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(57) Abstract: The present invention refers to a targeted receptor molecule based on a receptor of soluble inflammatory antigens, binding specifically to a membrane bound marker of inflammation, methods of producing said targeted receptor molecule and methods of anti-inflammatory treatment.



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Targeted Receptor

The present invention refers to a targeted receptor molecule and methods of anti-inflammatory treatment.

Chronic inflammatory disorders are characterized by an abundant leucocyte infiltration in the affected tissue due to the continuous recruitment of these inflammatory cells. Although these events are part of the body's defense mechanisms, excessive responses occur in the vicious circle of cell activation and cell destruction, leading to acute and chronic inflammatory disorders. Many current therapeutic approaches are based on attempts to control leucocyte activation. There has been a growing interest in the role of the endothelial cells in leucocyte recruitment and the possibility of manipulating endothelial cell activation as a therapeutic approach. The endothelium plays an important role in the inflammatory cascade, and has the advantage of being easily accessible for drug targeting preparations due to direct contact with the blood as well as the presence of endocytotic processes. Therefore endothelial cells are attractive targets for cell-selective pharmacological intervention employing drug targeting strategies. Adequate cell-selective delivery of potent anti-inflammatory agents may provide an important tool for increasing the efficacy and reducing the side-effects of such agents in the treatment of chronic inflammatory diseases.

Cell adhesion molecules are glycoproteins expressed on the cell surface and play an important role in inflammatory as well as neoplastic diseases. There are four main groups: the integrin family, the immunoglobulin superfamily, selectins, and cadherins.

The integrin family has eight subfamilies, designated as $\beta 1$, through $\beta 8$. The most widely studied subfamilies are $\beta 1$, (CD29, very late activation [VLA] members), $\beta 2$ (leukocyte integrins such as CD11a/CD18, CD11b/CD18, CD11c/CD18, and $\alpha_d\beta_2$), $\beta 3$ (CD61, cytoadhesions), and $\beta 7$ ($\alpha_4\beta_7$ and $\alpha_E\beta_7$). Integrins are cell surface receptors that interact with the extracellular matrix (ECM) and mediate various intracellular signals. They define cellular shape, mobility, and regulate the cell cycle. These integral membrane proteins are attached to the cellular plasma membrane through a single transmembrane helix.

Integrin plays a role in the attachment of cells to other cells, and also plays a role in the attachment of a cell to the material part of a tissue that is not part of any cell (the extracellular

matrix). Besides the attachment role, integrin also plays a role in signal transduction, a process by which a cell transforms one kind of signal or stimulus into another.

The immunoglobulin superfamily (IgSF) is a large group of cell surface and soluble proteins that are involved in the recognition, binding, or adhesion processes of cells. Molecules are categorized as members of this superfamily based on shared structural features with immunoglobulins (also known as antibodies); they all possess a domain known as an immunoglobulin domain or fold. Members of the IgSF include cell surface antigen receptors, co-receptors and co-stimulatory molecules of the immune system, molecules involved in antigen presentation to lymphocytes, cell adhesion molecules and certain cytokine receptors. They are commonly associated with roles in the immune system. The immunoglobulin superfamily includes leukocyte function antigen-2 (LFA-2 or CD2), leukocyte function antigen-3 (LFA-3 or CD58), intercellular adhesion molecules (ICAMs), vascular adhesion molecule-1 (VCAM-1), platelet-endothelial cell adhesion molecule-1 (PE-CAM-1), and mucosal addressin cell adhesion molecule (MAdCAM-1). MAdCAM-1, which belongs to the group of addressins, is the gateway molecule for cellular migration into the mucosal immune system. Another Important addressin, apart from MadCAM is PNAd (peripheral node addressin). PNAd interacts with L-selectin and is the homing receptor lymphocytes to peripheral lymph nodes.

Selectins are a family of cell adhesion molecules (or CAM's). All selectins are single-chain transmembrane glycoproteins which share similar properties to C-type lectins due to a related amino terminus and calcium-dependent binding. Selectins bind to sugar moieties and so are considered to be a type of lectin, cell adhesion proteins that bind sugar polymers. The selectin family includes E-selectin (CD62E), P-selectin (CD62P), and L-selectin (CD62L).

Cadherins are a major class of membrane proteins with prominent roles in cell-cell adhesion, and the regulation of tissue organisation and morphogenesis. They include epithelial (E), placental (P), and neural (N) subclasses. The binding sites (ligands/receptors) are different for each of these cell adhesion molecules (e.g., ICAM binds to CD11/CD18; VCAM-1 binds to VLA4). E-cadherin (epithelial) is probably the best understood cadherin. It consists of 5 cadherin repeats (EC1 ~ EC5) in the extracellular domain, one transmembrane domain, and an intracellular domain that binds p120-catenin and beta-catenin. The intracellular domain

contains a highly-phosphorylated region vital to beta-catenin binding and therefore to E-cadherin function. Beta-catenin can also bind to alpha-catenin. Alpha-catenin participates in regulation of actin-containing cytoskeletal filaments. In epithelial cells, E-cadherin-containing cell-to-cell junctions are often adjacent to actin-containing filaments of the cytoskeleton. E-cadherin is first expressed in the 2-cell stage of mammalian development, and becomes phosphorylated by the 8-cell stage, where it causes compaction. In adult tissues, E-cadherin is expressed in epithelial tissues, where it is constantly regenerated with a 5-hour half-life on the cell surface.

Loss of E-cadherin function or expression has been implicated in cancer progression and metastasis. E-cadherin downregulation decreases the strength of cellular adhesion within a tissue, resulting in an increase in cellular motility. This in turn may allow cancer cells to cross the basement membrane and invade surrounding tissues.

The specific cell adhesion molecules and their ligands may be involved in pathologic conditions and potential therapeutic strategies by modulating the expression of these molecules.

The recruitment and migration of leucocytes into inflamed tissues is a carefully orchestrated process. It consists of sequential steps mediated by different families of adhesion molecules expressed by both the leucocytes and the endothelial cells at the site of inflammation. Of these adhesion molecules, the selectin family mediates the initial contact and subsequent rolling of the leucocyte on the endothelium. It consists of three members, i.e. E- (endothelial), P- (platelet) and L- (leucocyte) selectin. Activated endothelial cells express E- and P-selectin. P-selectin is also expressed on platelets, whereas L-selectin is only expressed on subsets of leucocytes.

Both E and P-selectin are known to bind with Sialyl Lewis x (sLex)-like glycans expressed by circulating leukocytes. Furthermore, P-selectin (and E) also binds to P-selectin glycoprotein ligand-1 (PSGL-1) expressed by human leukocytes. The E-selectin-ligand ESL-1 is a variant of a receptor for fibroblast growth factor. L-selectin binds to PNad (peripheral node addressin), a set of sialomucins that are constitutively displayed on high endothelial venules of lymph nodes.

Selectins are also involved in the adaptive immune response with the recruitment of activated lymphocytes to sites of inflammation. One of their remarkable features is the ability to recruit T helper 1 (Th1) cells and exclude T helper 2 (Th2) cells via some sort of receptor-associated mechanism involving E and P-selectin that allows endothelial cells to discriminate between Th1 and Th2 cells. Th1 cells bind to P-selectin by means of PSGL-1, however, both Th1 and Th2 cells express PSGL-1, but Th1 cells have the "binding form of PSGL-1."

If during the rolling process the leucocyte is correctly activated, the affinity of the members of the integrin family of adhesion molecules on the leucocyte membrane increases. Examples of activating factors are cytokines such as interleukin IL-6 and IL-8, which can be produced by the activated endothelial cells; and chemokines such as monocyte chemotactic proteins (MCPs), growth related proteins (GROs) and interferon γ -inducible protein 10 (IP-10). The so-called counter receptors for integrins on the endothelium are members of the immunoglobulin superfamily (IgSF) and encompass Intercellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1). These molecules are highly expressed by activated endothelial cells in inflammatory sites. The interaction of integrins on the leucocyte with the immunoglobulin superfamily members on the endothelium mediates the firm attachment of the leucocyte, followed by transmigration into the tissue. In this latter process Platelet Endothelial Cell Adhesion Molecule-1 (PECAM-1 or CD31) and a variety of matrix metalloproteases (MMPs) exert important functions. Although initially identified as an IgSF member with a main function in cell-cell contact, PECAM-1 was recently shown to be a modulator of vascular cell activation as well.

Several targeted drug delivery approaches mediated by E-Selectin have been described. Specific binders to selectin include Aptamers (short synthetic DNA), peptides, carbohydrates, antibodies and natural ligands (Mol Interv. 2006 Apr; 6(2):98-112). Jamer et al (Rheumatology 2002;41:53-61) radio labelled murine Fab fragments generated against human E-selectin and applied to patients with RA, as a visualization study of active joint inflammation. A conjugate of an anti-thrombin agent with anti-E-selectin antibody has been described by Kiely et al (Arterioscler Thromb Vasc Biol. 1995 Aug;15(8):1211-8). Everts et al (Pharm Res. 2003 Jan;20(1):64-72) described a toxin liposome conjugate with an anti-selectin or anti-ICAM1 antibody for drug delivery to inflamed endothelium. Sialyl Lewis(x)-liposomes has been described as vehicles for site-directed, E-selectin-mediated drug transfer into activated endothelial cells (Stahn et al. Cell Mol Life Sci. 2001 Jan;58(1):141-7).

Pharmacologically active dexamethasone was delivered into activated endothelial cells by an dexamethasone-anti-E-selectin immunoconjugate (Asgeirsdottir et al. *Biochem Pharmacol.* 2003 May 15;65(10):1729-39).

Cytokines are messenger molecules that can communicate signals from one cell type to another and can instruct the cell receiving the signal to proliferate, differentiate, secrete additional cytokines, migrate etc.

One of the most important cytokine groupings, is the interleukine family. Interleukines act as communicators between leukocytes. Members of the interleukin family are diverse.

Approximately 30 interleukines have been described to date (IL-1 to IL-33). Other cytokine families have been established on the basis of their ability to support proliferation of hematopoietic precursors (colony stimulating factors), or cytotoxic activity towards transformed cell types (tumor necrosis factors), or the ability to interfere with viral replication (interferons) or their ability to recruit e.g. memory t cells and macrophages (chemokines).

Cytokines are typically low molecular weight (15-25kD) secreted proteins that mediate cell division, inflammation, immunity, differentiation, migration and repair. Cytokine production can occur in response to the release of endogenous "danger signals" that betray the presence of cells dying by necrosis, a mode of cell death that is typically seen in pathological situations and is typically provoked by infectious agents or tissue injury. It is relevant that the AU-rich sequences in the 3' untranslated regions of mRNA of many cytokines prime these mRNAs for rapid degradation thereby ensuring that cytokine production rapidly declines in the absence of appropriate stimulation. Unlike endocrine hormones, the majority of cytokines normally act locally in the paracrine or even autocrine fashion. Thus cytokines derived from lymphocytes rarely persist in the circulation, but nonlymphoid cells can be triggered by bacterial products to release cytokines which may be detected in the blood-stream, often to the detriment of the host. Septic shock for example, is a life-threatening condition that largely results from massive overproduction of cytokines such as TNF and IL-1 in response to bacterial infection and highlights the necessity to keep a tight rein on cytokine production. Certain cytokines, including IL-1 and tumor necrosis factor (TNF), also exist as a membrane-anchored forms and can exert their stimulatory effects without becoming soluble.

Cytokines are highly potent, often acting at femtomolar (10^{-15} M) concentrations, combining with small numbers of highly affinity cell surface receptors to produce changes in the pattern of RNA and protein synthesis in the cells that act upon.

Cytokine receptors typically possess specific protein-protein interaction domains or phosphorylation motifs within their cytoplasmic tails to facilitate recruitment of appropriate adaptor proteins upon receptor stimulation. A recurring theme in cytokine receptor activation pathways is the ligand-inducer dimer- or trimerization of receptor subunits; this facilitates signal propagation into the cell through the interplay of the transiently associated receptor cytoplasmic tails. There are six major cytokine receptor structural families: hematopoietin receptors, interferon receptors, TNF receptors, IgSF cytokine receptors, chemokine receptors, TGF receptors.

Cytokines are responsible for generating antigen-specific chronic inflammatory reactions which deal with intracellular parasites, although there is a different emphasis in the pattern of factors involved. The initiating event is probably a local inflammatory response to tissue injury caused by the infectious agent which provokes the synthesis of adhesion molecules such as VCAM-1 (vascular cell adhesion molecule) and ICAM-1 on adjacent vascular endothelial cells. These adhesion molecules permit entry of memory T-cells to infected site through their VLA-4 and LFA-1 homing receptors. Contact with processed antigen derived from the intracellular parasite will activate the specific T-cell and induce the release of secreted cytokines. TNF will further enhance the expression of endothelial accessory molecules and increase the chances of other memory cells in the circulation homing in to meet the antigen provoking inflammation.

The recruitment of T-cells and macrophages to the inflammatory site is greatly enhanced by the action of chemotactic cytokines termed chemokines (chemoattractant cytokine). These can be produced by a variety of cell types and are divided into four families based on the disposition of the first (N-terminal) two of the four canonical cysteine residues. CXC chemokines have one amino acid and CX3C have three amino acids between the two cysteines. CC chemokines have adjacent cysteines at this location, whereas C chemokines lack cysteines 1 and 3 found in other chemokines. Chemokines bind to G-protein-coupled seven transmembrane receptors. Despite the fact that a single chemokine can sometimes bind to more than one receptor, and a single receptor can bind several chemokines, many chemokines exhibit a strong tissue and receptor specificity. They play important roles in inflammation, lymphoid organ development, cell trafficking, cellular compartmentalization within lymphoid tissues, Th1/Th2 development, angiogenesis and wound healing.

