细胞结合化合物具有至少一个对 FcR 特异的结合区域并能引发至少一种效应功能，那么任何修饰都是在本发明的范围内的．

也可通过重组 DNA 技术领域中的技术熟练人员已知的其它方法产生单克隆抗体．已经发展了一种被称为“组合抗体呈现”（combinatorial antibody display）的替代方法以鉴定和分离具有特定抗原特异性的抗体片段，并可被用于产生单克隆抗体（组合抗体呈现的描述参见，例如，Sastry et al. (1989) PNAS 86: 5728; Huse et al (1989) Science 246: 1275 and Orlandi et al. (1989) PNAS 86: 3833）．按照上文描述的方法用免疫原免疫动物后，克隆了得到的 B 细胞库的抗体库．利用寡聚引物混合物和 PCR 得到多种免疫球蛋白群可变区 DNA 序列的方法大体上是已知的．例如，可利用相应于 5’引导（信号肽）序列和/或框架（FR1）序列的寡聚核苷酸引物混合物以及相应于保守的 3’恒定区的引物通过 PCR 从许多鼠类抗体中扩增重链和轻链可变区(Larrick et al., 1991, Biotechniques 11: 152-156)．类似的测量可被用于从人类抗体中扩增重链和轻链可变区(Larrick et al., 1991, Methods: Companion to Methods in Enzymology 2: 106-110)．

在一个示例性的实施方案中利用常规方法从 B 淋巴细胞，例如外周血细胞、骨髓或脾脏制备物，中分离了 RNA（例如，美国专利第 4, 683,202; Orlandi et al. PNAS(1989) 86 : 3833-3837; Sastry et al., PNAS (1989) 86: 5728-5732; and Huse et al. (1989) Science 246: 1275-1281）．用对重链、κ轻链和λ轻链恒定区特异的引物以及对信号序列特异的引物合成了第一条 cDNA 链．用可变区 PCR 引物单独或联合扩增了重链和轻链的可变区，并连接到适当的载体上以进一步操作以产生呈现包（display package）．用于扩增方法的寡聚核苷酸引物可以是独特的或简并的，或在简并位置掺入了次黄嘌呤．也可在引物中引入限制性内切酶位点以将扩增片段克隆到载体的预先决定的阅读框中进行表达．

从源自免疫的抗体库克隆得到的 V 基因文库可由一群呈现包表达，以形成抗体呈现文库，上述呈现包优选地来自丝状噬菌体．理想的呈现包包含一个体系，该体系允许非常多样化的抗体呈现文库的采样，

经过每轮亲和分离后能快速归类，并且很容易从纯化的呈现包中分离抗体基因. 除了可由商业途径获得的制备噬菌体呈现文库的试剂盒外（例如，the Pharmacia Recombinant Phage Antibody System, catalog no. 27-9400-01; and the Stratagene SurfZAP™ phage display kit, catalog no. 240612），可以找到特别适合用于生产噬菌体呈现文库的方法和试剂的例子，例如，Ladner et al. 美国专利第 5,223,409 号； Kang et al. 国际公开 No. WO 92/18619; Dower et al. 国际公开 No. WO 91/17271; Winter et al. 国际公开 WO 92/20791; Markland et al. 国际公开 No. WO 92/15679; Breitling et al. 国际公开 WO 93/01288; McCafferty et al. 国际公开 No. WO 92/01047; Garrard et al. 国际公开 No. WO 92/09690; Lader et al. 国际公开 No. WO 90/02809; Fuchs et al. (1991) Bio/Technology 9:1370-1372; Hay et al. (1992) Hum Antibod Hybridomas 3:81-85; Huse et al. (1989) Science 246:1275-1281; Griffths et al. (1993) EMBO J12:725-734; Hawkins et al. (1992) J Mol Biol 226:889-896; Clackson et al. (1991) Bio/Technology 9:1373-1377; Hoogenboom et al. (1991) Nuc Acid Res 19:4133-4137; and Barbas et al. (1991) PNAS 88:7978-7982.

在一些实施方案中，可以在同一多肽上表达重链和轻链的 V 区结构域，上述结构域通过柔性的连接序列连接形成单链 Fv 片段，并且随后 scFv 基因被克隆到合乎需要的表达载体或噬菌体基因组中. 如 McCafferty et al., Nature (1990) 348: 552-554 中所描述，通过柔性的（Gly_4-Ser）$_3$ 连接序列连接在一起的抗体的完整 V_H 和 V_L 结构域可被用于产生单链抗体，上述单链抗体可根据抗原亲和性赋予呈现包可分离性. 然后分离的对抗原具有免疫反应性的 scFV 抗体可被配制成药用制备物以用于目的方法中.

一旦抗体库呈现于呈现包（例如，丝状噬菌体）的表面，就用 FcγR 或其肽片段筛选，以鉴定和分离能表达对 FcγR 特异的抗体的呈现包. 可以从呈现包中回收编码选中的抗体的核酸（例如从噬菌体基因组中）并通过常规重组 DNA 技术亚克隆到其它表达载体中.

本发明的巨噬细胞结合化合物中对靶抗原具有高亲和性的抗 Fc 受体结合试剂和/或其它结合试剂的制备可根据本领域中的人员已知

的方法，例如涉及文库筛选的方法（Ladner, R. C., et al., 美国专利 5, 233, 409; Ladner, R.C., et al., 美国专利 5, 403, 484）。进而，这些文库中的方法可被用于筛选以得到模拟抗体的结构决定簇的结合决定簇。特别是，特定抗体分子的 Fv 结合表面依照蛋白-蛋白相互作用的原则与其表位相互作用，因此，V_H和 V_L（后者可能是κ或λ链型）的序列数据是本领域的技术人员已知的蛋白工程技术的基础。利用先前已确定的其它抗体的三维结构通过建模的方法可以从抗体的序列信息得到包含结合决定簇的蛋白表面细节，上述三维结构是从 NMR 研究或晶体学数据得到的。例子参见 Bajorath. J. and S. Sheriff. 1996. Proteins:Struct., Funct., and Genet. 24(2),152-157; Webster, D. M. and A. R. Rees, 1995. "抗体结合位点的分子模型", in S. Paul, Ed., 分子生物学方法 Molecular Biol. 51, 抗体工程方法. Humana Press , Totowa, NJ, pp 17-49; and Johnson, G., Wu, T.T. and E.A. Kabat, 1995, "Seqhunt: 顺序排列的核苷酸和氨基酸序列的筛选程序"分子生物学方法 Biol. 51, op. cit., pp 1-15.

在一个实施方案中，抗－Fc 受体结合试剂包含源自抗体的，并且移植到非抗体分子中的抗原结合位点。例如，抗原结合区域可以移植到肽或蛋白上。在一个实施方案中，抗体结合区域的一部分，例如，类似源自抗体轻链的抗原结合区域的部分，被移植到蛋白或肽上，并且抗原结合区域的另一部分，例如，类似源自抗体重链的抗原结合区域的部分，被移植到另一个蛋白或肽上。在本发明的一个优选的实施方案中，分别具有抗原结合区域的一部分的两种蛋白或肽通过例如化学连接、重组方法、或非共价作用，连接，以便产生具有对人免疫球蛋白的 FcR 特异的抗原结合位点的蛋白，该蛋白引起至少一种 Fc 受体介导的效应细胞功能。

也可以通过筛选多种类型的组合文库以获得具有所需结合活性的抗原结合区域，并且通过已经描述的方法鉴定活性种类。例如，可以用噬菌体呈现技术(Marks et al. (1992) J Biol Chem 267:16007-16010)鉴定结合 FcγRs 的蛋白。噬菌体呈现文库已经在上面描述过了。例如，可以用呈现包种群表达多样化的肽文库以建立肽呈现文库。理想的呈现包包含一个体系，该体系允许非常多样化的抗

体呈现文库的采样，经过每轮亲和分离后能快速归类，并且很容易从纯化的呈现包中分离抗体基因．肽呈现文库可以在，例如，原核生物和病毒中，它们可以快速扩增，相对容易操纵，并且可以产生大量克隆．优选的呈现包包括，例如，植物细菌细胞、细菌芽孢，并且最优选的是细菌病毒（特别是 DNA 病毒）．然而，本发明也考虑使用真核细胞，包括酵母和其孢子，作为潜在的呈现包．噬菌体呈现文库在上文已有描述．

其它技术，包括利用适当“受体”， 例如，FcγRI 或 FcαR，进行亲和层析以分离结合试剂，然后利用传统技术（例如质谱或 NMR）鉴定分离的结合试剂或配基．优选地，可溶性受体被偶联到可被探测的标记（如荧光色素、显色酶类、放射性同位素或发光化合物）上以显示配基的结合．替代地，固定的化合物可被选择性地释放以允许其扩散通过膜和受体相互作用．

化合物组合文库可被合成得带有“标签”以编码文库每个成员的身份（参见，如，W. C. Still et al.，国际申请 WO94/08051）．总的来说，这种方法的特征为连接到固相支持物上或化合物上的惰性但容易检测的标签．当检测到活性化合物时通过鉴定该化合物所伴随的独特标签鉴定该化合物的身份．这种标签方法允许合成很大的化合物文库，可以在文库中的全套所有化合物中鉴定含量很低的化合物．

II 花青苷组分

在本发明的另一实施方案中巨噬细胞结合化合物的 Fc 受体结合试剂是化学部分，如花青苷组分，包括但不限于，荧光染料 Cy5.18OSu（称为 Cy5）以及其偶联与衍生物．已知花青苷组分以很高的亲和力和特异性和 FcγRI 结合．在一些情况下花青苷组分可含有两个或多个部分，如花青苷琥珀酰亚胺基酯和藻胆体蛋白，如，PE．此处所用的术语“PE-Cy5”指由藻红蛋白和 Cy5.18OSu 组成的特定色素串联；术语“PE-Cy5 试剂”指，例如但不限于， 和抗体、遗传工程结合蛋白和肽（美国专利地 5,233,409 号和第 5,403,484 号）、亲和素、生物素或其它分子实体偶联的 PE-Cy5．PE-Cy5 偶联物可被用于治疗或诊断用途．

从矢车菊（Centaurea cyanus）分离得到的花青苷结构上是花青素（cyanidin）的 3,5－二糖苷，花青素是从香蕉分离得到的 2-(3,4-

二羟基苯基)-3,5,7-三羟基-1-苯并吡喹氯化镓(benzopyrylium chloride)(Merck Index). 另一种花青素衍生物，从酸莓中分离得到的3-鼠李糖苷,被认为对夜盲症具有治疗作用.越桔(Vaccinium myrtillus)果实分离得到的花青糖苷(anthocyanoside)在市场上是作为食疗(nutraceutical)食品添加剂，据一个制造商说，口服后它能促进血管舒张，降低毛细管渗透性，保护血管中的胶原，能作为抗氧化剂发挥作用，并支持对炎症过程的控制，改善整体视力，胃内壁(stomach lining)，血脑屏障和腿以及结肠的血管(Gen. Engin. News 16 (11), p 27, 1996).

花青素的衍生物染料Cy5，也称为Cy5.18OSu，的化学结构为5,5'-双磺基-1,1'-(ε-羧基苯基)-3,3,3',3'-四甲基吲哚二羧基花青苷二琥珀酰亚胺基酯(A.S Waggoner et al., In: Clinical Flow Cytometry, p. 185 (Eds) A. Landay et al. The New York Academy of Sciences, New York, New York, 1993).用于巯基(Ernst, L., A. et al., 1989, Cytometry 10:3)和羧甲基吲哚花青苷琥珀酰亚胺基酯(South wick, P.L. et al., 1990 Cytometry 11:418)的花青苷染料标记试剂已有描述，并且组分已经申请专利(美国专利第4,981,977和5,268,486号)，上述专利的内容在此引入作为参考. Mujumdar, R. B., 1993, Bioconj. Chem 4: 105 中给出了Cy5的结构、其合成已经光吸收和发射光谱. Cy5是硫代吲哚花青苷琥珀酰亚胺基酯，是在吲哚花青苷荧光色素的芳香核带有带负电的磺酸基的氨基反应活性花青苷染料. 这个家族的Cy5成员的特征为5-碳，连接两个取代环的不饱和次甲桥. Cy5可为633nm的氦氖激光或647nm的Dr激光所激发. Cy5及其衍生物以其光稳定性而闻名，可以与荧光素相比或更好. 250,000的消光系数(L/mol cm)是非常高的. 此处表达方式"Cy5"包括了结构和合成方式类似的相关染料(Mujumdar et al., supra)，以便该表达方式大体上包括了花青苷染料标记试剂的硫代吲哚花青苷琥珀酰亚胺基酯，例如，Cy3.29.OSu(被称为Cy3)和Cy7.18.OH. 术语Cy5试剂、Cy5偶联物和Cy5衍生物的意思应为含有至少一个Cy5部分和其它分子实体的偶联物. 这种基础结构的其它性衍生物已有描述，花青苷试剂的硫代苯基吲哚花青苷琥珀酰亚胺基酯和Cy5以及其它硫代吲哚花青苷琥珀酰

亚胺基酯具有共性，并且被预期能特异地亲和地结合 FcγRI.

在 Waggoner et al.,1993, 上文描述了用单一 488nm 氩离子激光激发 Cy5 和 PE 串联组成的 Cy5 试剂 PE－Cy5 产生三色荧光，还描述了优化条件. 基于德克萨斯红的串联染料的主要问题是由于一个组成部分的不稳定性，导致了在使用过程中一部分发射泄漏到另一组成部分的光谱，从而当德克萨斯红发射光谱位于或接近于第二部分的发射光谱时其应用受到限制. 然而，Cy5 和它的染料家族试剂发射光波长大于德克萨斯红，因此通过使用 Cy5 和其它染料得到的数据的分析时所要求的检测窗口设置频道补偿和下游计算频道补偿极小. Cy5 试剂最佳使用方式所考虑的因素包括从组分合成 Cy5 试剂的合成方法，因为每分子偶联物结合的 Cy5 分子的数量比率影响合成产物的相对发射波长光谱. 因此，对于 PE－Cy5，当更多的 Cy5 分子结合到每个 PE，从 PE 到 Cy5 的能量转化效率提高，直至最佳范围，超过这个范围就观察到过多的 Cy5 组成部分的相互淬灭作用. 在 PE－Cy5 串联染料中的最佳比率为每个 PE 对 4 至 8 个 Cy5 分子(Waggoner et al., 1993. supra）. 串联染料是光敏的，如果染料的储存和操作以及实验是在暗处进行的，那么染料在使用中的稳定性将提高.

