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(54) Title: RECOMBINANT ANTIBODIES AGAINST HUMAN TYPE II TRANSGLUTAMINASE AND USES THEREOF

(57) Abstract: The present invention provides specific antibodies that are able to inhibit TGase II enzymatic activity. The present invention further provides a pharmaceutical composition comprising as an active ingredient the antibody of the invention useful in treating or preventing fibrosis diseases, scarring, or fibrosis-related pathologies. The pharmaceutical composition may be useful for treating both acute and chronic forms of fibrosis of organs.



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RECOMBINANT ANTIBODIES AGAINST HUMAN TYPE II TRANSGLUTAMINASE AND USES THEREOF

This application claims priority of US Provisional patent application No 60/664238,
5 filed March 22, 2005, and of US Provisional patent application No 60/689006, filed
June 8, 2005, both of which are hereby incorporated by reference in their entirety.

Throughout this application various patent and scientific publications are cited. The
disclosures for these publications in their entireties are hereby incorporated by reference
into this application to more fully describe the state of the art to which this invention
10 pertains.

FIELD OF THE INVENTION

The present invention relates to immunoglobulins and functional fragments
thereof, useful for inhibiting the enzymatic activity of type II transglutaminase (TGase
15 II), compositions comprising said immunoglobulins and methods of using same for
treating or inhibiting diseases or disorders, such as fibrosis, scarring, or fibrosis-related
pathologies.

BACKGROUND OF THE INVENTION

Transglutaminases are a group of calcium-dependent enzymes that catalyze the
20 formation of ϵ -(γ -glutaminy) lysine isopeptide bonds between protein-bound glutamine
and lysine residues. These bonds are responsible for the crosslinking of large proteins
and the incorporation of small primary amines into proteins (Greenberg, C. S., et al.,
1991, FASEB J. 5, 3071- 3077). Transglutaminases catalyze protein aggregation
reactions in blood coagulation, skin maturation and the clotting of seminal secretions.
25 The most widespread member of the family is the cellular form of the enzyme, tissue
transglutaminase (TGase II), which is expressed in varying amounts in many cell types.
TGase II is unique in the transglutaminase family of enzymes in that it is able to bind
and hydrolyze GTP and ATP, and to bind to fibronectin. Tissue TGase II is
predominantly located in the cytosol, although TGase II has also been reported to exist
30 in the nucleus, at the cell surface and in the extracellular matrix. The enzyme is highly
expressed in endothelial cells and its activity at the surface of such cells is thought to
enhance basement membrane stabilization, cell spreading and cell adhesion. However,

the overall significance of the high amount of enzyme in this cell type and its biological function is poorly understood.

Protein modification mediated by tissue transglutaminases has been implicated in the pathology and etiology of numerous diseases and processes (see review by Aeschlimann & Thomazy, 2000, *Connective Tissue Research* 41(1):1-27). For example, TGase II-mediated protein modification has been shown to occur in fibrosis and tissue scarring (Johnson et al., 1999, *J. Soc. Neph.* 10: 2146-2157), neurodegenerative diseases, celiac disease (CD) (Sblattero et al., 2004, *J. Autoimmun.* 22(1): 65-72; Sblattero et al., 2002, *Eur. J. Biochem.* 269(21): 5175-81; Esposito et al., 2002 *Gut*; 2: 177-181; Sblattero et al., 2000, *Hum Antibodies.* 9(4):199-205), thrombosis, cancer, psoriasis and inflammatory diseases of the joints. Tissue TGase II has also been implicated in a number of diseases involving angiogenesis, such as the development of solid tumors and rheumatoid arthritis. Hence, TGase II represents a potential target in the development of new treatments of such diseases and disorders. At the present time, no effective therapies are available to prevent fibrosis and scar formation.

The inventors of the present invention and their co-workers have recently reported the tissue transglutaminase autoantibody response in Celiac disease (CD) (Marzari et al., 2001, *J. Immunol.* 166: 4170-4176) and suggested the possible role of these anti- TGase II antibodies in the onset of CD.

Several classes of transglutaminase inhibitor compounds are known in the art, including competitive amine inhibitors, competitive glutamine inhibitors and irreversible inhibitors. Competitive amine inhibitors include dansylcadaverines and N-phenyl-N'-(O-aminoalkyl) thioureas. Competitive glutamine inhibitors include aliphatic amides, dipeptides and polypeptides. Irreversible inhibitors include iodoacetamide, phenol-containing halomethyl ketones, alkyl isocyanates, ahalomethylcarbonyl inhibitors, dihydroisoazoles (US 4,912,120), azoles, azolium salts (US 4,968,713), thiadiazoles, and epoxides.