Rheumatoid arthritis (RA) is an inflammatory disease of the synovium which results in erosion, deformity and finally the destruction of joints. Inflammation of the joints is associated with a villous hypertrophy of the synovial membrane, which on microscopy shows proliferation of the lining layer with an inflammatory infiltrate. There is extensive expression of HLA-7.3 Chronic Inflammatory Disorders 173 DR on T cells, B cells and synovial lining cells, indicating strong immunological activity. RA is thought to be an autoimmune reaction, caused by an interaction between constitutional and environmental factors.

In human biopsies of patients with RA, an increased expression of the adhesion molecules E-selectin, VCAM-1, ICAM-1 and the CD11c integrin has been demonstrated. Also, soluble mediators produced by perivascular cells (e.g. the chemokines Macrophage Inflammatory Protein (MIP)-1 α and MIP-1 β) or by endothelial cells themselves (e.g. IL-8 and IL-15) are important in the regulation of leucocyte infiltration in RA. Tumour Necrosis Factor α (TNF α or TNFa) is thought to be a particularly important inflammatory mediator contributing to the pathology of arthritis, as demonstrated by the beneficial effects of the TNF α -neutralizing therapies that are currently being explored. However, present therapies for RA mainly aim at the inhibition of cyclooxygenase enzymes which are responsible for the overproduction of inflammatory mediators like prostaglandin E2 in arthritis-affected joints.

Patients have been treated with TNFa antagonists for indications that include rheumatoid arthritis, juvenile rheumatoid arthritis, inflammatory bowel disease, psoriatic arthritis and ankylosing spondylitis. Since the approval of TNFa blockers, concerns regarding safety have been raised. There is a risk of re-activation of granulomatous diseases, especially tuberculosis, and measures should be taken for detection and treatment of latent tuberculosis infections. TNFa antagonists have resulted in re-activation of chronic hepatitis B if not given concurrently with antiviral therapy. Variable rates of increased lymphoma risk have been described with anti-TNF therapy compared with the general population, although no increased risk was found compared with a rheumatoid arthritis population. Large phase II and III trials with TNF-alpha antagonists in advanced heart failure have shown trends towards a worse prognosis, and should therefore be avoided in this population. Both etanercept and infliximab are associated with the formation of autoantibodies, and these autoantibodies are rarely associated with any specific clinical syndrome. Rare cases of aplastic anaemia, pancytopenia, vasculitis and demyelination have been described with anti-TNF therapy. The safety profile

and adverse events of the three commercially available TNF-alpha antagonists, etanercept, infliximab and adalimumab, is continuously watched.

Antibodies are a valuable molecule class for therapy, in particular for therapy of rheumatoid arthritis. Antibodies are naturally occurring molecules and as such a major part of the immune system. They can bind specifically to a molecular structure – an antigen – and subsequently trigger elimination and/or destruction of the antigen. The prototype antibody molecule, IgG, consists of four polypeptide chains, two identical heavy chains and two identical light chains (see fig.1). The polypeptide chains are linked by disulfide bonds and non-covalent interactions. Heavy chains and light chains of the molecule consist of independently folded small domains of approximately 100 amino acids (called VH, VL, CH1, CH2, CH3, CL in IgG). All domains display very similar three-dimensional structure. Only one domain in heavy and light chain respectively is of highly variable sequence in different antibodies, these two domains together form the specific antigen-binding site of the antibody. Even in those variable domains, only a few amino acid loops (Complementary-Determining Region, called CDR-loops) are different with each antibody.

Due to the modular domain architecture of an antibody a number of various antibody format derivatives have evolved, e.g. Fabs, single-chain Fv antibodies (ScFv), minibodies, single domain antibodies, Fc fragments and combinations thereof that span a broad range of size, antigen binding sites and specificities. The repertoire of antibody-based proteins has been further expanded and endowed with novel functions by fusion to other proteins such as enzymes and toxins. Antibodies have been chemically conjugated to drugs and can be engineered for site specific coupling of drugs.

While those various formats are mainly created by new combinations of antibody domains, the recent trend in antibody engineering focuses on the engineering of the domains itself. Besides engineering variable regions around the CDR-loops, engineering of conserved regions in antibody domains has evolved as a useful means for the purpose of building new or improved functions into antibodies of various formats. Thereby all protein engineering experience gathered for manipulating variable domains can be utilized by analogy.

In general such technologies can be divided into Directed Evolution techniques and Rational Design techniques.

Directed Evolution of antibodies starts with random mutations in the molecule in order to generate a library of antibodies which can be selected for desired properties. Screening of such libraries can be performed by high-throughput techniques as known from small molecule drug screening. Alternatively, desired antibody mutants can be displayed on cells, phages or ribosome-particles followed by selection via repeated binding and propagation steps. The major limitation of directed evolution technologies is the usually huge number of possible mutants which often outnumbers technical synthesis capacity. This means that a large number, but not all possible mutants can be synthesized and screened.

Rational Design on the other hand allows for certain changes in the molecule based on structure/function knowledge. As more and more antibody molecular structures are known in detail and antibody structure determination with or without antigen has become a standard technology, Rational Design techniques can be utilized to improve antibody molecules. The limitation of rational design technologies is a less than 100% predictability of the result and therefore a number of alternative designs have to be tested.

Usually, a combination of both technologies is applied in order to overcome the limitations of both approaches and to optimize outcome of antibody engineering efforts.

Due to the size of antibody molecules they can be potentially recognized by the immune system of the patient. Such immune responses might compromise antibody efficacy by direct neutralization, may accelerate clearance and prevent further dosing or induce serum sickness. To mitigate this concern, antibody therapeutics today are being designed as far as possible identical to naturally occurring antibodies in the human organism. Mouse antibodies have been used and are still being used as a starting point for the development of antibody therapeutics. Such antibodies can be humanized by either combining the antigen-specific mouse variable domains with human constant domains (a technique called chimerization) or by exchanging CDR-regions of a human antibody by the antigen-specific CDR-loops of the mouse antibody (a technique called CDR-grafting). In fact, most of the antibodies currently on the market are chimeric and CDR-grafted humanized IgG molecules.

Many of the antibodies being newly developed are derived from single antibody-producing cells (B-cells) from human donors or from animals transplanted with human immunoglobulin

genes or human B-cells. Antibodies derived from these in vivo sources have potentially low immunogenicity. Other sources of specific antibodies are libraries of human immunoglobulin repertoires from donors or from artificial synthetic genes. It has been shown that very large libraries of such molecules allow the selection of virtually any specificity and antigen binding affinity. The first antibody derived from such a library is already on the market (Humira™, a TNFa specific human antibody).

Engineering of CDR-loops has been used extensively to modulate antigen binding sites of variable domains in terms of affinity and specificity. Additional antigen binding sites have been introduced into antibodies or antibody fragments by new combinations of variable domains in order to create bispecific or multivalent antibodies.

Recent work described in WO2006/072620A1 refers to a method for engineering an immunoglobulin comprising modifications in a structural loop region and determining the binding of said immunoglobulin to an antigen. This method employs randomization techniques to modify structural loops and to create new binding sites for antigens.

It is the object of the present invention to provide an improved anti-inflammatory therapy with reduced side effects.

Summary of the Invention

The object is solved by the subject of the claims of the present invention.

According to the invention there is provided a targeted receptor molecule based on a receptor of soluble inflammatory antigens, which - in addition to its receptor function - is binding specifically to an adhesion molecule or a membrane bound marker of inflammation. The soluble inflammatory antigens are also called self-antigens associated with inflammatory conditions. In particular, molecules with at least two specificities are provided, preferably one for soluble mediators of inflammation processes, and the other for a membrane bound molecules overexpressed at the site of inflammation.

A targeted antagonist of inflammatory processes has not been described before. It is a completely new approach to provide for instance a cytokine antagonist, such as a TNFa

blocker, directly at the site of inflammation, thus with local efficacy to endothelial cells lining the vascular lumen. This will provide effective, precise and safe therapeutic interventions for treatment of diverse inflammatory disease conditions at low dosages. Systemic side effects or effects at sites, where the antagonizing effect is not desired, such as the lung or the liver, are thus reduced.

By the targeted approach it is the first time possible to bind specifically an adhesion molecule such as an immobilized marker of inflammation and at the same time to antagonize the effects of inflammation mediated by the respective soluble inflammatory antigens. Thereby pro-inflammatory and anti-inflammatory processes would be rebalanced by antagonizing inflammatory antigens as biological agents or agonizing the anti-inflammatory processes, including the downregulation of overexpression of inflammatory factors, such as initiators or mediators of inflammation, including initiators of acute inflammatory processes.

Preferably the marker of inflammation is a cell adhesion molecule, like an adhesion molecule of epithelial or leukocyte cells. Also preferred are receptors of cytokines, growth factors or chemokines. Any cell-bound marker overexpressed at the site of inflammation would be appropriate as a target of inflammation antagonizing therapy. Those markers are preferably used, which are expressed by both the leucocytes and the endothelial cells at the site of inflammation.

The preferred marker targeted is selected from the group consisting of a selectin, an integrin, an immunoglobulin superfamily member, a catherin, a protocatherin, a catenin, and receptors thereof, or other modulators of vascular cell activation.

Exemplary molecules of the selectin family are selected from the group consisting of E-selectin, P-selectin and L-selectin, either the soluble form or membrane bound, the membrane bound markers being preferred.

Further preferred markers are receptors of interleukins, colony stimulating factors, interferons, tumor necrosis factors and endothelial growth factors. Particularly preferred markers are selected from the group consisting of cytokine receptors, produced by the activated endothelial cells, such as receptors of IL-1, IL-6, IL-8, VEGF and TNF α . Further preferred markers are chemokine receptors, such as receptors of monocyte chemotactic proteins

(MCPs), growth related proteins (GROs) and interferon gamma-inducible protein 10 (IP-10). Also preferred are immobilized Eicosanoide, such as Leukotriene B4 and Prostaglandins, acute phase proteins, like C-reactive Protein, and their receptors.

Further preferred receptor molecules are targeting an immunoglobulin superfamily member selected from the group consisting of ICAM-1, PECAM-1, VCAM-1, MAdCAM-1 and PNAd.

Other preferred receptor molecules according to the invention are targeting a marker, which is a cadherin selected from the group consisting of E-cadherin, P-cadherin and N-cadherin. It is understood, that the markers of inflammatory processes can be targeted directly or by their biological ligands, such as cells migrating to the site of inflammation, so to interfere with the inflammatory process.

Those receptors are preferred that contain at least one binding structure of a molecule selected from the group consisting of biological receptors or ligands, peptides, polypeptides, chemical structures, Aptamers, immunoglobulins, immunoglobulin-like molecules, modular antibodies, fragments and derivatives thereof. A particularly preferred receptor molecule according to the invention is one of a bispecific, oligospecific or multispecific molecule. It is most preferred to use modular antibodies because those molecules can be easily engineered to be multispecific in a modular way.

The soluble inflammatory antigens recognized specifically by the receptor according to the invention are preferably proinflammatory proteins or anti-inflammatory proteins, and receptors thereof. In some cases receptors can be presented in the soluble or immobilized form. If the therapy provides for local enrichment of anti-inflammatory proteins, the preferred receptor targets are anti-inflammatory proteins. An alternative therapy according to the invention provides for local neutralization of pro-inflammatory agents, thus pro-inflammatory proteins are preferably targets of the receptor molecule in this case.

Preferred receptor molecules according to the invention are binding said inflammatory antigen with a high affinity or medium affinity, in particular with a high on and/or a high off rate.

Whereas receptor molecules are preferred that bind proinflammatory proteins with a low off rate, other receptor molecule according to the invention that bind anti-inflammatory proteins with a high off rate.

On the other hand the preferred receptor according to the invention is binding said marker is with low or medium affinity.

The preferred receptor molecule according to the invention specifically targets a soluble inflammatory antigen selected from the group consisting of a interleukins, colony stimulating factors, interferons, tumor necrosis factors and endothelial growth factors.

Preferred receptor molecules according to the invention would bind and eventually neutralize an inflammatory antigen selected from the group consisting of Blys, IL-6, LIF, OSM, IL-1, TNFalpha, IL-12, IL-23, IL-15, GM-CSF, M-CSF, IL-17, IL-18, VEGF, RANKL, DARC, MCP1, MIP1beta, Interferon beta, OPG, TGFbeta, IL-10, IL-13, IL-11, IL-4, staff-R and IL-1ra.

According to the invention there is further provided a method of producing a preparation of a targeted receptor molecule according to the invention, which comprises the steps of

- a. preparing a library containing said receptor and variants thereof, by mutagenizing the amino acid sequence apart from the receptor region binding to said inflammatory antigen to provide new additional binding sites,
- b. contacting said library with a test sample containing said marker,
- c. selecting the member of the library binding to said marker,
- d. determining the binding property of the selected molecule to said inflammatory antigen and said marker, and
- e. treating the selected molecule to manufacture said targeted receptor molecule.

The preferred method according to the invention employs a library containing modular antibodies binding to said inflammatory antigen, which modular antibodies are randomized in the structural loop region to provide said new binding sites.

In an alternative method according to the invention the method comprises the steps of:

- a. preparing a library containing a binding moiety specifically binding to said marker of inflammation, by mutagenizing the binding moiety to provide new additional binding sites,
- b. contacting said library with a test sample containing said inflammatory antigen,
- c. selecting the member of the library binding to said inflammatory antigen,
- d. determining the binding property of the selected molecule to said inflammatory antigen and said marker, and
- e. treating the selected molecule to manufacture said targeted receptor molecule.