与先前合成的串联染料相比，PE-Cy5 由于没有背景信号，由于荧光强度增强，其信号大小得以改善，这使得它在用抗体染料偶联物进行细胞分析研究时成为一种成功的分析工具. 但是，至少有一个报道认为由于 Cy5 组成部分，来自不同供应商的多种 PE－Cy5 都和骨髓细胞“非特异性”结合（Stewart SJ, et al. supra），因为 PE－德克萨斯红偶联物没有这种性质. 与之相反，Takizawa et al.报道了 PE 及其 mAb 偶联物与低亲和性小鼠 IgG 受体 FcγRII 和 FcγRIII 的结合（J. Immunol. Methods, 1993, 162: 269）

杀伤巨噬细胞或降低巨噬细胞活性的细胞毒性试剂的生产

1. 细胞毒素

许多细胞毒性试剂可通过本发明的化合物被导向到巨噬细胞上（即通过与结合巨噬细胞上 Fc 受体的试剂连接）. 如此处所用，术语“细胞毒素”和“细胞毒性试剂”包括任何能杀伤巨噬细胞或降低其活性的化合物(例如药物).例如，该化合物可以是毒素，如 Gelonin，皂草素、外毒素 A、癌酶或蓖麻蛋白 A，或药物，如二氯亚甲基二膦

酸酯（CL2MDP）或其衍生物．用于本发明的细胞毒性可进而包括增强该化合物治疗活性的试剂或组成部分．

例如，细胞毒素可包括细胞调亡促进剂、有丝分裂抑试剂、烷化剂、抗代谢物、核酸嵌入剂、拓扑异构酶抑试剂、巨噬细胞特异性药物或放射性核素．本发明优点是将这些细胞毒素定向到巨噬细胞高亲和性的Fcγ受体（例如，使用如Mab22、Mab32或其人源化形式的抗体）上，在那里细胞毒素被巨噬细胞，例如，内化．因此，这些细胞毒素在杀伤细胞或调节细胞功能上比其它不被内化的试剂或内化速度较慢的试剂更有效．

细胞毒素试剂可以是细菌或植物起源的毒性药物或具有酶活性的毒素或它们的生物活性片段（“一条链”）．酶活性毒素及其片段的例子包括白喉A链、白喉毒素的非结合活性片段、外毒素A链（来自Pseudomonas aeruginosa）、蓖麻毒素A链、相思豆毒蛋白A链、modeccinA链、α－八叠球菌、Aleurites fordii蛋白、dianthinproteins、phytolacca americana蛋白（PAPI、PAPII和PAP－S）、momordicacharantia抑试剂、麻疯树毒蛋白、巴豆毒素、saponaria officinalis抑试剂、gilonin、mitogellin、局限曲菌素、酚霉素和伊诺霉素．优选的可用毒素包括Gelonin、皂草素、外毒素A、癌酶、蓖麻毒素A、白喉毒素、假单孢杆菌属外毒素或这些毒素的亚基．这些毒素的制备研究、体内用途、和药物动力学已有描述，例如，Vitetta et al. (1987) Science 238: 1098-1104; Spitlet, L. et al. (1987) Clin. Chem. 33(b): 1054; Uhr et al. Monoclonal Antibodies and Cancer, Academic Press, Inc., PP. 85-98 (1983)．本发明化合物和上述毒素试剂的偶联物可以通过使用多种的双功能蛋白偶联试剂制备，下文题为“巨噬细胞结合偶联物的制备方法”的部分详细描述了双功能蛋白偶联试剂．这些试剂的例子包括SPDP、IT、酰亚胺酯的双功能衍生物如己二酸二甲酯、HCl、活性酯如辛二酸二琥珀酰亚胺基酯、醛类如戊二醛、双叠氮化合物如双－（对叠氮苯甲酰基）己二胺、二重氮化合物衍生物如双－（对氮基苯甲酰基）乙二胺、二异氰酸酯如甲苯2，6－二异氰酸酯和双活性氟化合物如1，5－二氟－2，4－二硝基苯．

在其它实施方案中细胞毒素是一种药物．药物的例子包括二氯亚

甲基二膦酰酯（CL2MDP）或其它 chlodronate 衍生物（Bogers et al.(1991) Clin. Exp. Immunol. 86: 328-333）. 替代地，细胞毒素可以是细胞调亡促进剂、有丝分裂抑试剂、烷化剂、抗代谢物、核酸插入剂、拓扑异构酶抑试剂. 可用于本发明化合物试剂的例子包括拓扑异构酶 II 抑试剂椭圆素（它插入 DNA 或在 DNA 上形成缺口）. 运送这些药物的方法，例如，脂质体，在下文有所描述.

在一些实施例中细胞毒素可包括光敏组成部分（例如光敏药物）. 包括光敏这样的组成部分的细胞毒素可被用于敏化目标，例如巨噬细胞，使之在受到光激活时，例如通过可见光的辐射激活，被摧毁. 优选地，光敏组成部分在光激活之前没有直接的生物学活性. 包含这样的组成部分的化合物可被，例如局部地或通过注射，施用到客体上. 当这些化合物暴露于特定波长的光，例如可见光，而被光激活时该组成部分变成毒性的（自身变成毒性或通过激活与该组成部分相连的细胞毒素）并选择性地摧毁巨噬细胞. 光激活的机制不局限于任何特定理论，一般认为它包括能量从光敏部分向内源氧的转移，从而将其转化为单线态氧. 单线态氧被认为是对细胞毒性效应负责的. 含有光敏组成部分的巨噬细胞结合化合物在治疗皮肤疾病时尤其有用.

可用于本发明的光敏试剂的例子包括卟啉类化合物，例如，血卟啉衍生物（Lipson, R.L. et al. (1961), J. National Cancer Inst. 26:1-8; Photophrin II Compositions (US 4,649,151, Dougherty, T. J. (1983) Adv. Exp. Med. Bio. 160: 3-13 Kessel, D. et al. (1987) Photochem. Photobiol. 46: 463-568 and Scourides, P. A. et al. (1987) Cancer Res. 47: 3439-3445）焦脱镁叶绿酸化合物（US 5,459,159; US 4,996,312, 和 US 4,849,207, 和 EP 220686）; 叶绿素和细菌叶绿素衍生物（EPA 93111942.4）; porphycene 衍生物（WO 96/31451）; phrobine 衍生物（WO 95/08551）以及氯、酞菁染料、和卟吩（综述见 Harvey, I. Pass. (1993) J. Natl. Canc. Inst 85:443-457）. 能发出荧光信号的光敏试剂的光激活形式也可用于标记本发明的巨噬细胞结合化合物以用于诊断目的.

在其它实施方案中本发明的巨噬细胞结合化合物可包含通过光裂解连接与治疗或诊断试剂，例如细胞毒性试剂，偶联的 Fc 受体结

合试剂．优选地，上述连接是通过光激活试剂．如一种生色基，介导的，在暴露于光时上述光激活试剂释放治疗或诊断试剂（Goldmacher et al. (1992) Bioconj. Chem 3: 104-107）．例如，在皮肤病的应用中光将诱导连接的降解，在局部区域（例如皮肤）释放活性毒素．适于释放所结合的治疗或诊断试剂的光激活试剂包括任何能连接到治疗或诊断试剂的功能基团（例如苯酚）上的试剂，上述光激活试剂在暴露于光时释放功能形式的治疗或诊断试剂．作为一个例子，上述光激活试剂可以是生色基团．通常所选择的吸收光可由常见的辐射源（例如 240－370nm 的紫外光）发的出生色基团．对这样的波长具有光反应性的生色基团包括，但不限于，吖啶类、硝基芳香族类化合物和芳基磺胺类．

当使用生色基团时生色基团被光激活并释放或“解放”治疗试剂的波长和效率将根据生色基团所连接的特定功能基团而有所不同．例如，当使用硝基芳香类化合物如邻硝基苯甲基化合物时吸收波长可由于添加甲氧基而明显变长．在一个实施方案中硝基甲基苯（NB）和硝基乙基苯(NPE)分别被添加两个甲氧基而被修饰成 4,5－二甲氧基－2－硝基苯（DMNB)和 1－（4,5－二甲氧基－2－硝基苯）乙基（DMNPE），从而将它们吸收波长范围增加到 340－360nm（λ_{max}=355nm）．促进治疗或诊断试剂光释放的辐射可由多种辐射源提供，这些辐射源包括，但不限于，非相干紫外光源和受激准分子．在一个实施方案中，可以使用在 248nm 工作的 KrF 受激准分子激光．替代地，也可以使用在 266nm 工作的四倍频固态掺钕（neodymium-doped）YAG 激光或其类似物，或使用在 257nm 或 275nm 工作的氩离子激光．光激活试剂可与治疗试剂反应产生一种光释放连接物．当使用生色基团作为光激活试剂时，可以选择激发波长以便选择性地激发特定的生色基团．例如，可以将两种不同的药物或两种不同的生色基团以光释放的方式连接到底物上，然后通过选择与相应生色基团匹配的激发波长独立释放或先后释放这两种药物．可以进而选择生色基团和激发波长以避免药物（例如，失活）或周围组织的不合乎需要的光反应．例如，核酸的光敏性是众所周知的．当药物是核酸时，必须避免破坏核酸的激发能量（例如，波长小于 280nm）．

此外，物可以通过将本发明的巨噬细胞结合化合偶联到放射性核

素，例如，131I、90Y、105Rh、47Sc、67Cu、212Bi 和 211At，以标记该化合物，如 Goldenberg, D. M. et al. (1981) Cancer Res. 41: 4354-4360; in EP 0365 997 ; Carrasquillo et al., Cancer Treat. Rep., 68: 317-328 (1984); Zaloberg et al., J. Natl. Cancer Institute 72: 697-704 (1984); Jones et al., Int. J. Cancer 35:715-720 (1985); Lange et al., Surgery 98: 143-150 (1985); Kaltovich et al., J. Nucl. Med. 27:897 (1986); Order et al., Intl. J. Radiother. Oncl. Biol. Phys. 8:259-261 (1982); Courtenay-Luck et al. Lancet 1:1441-1443(1983); Ettinger et al., Cancer Treat. Rep. 56:289-297 (`1982)中所描述；所有这些专利的公开内容在此引入作为参考。这样的放射性核素也能增强光敏组成部分的细胞毒性效应。

在这些诊断应用中，连接一个标记基团到巨噬细胞结合化合物上以便利它们的检测是合乎需要的（例如，在一个样品中它们结合到巨噬细胞上）。相应地，除了上文所列的核素外，适合的标记基团包括，例如，荧光色素、显色酶、放射性同位素或发光的化合物。例如，当标记基团是酶时，连接到巨噬细胞结合化合物上的酶将与适当的底物，优选地与产色素的底物，反应，这样以便产生可探测的化学信号，例如，可通过分光光度计、荧光光度计或通过视觉手段探测的信号。可用于抗体的可探测性标记的酶包括，但不限于，苹果酸脱氢酶、葡萄球菌核酸酶、，δ-5-类固醇异构酶、酵母乙醇脱氢酶、α-磷酸甘油脱氢酶、磷酸丙糖异构酶、辣根过氧化物酶、碱性磷酸酶、天冬酰胺酶、葡萄糖氧化酶、β-半乳糖苷酶、核糖核酸酶、脲酶、 过氧化氢酶、葡萄糖-6-磷酸脱氢酶、葡萄糖淀粉酶和乙酰胆碱酯酶。检测可以通过比色法完成，在比色法中使用了上述酶的产颜色底物。检测也可以通过目测底物的酶促反应的程度，并与类似方法制备的标准进行比较而进行。

巨噬细胞结合化合物与巨噬细胞结合的检测也可以通过使用多种免疫测定方法中的任何一种方法来完成。例如，可以使用放射性免疫检测（RIA）（参见，例如，Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986, 在此

引入作为参考）. 替代地，可以使用酶免疫分析（EIA）（Voller. "The Enzyme Linked Immunosorbent Assay (ELISA)", Diagnostic Horizons 2:1-7, 1978, Microbiological Associates Quarterly Publication, Walkersville, MD; Voller, et al., J. Clin. Pathol. 31: 507-520 (1978); Butler, Meth. Enzymol. 73:482-523 (1981); Maggio, (ed.) Enzyme Immunoassay, CRC Press, Boca Raton, FL, 1980; Ishikawa, et al., (eds.) Enzyme Immunoassay, Kgaku Shoin, Tokyo, 1981）. 放射性同位素的检测可以通过这些方法，例如使用γ计数器或闪烁计数器或通过放射性自显影.

也可以用荧光化合物标记巨噬细胞结合化合物. 当荧光标记的化合物暴露于合适波长的光时，就可以检测到它的存在. 在多数普遍应用的荧光标记化合物中有异硫氰酸荧光素、罗丹明、藻红蛋白、藻青蛋白、藻青蛋白同分异构体、邻苯二醛和荧光胺.

可以用荧光发射金属，如 152Eu 或其它镧系元素，标记本发明的化合物. 这些金属可以通过例如二乙基三胺五乙酸(DTPA) 或乙二胺四乙酸 (EDTA)金属螯合基团被连接到抗体上. 替代地，这些化合物也可以通过与化学发光化合物的偶联而被标记. 然后，化学发光标记的化合物的存在是通过检测化学反应中发出的光. 特别有用的化学发光标记化合物的例子有发光氨、异发光氨、theromatic acridinium 酯、咪唑、吖啶鎓盐和草酸酯.