There is an unmet need for highly selective molecules capable of inhibiting aberrant TGase II enzymatic activity thereby addressing the clinical manifestations associated with its expression.

SUMMARY OF THE INVENTION

The present invention provides specific antibodies that are able to inhibit TGase II enzymatic activity. The present invention further provides a pharmaceutical composition comprising as an active ingredient the antibody of the invention useful in treating or preventing fibrosis diseases, scarring, or fibrosis-related pathologies. The present invention also provides a method for treating both acute and chronic forms of fibrosis of organs. The present invention additionally provides a method for inhibiting TGase II enzymatic activity in the cells of subjects in need thereof by exposure to antibodies capable of inhibiting TGase II function.

The present invention provides a molecule comprising at least one of the CDR3 variable regions of SEQ ID NO: 9 or SEQ ID NO: 10 of a recombinant antibody which has specific binding affinity for TGase II and which inhibits the enzymatic activity of said enzyme. Generation of inhibitory molecules would be useful for developing medicaments for use in treating or preventing fibrosis diseases, scarring, or fibrosis-related pathologies associated with the enzymatic activity of TGase II.

The molecules of the present invention include antibodies or antigen binding fragments thereof. According to one embodiment, the present invention provides a cloned human single-chain antibody fragment (scFv), which binds to TGase II and inhibits its enzymatic activity. According to another embodiment, the present invention provides a molecule which binds to TGase II and inhibits its enzymatic activity comprising V_L and V_H regions having SEQ ID NO:2 and 6, respectively, and optionally a linker (SEQ ID NO:4). According to the principles of the present invention, molecules that bind to TGase II and inhibit its enzymatic activity are provided. These molecules are useful in treating disorders and diseases associated with TGase II enzymatic activity including fibrotic diseases, scarring or diseases in which fibrosis is evident (fibrosis-related pathologies).

Another aspect of the present invention provides a pharmaceutical composition comprising as an active ingredient a molecule of the present invention useful for preventing or treating fibrotic diseases, scarring or diseases in which fibrosis is evident.

In one embodiment of the present invention, said fibrotic disease is selected from pulmonary fibrosis, liver fibrosis, cardiac fibrosis, kidney fibrosis, skin fibrosis and

myelofibrosis. In another embodiment of the present invention, said scarring is selected from scleroderma, keloids and hypertrophic scars, ocular scarring, inflammatory bowel disease, macular degeneration, Grave's ophthalmopathy, drug induced ergotism and psoriasis. In a further embodiment of the present invention, said fibrosis-related pathology is selected from glioblastoma in Li-Fraumeni syndrome, sporadic glioblastoma, myleoid leukemia, acute myelogenous leukemia, myelodysplastic syndrome, myeloproliferative syndrome, gynecological cancer, Kaposi's sarcoma, Hansen's disease, and collagenous colitis. In still another embodiment of the present invention said fibrosis-related pathology is selected from ocular disease, a cardiovascular disease, atherosclerosis / restenosis, and a neurological disease. In one embodiment, said pulmonary fibrosis is selected from interstitial lung disease and fibrotic lung disease. In another embodiment, said ocular scarring is selected from proliferative vitreoretinopathy (PVR) and scarring resulting from surgery to treat cataract or glaucoma. In a further embodiment, said neurological disease is selected from polyglutamine disease, spinobulbar muscular atrophy, dentatorubral-pallidoluysian atrophy, spinocerebellar ataxias (SCAs) 1, 2, 3, 6, 7 and 17, Alzheimer's disease and Parkinson's disease.

A further aspect of the present invention provides methods for treating or inhibiting the aforementioned diseases and disorders of both acute and chronic forms of fibrosis of organs by administering a therapeutically effective amount of a pharmaceutical composition comprising a molecule of the present invention to a subject in need thereof.

BRIEF DESCRIPTION OF THE FIGURES

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Figure 1 sets forth competitive ELISA results. Celiac disease (CD) patient and healthy donor sera diluted 1:50, 1:100, 1:200, 1:400 (x -axis, values not in scale) mixed with scFvs (groups 2/A and 2/D) diluted 1:10 and tested on human TGase II. Secondary Abs: mouse mAb anti-tag SV5 and anti-mouse Ig conjugated with peroxidase.

30

Figure 2 sets forth schematic representation of the cloning procedures.

Figure 3 sets forth ELISA of supernatants of cultured HEK 293T cells transfected with seven constructs of scFv 2.8 TGase II fused to Fc domains CH3 or CH2-CH3 from man,

mouse and rat. Antigens: human TGase II, mouse TGase II and BSA. Secondary antibodies: mAb SV5 and anti-mouse Ig conjugated with peroxidase.