Preferred methods according to the invention refer to modular antibodies binding to said marker, which modular antibodies are randomized in the structural loop region to provide said new binding sites.

There is also a kit provided according to the invention, which may be used in conjunction with such methods employing a library, which kit enables selecting a targeted receptor molecule by a method comprising

- a. a library of said receptor molecule,
- b. a test sample containing said inflammatory antigen, and
- c. a test sample containing said marker.

An alternative method of producing a preparation of a targeted receptor molecule according to the invention provides for associating a moiety binding to said inflammatory antigen with a moiety binding to said marker by one of conjugation, recombinant or fusion techniques. Thus, the binding moieties may be first derived from a parent molecule, a library of binders or synthesized as individual components and combined thereafter.

The receptor molecule according to the invention may be particularly useful in the treatment of inflammatory disease or associated inflammatory conditions, such as chronic inflammatory disorders, autoimmune disease, chronic inflammation, chronic prostatitis, glomerulonephritis, hypersensitivities, pelvic inflammatory disease, reperfusion injuries, vasculitis, rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, psoriasis, Crohn's Disease, ulcerative colitis, atherosclerosis, inflammatory bowel disease, asthma, psoriasis, transplant rejection, or Alzheimer's disease.

The receptor molecule according to the invention is preferably provided either for the targeted delivery of receptors of inflammatory antigens associated with inflammatory conditions, for localizing therapeutic receptors of inflammatory antigens in patients at the site of inflammation, or for accumulating inflammatory antigens in patients at the site of inflammation.

Detailed Description of the Invention

The term “immunoglobulin” as used according to the present invention is defined as polypeptides or proteins that may exhibit mono- or bi- or multi-specific, or mono-, bi- or multivalent binding properties, preferably at least two, more preferred at least three specific binding sites for epitopes of e.g. antigens, effector molecules or proteins either of pathogen origin or of human structure, like self-antigens including cell-associated or serum proteins. The term immunoglobulin as used according to the invention also includes functional fragments, such as Fc, Fab, scFv, single chain dimers of CH/CL domains, Fv, Fc, or other derivatives or combinations of the immunoglobulins. The definition further includes domains of the heavy and light chains of the variable region (such as dAb, Fd, Vl, Vk, Vh, VHH) and the constant region or individual domains of an intact antibody such as CH1, CH2, CH3, CH4, Cl and Ck, as well as mini-domains consisting of at least two beta-strands of an immunoglobulin domain connected by a structural loop.

“Modular antibodies” as used according to the invention are defined as antigen-binding molecules, like human antibodies, composed of at least one polypeptide module or protein domain, preferably in the natural form. The term “modular antibodies” includes antigen-binding molecules that are either immunoglobulins, immunoglobulin-like proteins, or other proteins exhibiting modular formats and antigen-binding properties similar to immunoglobulins or antibodies, which can be used as antigen-binding scaffolds, preferably based on human proteins.

The term “immunoglobulin-like molecule” as used according to the invention refers to any antigen-binding protein, in particular to a human protein, which has a domain structure that can be built in a modular way. Immunoglobulin-like molecules as preferably used for the present invention are T-cell receptors (TCR), fibronectin, transferrin, CTLA-4, single-chain

antigen receptors, e.g. those related to T-cell receptors and antibodies, antibody mimetics, adnectins, anticalins, phylomers, repeat proteins such as ankyrin repeats, avimers, VersabodiesTM, scorpio toxin based molecules, and other non-antibody protein scaffolds with antigen binding properties.

Ankyrin repeat (AR), armadillo repeat (ARM), leucine-rich repeat (LRR) and tetratricopeptide repeat (TPR) proteins are the most prominent members of the protein class of repeat proteins. Repeat proteins are composed of homologous structural units (repeats) that stack to form elongated domains. The binding interaction is usually mediated by several adjacent repeats, leading to large target interaction surfaces.

Avimers contain A-domains as strings of multiple domains in several cell-surface receptors. Domains of this family bind naturally over 100 different known targets, including small molecules, proteins and viruses. Truncation analysis has shown that a target is typically contacted by multiple A-domains with each domain binding independently to a unique epitope. The avidity generated by combining multiple binding domains is a powerful approach to increase affinity and specificity, which these receptors have exploited during evolution.

Anticalins are engineered human proteins derived from the lipocalin scaffold with prescribed binding properties typical for humanized antibodies. Lipocalins comprise 160–180 amino acids and form conical b-barrel proteins with a ligand-binding pocket surrounded by four loops. Small hydrophobic compounds are the natural ligands of lipocalins, and different lipocalin variants with new compound specificities (also termed 'anticalins') could be isolated after randomizing residues in this binding pocket.

Single chain antigen receptors contain a single variable domain and are 20% smaller than camelid single domain antibodies.

Phylomers are peptides derived from biodiverse natural protein fragments.

It is understood that the term "modular antibody", "immunoglobulin", "immunoglobulin-like proteins" includes a derivative thereof as well. A derivative is any combination of one or more modular antibodies of the invention and or a fusion protein in which any domain or

minidomain of the modular antibody of the invention may be fused at any position of one or more other proteins (such as other modular antibodies, immunoglobulins, ligands, scaffold proteins, enzymes, toxins and the like). A derivative of the modular antibody of the invention may also be obtained by association or binding to other substances by various chemical techniques such as covalent coupling, electrostatic interaction, di-sulphide bonding etc. The other substances bound to the immunoglobulins may be lipids, carbohydrates, nucleic acids, organic and inorganic molecules or any combination thereof (e.g. PEG, prodrugs or drugs). A derivative is also an antibody with the same amino acid sequence but made completely or partly from non-natural or chemically modified amino acids.

The term "antigen" as used herein shall comprise molecules selected from the group consisting of allergens, tumor associated antigens, self antigens including cell surface receptors, enzymes, Fc-receptors, FcRn, HSA, IgG, interleukins or cytokines, proteins of the complement system, transport proteins, serum molecules, bacterial antigens, fungal antigens, protozoan antigen and viral antigens, also molecules responsible for transmissible spongiform encephalitis (TSE), such as prions, infective or not, and markers or molecules that relate to inflammatory conditions, such as pro-inflammatory factors, multiple sclerosis or alzheimer disease, or else haptens.

The term antigen as used according to the present invention shall in particular include all antigens and target molecules capable of being recognised by a binding site of a modular antibody. Specifically preferred antigens as targeted by the receptor molecule according to the invention are those antigens or molecules, which have already been proven to be or are capable of being immunologically or therapeutically relevant, especially those, for which a clinical efficacy has been tested.

The antigen is either recognized as a whole target molecule or as a fragment of such molecule, especially substructures of targets, generally referred to as "epitopes".

The term "epitope" as used herein according to the present invention shall mean a molecular structure which may completely make up a specific binding partner or be part of a specific binding partner to a binding site of modular antibody or an immunoglobulin of the present invention. The term epitope may also refer to haptens. Chemically, an epitope may either be composed of a carbohydrate, a peptide, a fatty acid, an organic, biochemical or inorganic

substance or derivatives thereof and any combinations thereof. If an epitope is a polypeptide, it will usually include at least 3 amino acids, preferably 8 to 50 amino acids, and more preferably between about 10-20 amino acids in the peptide. There is no critical upper limit to the length of the peptide, which could comprise nearly the full length of a polypeptide sequence of a protein. Epitopes can be either linear or conformational epitopes. A linear epitope is comprised of a single segment of a primary sequence of a polypeptide chain. Linear epitopes can be contiguous or overlapping. Conformational epitopes are comprised of amino acids brought together by folding of the polypeptide to form a tertiary structure and the amino acids are not necessarily adjacent to one another in the linear sequence. Specifically, epitopes are at least part of diagnostically relevant molecules, i.e. the absence or presence of an epitope in a sample is qualitatively or quantitatively correlated to either a disease or to the health status of a patient or to a process status in manufacturing or to environmental and food status. Epitopes may also be at least part of therapeutically relevant molecules, i.e. molecules which can be targeted by the specific binding domain which changes the course of the disease.

As used herein, the term "specifically binds" or "specific binding" refers to a binding reaction which is determinative of the cognate ligand of interest in a heterogeneous population of molecules. Thus, under designated conditions (e.g. immunoassay conditions), the modular antibody binds to its particular "target" and does not bind in a significant amount to other molecules present in a sample. The specific binding means that binding is selective in terms of target identity, high, medium or low binding affinity or avidity, as selected. Selective binding is usually achieved if the binding constant or binding dynamics is at least 10 fold different, preferably the difference is at least 100 fold, and more preferred a least 1000 fold.

The term "expression system" refers to nucleic acid molecules containing a desired coding sequence and control sequences in operable linkage, so that hosts transformed or transfected with these sequences are capable of producing the encoded proteins. In order to effect transformation, the expression system may be included on a vector; however, the relevant DNA may then also be integrated into the host chromosome. Alternatively, an expression system can be used for in vitro transcription/translation.

A "structural loop" or "non-CDR-loop" according to the present invention is to be understood in the following manner: modular antibodies or immunoglobulins are made of domains with a so called immunoglobulin fold. In essence, anti-parallel beta sheets are connected by loops to

form a compressed antiparallel beta barrel. In the variable region, some of the loops of the domains contribute essentially to the specificity of the antibody, i.e. the binding to an antigen. These loops are called CDR-loops. All other loops of antibody variable domains are rather contributing to the structure of the molecule and/or interaction with other domains. These loops are defined herein as structural loops or non-CDR-loops.

All numbering of the amino acid sequences of the immunoglobulins is according to the IMGT numbering scheme (IMGT, the international ImMunoGeneTics, Lefranc et al., 1999, Nucleic Acids Res. 27: 209-212).

The term "adhesion molecule" as used according to the invention includes all cell adhesion molecules and receptors of cytokines or chemokines, as well as receptors of growth factors, e.g. VEGF that play a significant role in inflammatory processes. Exemplary cell adhesion molecules targeted according to the invention are all members of the groups of the integrin family, like Integrin $\alpha 1\beta 1$, $\alpha 2\beta 1$, $\alpha 3\beta 1$, $\alpha 4\beta 1$, $\alpha 5\beta 1$, $\alpha 6\beta 1$, $\alpha 7\beta 1$, $\alpha 8\beta 1$, $\alpha 9\beta 1$, $\alpha 10\beta 1$, $\alpha 11\beta 1$, $\alpha V\beta 1$, $\alpha L\beta 2$, $\alpha X\beta 2$, $\alpha D\beta 2$, $\alpha IIb\beta 3$, $\alpha V\beta 3$, $\alpha 6\beta 4$, $\alpha V\beta 5$, $\alpha V\beta 6$, $\alpha 4\beta 7$, $\alpha E\beta 7$ and $\alpha V\beta 8$, the immunoglobulin superfamily, e. g. LFA-2, LFA-3, ICAMs, VCAM-1, PE-CAM-1, MAdCAM-1, selectins like L-selectin, E-selectin and P-selectin, and cadherins, such as N-cadherin, E-cadherin and P-cadherin.

The term "cytokines" as used according to the invention means soluble and membrane bound cytokines as well as receptor bound cytokines. Exemplary cytokines targeted according to the invention are all members of the group of the interleukins, e.g. IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IL-16, IL-17, IL-18, IL-19, IL-20, IL-21, IL-22, IL-23, IL-24, IL-25, IL-26, IL-27, IL-28, IL-29, IL-30, IL-31, IL-32, and IL-33, the group of colony stimulating factors, such as GM-CSF, G-CSF, M-CSF, SLF, the group of tumor necrosis factors, like TNF α and TNF β , and the group of interferons, e.g. IFN α , IFN β and IFN γ , and others like TGF β , LIF, Eta-1, MIF and Oncostatin M.

Exemplary receptors of cytokines targeted according to the invention are type 1 interleukin receptors, erythropoietin receptor, GM-CSF receptor, G-CSF receptor, growth hormone receptor, prolactin receptor, Oncostatin M receptor, Leukemia inhibitory factor receptor, type II cytokine receptor, type II interleukin receptors, interferon-alpha/beta receptor, interferon-gamma receptor, Interleukin-1 receptor, CSF1, C-kit receptor, Interleukin-18 receptor, CD27,

CD30, CD40, CD120, receptors of the migration inhibition factor MIF, such as CD74, CXCR2 and CCR4, lymphotoxin beta receptor, TGF beta receptors TGF beta receptor 1, TGF beta receptor 2.

The term "chemokines" as used according to the invention means soluble, receptor bound or and membrane bound chemokines. Exemplary chemokines targeted according to the invention are all members of the group of the CXC-family, like CXCL1, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14, CXCL15 and CXCL16, the group of the C-family, such as XCL1 and XCL2, the group of the CX3C-family, like CX3CL1, and the group of the CC-family including CCL1, CCL2, CCL3, CCL4, CCL5, CCL6, CCL7, CCL8, CCL9/10, CCL11, CCL12, CCL13, CCL14, CCL15, CCL16 CCL17, CCL18, CCL19, CCL20, CCL21, CCL22, CCL23, CCL24, CCL25, CCL26, CCL27 and CCL28.

Exemplary chemokine-receptors targeted according to the invention are CXCR1, CXCR2, CXCR3-A, CXCR3-B, CXCR4, CXCR5, CXCR6, XCR1, CX3CR1, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9, CCR10, MCAF receptor, NAP-2 receptor. CXCR2 and CXCR4 may also bind the cytokine MIF (macrophage migration inhibition factor).