类似地，可以用生物发光的化合物标记本发明的巨噬细胞结合化合物. 生物发光是在生物体系中的一类化学发光，生物体系中的催化蛋白提高了化学发光反应的效率. 通过检测发光的存在鉴定生物发光蛋白的存在. 用于标记的重要生物发光化合物有虫萤光素、萤光素酶和发光蛋白质.

抗－Fc 受体结合试剂与细胞毒素的偶联

本发明的巨噬细胞结合化合物除了其它供选成分外还包含一种与细胞毒素连接的，能结合到巨噬细胞 Fc 受体上的试剂. 相应地，为了产生这样的化合物，通过多种已知技术，或通过将抗－Fc 受体结合试剂和细胞毒素一起作为融合分子进行重组表达，抗－Fc 受体结合试剂被偶联（例如，通过共价偶联）到细胞毒素上（参见例如，D.

M. Kranz et al. (1981) Proc. Natl. Acad. Sci. USA 78: 5807, 美国专利 4, 474, 893）.

适用于上述目的的试剂，例如偶联试剂，在本领域中是众所周知的. 术语“偶联试剂”和“交联剂”用于包括具有两个反应功能基团的能在两个其它分子之间起桥梁作用的分子，上述两个功能基团中，其中一个与第一个分子反应形成共价键并且另一个与第二个分子反应形成共价键，因此有效地将两个分子连接在一起. 优选地，交联剂的两个反应功能基团的功能组成部分不同. 合适的功能基团的例子包括氨基、羧基、巯基和羟基. 当交联剂的一个功能基团与一个分子（例如，Fc 受体结合试剂）反应时，另一个功能基团可以，如果需要，通过保护基团防止与上述分子的反应，该保护基团是修饰交联剂的第二个功能基团的以便第二个功能基团不会与上述分子反应. 第一次反应完成后，保护基团可以被除去，恢复了第二个功能基团，然后第二个功能基团可以与另一个分子（例如，毒素）反应.

本发明的巨噬细胞结合化合物的制备可以通过本领域已知的方法将它们的组分试剂，例如，抗－FcR 和细胞毒素，进行交联. 例如，巨噬细胞结合化合物的每个试剂可以分别生产，然后彼此交联. 当结合特异性为蛋白或肽时，可以用多种的偶联或交联试剂进行共价交联. 交联剂的例子包括蛋白 A、碳二亚胺、N-琥珀酰亚胺基-S-乙酰基-硫代乙酸(SATA)、N-琥珀酰亚胺基-3-（2-吡啶二硫代）丙酸(SPDP)和硫代琥珀酰亚胺基 4-（N-马来酰亚胺甲基）环己烷-1-羧酸(sulf-SMCC)（例子参见，Karpovsky et al. (1984) J. Exp. Med. 160: 1686; Liu, MA et al. (1985) Proc. Natl. Acad. Sci. USA 82: 8648). 其它方法包括 Paulus (Behring Ins. Mitt. (1985) No. 78, 118-132); Brennan et al. (Science (1985) 229:81-83), 和 Glennie et al. (J. Immunol. (1987) 139: 2367-2375) 所描述的. 优选的交联剂是 SATA 和 sulfo-SMCC, 两者都可以从 Pierce Chemical 公司(Rockford, IL)获得.

在巨噬细胞结合分子包含两种抗体（例如，双特异抗体）的情况下，这些抗体可以通过两个重链 C－末端铰链区域的巯基键而交联. 在特定的优选的实施方案中，在交联之前铰链区域被修饰以包含奇数个巯基残基，优选的是一个巯基. 替代地，两种试剂都可以在同一载

体中编码并且在同一宿主细胞中表达和组装．这种方法在巨噬细胞结合化合物为 mAb ×mAb、mAb ×Fab、Fab ×$(Fab)_2$或配基×Fab 融合蛋白的情况下特别有用．本发明的巨噬细胞结合化合物，例如，双特异分子可以是单链分子，例如单链双特异抗体，包含一个单链抗体和一个结合决定簇的单链双特异分子，或包含两个结合决定簇的单链双特异分子．巨噬细胞结合化合物也可以是单链分子或可以包含至少两种单链分子．制备双特异和多特异分子的方法在，例如，美国专利第 5，260，203 号、美国专利第 5，455，030 号、美国专利第 4，881，175 号、美国专利第 5，132，405 号、美国专利第 5，091，513 号、美国专利第 5，476，786 号、美国专利第 5，013，653 号、美国专利第 5，258，498 号和美国专利第 5，482，858 号中有所描述．

一旦产生符合上述指导方针的巨噬细胞结合化合物，就可以利用已知的技术，例如酶联免疫吸附检测（ELISA）、放射性免疫检测（RIA）或 Western Blot 检测，检测该化合物与巨噬细胞的结合．这些检测方法一般通过运用对感兴趣复合物特异的标记试剂（例如，抗体）检测特别感兴趣的蛋白－抗体复合物的存在．例如，可以使用例如酶联抗体或识别抗体－FcR 复合物并且与之特异性结合的抗体片段检测 FcR－抗体复合物．替代地，可以通过使用多种其它免疫检测的任何一种方法检测上述复合物．例如，抗体可以被放射性标记并且用于放射性免疫检测中（RIA）（参见，例如，Weintraub，B.，Principles of Radioimmunoassays，Seventh Training Course on Radioligand Assay Techniques，The Endocrine Society，March，1986，在此引入作为参考）．放射性同位素的检测可以通过这些方法，例如使用γ计数器或闪烁计数器或通过放射性自显影．

药用组合物和施用途径

本发明的巨噬细胞结合化合物优选地和载体或稀释剂一起存在于组合物中．对于患者的体内施用而言（例如，治疗或诊断一种病症），该化合物优选地存在于药用上可接受的载体或稀释剂中．如下文详细描述的，本发明的药用组合物可以被特定地配制成固体或液体的形式以便施用，包括适合以下施用形式的配制方式：（1）口服施用，例如，灌服（水或非水溶液或悬浮液）、药片、大丸药、粉剂、

颗粒、糊剂；（2）肠胃外的施用，例如，通过作为如无菌的溶液或悬浮液进行皮下的、肌肉的或静脉内的注射．（3）局部的运用，例如，施用于皮肤的油膏、药膏或喷雾．（4）阴道内或直肠内，例如，作为阴道栓剂、油膏或泡沫；（5）气溶胶，例如，作为水的气溶胶、脂质体的制备或包含该化合物的固体颗粒．

本发明的药用组合物也可用在联合治疗中，即与其它的试剂联合．例如，该联合治疗可以包括本发明的组合物与至少一种其它抗巨噬细胞试剂或其它常规治疗．抗巨噬细胞试剂的例子包括chlodronate 化合物，例如，二氯亚甲基二膦酸酯(CL2MDP)．

此处所用的短语“药用上可接受的载体”是指药用上可接受的材料、组分或载体，例如液体或固体的填充剂、稀释剂、赋形剂、溶解或形成胶囊的材料，这些材料参与将目的化学药品从一个器官或身体的一部分携带或运送到另一个器官或身体的一部分．每个载体必须在与其它的配制成分相容并且对病人无害的意义上是“可接受的”．可以用作药用上可接受载体的材料的例子包括：（1）糖类，例如乳糖、葡萄糖和蔗糖；（2）淀粉类，例如谷物淀粉和马铃薯淀粉；（3）纤维素及其衍生物，例如羧甲基纤维素钠、乙基纤维素和醋酸纤维素；（4）粉状黄蓍胶；（5）麦芽；（6）凝胶；（7）滑石粉；（8）赋形剂，例如可可粉黄油和栓剂蜡；（9）油，例如花生油、棉籽油、红花油、芝麻油、橄榄油、谷物油和豆油；（10）乙二醇类，例如丙烯乙二醇；（11）多羟基化合物，例如丙三醇、山梨醇、甘露醇和聚乙二醇；（12）酯，例如油酸乙酯和月桂酸乙酯；(13)琼脂；（14）缓冲试剂，例如氢氧化镁和氢氧化铝；（15）褐藻酸；（16）无热源水；（17）等渗盐水；（18）Ringer's溶液；（19）乙醇；（20）磷酸缓冲溶液；和（21）其它运用于药物配制的无毒相容物质．

“药用上可接受的盐”是指保留了原初化合物的合乎需要的生物活性并且不会带来任何不合乎需要的毒性效应的盐（参见，例如，Berge, S. M., et al. (1997) J. Pharm. Sci. 66: 1-19）．这些盐的例子包括酸加成盐和碱加成盐．酸加成盐包括来自无毒无机酸，例如盐酸、硝酸、磷酸、磷酸、氢溴酸、氢碘酸、磷等等的盐，也包括来自无毒有机酸，例如脂肪族的单羧基和双羧基酸、苯基取代链烷酸、羟基链烷酸、芳香酸、脂肪族和芳香族磺酸等等的盐．碱加成盐

包括来自碱土金属，例如钠、钾、镁、钙等等，也包括来自无毒的有机胺，例如 N,N'-二苯甲基乙二胺、N-甲基还原葡萄糖胺、氯化普鲁卡因、胆碱、二乙醇胺、乙二胺、普鲁卡因等等.

本发明的组合物可以通过本领域中多种已知的方法施用. 技术熟练的人员将认识到施用的途径和/或方式将根据所需要的结果而不同.

术语“施用”用于包括将本发明的巨噬细胞结合化合物引入到患者中并且允许该化合物执行其预定功能（即巨噬细胞减少和/被抑制）的任何途径. 可以使用的施用途径的例子包括注射（皮下的、静脉的、肠胃外的、腹膜内的、鞘内的等等）、口服、吸入、直肠和真皮内. 当然所提供的药用制备物是适用于各个施用途径的. 例如，这些制备物是通过注射、吸入、眼药水、药膏、栓剂等等以药片或胶囊的形式施用的；通过注射、灌输或吸入施用；通过洗剂或药膏的局部施用；和通过栓剂对直肠的施用. 注射可以是大药丸或可以是连续的灌输. 根据施用途径，巨噬细胞结合化合物可以被包上指定的材料或放置在指定的材料中以防止其和天然环境的接触，上述天然环境可能不利于该化合物执行预定的功能. 巨噬细胞结合化合物可以单独施用，或与上文描述的另一试剂或药用上可接受的载体联合施用，或都与两者联合施用. 巨噬细胞结合化合物可以在其它试剂施用以前施用、与其它试剂同时施用或在其它试剂施用之后施用. 更进一步地，该化合物可以以前体形式或无活性的形式（例如，包含光敏毒素的巨噬细胞结合化合物）施用，上述前体或无活性形式可以在体内被转化成活性代谢物或活性更高的代谢物，例如，当暴露于光时.

此处所用的短语“肠胃外的施用”和“施用于肠胃外”意思是除了通过肠道途径施用和局部的施用以外的施用方式，通常通过注射，包括，但不限于，静脉内的、肌肉内的、动脉内的、鞘内的、囊内的、眼窝内的、心内的、真皮内的、腹膜内的 、气管内的、皮下的、表皮下的、关节内的、囊下的、蛛网膜下的、脊柱内的和胸骨内的注射和灌输.

此处所用的短语“全身的施用”“施用于全身”“肠胃外的施用”和“施用于肠胃外”是指施用巨噬细胞结合化合物以便其患者系统中并且，因此，受到新陈代谢和其它类似过程的代谢，例如，皮下的施

用.

通常，本发明的巨噬细胞结合化合物用于局部施用以治疗或诊断疾病，上述疾病的特征是身体特定范围或区域（例如，皮肤、肺、关节或肌肉/神经组织）内巨噬细胞的数量和/或功能异常．对于皮肤病学的运用，该化合物优选地通过局部途径或透皮贴片（transdermal patch）运送或施用．在皮肤损伤的治疗中局部施用是优选的，包括头皮损伤、角膜损伤（角膜炎）和粘膜损伤，在这些情况下这种直接运用是可行的．香波配制物有时有利于治疗头皮损伤例如脂溢性皮炎和头皮牛皮癣．漱口药和口服糊剂配制物能有利于粘膜损伤，例如口损伤和黏膜白斑病．实践本发明的一种优选途径是将含巨噬细胞结合化合物的药膏或基于油的载体，直接施用于伤口，例如，牛皮癣的伤口．通常在药膏或油中的巨噬细胞结合化合物的浓度是 1－2％．此外，真皮内施用是真皮损伤例如真皮牛皮癣和伤口的一种选择．替代地，可以局部地使用气溶胶．在局部直接运用不可行的情况下口服施用是治疗上述皮肤损伤和其它损伤的优选的选择，并且对其它运用也是优选的途径．

进而，该组合物可以通过肠胃外的途径运送，尤其是在治疗关节炎，如牛皮癣关节炎或类风湿性关节炎时以及皮肤损伤的直接注射时．肠胃外治疗通常是真皮内、关节内、肌肉内或静脉内的．在治疗一个或只有少数（例如 2－6）关节时，关节内的注射是优选的选择．此外，在适当的情况下治疗组合物被直接注射到损伤部位（损伤部位内施用）．作为治疗关节炎的选择，本发明的组合物可以全身施用．

对于治疗呼吸道疾病，本发明的组合物通过鼻的气溶胶或吸入而被施用．这样的组合物可以被制备成盐溶液，并使用苄醇或其它的合适的防腐剂，提高生物利用率的吸收促进剂、碳氟化合物和/或常规的增溶或分散剂．

在一些实施方案中，包含上述化合物的组合物可以被全身或局部施用．例如，包含光敏组成部分，如毒素或连接物，的巨噬细胞结合化合物的组合物，可以以这样的方式被施用．更进一步地，优选地通过本发明组合物的局部或全身的施用治疗一些自免疫疾病，例如多发性硬化症．