Figure 4 sets forth ELISA of supernatants of cultured HEK 293T cells transfected with seven constructs of scFv 2.8 TGase II fused to Fc domains CH3 or CH2-CH3 from human, mouse and rat. Antigens: human TGase II and mouse TGase II. Secondary antibodies: goat anti-human IgG and IgA and anti mouse or rat IgG conjugated with peroxidase

Figure 5 sets forth Western blotting of the miniantibodies 2.8 scFv MoIgGCH3 (1-3) and 2.8 HuIgG CH2-CH3 (4-6) with reducing agents (2 and 5), treated with glycosidase PNGase F (3 and 4), and in non-reducing, non- denaturing conditions (1 and 6). Secondary antibodies: mAb SV5 and anti-mouse Ig conjugated with alkaline phosphatase.

Figure 6 sets forth the inhibitory effect of purified 2.8 TGase II/HuG1CH2CH3 miniantibodies TGase II on activity. ELISA plates coated with the TGase II substrate gliadin were incubated with 0.2 mM Biopentilamine, 0.25 µg of TGase II and increasing amount of miniantibody (X axis). The incorporation of Biopentilamine is revealed by streptavidine conjugated with peroxidase.

Figure 7 sets forth ELISA time course of the serum anti TGase II miniantibody titer in 4 mice injected with scFv 3.7 TGase II/HuG1CH2CH3 pCDNA purified DNA. Serum dilution 1:50. Secondary antibodies: mAb SV5 and anti-mouse Ig conjugated with peroxidase. The control is a normal mouse serum.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to antibodies comprising at least an antigen-binding portion of an anti-TGase II antibody. The present invention relates specifically to a recombinant cloned human single-chain antibody fragment (scFv) against TGase II with inhibitory properties of TGase II enzymatic activity. The present invention is based on the discovery that human antibodies against TGase II are useful in treating disorders and diseases associated with TGase II enzymatic activity.

Molecules, including antibodies and fragments thereof, comprising at least an antigen binding portion of an anti-TGase II antibody are useful for the treatment of various pathological conditions including fibrotic diseases, scarring or diseases in which

fibrosis is evident (fibrosis-related pathologies) include both acute and chronic forms of fibrosis of organs, including all etiological variants of the following: pulmonary fibrosis, including interstitial lung disease and fibrotic lung disease, liver fibrosis, cardiac fibrosis including myocardial fibrosis, kidney fibrosis including chronic renal failure, skin fibrosis including scleroderma, keloids and hypertrophic scars; myelofibrosis (bone marrow fibrosis); all types of ocular scarring including proliferative vitreoretinopathy (PVR) and scarring resulting from surgery to treat cataract or glaucoma; inflammatory bowel disease of variable etiology, macular degeneration, Grave's ophthalmopathy, drug induced ergotism, psoriasis, glioblastoma in Li-Fraumeni syndrome, sporadic glioblastoma, myeloid leukemia, acute myelogenous leukemia, myelodysplastic syndrome, myeloproliferative syndrome, gynecological cancer, Kaposi's sarcoma, breast cancer, Hansen's disease, and collagenous colitis.

Other diseases and conditions that are amenable to treatment by inhibition of TGase II include ocular diseases especially cataract, cardiovascular diseases especially cardiac hypertrophy, atherosclerosis / restenosis, thyroid diseases, inflammatory diseases such as Crohn's disease, sporadic inclusion body myositis, allergic conjunctivitis, inflammatory bowel disease, autoimmune diseases such as celiac's disease, sporadic inclusion body myositis, inflammatory myopathies, dermatomyositis, polymyositis, dermatitis herpetiformis, type I diabetes, systemic lupus erythematosus, rheumatoid arthritis, osteoarthritis, myasthenia gravis, hemolytic anemia, multiple sclerosis, autoimmune diseases with subepidermal blisters, bullous pemphigoid, goodpasture disease, Sjogren syndrome, neurological diseases, including polyglutamine disease, spinobulbar muscular atrophy, dentatorubral-pallidoluysian atrophy, spinocerebellar ataxias (SCAs) 1, 2, 3, 6, 7 and 17, Alzheimer's disease, Huntington's disease, Parkinson's disease, rubropallidal atrophy and spinocerebellar palsy.