The receptor molecules of the invention may specifically bind to any kind of self-antigens or markers that are associated with inflammatory processes, which have proven the anti-inflammatory effect in a cell-based assay employing activated cells. Targeted structures include biological receptors, ligands or a variety of targets.

The particular binding moieties are formed by proteinaceous molecules, proteins, peptides, polypeptides, nucleic acids, glycans, carbohydrates, lipids, small and large organic molecules or inorganic molecules. Typically, the receptor molecule according to the invention is a modular antibody, in particular an immunoglobulin comprising loop regions whereby some of the loop regions specifically bind to different molecules or epitopes, or an immunoglobulin-like molecule which is composed in a modular way, binding to different epitopes.

The invention preferably refers to complete antibody molecules or part of antibodies, such as IgG, IgA, IgM, IgD, IgE and the like. The modular antibodies of the invention may also be a

functional antibody fragment such as Fab, Fab₂, scFv, Fv, Fc, FcabTM, or parts thereof, or other derivatives or combinations of the immunoglobulins such as minibodies, domains of the heavy and light chains of the variable region (such as dAb, Fd, VL, including Vlambda and Vkappa, VH, VHH) as well as mini-domains consisting of two beta-strands of an immunoglobulin domain connected by at least two structural loops, as isolated domains or in the context of naturally associated molecules. A particular embodiment of the present invention refers to the Fc fragment, FcabTM, of an antibody molecule, either as antigen-binding Fc fragment through modifications of the amino acid sequence or as conjugates to receptors or other antigen-binding modules, such as scFv.

Antibody or immunoglobulin domains may be modified according to the present invention (as used herein the terms immunoglobulin and antibody are interchangeable) which modifications are preferably effected in immunoglobulin domains or parts thereof that contain a loop, either a CDR-loop or a non-CDR loop, structural loops being the preferred sites of modifications. In some cases it is preferable to use a defined modified structural loop or a structural loop region, or parts thereof, as isolated molecules for binding or combination purposes.

The immunoglobulins can be used as isolated peptides or as combination molecules with other peptides. The peptides are homologous to immunoglobulin domain sequences, and are preferably at least 5 amino acids long, more preferably at least 10 or even at least 50 or 100 amino acids long, and constitute at least partially the loop region of the immunoglobulin domain. The binding characteristics relate to predefined epitope binding, affinity and avidity.

The modular antibody according to the invention is possibly further combined with one or more modified immunoglobulins or with unmodified immunoglobulins, or parts thereof, to obtain a combination immunoglobulin. Combinations are preferably obtained by recombination techniques, but also by binding through adsorption, electrostatic interactions or the like, or else through conjugation or chemical binding with or without a linker. The preferred linker sequence is either a natural linker sequence or functionally suitable artificial sequence.

In general the modular antibody according to the invention may be used as a building block to molecularly combine other modular antibodies or biologically active substances or molecules. It is preferred to combine molecularly at least one antibody binding to the specific partner via

the variable or non-variable sequences, like structural loops, with at least one other binding molecule which can be an antibody, antibody fragment, a soluble receptor, a ligand or another antibody domain. Other combinations refer to proteinaceous molecules, nucleic acids, lipids, organic molecules and carbohydrates.

The engineered molecules according to the present invention will be useful as stand-alone proteins as well as fusion proteins or derivatives, most typically fused in such a way as to be part of larger antibody structures or complete antibody molecules, or parts or fragments thereof, such as Fab fragments, Fc fragments, Fv fragments and others. It will be possible to use the engineered proteins to produce molecules which are monospecific, bispecific, trispecific, and maybe even carry more specificities at the same time, and it will be possible at the same time to control and preselect the valency of binding at the same time according to the requirements of the planned use of such molecules.

According to the present invention, the receptor molecule or modular antibody optionally exerts one or more binding regions to antigens, besides the receptor site binding specifically to the inflammatory antigen and the binding sites specific for the targeted marker. Antigen binding sites to one or more antigens may be presented by the CDR-region or any other natural receptor structure, or introduced into a structural loop region of an antibody domain, either of a variable or constant domain structure. The antigens as used for testing the binding properties of the binding sites may be naturally occurring molecules or chemically synthesized molecules or recombinant molecules, either in solution or in suspension, e.g. located on or in particles such as solid phases, on or in cells or on viral surfaces. It is preferred that the binding of an immunoglobulin to an antigen is determined when the antigen is still adhered or bound to molecules and structures in the natural context. Thereby it is possible to identify and obtain those modified immunoglobulins that are best suitable for the purpose of diagnostic or therapeutic use.

It is particularly preferred that the receptor molecule according to the invention is a modular antibody binding to said antigens through at least part of a structural loop and/or CDR loop.

For example, the preferred method of producing the targeted receptor molecule according to the invention refers to engineering a modular antibody that is binding specifically to at least one first epitope and comprising modifications in each of at least two structural loop regions,

and determining the specific binding of said at least two loop regions to at least one second epitope, wherein the unmodified structural loop region (non-CDR region) does not specifically bind to said at least one second epitope. Thus, an antibody or antigen-binding structure specific for a first antigen may be improved by adding another valency or specificity against a second antigen, which specificity may be identical, either targeting different epitopes or the same epitope, to increase valency or to obtain bi-, oligo- or multispecific molecules.

Preferred epitopes of the immobilized markers of inflammation are extracellular epitopes of membrane bound transport proteins. In particular those epitopes accessible at the membrane binding site are preferred. Besides the binding site of the modular antibody as engineered according to the invention, further binding capacities may be present, constructed or introduced aside from or directly into the structural loop regions of constant or variable domains, e.g. binding capacities for other antigens, small molecules, for drugs or enzymes, catalytic sites of enzymes or enzyme substrates or the binding to a transition state analogue of an enzyme substrate.

The soluble inflammatory antigens as bound by the receptor molecule according to the invention preferably participate in the balance of pro-inflammatory and anti-inflammatory processes, thus are considered as proinflammatory proteins or anti-inflammatory proteins, and receptors thereof. A proinflammatory protein may be antagonized or neutralized by the receptor capturing the protein and eliminating its effect at the site of inflammation. Specific receptors are selected for the high or medium affinity binding, such as with a K_d of lower than 10^{-7} M, preferably lower than 10^{-8} M, more preferably lower than 10^{-9} M, or else for the high avidity of binding, or else the high on-rate, and preferably the low off-rate of binding.

Anti-inflammatory proteins, however, may be agonized by localizing, enriching or accumulating the proteins at the site of inflammation through binding by the receptor molecule according to the invention. The receptor molecule is usually designed to exert a weaker binding property to the anti-inflammatory protein than its biological ligand, thus is either prolonging or increasing the anti-inflammatory effect at the site of inflammation. This can specifically be achieved by a low binding affinity (K_d higher than mM)

Preferred receptor molecules according to the invention are further characterized by the binding affinity to said marker or inflammation, as used as a target for the targeted therapeutic approach. Binding affinity to said marker is preferably low or medium, usually with a K_d of at least 10^{-9} M, preferably a least 10^{-8} M, more preferred higher than 10^{-7} M.

Preferred inflammatory antigens captured or localized by the receptor molecule according to the invention are selected from the group consisting of a cytokine, a chemokine, and receptors thereof.

In particular the inflammatory antigens are selected from the group consisting of Blys, IL-6, LIF, OSM, IL-1, TNFalpha, IL-12, IL-23, IL-15, GM-CSF, M-CSF, IL-17, IL-18, VEGF, RANKL, DARC, MCP1, MIP1beta, Interferon beta, OPG, TGFbeta, IL-10, IL-13, IL-11, IL-4, staff-R and IL-1ra, and receptors thereof, preferably in the soluble form.

According to a preferred embodiment the pair of targets is selected that consists of the biological pair of ligand and receptor molecule. Thus, the receptor according to the invention would interfere with the biological interaction of the pair of targets from two sides, neutralizing the soluble ligand by the first specificity to the inflammatory antigen, and also blocking the immobilized marker of inflammation by the second specificity. In some cases it may however be preferred to bind targets not interfering with each other in the absence of the receptor molecule according to the invention.

The specific binding structure targeting the marker of inflammatory conditions can be provided as a separate binding moiety or component, or as part of the receptor molecule according to the invention. The binding structure is usually engineered into the receptor molecule, fused or conjugated therewith. It can also be a biological ligand of the targeted marker, or a derivative thereof. On the other hand the specific binding structure can also be synthetic and specifically designed to be used as the receptor according to the invention that binds to the self-antigens of inflammatory processes. Binding structures preferably used according to the invention are selected from the group consisting of biological receptors or ligands, peptides, polypeptides, chemical structures, Aptamers, immunoglobulins, immunoglobulin-like molecules, modular antibodies, fragments and derivatives of those binding structures. Thus, particular embodiments refer to bispecific constructs of those binding structures, like bispecific antibodies or antibody fragments.

Besides the bispecific molecules, further preferred receptor molecules according to the invention are oligospecific or even multispecific molecules binding to antigens in addition to the inflammatory antigens and the markers of inflammation. Particularly preferred embodiments refer to at least two specificities to the inflammatory antigens, to effectively deplete the pro-inflammatory proteins at the site of inflammation, thus, acting as a sponge protein.

Preferred receptor molecules according to the invention are based on modular antibodies, which are provided preferably as an immunoglobulin, immunoglobulin-like molecules, a member of the immunoglobulin superfamily, or proteins with immunoglobulin-like structure, or parts thereof. For instance, a modular antibody according to the invention may be a member of the immunoglobulin superfamily selected from the group consisting of intact IgG, Fab, Fc, scFv and dAb. Other specific embodiments relate to proteins with immunoglobulin-like modular structure, which are preferably selected from the group consisting of fibronectin, TCR, transferrin, CTLA-4, single-chain antigen receptors, e.g. those related to T-cell receptors and antibodies, antibody mimetics, adnectins, molecules based on anticalins, phylomers, avimers, affibodies, ankyrin repeats, Kunitz domains, PDZ-domains, scorpion toxins immunity proteins, Knottins, Versabodies, Green Fluorescent Protein and other non-antibody protein scaffolds with antigen binding properties.

The modular antibody according to the invention may be provided as a native molecule, or else conjugated to drugs, chemicals, biologicals, biologically active structures, or fused therewith. Recombinant techniques are preferred to engineer the additional properties or biological functions of the receptor molecule according to the invention.

The preferred modular antibody according to the invention is selected from the group of VH, VL, including Vkappa and Vlambda, VHH, dAb, and combinations thereof. It turned out that those modifications are advantageous when brought into the loop regions of a VH, a Vkappa, a Vlambda or a VHH, and combinations thereof. More specifically, the modified loop regions comprise at least one modification within amino acids 7 to 22, amino acids 39 to 55, amino acids 66 to 85, amino acids 89 to 103 or amino acids 106 to 117.

The structural loop regions of the modular antibody or immunoglobulin of human or humanized origin as modified according to the invention are selected preferably from the structural loops that comprise amino acids 8 to 20, amino acids 44 to 50, amino acids 67 to 76 and amino acids 89 to 101, most preferably amino acid positions 12 to 17, amino acid positions 45 to 50, amino acid positions 69 to 75 and amino acid positions 93 to 98.

In another preferred embodiment a modification in the structural loop region comprising amino acids 93 to 98 is combined with a modification in the structural loop region comprising amino acids 8 to 20.

The above identified amino acid regions of the respective immunoglobulins comprise loop regions to be modified. Preferably, a modification in the structural loop region comprising amino acids 93 to 98 is combined with a modification in one or more of the other structural loops.

In a preferred embodiment a modification in the structural loop region comprising amino acids 93 to 98 is combined with a modification in the structural loop region comprising amino acids 69 to 75.

Most preferably each of the structural loops comprising amino acids 93 to 98, amino acids 69 to 75 and amino acids 8 to 20 contain at least one amino acid modification.

In another preferred embodiment each of the structural loops comprising amino acids 93 to 98, amino acids 69 to 75, amino acids 44 to 50 and amino acids 8 to 20 contain at least one amino acid modification.

According to a preferred embodiment of the present invention the structural loop regions of a modular antibody or an immunoglobulin of murine origin are modified, e.g. within a VH, in the region of amino acids 6 to 20, amino acids 43 to 52, amino acids 67 to 79 and amino acids 92 to 101.

According to another preferred embodiment of the present invention the structural loop regions of a modular antibody or an immunoglobulins of camelid origin are modified, e.g.

within a VHH, in the region of amino acids 7 to 18, amino acids 43 to 55, amino acids 68 to 75 and amino acids 91 to 101.

The variable domains of camelid origin or humanized variants of camelid origin, have the advantage that they could easily be combined with other variable domains, for instance with other VHH of camelid origin, modified or native. The possible combination of VHH of camelid origin is the basis for multivalent immunoglobulins. Thus, according to the invention specific modified variable domains of camelid origin are multivalent combinations, preferably with at least 3, more preferably with at least 4 or 5 valencies or VHHs.

It is understood that the modular antibodies according to the invention can be reengineered or produced based on a known amino acid sequence, by any appropriate technique of recombinant production or synthesis methods.

The preferred techniques of producing the appropriate libraries containing binding moieties, including the receptor molecules according to the invention, in particular modular antibodies, are based on directed evolution and/or rational design techniques.

Libraries as used according to the invention comprise at least 10 receptor molecules or modular antibodies according to the invention, preferably at least 100, more preferred at least 1000, more preferred at least 10^4 , more preferred at least 10^5 , more preferred at least 10^6 , more preferred at least 10^7 , more preferred at least 10^8 , more preferred at least 10^9 , more preferred at least 10^{10} , more preferred at least 10^{11} , up to 10^{12} members of a library, preferably derived from a parent molecule containing at least one specific binding moiety and derivatives to engineer a new binding site apart from the original binding region of said parent moiety.