除了本发明的化合物外粉末和喷雾还可以包含载体，例如乳糖、

滑石、硅酸、氢氧化铝、硅酸钙和聚酰胺粉末，或这些物质的混合物。此外喷雾还包含通常的推进剂，例如氯氟烃和挥发性的非取代烃，例如丁烷和丙烷。

通常，水的气溶胶是由水溶液或试剂的悬浮液与常规的药用上可接受的载体和稳定剂一起配制成的。载体和稳定剂因特定混合物的需要而不同，但通常包含非离子表面活性剂（吐温、Pluronics、聚乙二醇）、无毒的蛋白如血清白蛋白、山梨聚糖酯、油酸、卵磷脂、氨基酸如甘氨酸、缓冲液、盐、糖或糖醇。气溶胶通常从等渗的溶液制备。

不论选用什么施用途径，本发明的巨噬细胞结合化合物和/或药用组合物都通过本领域的技术人员已知的常规方法被配制成药用上可接受的剂量形式；上述巨噬细胞结合化合物可以以水化的形式应用。

为了达到对特定病人、组合物以及施用方式的合乎需要的治疗效果而不至于对病人产生毒性，可以改变本发明药用组合物中活性组分的实际剂量水平以及施用的疗程。

活性化合物可以和能防止该化合物快速释放的载体一起制备，例如受调控的释放配方，包括植入、透皮贴片和装入微胶囊的运送系统。可以使用生物可降解的、生物兼容的聚合物，例如乙酸乙烯乙酯、聚酐类、聚乙二醇酸（polyglycolic acid）、胶原质、聚原酸酯类和聚乳酸。制备这些制备物的许多方法是授予专利的或对于本领域的技术人员通常是已知的。参见，例如，Sustained and Controlled Release Drug Delivery Systems, J. R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

为了通过某些施用途径施用本发明的化合物，也许有必要用一种材料将该化合物包上或和该化合物共同施用以防止该化合物的失活。例如，该化合物可以在合适的载体，例如脂质体或稀释液，中施用于患者。药用上可接受的稀释液包括盐和水的缓冲溶液。脂质体包括水包油包水（water-in-oil-in-water）CGF 乳浊液以及常规脂质体（Streijan et al., (1984) J. Neutoimmunol. 7:27）。药用上可接受载体包括无菌水溶液或分散液和用于临时制备无菌注射溶液或分散液的无菌粉末。这些介质和试剂在药用活性物质中的应用在本

领域是已知的．除了一些常规介质和试剂与活性化合物不相容外，它们在本发明的药用组合物中的应用是预期的．补充的活性化合物也可以被掺入到该组合物中．

治疗组合物通常必须是无菌的并且在生产和贮存的条件下是稳定的．该组合物可以被配制成溶液、微乳浊液、脂质体或其它适合用于高药物浓度的有序结构．载体可以是含有，例如，水、乙醇、多羟基化合物（例如，甘油、聚丙二醇和液态聚乙二醇等等）及它们的适当混合物，的溶剂或分散介质．可以通过，例如，使用外包物如卵磷脂，通过分散时维持合乎需要的颗粒大小以及通过使用表面活性剂来维持适当的流动性．在许多情况下，组合物中优选地包含等渗试剂，例如，糖类、多元醇类如甘露醇、山梨糖醇或氯化钠．通过在组合物中包含一种延迟吸收的试剂，例如单硬脂酸盐和明胶，可以延迟注射组合物的吸收．

可以根据需要通过将所需数量的活性化合物与上述列举的一种或多种组分合并到适当的溶剂中，然后进行除菌微孔过滤而制备无菌溶液．通常，分散液的制备是通过将活性化合物合并到无菌载体中，上述载体包含基础分散介质以及合乎需要的其它上述列举成分．对于用于制备无菌可注射溶液的无菌粉末的情况，优选的制备方法是真空干燥和冷冻干燥（冻干）以产生活性组分以及来自先前无菌过滤溶液中任何其它合乎需要的组分的粉末．

调节剂量服用方式以提供最佳合乎需要的反应（例如，治疗反应）．例如，可以施用单个的大药丸，可以随时间施用分开的剂量或可以根据治疗状况的紧急程度按比例减少或增加剂量．为了便于施用和使剂量一致，将组合物配制成剂量单位的形式是特别有利的．此处所用的剂量单位形式是指适于作为患者治疗时的单一剂量的物理分离的单元，每个单位包括根据计算能产生所需治疗效果的预定数量的活性化合物以及合乎需要的药用载体．本发明的剂量单位形式的规格的指导原则和直接根据是（a）活性化合物的独特特征和需要得到的特定治疗效果，(b)合成这样的活性化合物以治疗个体的敏感性时本领域的内在局限性．

药用上可接受的抗氧化剂的例子包括：（1）水溶性抗氧化剂，例如抗坏血酸、盐酸半胱氨酸、硫酸氢钠、偏亚硫酸钠、硫酸钠等等；

（2）脂溶性抗氧化剂，例如棕榈酸抗坏血酯、丁化羟基苯甲醚（BHA）、丁基化羟基甲苯(BHT) 、卵磷脂、没食子酸丙酯、α－生育酚等等；（3）金属螯合剂，例如柠檬酸、乙二胺四乙酸（EDTA）、山梨糖醇、酒石酸、磷酸等等.

对于治疗组合物，本发明的制备物包括适合局部、皮内或皮下施用的制备物．该制备物通常可以以单位剂量形式存在并且可以通过制药领域中任何已知方法制备．与载体材料结合产生单一剂量形式的活性组分的量可根据被治疗患者和特定施用方式而不同．与载体材料结合产生单一剂量形式的活性组分的量通常是能产生治疗效果的组合物的量．通常，没有百分之百，活性组分的数量范围大约从 0.01%至99%，优选地大约从 0.1% 至 70%，最优选地从 1% 至 30%．

本发明的组合物用于局部和皮内施用的剂量形式包括粉末、喷雾、油膏、糊剂、药膏、洗液、凝胶、溶液、贴片和吸入剂．可以在无菌的条件下将活性化合物与可能需要的药用上可接受的载体、任何防腐剂、缓冲液或推进剂一起混合.

可以用于本发明的药用组合物的适合的含水和无水载体的例子包括水、乙醇、多羟基化合物（例如，甘油、聚丙二醇和聚乙二醇等等）以及它们的适当混合物、植物油如橄榄油和可注射的有机酯如油酸乙酯．可以通过，例如，使用外包物例如卵磷脂，通过分散时维持合乎需要的颗粒大小以及通过使用表面活性剂以维持适当的流动性.

这些组合物也可以含有佐剂例如防腐剂、润湿剂、乳化剂和分散剂．防止微生物的存在可通过无菌过程，参照上文，和抗细菌及抗真菌试剂，例如，对羟基苯甲酸酯类、氯丁醇、苯酚山梨酸等等．也可以根据需要在组合物中包含等渗试剂，例如糖、氯化钠等等．此外，通过包含一种延迟吸收的试剂，例如单硬脂酸铝和明胶，可以延迟注射的药用形式的吸收.

当本发明的化合物作为药物施用于人和动物时，它们可以单独施用或作为药用组合物施用，该药用组合物包括，例如，0.01－99.5%（更优选的是 0.1－90%）的活性组分并且和药用上可接受的载体结合在一起.

可以改变本发明药用组合物中活性组分的实际剂量水平以便获

得活性组分的一定剂量，该剂量是有效达到对特定病人、组合物以及施用方式的合乎需要的治疗效果而不至于对病人产生毒性。剂量水平的选择依赖于许多药物动力学因素，这些因素包括使用的本发明特定组合物或它们的酯、盐或酰胺的活性、施用的途径、施用的时间、使用的特定化合物的排泄率、治疗持续的时间、其它药物、用于和使用的特定组合物结合的化合物和/或材料、受治疗的病人的年龄、性别、体重、状况、整体健康和先前的病史和在医学领域众所周知的类似因素。

具有本领域的普通技术的内科医生和兽医可以很快地决定并且开出所需的药用组合物的有效数量。例如，为了获得合乎需要的治疗效果，医生或兽医开始时开出的用于药用组合物中的本发明的化合物剂量水平低于所需的剂量水平，并且逐步地增加剂量直到达到所需的治疗效果。通常，本发明组合物的每日剂量为能有效产生治疗效果的最低剂量。这样的有效剂量通常由上述因素决定。局部施用是优选的，例如，局部的、皮下的、皮内的，优选地施用于接近目标的位点上。如果需要，治疗组合物有效的每日剂量可以在每日适当的间隔以二、三、四、五、六或更多个小剂量分别施用，供选地，可以以单位剂量形式施用。尽管本发明的化合物可以被单独施用，但优选地将该化合物作为药用制备物（组合物）施用。

可以通过本领域已知的医疗设备施用治疗组合物。例如，在一个优选的实施方案中，可以通过无针的皮下注射设备施用本发明的治疗组合物，这样的设备公布在美国专利第 5，399，163，5，383，851，5，312，335，5，064，413，4，941，880，4，790，824，或 4，596，556 号。用于本发明的众所周知的移植片和模块的例子包括：美国专利第 4，487，603 号公布的用于分配药物的可调速可植入微灌输泵；美国专利第 4，486，194 号公布的通过皮肤施用药物的治疗设备；美国专利第 4，447，233 号公布的用于运送药物的灌输速度精确的药物灌输泵；美国专利第 4，447，224 号公布的用于连续运送药物的可变流可植入灌输装置；美国专利第 4，439，196 号公布的具有多室分隔间的渗透性药物运送系统；美国专利第 4，475，196 号公布的渗透性药物运送系统。这些专利在此引入作为参考。许多其它这样的植入、运送系统和模块已被本领域技术人员所知。

在一些实施方案中，本发明的化合物可以被制备成能确保其在体内适当的分配．在一个实施方案中，巨噬细胞结合分子可以被包入脂质体中．制造脂质体的方法参见，例如，美国专利第 4，522，811；5，374，548 和 5，399，331 号．上述脂质体可以包含一个或更多个被选择性运送到特定细胞或器官中的组成部分，从而增进目的药物的运送（参见，例如 V. V. Ranade (1989) J. Clin. Pharmacol. 29:685）．例如，某些实施方案，优选地使用抗 Fc 受体的单链抗体（scFv）如 H22 scFv 抗体，将本发明的化合物导向 Fc 承载（Fc-bearing）巨噬细胞．包有 scFv 片段的脂质体的制备方法在 de Kruif, J. et al. (1996) FEBS 399:232-236 中有所描述．例如，通过稀释含有正辛基 β－D－葡萄糖苷，脂类和脂类修饰的 scFv 的混合物微团使得去污剂浓度远远低于去污剂的临界微团浓度，可以将脂类修饰的 H22 scFv 偶联到含有蛋黄磷脂酰胆碱（EPC）、蛋黄磷脂酰甘油（EPG）、胆固醇以及，供选地，作为双分子层荧光标记物的罗丹明－磷脂酰乙醇胺（罗丹明），的脂质体上，上述脂质体成分的摩尔比为 10: 1: 5: 0.01．可以通过 SDS－PAGE 鉴定脂质体中 scFv 分子的掺入．

“有效治疗剂量”是指该剂量能使指定治疗的区域与未治疗的对照区域相比，巨噬细胞数量减少，或该剂量能在指定的区域抑制巨噬细胞的活性，例如，该区域的巨噬细胞不再增生或参与该区域的炎症反应．因此，巨噬细胞介导的疾病的症状得到改善．可以在动物模型系统中评价本发明的化合物杀伤巨噬细胞群或抑制巨噬细胞群活性的能力，上述动物例如本文例子中所描述的表达人 Fc 受体的转基因动物．替代地，可以通过专业人员已知的体外实验评估这些功能．治疗化合物的有效治疗剂量可以减少巨噬细胞群或抑制巨噬细胞群的活性，或者改善患者的症状．本领域普通技术人员可以根据这些因素例如患者的大小、患者症状的严重性和特定的组合物或选择施用的途径，确定这样的剂量．

组合物必须是无菌的和流动性的，流动的程度是使组合物可以通过注射运送．除了水外，载体可以是等渗缓冲盐溶液、乙醇、多羟基化合物（例如，甘油、聚丙二醇和液态聚乙二醇等等）以及它们的适当混合物．可以，例如，通过使用外包物如卵磷脂，通过分散时维持合乎需要的颗粒大小以及通过使用表面活性剂以维持适当的流动

性．在许多情况下，组合物中优选地包含等渗试剂，例如，糖类、多元醇类如甘露醇、山梨糖醇或氯化钠．可以通过在组合物中包含一种延迟吸收的试剂，例如单硬脂酸铝和明胶，以延迟注射组合物的吸收．

发明用途和方法

本发明的巨噬细胞结合化合物具有一些诊断、治疗和研究的用途．它们可以施用于体外细胞（培养）、来自体内的细胞、或体内细胞（患者中）以治疗、诊断或研究各种病症．

在一个实施方案中，提供了在指定治疗区域或诊断区域减少（例如减少数量）或抑制巨噬细胞活性的方法．该方法是通过让足够数量的巨噬细胞结合化合物接触指定的区域以获得上述的效果．此处所用的术语“指定的区域”或“局部区域”都指包含或可能包含导致病症的巨噬细胞的组织或细胞的任何指定样品（体外或体内），例如人体的局部区域（皮肤、肺、关节等等）或培养样品的组织．接触可以发生在体外（例如培养细胞）或体内（例如，通过对患者施用本发明的化合物）．

此处所用的术语“患者”用于包括人和非人类动物．优选的人类包括具有疾病的病人，该疾病的特征是巨噬细胞，例如，皮肤巨噬细胞，的活性异常．术语“活性”用于包括巨噬细胞的所有生物功能，包括增殖、分化、生存、生长因子或细胞因子分泌作用，还有其它．本发明的术语“非人类动物”包括所有脊椎动物，例如，哺乳动物和非哺乳动物，例如，非灵长类、绵羊、狗、牛、鸡、两栖动物、爬行动物等等．