Usually the libraries according to the invention further contain variants of the modular antibody, resulting from mutagenesis or randomization techniques. These variants include inactive or non-functional antibodies. Thus, it is preferred that any such libraries be screened with the appropriate assay for determining the inflammation modulating effect. Preferred libraries, according to the invention, comprise at least 10^4 variants of such modular antibodies, more preferred at least 10^5 , more preferred at least 10^6 , more preferred at least 10^7 , more preferred at least 10^8 , more preferred at least 10^9 , more preferred at least 10^{10} , more preferred at least 10^{11} , up to 10^{12} variants to provide a highly diverse repertoire of antibodies

for selecting the best suitable binders. Any such synthetic libraries may be generated using mutagenesis methods as disclosed herein.

Particular embodiments refer to libraries that contain modular antibodies with modifications in at least two structural loops, more preferred at least three structural loops.

A library according to the invention may be designed as a dedicated library that contains at least 50% specific formats, preferably at least 60%, more preferred at least 70%, more preferred at least 80%, more preferred at least 90%, or those that mainly or essentially consist of specific antibody formats or modular antibodies. Specific antibody formats are preferred, such that the preferred library according to the invention it is selected from the group consisting of a VH library, VHH library, Vkappa library, Vlambd library, Fab library, scFv library, a CH1/CL library and a CH3 library. Libraries characterized by the content of composite molecules containing more than one antibody domains, such as an IgG library or Fc library, in particular an Fcab™ library, are specially preferred. Other preferred libraries are those containing T-cell receptors, forming T-cell receptor libraries.

The particular libraries according to the invention contain the respective receptor of inflammatory antigens, or parts thereof, as a scaffold for designing the targeted receptor molecule according to the invention. Thus, any member of the library binding to the respective marker may be selected to produce the targeted receptor molecule binding to both the inflammatory antigen and the marker. On the other hand, it may be preferred to use the binder to the marker or parts thereof used as a binding moiety, as a scaffold to design the dedicated library. Members of such a library are screened for their binding property to the respective inflammatory antigens, to obtain the receptor molecules according to the invention. Binding moieties that can be used to prepare appropriate libraries are preferably selected from variable binding regions.

The term "variable binding region" as used herein refers to molecules with varying structures capable of binding interactions with antigens. Those molecules can be used as such or integrated within a larger protein, thus forming a specific region of such protein with binding function. The varying structures can be derived from natural repertoires of binding proteins such as immunoglobulins or phylomers or synthetic diversity, including repeat-proteins, avimers and anticalins. The varying structures can as well be produced by randomization

techniques, in particular those described herein. These include mutagenized CDR or non-CDR regions, loop regions of immunoglobulin variable domains or constant domains.

In some cases libraries are preferred that contain a significant portion of library members with specific mutations. Those specifically designed libraries preferably contain members with selected amino acids in structural loops, preferably the C-terminal loops, which portion or share is higher than 10%, preferably at least 20%, more preferred at least 30%, more preferred at least 40%, more preferred at least 50%, more preferred at least 60%, more preferred at least 70%, more preferred at least 80%, more preferred at least 90%, more preferred at least 95%, up to 99%.

The preferred Fc library according to the invention further contains a significant portion of stable library members with specific mutations. Those specifically designed libraries preferably contain members with binding properties to Protein A, which portion is higher than 10%, preferably at least 20%, more preferred at least 30%, more preferred at least 40%, more preferred at least 50%, more preferred at least 60%, more preferred at least 70%, more preferred at least 80%, more preferred at least 90%, more preferred at least 95%, up to 99%.

Further embodiments relate to libraries of immunoglobulin-like molecules or other modular protein scaffolds, for instance, libraries selected from the group consisting of T-cell receptor library, fibronectin library, avimer library, anticalin library, ankyrin repeat library, Kunitz domain libraries, scorpio toxin based libraries and VersabodyTM based libraries.

Each of the above mentioned libraries according to the invention contains members having the property of the targeted receptor molecule according to the invention, either alone or combined with another binding domain.

According to the invention there is further provided a method of producing a preparation of a targeted receptor molecule based on a receptor of a soluble inflammatory antigen, binding specifically to a marker of inflammation, which comprises the steps of

- a. preparing a library containing said receptor and variants thereof, by mutagenizing the amino acid sequence apart from the receptor binding region to provide new binding sites,
- b. contacting said library with a test sample containing said marker,

- c. selecting the member of the library binding to said marker,
- d. determining the binding property of the selected molecule to said inflammatory antigen and said marker, and
- e. treating the selected molecule to manufacture said targeted receptor molecule.

The mutagenesis site apart from the parent binding region is usually at the opposite site of a protein domain, such as an antibody domain, or else located at a different domain or moiety, not to interfere with the parent binding properties. It is therefore possible to keep the original binding properties of the molecule essentially unchanged as determined in an appropriate assay for bispecific binding.

The binding properties usually are determined by standard in vitro assays, employing ELISA techniques. It may be preferably to further use a functional assay to select appropriate binders for producing a therapeutic molecule. Useful functional assays are usually cell based, to determine the effect of an anti-inflammatory molecule on the activation of epithelial cells, in vitro, or ex vivo assays. The functional activity may be determined by inhibition of activation in the presence of an inflammatory stimulus, or the downregulation of inflammatory proteins that are overexpressed by activated epithelial cells. Likewise the interference with T-cell or leukocyte functions may be determined to prove the anti-inflammatory effect.

The preferred method employs a library containing modular antibodies binding to said inflammatory antigen, which modular antibodies are randomized to build the binding sites to said marker of inflammation. Exemplary libraries contain antibodies which are TNF α blockers or antibody fragments or binding regions as parts of the TNF α blockers, such as Fab or Fc α TM modular antibodies binding to TNF α .

According to one embodiment of the invention a receptor molecule obtainable by the method according to the invention is used again as a scaffold or as a combined protein scaffold for the preparation of a new protein library of modular antibodies.

The receptor molecule according to the invention may be produced according to an alternative method, wherein a variable binding region or binding domains with specificity to an inflammatory marker is used as a parent molecule to prepare mutants and variants with additional binding sites with receptor function for the inflammatory antigen.

This alternative method according to the invention refers to producing a preparation of a targeted receptor molecule based on a receptor of a soluble inflammatory antigen, binding specifically to a marker of inflammation, and comprises the steps of:

- a. preparing a library containing a binding moiety specifically binding to said marker of inflammation, by mutagenizing the binding moiety to provide new binding sites,
- b. contacting said library with a test sample containing said inflammatory antigen,
- c. selecting the member of the library binding to said inflammatory antigen,
- d. determining the binding property of the selected molecule to said inflammatory antigen and said marker, and
- e. treating the selected molecule to manufacture said targeted receptor molecule.

The preferred method employs a library containing modular antibodies binding to said marker, which modular antibodies are randomized to build the binding sites to said inflammatory antigen. Exemplary libraries contain antibodies which are antibodies against a marker of inflammation, such as E-selectin, or antibody fragments as parts of the antibody, such as Fab or Fcab™ modular antibodies binding to the marker, like E-selectin.

The test samples as employed in the screening methods as described above may contain said inflammatory antigens and/or the markers either in soluble form or on a solid phase, including those bound to carriers or cell-bound proteins, like membrane proteins in the natural context.

According to the invention there is thus provided a kit for selecting receptor molecules that comprises both the suitable library of receptor molecules or ligand to markers of inflammation, and the test samples for the panning steps.

Accordingly both alternative kits are provided according to the invention.

A first kit for selecting a targeted receptor molecule based on a receptor of a inflammatory antigen in the soluble form, which is associated with inflammatory conditions, binding specifically to a marker of inflammation, comprises

- a. a library containing members presenting said receptors, and

- b. a test sample containing said marker.

An alternative kit for selecting a targeted receptor molecule based on a receptor of an inflammatory antigen associated with inflammatory conditions, binding specifically to a marker of inflammation, comprises

- a. a library containing members presenting a binding moiety specifically binding to said markers, and
- b. a test sample containing said inflammatory antigen.

Another alternative method of producing a preparation of a targeted receptor molecule based on a receptor of a inflammatory antigen associated with inflammatory conditions, binding specifically to a marker of inflammation, comprises associating a moiety binding to said inflammatory antigen with a moiety binding to said marker by one of conjugation, recombinant or fusion techniques. By the combination of the two moieties of either the same or different type, a bispecific construct is obtained having the desired binding characteristics. Thus, a combination of two antibody domains, fragments or derivatives, in particular a combination of two modular antibodies with different binding specificity is preferred according to this embodiment.

It is understood that the method according to the invention may comprise the steps of isolating and manufacturing a preparation of the receptor molecule, in particular a pharmaceutical preparation, as used in common practise.

Modular antibody engineering techniques are preferred, which keep the native antibody structure, preferably without losing advantageous antibody properties, like antigen binding, Fc receptor binding and/or Protein A binding. It is surprising that the modification of only a few amino acids within the native structure of an antibody can bring about the specific binding properties. In particular when structural loops of immunoglobulins or immunoglobulin-like molecules are mutated, it surprisingly turns out that only a few structure sparing mutations are necessary to create the novel or extra binding site for inflammatory antigen and/or a marker. By means of only a few conservative mutations, the novel binding site can be created while preserving the overall structure of the modular antibody. Preferred sites of mutation involve the randomization of less than 15, more preferable less than 14, 13,

12, 11, 10, 9, 8, 7, 6 or less than 5 to a minimum of 3 amino acid position within a binding region.

According to a preferred embodiment of the present invention the receptor molecule is a modular antibody of human, camelid, rabbit, chicken, rat, dog, horse, sheep or murine origin, or a humanized antibody.

Since the receptor molecule according to the invention may be employed for various purposes, in particular in pharmaceutical compositions, the receptor molecule is preferably of human, camelid or murine origin. Of course, the receptor molecule may also be a humanized or a chimeric immunoglobulin. In the most preferred embodiment of the invention the receptor molecule is a modular antibody of human origin or a humanized version of a variable domain of any species.

A humanized modular antibody has at least about 50% amino acid sequence identity, preferably at least about 55% amino acid sequence identity, more preferably at least about 60% amino acid sequence identity, more preferably at least about 65% amino acid sequence identity, more preferably at least about 70% amino acid sequence identity, more preferably at least about 75% amino acid sequence identity, more preferably at least about 80% amino acid sequence identity, more preferably at least about 85% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, more preferably at least about 95% amino acid sequence identity to a native human modular antibody sequence.

A humanized modular antibody has furthermore at least about 50% amino acid sequence identity, preferably at least about 55% amino acid sequence identity, more preferably at least about 60% amino acid sequence identity, more preferably at least about 65% amino acid sequence identity, more preferably at least about 70% amino acid sequence identity, more preferably at least about 75% amino acid sequence identity, more preferably at least about 80% amino acid sequence identity, more preferably at least about 85% amino acid sequence identity, more preferably at least about 90% amino acid sequence identity, more preferably at least about 95% amino acid sequence identity when comparing all surface accessible amino acids to the surface accessible amino acids of a native human modular antibody sequence.

The preferred homology or sequence identities specifically relates to those sequences of the framework region.

According to a preferred embodiment of the present invention the modified loop regions of CH1, CH2, CH3 and CH4 comprise amino acids 7 to 21, amino acids 25 to 39, amino acids 41 to 81, amino acids 83 to 85, amino acids 89 to 103 and amino acids 106 to 117.

The loop regions of Igk-C and Igl-C of human origin that can be modified comprise preferably amino acids 8 to 20, amino acids 26 to 36, amino acids 41 to 82, amino acids 83 to 88, amino acids 92 to 100, amino acids 107 to 124 and amino acids 123 to 126.

The loop regions of Igk-C and Igl-C of murine origin that can be modified comprise preferably amino acids 8 to 20, amino acids 26 to 36, amino acids 43 to 79, amino acids 83 to 85, amino acids 90 to 101, amino acids 108 to 116 and amino acids 122 to 125.

The further preferred immunoglobulin of camel origin comprises preferably at least one constant domain selected from the group consisting of CH1, CH2 and CH3. According to a preferred embodiment of the present invention the loop regions of CH1, CH2 and CH3 of the camel immunoglobulin that can be modified comprise amino acids 8 to 20, amino acids 24 to 39, amino acids 42 to 78, amino acids 82 to 85, amino acids 91 to 103 and amino acids 108 to 117.

Preferably, the new binding sites in the structural loops are introduced into the modular antibody encoding the selected nucleic acid by substitution, deletion and/or insertion of at least one nucleotide.

According to another preferred embodiment of the present invention the modification of at least one nucleotide in each of at least two structural loop regions results in a substitution, deletion and/or insertion of an amino acid in the modular antibody or immunoglobulin encoded by said nucleic acid.

The modification of modular antibody may result in a substitution, deletion and/or insertion of 2 or more amino acids, preferably point mutations, change of amino acids of whole loops, more preferred the change of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, up to 30 amino

acids. However, the maximum number of amino acids inserted into a structural loop region of an immunoglobulin or immunoglobulin variable domain may in specific cases not exceed the number of 30, preferably 25, more preferably 20, amino acids, to maintain the native structure of the modular antibody.

A preferred method to introduce modifications is site directed random mutation. With this method one, two or more specific amino acid residues of the loops are exchanged or introduced using randomly generated inserts into such structural loops. Alternatively preferred is the use of combinatorial approaches.

The modular antibody loop regions are preferably mutated or modified by random, semi-random or, in particular, by site-directed random mutagenesis methods. The randomization of at least two, or even three or more structural loops are most preferred to create the novel binding sites to bind the inflammatory antigens and/or the markers of inflammation.