本发明的巨噬细胞结合化合物最初可以在体外检测．例如，可以在源自巨噬细胞的细胞系、培养分化的血液单核细胞和原代培养体系中检测这些分子杀死巨噬细胞和/或调节，例如降低，巨噬细胞活性的活性．可以在，例如， Immunopharmacology of Macrophages and Other Antigen-presenting Cells (ISBN 0-12-137800-4, 1994, Academic Press Limited)中找到体外检测巨噬细胞活性的方法．例如，从来自健康受检者和皮肤病患者的皮肤细胞中可以建立原代皮肤巨噬细胞培养物．可以在经过掺入一系列浓度的本发明的化合物后，按特定的时间间隔检测巨噬细胞的活性，例如细胞增殖或细胞因子分

泌．在一个实施方案中，可以利用来自健康受检者和皮肤病患者的“穿刺活组织”．穿刺活组织培养时可以被浸没在培养基中或表皮的一面在培养基中，该培养基可以掺入本发明的化合物．用本发明的巨噬细胞结合化合物培养之后，可以通过免疫组织化学或 ELISA、RIA、EIA 检测这些化合物对巨噬细胞活性的影响．

检测细胞增殖过程中发生的变化的方法，例如，掺入胸腺嘧啶核苷或 BrdU 的检测方法，在本领域中是已知的．本发明优选的巨噬细胞结合化合物降低或消除巨噬细胞的活性．可以通过多种的免疫检测，例如酶联免疫检测（ELISA）、酶免疫检测（ESA）或放射性免疫检测（RIA），检测细胞因子浓度的变化（参见，例如，Keler, T. et al. (1997) Cancer Research 57:4008-14），这些免疫检测方法在本领域中是已知的．可以检测的细胞因子的例子包括：粒细胞/巨噬细胞集落刺激因子（GM－CSF）、粒细胞集落刺激因子（G－CSF）、巨噬细胞集落刺激因子（M－CSF）、白细胞介素 1－12（IL－1 至 IL－12）和 TNF－α．利用 EIA 通过检测细胞因子和一种酶联抗体的相互作用可以测量细胞因子的浓度．检测酶活性是通过酶与适当的底物，优选的是产色的底物，反应，这样以便产生可检测的化学组成部分，该化学组分可以通过，例如，分光光度计、荧光光度计或可见的方法检测（Voller, “The Enzyme Linked Immunosorbent Assay (ELISA) .” Diagnostic Horizons 2:1-7, 1978, Microbiological Associates Quarterly Publication. Walkersville, MD; Voller, et al., J. Clin. Pathol. 31:507-520 (1978); Butler. Meth. Enzymol. 73:482-523 (1981); Maggio, (ed.) Enzyme Immunoassay, CRC Press. Boca Raton, FL, 1980; Ishikawa, et al., (ed.) Enzyme Immunoassay, Kgaku Shoin. Tokyo, 1981）．可用作抗体的可检测性标记的酶在上文有描述．检测可以通过比色方法来完成，该方法运用了该酶的产色底物．检测也可以通过目测底物酶促反应的程度，并与类似制备的标准物相比较而进行．

细胞因子的检测也可以通过放射性免疫检测（RIA）来完成（参见，例如，Weintraub, B., Principles of Radioimmunoassays, Seventh Training Course on Radioligand Assay Techniques, The Endocrine Society, March, 1986,在此引入作为参考）．检测放射

性同位素可以通过这些方法例如γ计数器或闪烁计数器或放射性自显影探测.

也可以用荧光化合物标记抗细胞因子抗体. 当荧光标记的抗体暴露于合适波长的光时, 就可以检测它的的存在. 在最普遍应用的荧光标记化合物包括异硫氰酸荧光素、罗丹明、藻红蛋白、藻青蛋白、藻青蛋白同分异构体、邻苯二醛和荧光胺. 可以用荧光发射金属, 如152Eu 或其它镧系元素, 可探测地标记该抗体. 这些金属可以通过例如二乙三胺五乙酸(DTPA) 或乙二胺四乙酸 (EDTA)金属螯合基团被连接到抗体上. 该抗体也可以通过与化学发光化合物的偶联而被探测地标记. 然后, 化学发光标记的抗体的存在是通过检测化学反应中发出的光. 特别有用的化学发光标记化合物的例子有发光氨、异发光氨、theromatic acridinium 酯、咪唑、吖啶锆盐和草酸酯. 类似地, 可以用生物发光的化合物标记该抗体. 生物发光是在生物体系中的一类化学发光, 生物体系中的催化蛋白提高了化学发光反应的效率. 通过检测发光的存在鉴定生物发光蛋白的存在. 用于标记的重要生物发光化合物有虫萤光素、萤光素酶和发光蛋白质.

也可以在体内检测巨噬细胞结合化合物. 例如, 可以用本文实施例中所描述的表达 Fc 受体的小鼠检测这些化合物. 在一个实施方案中, 巨噬细胞结合化合物被注射到这些转基因小鼠的真皮中. 同时平行进行注射载体的对照实验. 通过对小鼠的局部反复施用 5% 的月桂硫酸钠可以产生慢性皮肤炎. 这些化合物的效果可以在注射后的不同时间间隔用免疫组织化学的方法, 例如, 宏观地或临床地, 监测.

本发明的巨噬细胞结合化合物可以用于治疗疾病, 上述疾病的特征为巨噬细胞活性或数量异常. 术语“异常”是指在指定区域的巨噬细胞的密度不同于(例如, 高于)正常健康人的相同区域的巨噬细胞的密度. 术语“异常”也包括异常的巨噬细胞活性, 例如异常高的细胞增殖或细胞因子分泌. 相应地, 在一个实施方案中, 本发明提供了治疗或预防疾病的方法, 该疾病的特征是在指定区域巨噬细胞的数量或活性异常, 该方法包括通常在需要治疗的局部区域施用于患者, 药用组合物包含一个或更多的巨噬细胞结合化合物.

巨噬细胞结合化合物通常作为定位试剂将细胞毒素(例如, 药物)运送到承载 Fc 受体的巨噬细胞上. 在本发明的一个实施方案中,

细胞毒素被包入脂质体中，该脂质体本身被导向承载 Fc 受体的巨噬细胞上．因此，巨噬细胞结合化合物包含连接到脂质体上的抗 Fc 受体结合部分，上述脂质体含有细胞毒素．在一个优选的实施方案中，抗 Fc 受体结合部分是抗 Fc 受体的单链抗体 (scFv)如 H22 scFv．抗-FcR scFv 连接或插入到脂质体磷脂双分子层中的方式允许 scFv 仍然识别和结合脂质体外的 Fc 受体．这可以通过已知的方法来完成，例如 de Kruif, J. et al. (1996) FEBS 399:232-236 中所描述的方法．最终结果是定位于 FcR 的细胞毒素以脂质体的形式被运送到细胞上．

此处所用的巨噬细胞结合化合物的“有效治疗剂量”是指该化合物以一次或多次施用于患者时，能抑制细胞的生长或缺少这样的治疗就会增进临床症状的有效剂量．

此处所用的化合物的“有效预防剂量”是指巨噬细胞结合化合物以一次或多次施用于病人时，能防止或延迟巨噬细胞介导疾病的发生或复发的有效剂量．

“诱导”、“抑制”、“加强”、“提高”、“增加”、“减少”等等，例如，表示两种状态的数量差别的术语，至少表明两种状态的统计学上的显著差别．例如，“抑制巨噬细胞生长的有效剂量”表示细胞的生长速度将至少在统计学上显著不同于未治疗的细胞．

本发明的巨噬细胞结合化合物可以用于治疗各种巨噬细胞介导的疾病．这些疾病的特征不必仅仅是巨噬细胞数量和/或活性异常，但它们各自涉及不合乎需要的对病人有害的巨噬细胞活性．在一个实施方案中，该化合物用于治疗自免疫疾病，该自免疫疾病包括，例如，糖尿病、关节炎（包括类风湿性关节炎、幼年类风湿性关节炎、骨关节炎、牛皮癣关节炎）、多发性硬化症、脑脊髓炎、糖尿病、重症肌无力、系统性红斑狼疮、自免疫甲状腺炎、皮炎（包括遗传过敏性皮炎和湿疹性皮炎）、牛皮癣、Sjogren's 综合症、Sjogren's 综合症继发干性角膜结膜炎、斑秃、由于节肢动物咬伤反应引起的过敏反应、Crohn's 疾病、口腔溃疡、虹膜炎、结膜炎、角膜结膜炎、溃烂大肠炎、哮喘、过敏性哮喘、皮肤红斑狼疮、硬皮病、阴道炎、直肠炎、药疹、麻疯病逆向反应、结节性红斑皮屑、自免疫葡萄膜炎、过敏性脑脊髓炎、急性坏死性出血性脑病、自发性两侧渐进性感觉神经

听力丧失、再生障碍性贫血、纯红细胞贫血、慢性活动肝炎、Stevens-Johnson 综合症、自发性口炎性腹泻、扁平地衣症、Crohn's 疾病、Graves 眼病、肉状瘤病、原发性胆硬化、晚期葡萄膜炎和肺间质纤维症）．在过敏症，例如遗传性过敏症中免疫活性的下调也是合乎需要的．

可以将上述方法作为疗法的一部分的优选自免疫/皮肤疾病的例子包括：牛皮癣、免疫遗传过敏性皮肤炎、多发性硬化症、硬皮病和皮肤红斑狼疮．例如，本发明的方法和组合物可用于治疗遗传过敏性皮肤炎（AD）．不局限于理论，有人认为在 AD 的皮肤炎症急性期局部 T 细胞的表型从 Th2 型转变成慢性期的 Th1 型．在此时间点在损伤部位发现产生的 IL－12 的量增加，同时大量流入激活的巨噬细胞．巨噬细胞是诱导 T 细胞产生 IFN－γ的有效生产者，而 IFN－γ反过来又是巨噬细胞的有效激活剂（Thepen, T. et al. (1996) J Allergy Clin. Immunol. 97: 828-837; Grewe, M. et al. (1998) Immunol. Today 19: 359361）．这样的正反馈可能产生恶性循环，这种恶性循环自身就能够维持局部炎症而不需要外来刺激．考虑到巨噬细胞的调控潜能，其它这样的由于巨噬细胞的异常调控而导致连续免疫原非特异性反应的机制也似乎是可能的．如下文的实施例所描述，通过定位 Fc 受体，例如 FcγRI，在局部区域特异性地消除巨噬细胞炎症使得本发明可用于减小或消除由于巨噬细胞分泌而导致的正反馈循环，从而治疗诸如 AD 这样的疾病．

可用本发明的治疗方法治疗的疾病的例子进而包括传染性疾病，例如，HIV 感染、呼吸道疾病，例如，慢性多型性光皮肤炎（CPLD），慢性阻塞性肺病（COPD），例如，过敏性哮喘和类肉状瘤病以及炎症反应如在开放伤口或烧伤中观察到的炎症．

在其它实施方案中本发明的组合物和方法可用于化妆品．例如，巨噬细胞结合化合物可被局部施用到皮肤以延迟和/或防止皮肤的衰老过程．

本发明的治疗方法可以与其它去除巨噬细胞的技术联合使用．例如，本发明的巨噬细胞结合化合物可以和外科手术、化学疗法或放射性疗法联合使用．

本发明的巨噬细胞结合化合物可用于调节效应细胞上的 FcγR 水

平，例如通过覆盖和消除细胞表面的受体。抗 Fc 受体抗体的化合物也可被用于此目的。

本发明进而提供了一种试剂盒，该试剂盒中包含了单一剂量或多剂量的巨噬细胞结合化合物以及使用说明。

在其它实施方案中本发明的巨噬细胞结合化合物的复合物可被用于选择性地杀伤巨噬细胞或降低巨噬细胞的活性，例如，由第一种化合物和第二种化合物组成的复合物，上述第一种化合物具有至少一个对 FcR 特异的抗原结合区域以及毒素，，上述第二种化合物具有针对 FcR 受体的不同表位的抗原结合区域或针对不同 Fc 受体，例如 Fcα 受体的抗原结合区域。在一些实施方案中本发明的第二种巨噬细胞结合化合物可以和第一种联合使用。例如，该第二种巨噬细胞结合化合物可以具有至少一个对 IgA 受体，例如，Fcα受体，和 IgE 受体，例如，Fcε受体，Fcδ受体和/或 Fcμ受体，特异的抗原结合区域。

在对患者施用巨噬细胞结合化合物之前可以预先用一种调节，例如增强或抑制，Fcγ受体的表达或活性的试剂处理，例如对患者用细胞因子处理。在用巨噬细胞结合化合物治疗期间施用的优选细胞因子包括粒细胞集落刺激因子（G－CSF）、粒细胞巨噬细胞集落刺激因子（GM－CSF）、γ干扰素（IFN－γ）和肿瘤坏死因子（TNF）。

本发明的巨噬细胞结合化合物也可被用于体内和体外诊断以通过测量结合 Fc 受体的水平探测和/或测量巨噬细胞群。例如，如本文所提供的实施例中所显示，在人类急性或慢性皮肤炎的皮肤炎症中都检测到了 FcγRI 的大量表达。因此，此处所描述的巨噬细胞结合化合物可被用于诊断诸如炎症这样的情况。对于这样的用途，该化合物可以与一个可检测的分子连接。该可检测的标记可以是，例如，放射性同位素、荧光化合物、酶或酶辅助因子。相应地，在其它实施方案中本发明提供了一种用于体内或体外诊断疾病的方法，上述疾病的特征为巨噬细胞数量（例如，巨噬细胞异常）和/或 Fc 受体表达的异常（例如，表达 Fc 受体的细胞数增加和/或在一定细胞上的 Fc 受体表达水平升高）。通过测量给定样品中或局部区域中本发明化合物的结合水平可以推算出该区域中或样品中巨噬细胞的存在，如果该化合物中的抗 Fc 受体组分是对 Fc 受体特异的话。这可以这样完成：（i）取得身体样品，如体液、组织（例如皮肤样品）或病人的活体组织（ii）

将身体样品与本发明的巨噬细胞结合化合物或其片段接触；（iii）测定上述巨噬细胞结合化合物与身体样品结合的水平（iv）比较身体样品和对照样品，例如源自健康受检者的生物样品，与分子结合的量，或者与预先确定的基础水平进行比较，使得比对照水平高的结合能显示巨噬细胞疾病的存在，例如皮肤病的存在．优选地，相对于其它表达 Fc 受体的细胞，检测到的 Fc 受体的表达主要是在巨噬细胞上．体内和体外诊断的方法参见 PCTUS88/01941，EP 0 365 997 和 US 4,954,617．

下文将通过实施例进一步阐述本发明，这不应被理解为进一步的限制．所有在本申请中引用的所有参考文献、未定专利申请以及已发表专利的内容都在此引入作为参考．

实施例

材料与方法

以下的方法将用于下文所描述的研究．术语巨噬细胞结合化合物，CD64 免疫毒素（CD64IT）或免疫毒素（IT）在此可以互换使用．

单克隆抗体

下文的实施例描述相应于单克隆抗体 22 的人源化形式（H22）的抗 CD64（抗 FcR）抗体，上述单克隆抗体 22 的人源化形式在美国专利第 5，635，600 中有所描述，该专利在此引入作为参考．产生 H22 抗体的细胞系 1992 年 11 月 4 日储存于美国典型培养物保藏中心，命名为 HA022CL，ATCC 登记号为 CRL11，177．

其它可以用于本发明的组合物和方法的特异性抗 CD64 抗体为鼠抗体 mAb32.2、mAb44、mAb62 和 mAb197．产生 mAb32.2 的杂交瘤可从美国典型培养物保藏中心得到．ATCC 登记号 HB9469． mAb 197-Ricin A 偶联物的制备在以下的实施例中有所描述．

抗 FcR mAb 可通过蛋白 A 亲和层析（Bio-Rad，Richmond，CA）纯化各自的杂交瘤上清．

免疫组织化学染色

A: CD－64 染色

活组织样品在冰冻切片机上被切成 6μM 的切片并固定到包被的载玻片上．干燥过夜后切片用无水丙酮固定 10 分钟然后在空气中干燥．在 PBS，2％正常小鼠血清（NMS）中切片与偶联 FITC10.1（Serotec

1:40）偶联物温育 45 分钟. 用 PBS, 0.05%吐温洗涤切片 3 次，每次 5 分钟，然后与碱性磷酸酶（AP）偶联的绵羊抗 FITC 抗体（Boehringer Mannheim, 1:400）在 PBS 中温育（1％人 AB 血清，1％NMS 30 分钟）. 用 PBS/吐温洗涤两次和 Tris-HCl（0.1M, pH 8.5）洗涤一次后用溶于 0.1M Tris-HCl, pH8.5 的萘酚 AS－BI 磷酸盐（钠盐，50mg/100ml; Sigma）作为底物，新品红（10mg/100ml; Merck, Witehouse Station, N. J.）作为显色剂显示 AP 的活性，得到粉红色/红色的染色. 内源性 AP 活性通过在反应混合物中加入左旋咪唑（35mg/100ml, Sigma）得以抑制. 切片用苏木精轻微染色作为背景.

B: 标记

切片在无水丙酮中用过氧化氢（30%, 100μl/100ml）固定 7 分钟. 切片与最佳稀释度的第一大鼠抗体在 PBS, 2％NMS 中温育 45 分钟. 以下的抗体用于染色巨噬细胞: MOMA－2（Krall, G. et al, (1987) Scand. J. Immunol. 26: 653-661）; 树突状细胞: NLDC145（Krall, G. et al (1986) J. Exp. Med. 163: 981-997）; T 细胞: KT3（Tomonari, K. (1988) Immunogenetics 28:455-458）. 洗涤 3 次后（PBS, 0.05% 吐温 20, 5 分钟）在 PBS（1％人 AB 血清，1％NMS）中与过氧化物酶标记的兔抗鼠偶联物（DAKO）温育 30 分钟. 用 PBS 漂洗两次和用 NaAc（0.1M, pH5.0）漂洗 1 次后用过氧化氢作为底物，DAB 作为显色剂显示 PO 的活性，得到褐色的染色.

动物研究

皮肤炎症的诱导，免疫毒素的注射和活组织检查. 在此处所描述的实施方案中使用了表达人 FcγRI 的转基因 FVB/N 小鼠（Heijnen, I. A., et al.(1996) J. Clin. Invest. 97:331 338）. 以同窝出生的未转基因小鼠作为对照. 小鼠的两侧腹 1.5×1.0 的区域的毛被剃光并且每天在表皮上施用刺激性的月桂基硫酸钠（SLS）（5％于盐水中），连续刺激 10 天以诱导慢性皮肤炎症.

用 20μl Aescoket (Aesculaap, Gent, Belgium) 和 Rompun（Bayer, Leverkussen, Germany）4: 3 的混合物注射动物肌肉使之麻醉. 连续施用两次真皮注射（每次 10μl, 2×10^{-8}M, 指 Ricin A 部分，于盐水中）. 用相同的盐水注射动物对侧以用于对照.

按照上述方法麻醉动物并取出 3mm 穿刺活体组织，在液氮中速冻

并且储存在－70 ℃备用．该处皮肤用缝合线缝合．

从局部麻醉(1％利多卡因)的AD损伤皮肤(n=3)，24hAPT (n=3)，48h SLS (n=2)取出穿刺活体组织(3mm)，并且72h WB激发PLE皮肤．活体组织在液氮中速冻并且储存于－70 ℃备用．

实施例1: CD 64免疫毒素的制备

利用合适的连接物(例如利用杂合的双功能可切割的交联剂N-琥珀酰亚胺基-3-(2-吡啶基二硫代)丙酸(SPDP)(Pierce)按照产品说明书在GLP条件下进行偶联)将CD64单克隆抗体197(Guyre, P. M., et al. 1989. J. Immunol. 143: 1650-1655)和H22(Graziano, R. F., et al. 1995. J. Immunol. 155: 4996-5002)偶联到去糖基化的Ricin A上(30KDa, Sigma)．简言之，SPDP被偶联到CD64 mAb，例如H22，上，然后确定mAb-PDP的摩尔比．在确定mAb-PDP的摩尔比后，加入Ricin A. PDP自由基团和Ricin A自由链失活后该混合物用分子排阻层析纯化.进而用SDS－PAGE鉴定了H22－Ricin A偶联物的纯度．用0.2μm的滤膜将H22－Ricin A偶联物除菌．所有的制备步骤都在良好的药品管理规范的条件下进行．

实施例II: 利用CD64细胞毒素有效地杀伤巨噬细胞

FcγRI的组成性表达主要限制于骨髓细胞系，并且在前炎症和炎症条件下被强烈上调(Velde, A. A., et al. (1992) J. Immunol. 149:4048-4052; Schiff, D. E., et al. (1997) Blood 90:3187-3194)．FcγRI快速而有效地介导内吞作用的能力使该受体成为激活炎症巨噬细胞上的一个有效目标(Heijnen, I. A et al. (1996) J. Clin. Invest. 97:331 338)．按照实施例I中所描述的方法用毒素Ricin -A和CD64抗体的偶联物制备了一些抗h FcγRI的免疫毒素．

为了得知偶联物在诱导杀伤巨噬细胞上的效率，在存在与不存在本发明组合物条件下检测了干扰素－γ刺激的或未经干扰素－γ刺激的人类前单核细胞系U937培养物(图1, A和B)．Guyre, P. M., et al. (1983) J, Clin. Invest. 72: 393-397描述了U937细胞的培养条件和用细胞因子刺激U937细胞．简言之，U937细胞在300U/ml干扰素－γ存在的条件下培养24小时以便上调FcγRI的表达．用流式细胞仪监测FcγRI的水平．此外，还检测了转染或未转染FcγRIa cDNA的IIA1.6细胞．IIA1.6细胞来自鼠的A20 B淋巴细胞并且最近表明

属于 CD5+B 细胞/巨噬细胞的独特亚群（van Vugt, M. J., et al. 1998. Clin. Exp. Immunol. 113:415-422）.

通过测量对[^{3}H]胸苷的浓度依赖性掺入的抑制来评判 CD64 免疫毒素（IT）的细胞毒素功效（Post, J et al. Leuk. Res. 19:241-247）. 简言之，细胞被接种到 5×10^4 个细胞/每孔的 96 孔圆底板上并且与 ricin 部分的浓度为 10^{-12} 至 10^{-7}M 的 CD64 IT 一起温育 72 小时. 细胞用[^{3}H]胸苷（1μCi）脉冲掺入 4 小时，随后用玻璃棉滤膜收集并且在β平面扫描仪计数. 所有的温育都在补充了 2%人 AB 血清的培养基中进行以阻断 FcγRI 的 Fc 结合位点，因此只允许 IT 通过抗原识别位点结合. 选择接种的细胞数，使得掺入的[^{3}H]胸苷是细胞数的线性函数. [^{3}H]胸苷掺入的本底值是通过与 0.1mM 的放线酮一起温育得到的.

结果表示为与模拟处理的细胞相比，[^{3}H]胸苷掺入的百分比. 在图 1A－1B 中，柱形图表示与培养基对照（±SEM）相比[^{3}H]胸苷掺入的百分比. [^{3}H]胸苷掺入量随 H22－R 或 197－R 浓度的增加而剂量依赖性地减少表明了免疫毒素对刺激的 U937 细胞的细胞毒性. 对于图 1C－1D，柱形图表示与培养基对照（±SEM）相比[^{3}H]胸苷掺入的百分比. [^{3}H]胸苷掺入随 H22－R 或 197－R 浓度的增加而剂量依赖性地减少表明了免疫毒素对 hFcγRI-转染的 IIA1.6 细胞的细胞毒性. 这表明两种 IT 对 hFcγRI 表达细胞的特异性.

上述检测的两种免疫毒素是细胞杀伤的有力诱导剂. 然而，在诱导细胞杀伤时，H22 Ricin-A (H22-R)整体上比 197 Ricin-A (197-R)更有效，特别是对未刺激的细胞. 单独与 Ricin-A 在 10^{-8} 和 10^{-9}M 时没有明显的效果（88.9±14.2 和 100.4±13.5%，分别地）. 更进一步地，与使用这两种 ITs 的任何一种都能有效杀伤 hFcγRI-转染的 IIA1.6 细胞相比，在未转染的 IIA1.6 细胞上没有发现哪种 IT 有明显的效果（图 1，图 C 和 D）.

这些结果表明了 CD 64 IT 在体外杀伤 hFcγRI-表达细胞的有效性和特异性. 基于这些实施方案，H22－R 以 2×10^{-8}M 的浓度被用于下文所描述的体内实施方案中.

实施例 III：通过 CD 64－免疫毒素诱导细胞调亡

为了明确 H22 Ricin-A 的细胞毒性效应是否因为诱导了细胞调

亡，在低渗缓冲液中进行了碘化丙啶染色．在这个检测中，通过亚二倍体 DNA 内容物识别细胞调亡核碎片．为进行这些实验，按照 Nicoletti, I., et al. (1991) J. Immunol. Methods 139: 272-279 所描述的方法用碘化丙啶染色检测了核碎片．简言之，细胞与 IT 一起温育并在不同所时间收集．细胞用乙醇在－20°C 固定，与提取缓冲液(0.05M Na_2HPO_4; 0.0025M 柠檬酸; 0.01% Triton X-100; 20μg/ml 碘化丙啶）一起温育．碘化丙啶的荧光分析用的是荧光激活细胞分选器（FACScan）流式细胞术（Beckon and Dickinson, San Jose, CA）．

如图 2 所示，相对于对照，在 IT－处理的培养物中检测到了调亡核．在这个实验中，U937 细胞用 IFNγ 刺激并且与不同浓度的 H22－R 一起温育 6 小时．在暴露于 IT 后 2 小时即检测到了调亡核，并且该调亡核经过 16 小时处理后仍然是明显的．这个发现表明 H22 Ricin-A IT 的细胞毒性效应来自细胞调亡的诱导．细胞调亡介导的细胞杀伤通过减少体内的 hFcγRI-表达细胞而限制了潜在的破坏作用．此外，H22－R 诱导的持续细胞杀伤（甚至在 16 小时后）意味着 H22－R 作为 IT 减少体内 hFcγRI-表达细胞的可行性．

实施例 IV:　在人类慢性皮肤炎中检测 hFcγRI-表达细胞

在切片与 H22 抗体预温育后并且在不同浓度 H22 抗体存在的情况下，检测了另一种 CD 64 单克隆抗体，10.1(Dougherty, G. J et al. 1987. Eur. J. Immunol. 17:1453-1459) 的染色能力．由于 10.1 和 H22 识别 hFcγRI 上的不同表位，同时温育时染色强度或样式没有显著的变化．基于这些结果，在评判所收集的组织的所有涉及免疫组化的实验中都使用了 10.1 抗体．

为了检测人类慢性皮肤炎中 hFcγRI-表达细胞的存在，收集了过敏性皮炎(AD)病人的慢性炎症皮肤活体组织．按照 Hanifin 和 Raika 的标准诊断了 AD(Hanifin, J. M., and Rajka, G. (1980) Acta Derm. Venereol. (Stockholm) 92:44-47）．按照 Langeveld-Wildschut, E. G., et al. (1995) J. Allergy Clin. Immunol. 96:66-73 所描述的方法进行了遗传过敏症帖片测试（APT）．简言之，皮肤用胶带剥离 10 次并且在被诊断为 AD 的病人背部的临床上正常的皮肤上用 Leucotests(Beiersdorf, Hamburg, Germany) 施用了过敏原 Dermatophagoides pteronyssinus (Haarlem's Allergenen

Laboratorium, Haarlem, The Netherlands; 80μl, 10,000AU/ml）. 在相似的皮肤上以类似的方式施用了月桂基磺酸钠（SLS, Sigma, 0.1% 盐溶液）. 多形性光疹（PLE）的诊断是基于多形临床图中存在 WA 和/或 WB 照射后产生的丘疹和水泡以及严重骚痒和临床反应. 使用 Philips TL12 UVB 源，用 6 个最小红斑剂量照射了先前未暴露的皮肤.