These randomization methods may be used to make amino acid modifications at desired positions of the modular antibody or immunoglobulins, as preferably used according to the present invention. In these cases positions are chosen randomly, or amino acid changes are made using certain rules. For example certain residues may be mutated to any amino acids, whereas other residues may be mutated to a restricted set of amino acids. This can be achieved in a stepwise fashion by alternating of cycles of mutation and selection or simultaneously.

A preferred method according to the invention refers to a randomly modified nucleic acid molecule coding for a modular antibody or a part thereof which comprises at least one nucleotide repeating unit within a structural loop coding region having the sequence 5'-NNS-3', 5'-NNN-3', 5'-NNS-3' or 5'-NNK-3' or other nucleotide triplets. In some embodiments the modified nucleic acid comprises nucleotide codons selected from the group of TMT, WMT, RMC, RMG, MRT, SRC, KMT, RST, YMT, MKC, RSA, RRC, NNK, NNN, NNS or any combination thereof (the coding is according to IUPAC).

The randomly modified nucleic acid molecule may comprise the above identified repeating units, which code for all known naturally occurring amino acids or a subset thereof.

The modification of the nucleic acid molecule may be performed by introducing synthetic oligonucleotides into a larger segment of nucleic acid or by de novo synthesis of a complete nucleic acid molecule. Synthesis of nucleic acid may be performed with tri-nucleotide building blocks which would reduce the number of nonsense sequence combinations if a subset of amino acids is to be encoded (e.g. Yanez et al. *Nucleic Acids Res.* (2004) 32:e158).

Preferably the positions to be modified are surface exposed amino acids. Surface exposition of amino acids of structural loops can be judged from known protein structures of modular antibodies and by analogy or homology for such amino acid sequences for which no experimentally determined structure is available.

According to the invention it is preferred that specific amino acids foreign to the antibody region are introduced into the modular antibody to modulate the receptor function and/or the specific binding to the target, e.g. by substitution and/or insertion techniques.

In a preferred embodiment of the invention the modifications introduced into the modular antibody, in particular in the structural loops comprise at least 1, 2, 3, 4, 5, 6 foreign amino acids or amino acids not naturally occurring at the respective site of the structural loop of the non-modified immunoglobulin or immunoglobulin domain.

“Proximal distance” is preferably understood to be within 10 amino acid positions, or even closer, within 8 or 5 amino acids. It is also preferred to engineer mutants with mutations in proximal distance wherein the sites of mutagenesis are in sterical proximity. The tertiary structure of a binding region having a loop structure preferably provides for the proximity within 2000 Angström, more preferably within 1500 Angström. Most preferred are libraries with accumulated basic amino acids in the structural loop region or within the framework region, or most preferred within the binding site spanning over at least 2 loops of a modular antibody scaffold. Thereby the chances of successful panning and selecting molecules that bind the antigens and/or markers of inflammation are highly increased.

In one preferred embodiment, a library of polypeptide variants comprising receptor molecules according to the invention is used as a pool for selection wherein the modifications contain or introduce at least one, more preferably at least two amino acids per modified structural loop

out of the group of amino acids tryptophane, tyrosine, phenylalanine, histidine, isoleucine, serine, methionine, alanine and asparagine.

Further preferred specific mutations result in the introduction of other foreign amino acids to the native polypeptides. Any of the amino acids tryptophane, tyrosine, phenylalanine, histidine, isoleucine, serine, methionine, alanine and asparagines are not present in the structural loops of human native immunoglobulins, thus are considered as "foreign". A variant modular antibody as used according to the invention may contain at least two of said foreign amino acids within the framework region, preferably in the structural loops, to make up a binding site, preferably in proximal distance.

If the modified modular antibody is of human origin or a humanized immunoglobulin variable domain preferred modifications are the incorporation of least one tyrosine in any one of the positions 12 to 17, 45 to 50, 69 to 75 and 93 to 98, and/or at least one tryptophane in any one of the positions 12 to 17, 45 to 50, 69, 71 to 75, 93 to 94 and 96 to 98, and/or at least one histidine in any one of the positions 12 to 17, 46, 47, 49, 50, 69 to 74 and 93 to 98, and/or at least one asparagine in any one of the positions 12 to 17, 45 to 47, 49, 50, 70 to 73, 75, 94 to 96 and 98, and/or at least one methionine in any one of the positions 12 to 17, 46 to 50, 69 to 71, 73 to 75, 93, 95, 96 and 98, and/or at least one serine in any one of the positions 13, 71, 75, 94, 95 and 98, and/or at least one isoleucine in any one of the positions 12, 14 to 17, 45 to 50, 69, 70, 72 to 75, 93 and 96 to 98, and/or at least one phenylalanine in any one of the positions 15, 46, 48, 70 to 73, 75, 93, 95 and 98.

According to another preferred embodiment of the present invention at least two amino acid residues in positions 15 to 17, 29 to 34, 85 to 85, 92 to 94, 97 to 98 and/or 108 to 110 of a human or humanized single domain antibody are modified.

The modification of amino acids may preferentially be biased in order to introduce into structural loop regions amino acids which are known to be frequently involved in protein-protein interactions (e.g. Fellhouse et al. (2006) J. Mol. Biol. 357:100-114).

Further preferred methods employ the modification of the nucleic acid of modular antibodies by nucleotide sequence grafting or fusion techniques. However, other methods that relate to grafting of peptides or amino acid sequences onto the modular antibody, specifically within

the structural loop region, may be preferred as well. One of the preferred embodiments thus refers to a method of conjugating a peptide to a molecule containing an antibody domain, wherein the peptide is binding to either the marker or the inflammatory antigen, wherein the unmodified molecule does not significantly bind to said marker or inflammatory antigen. Suitable peptides can be selected from respective peptide libraries. It is preferred to provide those binders to inflammatory antigens that are not abundantly present in serum, but mainly located and enriched at the inflammation site or migrating thereto.

It is preferred to target those inflammatory antigens that are specifically present at a higher concentration in the area of the inflamed tissue, however, with a serum concentration of less than 20 mg/dl apart from the site of inflammation, preferably with a serum concentration of less than a 10mg/dl in healthy humans. Thus, the targeted receptor molecule according to the invention would not be neutralized by circulating serum proteins.

The nucleic acid molecules encoding the modular antibody according to the invention (and always included throughout the whole specification: immunoglobulins and immunoglobulin fragments comprising a modular antibody) may be cloned into host cells, expressed and assayed for their binding specificities. These practices are carried out using well-known procedures, and a variety of methods that may find use in the present invention are well-known in the art. The nucleic acids that encode the modular antibodies of the present invention may be incorporated into an expression vector in order to express said immunoglobulins. Expression vectors typically comprise an immunoglobulin operably linked - that is placed in a functional relationship - with control or regulatory sequences, selectable markers, any fusion partners, and/or additional elements. The modular antibody as used according to the present invention may be produced by culturing a host cell transformed with nucleic acid, preferably an expression vector, containing nucleic acid encoding the modular antibody, under the appropriate conditions to induce or cause expression of the modular antibody.

The methods of introducing exogenous nucleic acid molecules into a host are well known in the art, and will vary with the host used. Of course, also non-cellular or cell-free expression systems for the expression of immunoglobulins may be employed.

According to a preferred embodiment of the present invention the expression system comprises a vector. Any expression vector known in the art may be used for this purpose as appropriate.

The modular antibody is preferably expressed in a host, preferably in a bacterium, in yeast, in a plant cell, in an insect cell, in an animal cell or mammalian cell or in an organ of a plant or animal or in a complete plant or animal.

A wide variety of appropriate host cells may be used to express the polypeptides of the invention, including but not limited to mammalian cells (animal cells) plant cells, bacteria (e.g. *Bacillus subtilis*, *Escherichia coli*), insect cells, and yeast (e.g. *Pichia pastoris*, *Saccharomyces cerevisiae*). For example, a variety of cell lines that may find use in the present invention are described in the ATCC cell line catalog, available from the American Type Culture Collection. Furthermore, also plants and animals may be used as hosts for the expression of the immunoglobulin according to the present invention. The expression as well as the transfection vectors or cassettes may be selected according to the host used.

Of course also non-cellular or cell-free protein expression systems may be used. In vitro transcription/translation protein expression platforms, that produce sufficient amounts of protein offer many advantages of a cell-free protein expression, eliminating the need for laborious up- and down-stream steps (e.g. host cell transformation, culturing, or lysis) typically associated with cell-based expression systems.

In a preferred embodiment of the present invention, the receptor molecules are purified or isolated after expression. Receptor molecules may be isolated or purified in a variety of ways known to those skilled in the art. Standard purification methods include chromatographic techniques, electrophoretic, immunological, precipitation, dialysis, filtration, concentration, and chromatofocusing techniques. Purification can often be enabled by a particular fusion partner. For example, antibodies may be purified using glutathione resin if a GST fusion is employed, Ni²⁺-affinity chromatography if a His-tag is employed or immobilized anti-flag antibody if a flag-tag is used. For general guidance in suitable purification techniques, see e.g. Scopes, "Protein Purification: Principles and Practice", 1994, 3rd ed., Springer-Science and Business Media Inc., NY or Roe, "Protein Purification Techniques: A Practical Approach", 2001, Oxford University Press. Of course, it is also possible to express the modular antibodies

according to the present invention on the surface of a host, in particular on the surface of a bacterial, insect or yeast cell or on the surface of phages or viruses.

Modified receptor molecules of the invention such as modular antibody may be screened using a variety of methods, including but not limited to those that use in vitro assays, in vivo and cell-based assays, and selection technologies. Automation and high-throughput screening technologies may be utilized in the screening procedures. Screening may employ the use of a fusion partner or label, for example an enzyme, an immune label, isotopic label, or small molecule label such as a fluorescent or colorimetric dye or a luminogenic molecule.

In a preferred embodiment, the functional and/or biophysical properties of the receptor molecules are screened in an in vitro or ex vivo assay. In a preferred embodiment, the receptor molecule is screened for functionality, for example its ability to catalyze a reaction or its binding specificity, cross-linking properties, cross reactivity and/or affinity to its target.

In another preferred embodiment, the favourable receptor molecule may be selected in vivo, e.g. by introducing it into a cell or an organism. The specifically binding variants may be isolated either from body fluid such as blood or lymphatic liquid or from specific organs, depending on the required properties of the modified domains.

Assays may employ a variety of detection methods including but not limited to chromogenic, fluorescent, luminescent, or isotopic labels.

As is well known in the art, there are a variety of technologies that may be used for the identification and isolation of proteins with certain binding characteristics and affinities, including, for example, display technologies such as phage display, ribosome display, cell surface display, and the like, as described below. Methods for production and screening of antibody variants are well known in the art.

As is known in the art, some display and screening methods select for favourable members of a library. These methods are herein referred to as "selection methods", which find use in the present invention for displaying and screening modular antibodies. When variant receptor molecules or modular antibody libraries are screened using a selection method, only those members of a library that are favourable, that is which meet some selection criteria, are

propagated, isolated, and/or observed. As will be appreciated, because only the fittest variants are observed, such methods enable the screening of libraries that are larger than those screenable by methods that assay the fitness of library members individually. Selection is enabled by any method, technique, or fusion partner that links, covalently or non-covalently, the phenotype of molecules with its genotype, that is the function of e.g. an antibody with the nucleic acid that encodes it. For example the use of phage display as a selection method is enabled by the fusion of library members to a phage coat protein at the N- or at the C-terminus or on both termini (most frequently used is the filamentous phage gene III protein, however also other coat proteins such as protein VIII, protein VII, protein VI and protein IX can be used). In this way, selection or isolation of modified immunoglobulins that meet some criteria, for example binding affinity to the immunoglobulin's target, also selects for or isolates the nucleic acid that encodes it. Once isolated, the gene or genes encoding modular antibodies or immunoglobulins may then be amplified. This process of isolation and amplification, referred to as panning, may be repeated, allowing favourable antibody domain variants in the library to be enriched. Nucleic acid sequencing of the attached nucleic acid ultimately allows for gene identification.

A variety of selection methods are known in the art that may find use in the present invention for screening modular antibody libraries. These include but are not limited to phage display and its derivatives such as selective phage infection (Malmborg et al., 1997, *J Mol Biol* 273:544-551), selectively infective phage (Krebber et al., 1997, *J Mol Biol* 268:619-630), and delayed infectivity panning (Benhar et al., 2000, *J Mol Biol* 301:893-904), cell surface display (Wittrup, 2001, *Curr Opin Biotechnol*, 12:395-399) such as display on bacteria (Lee et al., 2000, *Nat Biotechnol* 18:645-648), yeast (Boder & Wittrup, 2000, *Methods Enzymol* 328:430-44), and mammalian cells (Whitehorn et al., 1995, *Biotechnology* 13:1215-1219), as well as in vitro display technologies (Amstutz et al., 2001, *Curr Opin Biotechnol* 12:400-405) such as polysome display (Mattheakis et al., 1994, *Proc Natl Acad Sci USA* 91:9022-9026), ribosome display (Hanes et al., 1997, *Proc Natl Acad Sci USA* 94:4937-4942), mRNA display (Nemoto et al., 1997, *FEBS Lett* 414:405-408), and ribosome-inactivation display system (Zhou et al., 2002, *J Am Chem Soc* 124, 538-543).