利用 FcγRI 抗体对来自人类皮肤的切片进行了免疫组化染色. 检测到的 FcγRI－表达细胞表现为粉红/红色的染色结果并且与苏木精复染色形成对比. 在正常未感染的皮肤中，很少细胞表达 FcγRI. 这些细胞主要位于真皮内. 相反，在慢性损伤的皮肤，例如，遗传性过敏皮肤炎的皮肤，中观察到了 FcγRI 在真皮中的大量表达. 染色的细胞同时位于真皮的渗透物中以及扩散通过了真皮. 在表皮没有观察到明显的染色. 接着，收集来自急性期模型的生物活体组织，例如遗传过敏症补片测试（APT）后 24 小时、多形性光疹（PLE）后 72 小时、月桂基磺酸钠（SLS）处理后 48 小时. 这些生物活体组织给出了相似的结果，然而，FcγRI 表达细胞的数量稍微高于慢性感染的组织. 在急性和慢性期都存在大量 FcγRI－表达细胞表明了在皮肤炎症反应中这些细胞有一定的作用.

实施例 V: 慢性皮肤炎症鼠模型的建立

为了确定从皮肤中消除炎症巨噬细胞是否可行并且对皮肤炎症是否有益，在实验动物中检测了 H22－R. 利用剃过毛的 hFcγRI－转基因小鼠以及它们未转基因的同窝出生小鼠在局部反复施用 SLS 后研究慢性皮肤炎症的诱导. 在这些小鼠中 hFcγRI 的表达模式、基因调控和功能反映了在人中 hFcγRI 的表达模式、基因调控和功能(heijnen, I. A., et al. (1996) J. Clin. Invest. 97: 331-338）. 如材料和方法部分中所描述，检测了几种方法并且证明了每天施用 5% SLS，施用 10 天就足够了.

在正常的未处理的皮肤中检测到的 T 细胞、树枝状细胞和巨噬细胞数量很少（分别为 5±4; 7±4 和 15±3 每 mm^2）. 此外，在正常的未处理的皮肤中（5±2 每 mm^2），检测到的 T 细胞、树枝状细胞和巨噬细胞表达细胞很少，并且它们的分布类似于正常的未感染的人皮肤. SLS 处理的结果是表皮变厚以及由 T 细胞、树枝状细胞和巨噬细胞组

成的大量真皮渗透物（图 3A）．对于这些实验，在慢性炎症皮肤中施用了一次 IT 真皮注射并且在不同的间隔取出刺穿活体组织并进行免疫组化染色．表达 hFcγRI 的细胞数量也显著地增加（图 3A）（75±11 每 mm^2）并且与在慢性感染的人皮肤中一样，这些细胞主要分布在真皮中．在 hFcγRI－转基因和未转基因的小鼠中，细胞组成没有明显的区别．然而在后者中，没有观察到明显的 hFcγRI 细胞染色．只用 H22－R 注射后没有检测到 hFcγRI－表达细胞或巨噬细胞的存在．

在慢性炎症病人皮肤和 SLS 诱导 hFcγRI －转基因小鼠的炎症之间在细胞组成以及 hFcγRI 表达上的相似性使之成为研究慢性皮肤炎症中 hFcγRI 表达细胞的作用的合适模型．该模型与 H22－R IT 一起用于下文描述的实施例中．

实施例 VI:　体内 FcγRI－表达巨噬细胞的有效减少

为了确定 H22－R 在体内对 hFcγRI－表达细胞的杀伤是否与体外所证明的一样有效，在 SLS 处理的小鼠中真皮注射了 H22－R．如上文材料和方法部分所描述，在表达人 FcγRI 的转基因小鼠中局部反复施用刺激物质 5％月桂基硫酸钠从而诱导了慢性皮肤炎症．对皮肤进行了 SLS 处理的 hFcγRI－转基因和未转基因小鼠的一次施用中连续进行了两次 10μl 2×10^{-8}M（3μg H22 和 0.6μg 的 Ricin A）的真皮注射．在对侧施用相同的载体作为对照．继续施用 SLS，同时在不同的时间点收集皮肤样品、导液淋巴结（draining lymph nodes）、肝脏和脾用于免疫组化分析．

通过检测巨噬细胞对碳颗粒的摄取检测到了真皮内注射定位特征．真皮注射碳颗粒的鼠的皮肤横截面表明了碳颗粒主要位于真皮，而不是在皮下的肌肉组织．这个分布表明了真皮内注射的定位特征．

经过月桂基硫酸钠的局部反复施用以及载体对照或 Ricin A-H22 的真皮注射后，表达人 FcγRI 的转基因小鼠皮肤的一个代表性免疫组化切片表明处理后 24 小时表皮变厚并且大量渗透细胞位于真皮．这种染色样式表明刺激物诱导了慢性炎症．大部分检测的渗透细胞是 FcγRI－阳性巨噬细胞（被染成粉红色）．相反，经过免疫毒素 Ricin A-H22 注射后 24 小时表达 Fcγ受体的渗透细胞的染色显著减少．

检测到了源自皮肤的表达 hFcγRI 的细胞在暴露于 IT 24 小时内就消失了（图 3A）．尽管继续施用 SLS，这种减少直到接近 96 小时

才结束，之后发生种群恢复．种群恢复只在 120 小时完成（图 3A）．在引流淋巴结、肝脏和脾中没有观察到明显的 hFcγRI 表达水平的变化．这个现象强调了该效应局限于注射位点的事实．在载体对照注射位点和未转基因的小鼠中没有观察到明显的变化．hFcγRI 表达细胞的快速且接近完全地消失以及它们在皮肤的持续缺乏表明用 H22－R IT 在慢性皮肤炎症中消除体内 hFcγRI－表达细胞的可行性．

实施例 VII：在局部皮肤炎症中 hFcγRI－表达细胞缺少的效果

在 hFcγRI－表达细胞减少的同时，MOMA－2－表达巨噬细胞的丰度也降低．这个发现表明 H22－R 注射导致感染皮肤中炎症巨噬细胞的有效减少（图 3A）．相反，在未转基因小鼠的巨噬细胞群中没有明显变化．这种有选择的减少确认了 H22－R 在皮肤中定位和减少巨噬细胞的特异性．

为了进一步地评价巨噬细胞减少的定位特征，检测了造血组织例如淋巴结、脾和肝脏．在其它的造血组织中没有检测到免疫毒素造成的细胞明显减少．对未转基因的同窝出生小鼠的处理没有导致任何细胞群的可探测变化．这些结果表明巨噬细胞的减少是对人 FcγRI－承载细胞特异的并且局限于注射位点．

在 24 小时内巨噬细胞即消失而在该检测时间点没有检测到树枝状细胞、T 细胞群或郎罕氏细胞的明显减少，进一步表明了该方法在局部减少巨噬细胞上的特异性．H22－R 的注射对 T 细胞和树枝状细胞的数量没有直接影响（图 3B）．然而，在 hFcγRI－表达巨噬细胞消失后，皮肤中 T 细胞和树枝状细胞的数量开始减少．T 细胞和树枝状细胞数量减少表明了局部炎症的消退，因而表明了甚至在炎症刺激物继续存在的情况下，局部炎症中巨噬细胞减少仍然具有有益的效果（图 3B）．

这些结果在组织学水平上表明了 CD 64 IT 在减少皮肤炎症巨噬细胞上的有效性和特异性．随后其它炎症细胞的消失表明了在慢性皮肤炎症中巨噬细胞的有害作用．

实施例 VIII：局部巨噬细胞的减少改善了皮肤的临床症状

为了确定局部巨噬细胞的减少是否改善了皮肤的临床症状，测量了两种参数：局部皮肤温度和红斑．红斑主要是由于微血管扩张增强并且直接关系到皮肤温度的升高．

为了检测施用 SLS 和 IT 注射导致的皮肤温度变化，利用温和的乙醚镇静剂将动物镇静并且用皮肤探针测量局部的温度(Ellab A-H1, Denmark)．为了说明作为炎症参数的微血管扩张和血管渗漏，用乙醚将动物镇静并且用 1％的 Evans 蓝溶液静脉注射动物．15 分钟后动物被致死并且取出动物皮肤用于测量．

利用小皮肤探针测量了 IT－处理的和对照动物的皮肤温度的局部变化．SLS 处理后检测到温度升高，这确认了局部炎症的诱导．图 4A 是柱状图，描述了真皮内注射 H22－R 对皮肤温度的影响随时间变化的函数．温度下降达到的水平可与未处理的未感染的皮肤相比．在 IT－处理的动物中检测到的温度下降通常持续 96 小时．之后，温度又升高，达到的水平可与 IT 注射以前相比．这些温度变化表明炎症的消退．载体对照和未转基因的小鼠都没有显示类似的温度下降．而且，发现巨噬的消失和局部皮肤温度下降之间有密切的时间关系．反之，巨噬细胞重新出现时检测到了温度升高．这个结果高度表明了在局部炎症中巨噬细胞的重要作用．

在小鼠中，由于鼠皮肤很薄很难评价皮肤红色．为了方便看清局部微血管的扩张，将 Evans 蓝注射到这些小鼠的静脉中．对于这些实验，通过表皮施用 SLS 在 hFcγRI－转基因小鼠（n＝9）或未转基因小鼠（n＝9）以诱导慢性皮肤炎症，并且在真皮内施用了 IT 或载体对照．在第 24 小时静脉注射 Evans 蓝，30 分钟后动物被杀死并且中间部分的皮肤被移走．利用这个技术检测到 SLS 处理后存在炎症反应．在未转基因小鼠或载体对照中没有检测到 IT 对微血管扩张的明显作用．然而，在后者的 H22－R 注射的一侧，该注射位点本身没有染成蓝色，表明了局部炎症的消退．而且，在所有染成蓝色的地方很少在 H22－R 注射的一侧．

实施例 IX：体内反复注射 CD64－IT 延长了对炎症的抑制

为了确定 IT 是否可以施用更长的时间，每天检测皮肤温度并且温度上升时在动物的同一部位再注射 IT．在这个实验中，继续施用 SLS．图 4B 表明只有在注射 H22－R 的 hFcγRI－转基因小鼠中炎症可以被控制至少 18 天．在任何时间点检测载体对照和未转基因小鼠没有显示出明显的温度下降．用 IT 反复注射表明长时间地抑制炎症是可能的．这个结果表明了在病人慢性皮肤炎症中延长 IT 处理的适用

性．总的来说，这些实验表明了在SLS诱导的慢性皮肤炎症中局部消除巨噬细胞的有益作用．

总而言之，本文实施例所描述的实验表明了利用本发明的组合物和方法可以选择性地并且有效地消除活性巨噬细胞，而对其它皮肤或造血细胞群没有显著的影响．而且，免疫毒素的作用主要位于运送的区域，因此减少了对全身的其它 FcγR 表达细胞的副作用．巨噬细胞消除时炎症减弱强调了炎症巨噬细胞在诱导和维持皮肤炎症中的重要作用．炎症的减弱是在组织学水平上并且通过临床参数例如局部皮肤温度和皮肤的红色的降低来检测的．而且，反复施用能延长对炎症的抑制．延长效力表明了本发明的方法和组合物在治疗患有慢性皮肤病的病人局部皮肤炎症上的潜在用途．本文所描述的方法可能有更广泛的用途，因为炎症巨噬细胞可能在其它类型的慢性炎症例如风湿性关节炎中起重要作用．

在人皮肤中的 CD64 染色显示了在急性和慢性皮肤炎症中存在大量的 FcγRI－表达细胞．这个结果表明通过 FcγRI 定位巨噬细胞可以真正提供治疗人皮肤炎症疾病的新方法．事实上，本文展示的SLS诱导的 hFcγRI－转基因小鼠慢性炎症的有效减弱支持了免疫毒素的潜在治疗用途．

等价方案

本领域的技术人员不需要常规实验以外的方法就能够辨别或确认此处所描述特定实施方案的等价方案．下文的权利要求将这些等价方案包括在内．

说 明 书 附 图

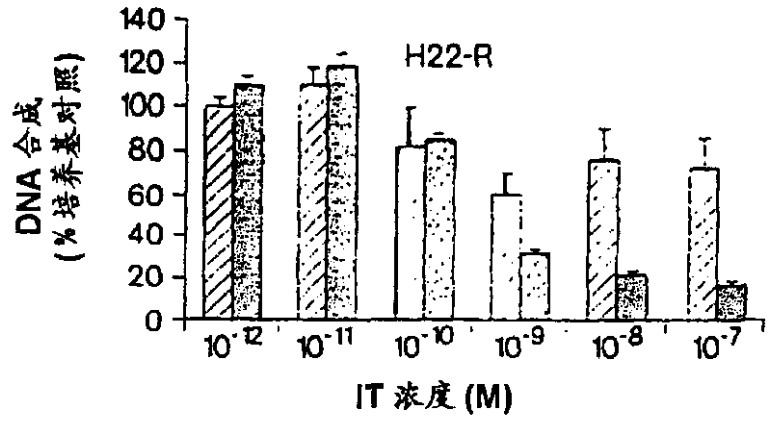


图 1A

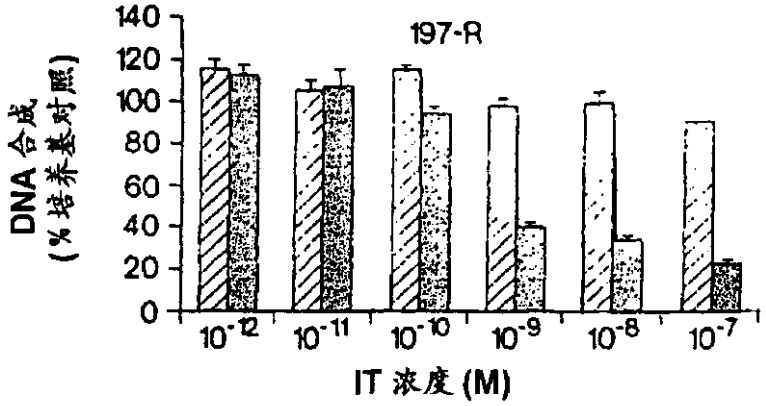


图 1B

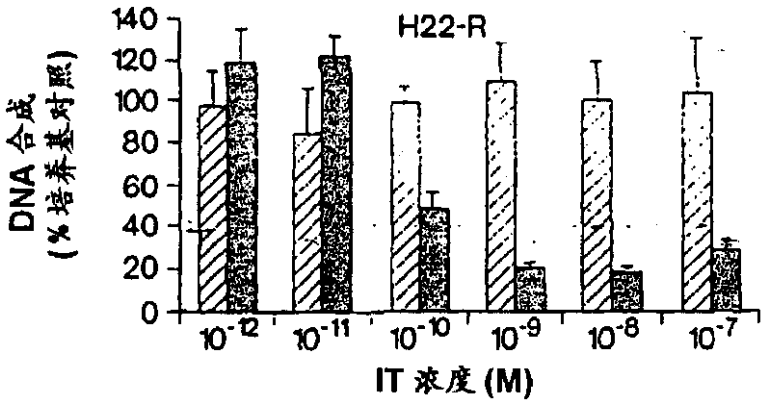


图 1C

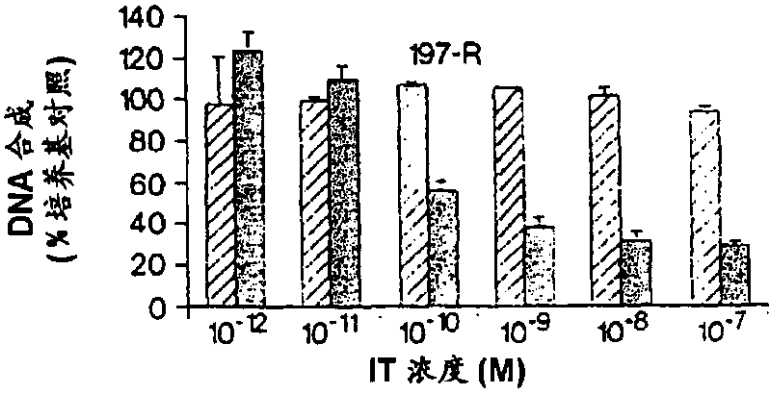
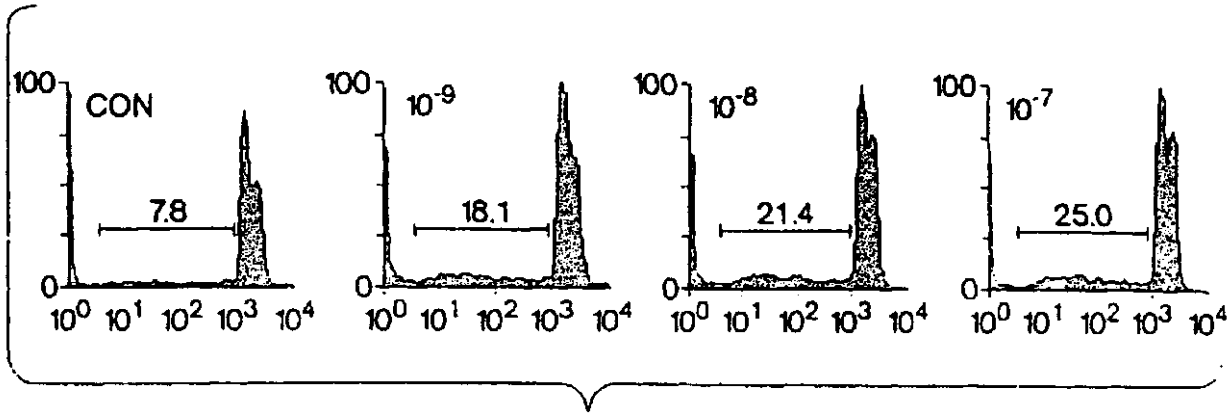


图 1D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0
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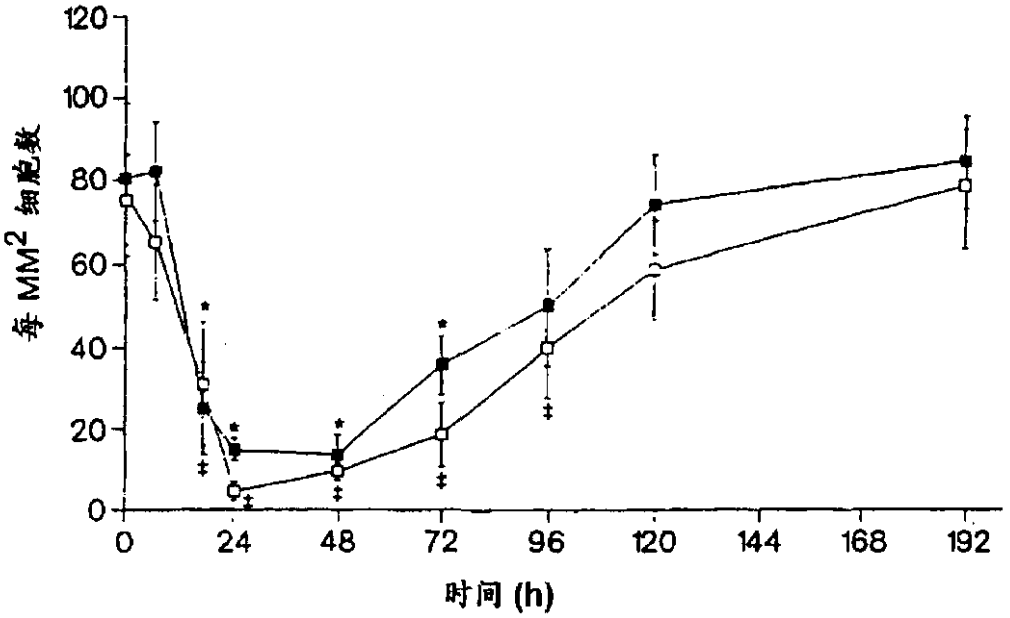
100
10^{-9}
18.1
0
10^0 10^1 10^2 10^3 10^4
100
10^{-8}
21.4
0
10^0 10^1 10^2 10^3 10^4
100
10^{-7}
25.0
0
10^0 10^1 10^2 10^3 10^4

图 2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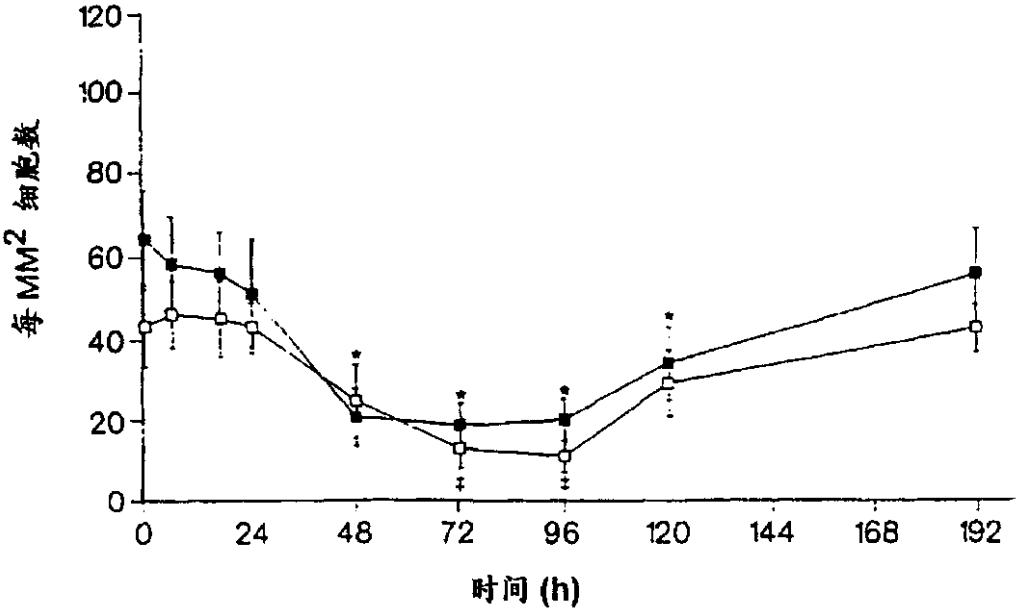
100
80
60
40
20
0
每 MM2 细胞数
0
24
48
72
96
120
144
168
192
时间 (h)

图 3A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60
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每 MM2 细胞数
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24
48
72
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120
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时间 (h)

图 3B

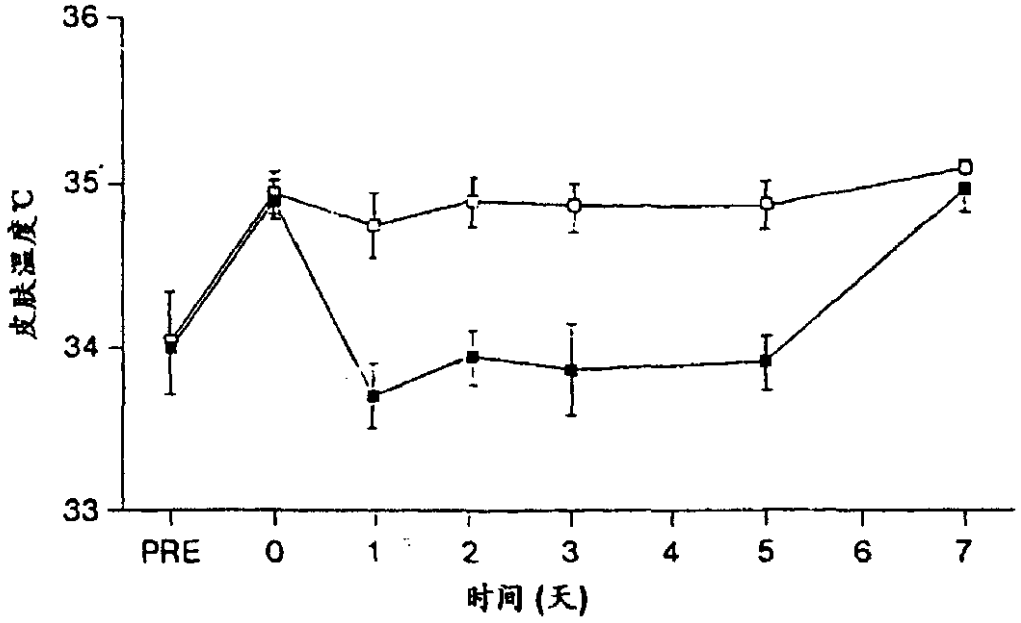
36
35
34
33
皮肤温度℃
PRE
0
1
2
3
4
5
6
7
时间(天)

图 4A

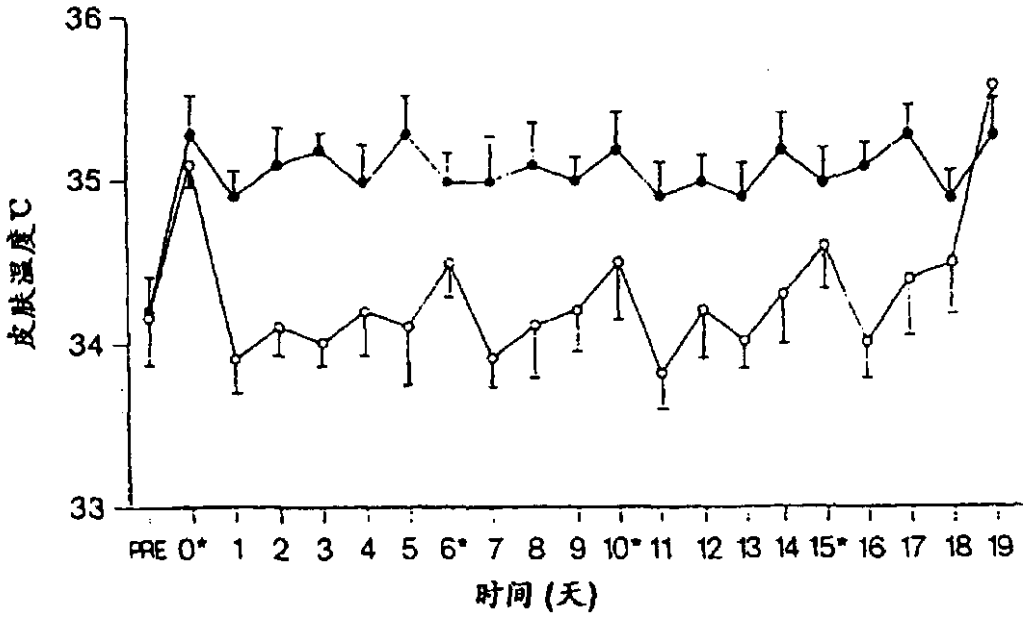
36
35
34
33
皮肤温度℃
PRE 0* 1 2 3 4 5 6* 7 8 9 10* 11 12 13 14 15* 16 17 18 19
时间(天)

图 4B