Other selection methods that may find use in the present invention include methods that do not rely on display, such as in vivo methods including but not limited to periplasmic expression and cytometric screening (Chen et al., 2001, *Nat Biotechnol* 19:537-542), the

antibody fragment complementation assay (Pelletier et al., 1998, Proc Natl Acad Sci USA 95:12141-12146), and the yeast two hybrid screen (Fields & Song, 1989, Nature 340:245-246) used in selection mode (Visintin et al., 1999, Proc Natl Acad Sci USA 96:11723-11728). In an alternate embodiment, selection is enabled by a fusion partner that binds to a specific sequence on the expression vector, thus linking covalently or noncovalently the fusion partner and associated immunoglobulin library member with the nucleic acid that encodes them. For example, WO9308278 describe such a fusion partner and technique that may find use in the present invention. In an alternative embodiment, in vivo selection can occur if expression of the antibody imparts some growth, reproduction, or survival advantage to the cell.

Some selection methods are referred to as "directed evolution" methods. Those methods include the mating or breeding of favourable sequences during selection, sometimes with the incorporation of new mutations. As will be appreciated by those skilled in the art, directed evolution methods can facilitate identification of the most favourable sequences in a plurality of polypeptides, and can increase the diversity of sequences that are screened. A variety of directed evolution methods are known in the art that may find use in the present invention for generating and screening antibody variable domain variants, or mutations thereof, including but not limited to DNA shuffling (PCT WO 01/70947), exon shuffling (Kolkman & Stemmer, 2001, Nat Biotechnol 19:423-428), family shuffling (Crameri et al., 1998, Nature 391:288-291), selective combinatorial randomization (WO2004/018674A1), Random Chimeragenesis on Transient Templates (Coco et al., 2001, Nat Biotechnol 19:354-359), molecular evolution by staggered extension process (StEP) in vitro recombination (Zhao et al., 1998, Nat Biotechnol 16:258-261), exonuclease mediated gene assembly (U.S. Pat. No. 6,352,842), Gene Site Saturation Mutagenesis (U.S. Pat. No. 6,358,709), Gene Reassembly (U.S. Pat. No. 6,358,709), SCRATCHY (Lutz et al., 2001, Proc Natl Acad Sci USA 98:11248-11253), DNA fragmentation methods (Kikuchi et al., Gene 236:159-167), single-stranded DNA shuffling (Kikuchi et al., 2000, Gene 243:133-137), and directed evolution antibody engineering technology (Applied Molecular Evolution) (U.S. Pat. No. 5,723,323).

In a preferred embodiment, modular antibody variants, or mutations thereof, are screened using one or more cell-based or in vivo assays. For such assays, purified or non-purified modular antibodies are typically added exogenously such that cells are exposed to modular antibodies or pools of modular antibody variants, or mutations thereof, belonging to a library. These assays are typically, but not always, based on the desired function of modular

antibodies; that is, the ability of the antibody or antibody domains as provided according to the invention to bind to its target and to mediate some biochemical event, for example effector function, ligand/receptor binding inhibition, apoptosis, and the like. Such assays often involve monitoring the response of cells to the modular antibody, for example cell survival, cell death, change in cellular morphology, or transcriptional activation such as cellular expression of a natural gene or reporter gene. For example, such assays may measure the ability of modular antibody variants, or mutations thereof, to elicit ADCC, ADCP or CDC. For some assays additional cells or components, that is in addition to the target cells, may need to be added, for example serum complement, or effector cells such as peripheral blood monocytes (PBMCs), NK cells, macrophages, and the like. Such additional cells may be from any organism, preferably humans, mice, rat, rabbit, and monkey. Immunoglobulins may cause apoptosis of certain cell lines expressing the target, or they may mediate attack on target cells by immune cells which have been added to the assay. Methods for monitoring cell death or viability are known in the art, and include the use of dyes, immunochemical, cytochemical, and radioactive reagents. For example, caspase staining assays may enable to measure apoptosis, and uptake or release of radioactive substrates or fluorescent dyes may enable cell growth or activation to be monitored. Alternatively, dead or damaged target cells may be monitored by measuring the release of one or more natural intracellular components, e.g. lactate dehydrogenase.

For use in anti-inflammatory therapy it is, however, preferred to employ molecules devoid of cytotoxic activity, such as ADCC or CDC.

Transcriptional activation may also serve as a method for assaying function in cell-based assays. In this case, response may be monitored by assaying for natural genes which may be upregulated, for example the release of certain interleukins may be measured, or alternatively readout may be via a reporter system. Cell-based assays may also involve the measure of morphological changes of cells as a response to the presence of modular antibody variants. Cell types for such assays may be prokaryotic or eukaryotic, and a variety of cell lines that are known in the art may be employed.

Alternatively, cell-based screens may be performed using cells that have been transformed or transfected with nucleic acids encoding the variant modular antibodies. In this case, receptor molecule variants of the invention are not added exogenously to the cells (e.g. Auf der Maur, 2004, *Methods*, 34:215-224). In another alternative method, the cell-based screen utilizes cell

surface display. A fusion partner can be employed that enables display of receptor molecules on the surface of cells (Wittrup, 2001, Curr Opin Biotechnol, 12:395-399). Other assays methods are based on the activation of cells through an inflammatory stimulus and determination of the release of cellular inflammatory products or determination of overexpression of inflammatory markers.

Receptor molecules according to the invention are preferably characterized by the native structure. It is further preferred that they are provided in a non-immunogenic form for passive immunotherapy. In the course of such passive immunotherapies the receptor molecules can be administered frequently as bolus injection or as continuous infusion, without inducing undesired side reactions, such as the induction of neutralizing antibodies to a level that requires higher dosing, e.g. doubling of the dose, than normal.

Another feature of the native structure is determined by the stability of the molecule, either in solution, buffer or serum, or stability after administration. Preferred modifications of the molecule would not significantly change the stability of the molecule. Thus, deterioration, dearrangement or destruction of its structure or conformation would be in principle avoided. A molecule would be considered significantly stable after modification, if its in vitro or ex vivo half-life in a buffer system or in human serum would not be significantly reduced, for instance by a factor of 10, preferably a factor of 5, even more preferred by a factor of 3 or 2.

If the parent receptor molecule or antibody comprises binding sites through CDR loops or non-CDR loops, its native structure is preferably represented by the CDR or non-CDR loop conformation, which binding site is optionally still biologically active after modification, so to enable the stable binding properties to a target. While in a preferred embodiment one or more structural loops are modified to engineer the new binding site to an inflammatory antigen and/or a marker of inflammation, it is understood that the loop conformation of binding CDR or non-CDR loops in the parent molecules is not significantly changed, so to keep the native, original binding property of the parent molecule.

The molecular stability of a modular antibody may be determined by its binding to Protein A or Protein G. It turned out that the natural binding site for Protein A, e.g. that is inherent to a native IgG, is an indicator for the molecular stability and expression stability of an antibody or an antibody Fc fragment, This is also important for practical reasons, because the modular

antibodies can be purified through affinity chromatography using those proteins as affinity ligands.

The biological or functional properties of the receptor molecules of the present invention may be characterized in cell, tissue, and whole organism experiments. As is known in the art, drugs are often tested in animals, including but not limited to mice, rats, rabbits, dogs, cats, pigs, and monkeys, in order to measure a drug's efficacy for treatment against a disease or disease model, or to measure a drug's pharmacokinetics, toxicity, and other properties. The animals may be referred to as disease models. Therapeutics are often tested in mice, including but not limited to nude mice, SCID mice, xenograft mice, and transgenic mice (including genetic knock-in and knock-out mutants). Such experimentation may provide meaningful data for determination of the potential of the polypeptide variant to be used as a therapeutic. Any organism, preferably mammals, may be used for testing. Because of their genetic similarity to humans, monkeys can be suitable therapeutic models, and thus may be used to test the efficacy, toxicity, pharmacokinetics, or other property of the receptor molecules of the present invention. Testing drug candidates in humans are most frequently required for approval as therapeutics, and thus of course these experiments are contemplated. Thus the receptor molecules of the present invention may be tested in humans to determine their therapeutic efficacy, toxicity, immunogenicity, pharmacokinetics, and/or other clinical properties.

The receptor molecules of the present invention may find use in a wide range of pharmaceutical products. In one embodiment the receptor molecules of the present invention is used for therapy or prophylaxis, for preparative or analytic use, as a diagnostic, an industrial compound or a research reagent, preferably a therapeutic. It is particularly preferred to prepare a therapeutic composition that can be used for therapeutic treatment of inflammatory disease, such as chronic inflammatory conditions. Through the localized effect of the therapeutic it is the first time possible to employ biological antagonists of inflammatory antigens at a very low dose, and with reduced systemic effects, such as adverse reactions. Likewise, a synergistic effect of both, blocking the inflammatory mediators and downmodulation of inflammation markers, will bring about the advantage of a short term treatment. It is assumed that the inflammatory disease may be effectively treated at an early stage, even by prophylactic treatment in the chronic disease cases.

The receptor molecule may find use in an antibody composition that is monoclonal, oligoclonal or polyclonal. In a preferred embodiment, the receptor molecules of the present invention are used to kill target cells that bear the target inflammatory antigen and/or marker of inflammation. In an alternate embodiment, the receptor molecules of the present invention are used to block, antagonize, or agonize the pro- and anti-inflammatory processes, for example by antagonizing a cytokine or a cytokine receptor thereof. In an alternately preferred embodiment, the receptor molecules of the present invention are used to block, antagonize, or agonize a target antigen and kill the target cells that bear the target antigen.

In an alternately preferred embodiment, the receptor molecules of the present invention are used to block, antagonize, or agonize growth factors or growth factor receptors and kill the target cells that bear or need the target antigen.

In an alternately preferred embodiment, receptor molecules of the present invention are used to block, antagonize, or agonize enzymes and substrate of enzymes.

In another alternatively preferred embodiment, the receptor molecules of the present invention are used to block, antagonize or agonize activated cells resulting from infection by infectious agents such as viruses, small viruses, prions, bacteria or fungi.

The receptor molecules of the present invention may be used for various therapeutic purposes. In a preferred embodiment, modular antibodies or respective domains are administered in an effective amount to a patient to treat a specific disorder associated with or caused by inflammatory disease, either chronic or sporadic conditions. A "patient" for the purposes of the present invention includes both, humans and other animals, preferably mammals and most preferably humans. By "specific disorder" herein is meant a disorder that may be ameliorated by the administration of a pharmaceutical composition comprising a receptor molecule of the present invention.

The preferred method according the invention refers to treatment of patients who are at risk of or suffering from chronic inflammatory disorders, like rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, psoriasis, Crohn's Disease, ulcerative colitis, atherosclerosis, inflammatory bowel disease, or other diseases characterized by leukocyte recruitment and

cytokine production, like asthma, psoriasis, organ transplant rejection episodes or Alzheimer's disease.

In one embodiment, a receptor molecule according to the present invention is the only therapeutically active agent administered to a patient. Alternatively, the receptor molecules according to the present invention are administered in combination with one or more other therapeutic agents, including but not limited to cytotoxic agents, chemotherapeutic agents, cytokines, growth inhibitory agents, anti-hormonal agents, kinase inhibitors, anti-angiogenic agents, cardioprotectants, anti-inflammatory agents, immunomodifiers, antibiotics, antivirals, anti-mycotics, or other therapeutic agents. The receptor molecules may be administered concomitantly with one or more other therapeutic regimens. For example, a modular antibody of the present invention may be formulated and administered to the patient along with chemotherapy, radiation therapy, or both chemotherapy and radiation therapy, anti-inflammatory therapy, immunotherapy, or antibiotic, antiviral, or anti-mycotic therapy. In one embodiment, the receptor molecules of the present invention may be administered in conjunction with one or more antibodies, which may or may not comprise a modular antibody. In accordance with another embodiment of the invention, the receptor molecules of the present invention and one or more other anti-inflammatory therapies are employed to treat inflamed tissue or joints. It is of course contemplated that the receptor molecules of the invention can be employed in combination with still other therapeutic techniques such as surgery.

A variety of other therapeutic agents may find use for administration with the receptor molecules of the present invention. In one embodiment, the receptor molecule is administered with an anti-angiogenic agent, which is a compound that blocks, or interferes to some degree with the development of blood vessels. The anti-angiogenic factor may, for instance, be a small molecule or a protein, for example an antibody, Fc fusion, or cytokine, that binds to a growth factor or growth factor receptor involved in promoting angiogenesis. The preferred anti-angiogenic factor herein is an antibody that binds to Vascular Endothelial Growth Factor (VEGF). In an alternate embodiment, the receptor molecule is administered with a therapeutic agent that induces or enhances adaptive immune response, for example an antibody that targets CTLA-4. In an alternate preferred embodiment, the receptor molecule is administered with a tyrosine kinase inhibitor, which is a molecule that inhibits to some extent tyrosine kinase activity of a tyrosine kinase. In an alternate embodiment, the modified

immunoglobulins of the present invention are administered with a cytokine. By "cytokine" as used herein is meant a generic term for proteins released by one cell population that act on another cell as intercellular mediators including chemokines.

Pharmaceutical compositions are contemplated wherein receptor molecules of the present invention and one or more therapeutically active agents are formulated. Formulations are prepared for storage by mixing said receptor molecules having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences, 1980, 16th edition, Osol, A. Ed.), in the form of lyophilized formulations or aqueous solutions. The formulations to be used for in vivo administration are preferably sterile. This is readily accomplished by filtration through sterile filtration membranes or other methods. The receptor molecules and other therapeutically active agents disclosed herein may also be formulated as immunoliposomes, and/or entrapped in microcapsules.

Administration of the pharmaceutical composition comprising a receptor molecule of the present invention, preferably in the form of a sterile aqueous solution, may be performed in a variety of ways, including, but not limited to local or systemic administration, orally, sublingual, subcutaneously, intravenously, intranasally, intraotically, transdermally, topically, e.g. in the form of gels, salves, lotions, creams, etc., intraperitoneally, intramuscularly, intrapulmonary, vaginally, parenterally, rectally, intraocularly or by the mucosal route.

Using the targeted receptor molecule according to the invention enables the targeted immunotherapy to reinstate the balance of pro-inflammatory and anti-inflammatory factors in situ. According to the invention a method of treatment is provided employing targeted delivery of the receptors of inflammatory antigens, but also localizing the therapeutic receptors by means of the receptor molecule that is targeted to the site of inflammation mediated by the binding property to the marker of inflammation. Either the therapeutic receptors are localized to increase the concentration of the receptor, or the inflammatory antigens themselves are localized and optionally accumulated at the site of inflammation, or the binding to the inflammatory antigen and/or the marker of inflamed tissue results in the biological reaction to downmodulate inflammation at the site of inflammation.

The local anti-inflammatory effect of the receptor molecule according to the invention results in the reduction of systemic side effects, or undesired side reactions at organs, such as liver or lung. Therapy with the receptor molecule according to the invention would also be preferred over the therapeutics that apply chemical drugs, as targeted therapy or used systemically. Contrary to chemicals or small molecules, the receptor molecule according to the invention is considered a biological having the advantage of tolerability and the possible reinstatement of the biological balance between proinflammatory and anti-inflammatory processes mediated by the patient's physiological molecules. Both, the marker and the inflammatory antigen addressed by the specificity of the receptor molecule according to the invention are originating from the patient, and can interact at the site of inflammation in a tolerable way. Rebalancing the inflammation system would bring about synergistic effects.

Binding to a marker of inflammation, such as E-Selectin, would downmodulate the expression of pro-inflammatory antigens, such as TNF α , and thus the effect of the receptor molecule that captures the TNF α would be increased.

Exemplary targeted receptor molecules according to the invention make use of endothelial cell anchoring anti-e-selectin (CD62E) or p-selectin. The second specificity targets soluble proteins, like MCP1 produced by e.g. keratinocytes. This targeted receptor protein binds to the endothelial cells, prevents contact with activated leukocytes and prevents activation of leukocytes. Though monospecific antibodies, like antibodies against CD62E or antibodies against MCP-1 proved to be unsuccessful in treating psoriasis or rheumatoid arthritis, the bispecific targeted therapy should provide for a significant improvement of anti-inflammatory therapy.

Likewise, the anti-MCP-1 β specificity may be combined with a receptor specifically binding to e-cadherin, interacting with integrins on T cells. This molecule will prevent both, docking of T cells and their activation. The binding is supposed to be stabilized whilst preventing internalization of the molecule.

Other examples refer to targeted receptor molecules that have a binding site to VCAM-1, a member of the IgSF binding to VLA4. Using a targeted receptor binding to MCP1 β , entrance of e.g. CD8 T cells through VCAM-1 will be inhibited.

Mad-CAM-1 is another exemplary membrane bound marker of inflammation located on endothelial cells in mucosal tissue, which is preferably bound by the targeted receptor according to the invention.

Among the combinations of binding properties those are most preferred that exhibit an anti-inflammatory effect as determined in e.g. a cellular assay of monocyte activation. The preferred assays refer to either inhibition of activation in the presence of a stimulus, like TNF α , resulting in the reduction of cytokine release or a reduced expression level of membrane bound inflammation markers. Other assays relate to the therapeutic potential to down-modulate inflammatory processes, preferably shown in a flow assay using a layer of activated epithelial cells, e.g. HUVEC cells, to which the targeted receptor molecule according to the invention is specifically bound. As soon as other cells, like leukocytes, are introduced into the test system, they would be activated, unless the targeted receptor molecule according to the invention provides for the local anti-inflammatory effect even under flow conditions.

The invention is further described by means of the following examples.

Examples

1. Design of Fc mutant libraries, Fcab™ libraries

Libraries of Fc fragments for display on filamentous phage are designed and synthesized chemically (Geneart, Germany). The synthetic gene libraries are cloned into a phage display vector such as pHEN1 for surface display by protein III fusion.

The wild type Fc is randomized in the loops marked with bold letters. Amino acids are written in capital letters in the single letter code. The underlined amino acids are sequences modified to result in the desired libraries. The codons for the random sequences are written in small letters. There are two randomization strategies: In one type of library the codons used are of the nnk type in order to cover all 20 amino acids. In another type of library kmt codons are used to cover only amino acids frequently involved in ligand binding (serine, alanine, tyrosine and aspartic acid).

EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVK
 FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP
 APIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQP
 ENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSL
 SPGK

Only the randomized loops are shown to characterize the respective libraries:

Library "CH3+5 kmt"

...RDEL-(kmt)₃-QVSL.....LTV-(kmt)₈-RW-(kmt)₂-GNV...

Library "CH3+5 kmt" with GS-----SG „stem“ loop

...RDEL-(kmt)₃-QVSL.....LTV-GS-(kmt)₈-SG-RW-(kmt)₂-GNV...

Library "CH3+5 kmt" with extended first loop

...RDE-(kmt)₅-VSL.....LTV-(kmt)₈-RW-(kmt)₂-GNV...

Library "CH3+5 nnb"

...RDEL-(nnb)₃-QVSL.....LTV-(nnb)₈-RW-(nnb)₂-GNV...

Library "CH3+5 nnb" with GS-----SG „stem“ loop

...RDEL-(nnb)₃-QVSL.....LTV-GS-(nnb)₈-SG-RW-(nnb)₂-GNV...

Library "CH3+5 nnb" with extended first loop

...RDE-kmt(nnb)₃ kmt-VSL.....LTV-(nnb)₈-RW-(nnb)₂-GNV...

The libraries can be prepared according to standard procedures as described (e.g. Phage Display: A Practical Approach (The Practical Approach Series, 266) by Tim Clackson (Editor), Henry B. Lowman (Editor) Oxford University Press, 2004)

2. Selection of TNFa binders and E-selectin binders

Phages Libraries are resuspended in 5 ml 20 mM MES, pH 6.0/5% skimmed milk/ 0.05% Tween 20 and added (100 μ l of 5×10^{12} cfu/ml/well) to 20 wells of a Maxisorp immunoplate (Nunc, Rochester, NY) coated with 1 μ g of human TNFa (BMS301; Bender Medsystems, Austria) or E-Selectin (R&D Systems, USA) and blocked with 5% skimmed milk. After incubation for 2 h at 37°C, wells are washed 10–30 times with 20 mM MES, pH 6.0/0.2% Tween 20/0.3 M NaCl and phage eluted by incubation in 100 μ l PBS, pH 7.4/well for 30 min at 37°C. Phages are used to reinfect exponentially growing E. coli TG1 according to standard procedures. Three to six rounds of panning are conducted. 20 to 100 clones of the panning rounds are sequenced for the specific inserts. The selected Fc mutants are cloned into a eukaryotic episomal vector (pCEP4) and expressed in HEK-293 cells. The proteins are purified and are tested for binding to TNFa and E-Selectin with selected Fc mutants in ELISA and FACS assays.

3. Reengineering the IgG antibody

Sequence information of the Fcab™ specific for E-selectin selected in Example 2 can be used to render an anti-TNFa antibody bispecific. The heavy and light chain genes for Humira and the respective heavy chain mutants thereof are synthesized (GenArt, Germany). In this example, the heavy chain gene for Humira (Abbott) is mutated to contain mutations in the Fc part which makes it binding to E-selectin (the sequence is obtained from selection of randomized Fc-display libraries of example 1, with E-selectin as described in Example 2 and subsequent sequencing of genes of those E-selectin specific binding clones. The various mutants are cloned into an appropriate expression vector (based on pCEP4) and expressed transiently in a mammalian cell expression system (HEK-293). The bispecific Humira variants are tested for binding to TNFa and E-selectin in ELISA. The bispecific antibody constructs are further characterized by their affinity against the two antigens and tested in vivo for accumulation of antibody in damaged joints.

VCAM-1 (Bender MedSystems, Austria) is coated to ELISA plates, after washing samples of Aptamer A, Aptamer B and hybridized Aptamers A and B are added in dilutions. After washing, VEGF-biotin (R&D Systems) is added and detected via Avidin-Enzyme conjugate.

Claims

1. Targeted receptor molecule based on a receptor of soluble inflammatory antigens, binding specifically to a membrane bound marker of inflammation.
2. Receptor molecule according to claim 1, wherein said marker is selected from the group of cell adhesion molecules and receptors of cytokines, growth factors or chemokines.
3. Receptor molecule according to any of claim 1 to claim 2, wherein said marker is expressed by both the leucocytes and the endothelial cells at the site of inflammation.
4. Receptor molecule according to any of claim 1 to claim 3, wherein said marker is selected from the group consisting of a selectin, an integrin, an immunoglobulin superfamily member, a catherin, a protocatherin, a catenin, and receptors thereof.
5. Receptor molecule according to any of claim 1 to claim 4, wherein said marker is a selectin selected from the group consisting of E-selectin, P-selectin and L-selectin.
6. Receptor molecule according to any of claim 1 to claim 4, wherein said marker is selected from the group consisting of receptors of interleukins, colony stimulating factors, interferons, tumor necrosis factors and endothelial growth factors.
7. Receptor molecule according to any of claim 1 to claim 4, wherein said marker is an immunoglobulin superfamily member selected from the group consisting of ICAM-1, PECAM-1, VCAM-1, MAdCAM-1 and PNAd.
8. Receptor molecule according to any of claim 1 to claim 4, wherein said marker is a cadherin selected from the group consisting of E-cadherin, P-cadherin and N-cadherin.
9. Receptor molecule according to any of claim 1 to claim 8, wherein said receptor is a binding structure selected from the group consisting of biological receptors or ligands, peptides, polypeptides, chemical structures, Aptamers, immunoglobulins, immunoglobulin-like molecules, modular antibodies, fragments and derivatives thereof.

10. Receptor molecule according to any of claim 1 to claim 9, wherein said inflammatory antigen is one of proinflammatory proteins or anti-inflammatory proteins, and receptors thereof.
11. Receptor molecule according to any of claim 1 to claim 10, wherein said inflammatory antigen is selected from the group consisting of a interleukins, colony stimulating factors, interferons, tumor necrosis factors and endothelial growth factors.
12. Receptor molecule according to any of claim 1 to claim 11, wherein said inflammatory antigen is selected from the group consisting of Blys, IL-6, LIF, OSM, IL-1, TNFalpha, IL-12, IL-23, IL-15, GM-CSF, M-CSF, IL-17, IL-18, VEGF, RANKL, DARC, MCP1, MIP1beta, Interferon beta, OPG, TGFbeta, IL-10, IL-13, IL-11, IL-4, staff-R and IL-1ra. .
13. Receptor molecule according to any of claim 1 to claim 12, which is a bispecific, oligospecific or multispecific molecule.
14. Method of producing a preparation of a targeted receptor molecule according to claim 1, which comprises the steps of
 - a. preparing a library containing said receptor and variants thereof, by mutagenizing the amino acid sequence apart from the receptor region binding to said inflammatory antigen to provide new additional binding sites,
 - b. contacting said library with a test sample containing said marker,
 - c. selecting the member of the library binding to said marker,
 - d. determining the binding property of the selected molecule to said inflammatory antigen and said marker, and
 - e. treating the selected molecule to manufacture said targeted receptor molecule.
15. Method according to claim 14, wherein said library contains modular antibodies binding to said inflammatory antigen, which modular antibodies are randomized in the structural loop region to provide said new binding sites.

16. Method of producing a preparation of a targeted receptor molecule according to claim 1, which comprises the steps of:

- a. preparing a library containing a binding moiety specifically binding to said marker of inflammation, by mutagenizing the binding moiety to provide new additional binding sites,
- b. contacting said library with a test sample containing said inflammatory antigen,
- c. selecting the member of the library binding to said inflammatory antigen,
- d. determining the binding property of the selected molecule to said inflammatory antigen and said marker, and
- e. treating the selected molecule to manufacture said targeted receptor molecule.

17. Method according to claim 16, wherein said library contains modular antibodies binding to said marker, which modular antibodies are randomized in the structural loop region to provide said new binding sites.

18. Kit for selecting a targeted receptor molecule according to claim 1, which comprises

- a. a library of said receptor molecule,
- b. a test sample containing said inflammatory antigen, and
- c. a test sample containing said marker.

19. Method of producing a preparation of a targeted receptor molecule according to claim 1, comprising associating a moiety binding to said inflammatory antigen with a moiety binding to said marker by one of conjugation, recombinant or fusion techniques.

20. Receptor molecule according to any of claims 1 to 13, for the treatment of inflammatory disease.

21. Receptor molecule according to claim 20, for the treatment of chronic inflammatory disorders, autoimmune disease, chronic inflammation, chronic prostatitis, glomerulonephritis, hypersensitivities, pelvic inflammatory disease, reperfusion injuries, vasculitis, rheumatoid arthritis, ankylosing spondylitis, psoriatic arthritis, psoriasis, Crohn's Disease, ulcerative colitis, atherosclerosis, inflammatory bowel disease, asthma, psoriasis, transplant rejection, or Alzheimer's disease.

22. Receptor molecule according to any of claims 1 to 13, for the targeted delivery of receptors of inflammatory antigens associated with inflammatory conditions.
23. Receptor molecule according to any of claims 1 to 13, for localizing therapeutic receptors of inflammatory antigens in patients at the site of inflammation.
24. Receptor molecule according to any of claims 1 to 13, for accumulating inflammatory antigens in patients at the site of inflammation.