Antibodies

Full length antibodies, or immunoglobulins, comprise two heavy chains linked together by disulfide bonds and two light chains, each light chain being linked to a respective heavy chain by disulfide bonds in a "Y" shaped configuration. Proteolytic digestion of an antibody yields Fv (Fragment variable) and Fc (fragment crystalline) domains. The antigen binding domains, Fab', include regions where the polypeptide sequence varies. The term F(ab')₂ represents two Fab' arms linked together by disulfide

bonds. The central axis of the antibody is termed the Fc fragment. Each heavy chain has at one end a variable domain (VH) followed by a number of constant domains (CH). Each light chain has a variable domain (VL) at one end and a constant domain (CL) at its other end, the light chain variable domain being aligned with the variable domain of the heavy chain and the light chain constant domain being aligned with the first constant domain of the heavy chain (CH1). The variable domains of each pair of light and heavy chains form the antigen-binding site. The domains on the light and heavy chains have the same general structure and each domain comprises four framework regions, whose sequences are relatively conserved, joined by three hypervariable domains known as complementarity determining regions (CDR1-3). These domains contribute specificity and affinity of the antigen-binding site.

The isotype of the heavy chain (gamma, alpha, delta, epsilon or mu) determines immunoglobulin class (IgG, IgA, IgD, IgE or IgM, respectively). The light chain is either of two isotypes (kappa, κ or lambda, λ) found in all antibody classes.

It should be understood that when the terms "antibody" or "antibodies" are used hererin, this is intended to include intact (full -length) antibodies, such as polyclonal antibodies or monoclonal antibodies (mAbs), miniantibodies (see Example 9), single chain antibodies as well as proteolytic fragments thereof such as the Fab or F(ab')₂ fragments. Further included within the scope of the term "antibody" or "antibodies" are chimeric antibodies; human and humanized antibodies; recombinant and engineered antibodies, and fragments thereof. Furthermore, the DNA encoding the variable region of the antibody can be inserted into the DNA encoding other antibodies to produce chimeric antibodies (see, for example, US patent 4,816,567). Single chain antibodies fall within the scope of the present invention. Single chain antibodies can be single chain composite polypeptides having antigen binding capabilities and comprising amino acid sequences homologous or analogous to the variable regions of an immunoglobulin light and heavy chain (linked VH-VL or single chain Fv (ScFv)). Both V_H and V_L may copy natural monoclonal antibody sequences or one or both of the chains may comprise a CDR-FR construct of the type described in US patent 5,091,513, the entire contents of which are hereby incorporated herein by reference. The separate polypeptides analogous to the variable regions of the light and heavy chains are held together by a polypeptide linker. Methods of production of such single chain antibodies, particularly where the DNA encoding the polypeptide structures of the V_H and V_L chains are known,

may be accomplished in accordance with the methods described, for example, in US patents 4,946,778, 5,091,513 and 5,096,815, the entire contents of each of which are hereby incorporated herein by reference.

5 Additionally, CDR grafting may be performed to alter certain properties of the antibody molecule including affinity or specificity. A non-limiting example of CDR grafting is disclosed in US patent 5,225,539.

10 A "molecule having the antigen-binding portion of an antibody" as used herein is intended to include not only intact immunoglobulin molecules of any isotype and generated by any animal cell line or microorganism, but also the antigen-binding reactive fraction thereof, including, but not limited to, the Fab fragment, the Fab' fragment, the F(ab')₂ fragment, the variable portion of the heavy and/or light chains thereof, Fab miniantibodies (see WO 93/15210, US patent application 08/256,790, WO 96/13583, US patent application 08/817,788, WO 96/37621, US patent application 08/999,554, the entire contents of which are incorporated herein by reference) and
15 chimeric or single-chain antibodies incorporating such reactive fraction, as well as any other type of molecule or cell in which such antibody reactive fraction has been physically inserted, such as a chimeric T-cell receptor or a T-cell having such a receptor, or molecules developed to deliver therapeutic moieties by means of a portion of the molecule containing such a reactive fraction. Such molecules may be provided
20 by any known technique, including, but not limited to, enzymatic cleavage, peptide synthesis or recombinant techniques.

The term "Fc" as used herein is meant as that portion of an immunoglobulin molecule (Fragment crystallizable) that without being bound by theory mediates phagocytosis, triggers inflammation and targets Ig to particular tissues; the Fc portion is
25 also important in complement activation.

The term "epitope" is meant to refer to that portion of any molecule capable of being bound by an antibody or a fragment thereof that can also be recognized by that antibody. Epitopes or antigenic determinants usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and have
30 specific three-dimensional structural characteristics as well as specific charge characteristics.

An "antigen" is a molecule or a portion of a molecule capable of being bound by an antibody that is additionally capable of inducing an animal to produce antibody capable of binding to an epitope of that antigen. An antigen may have one or more than one epitope. The specific reaction referred to above is meant to indicate that the antigen
5 will react, in a highly selective manner, with its corresponding antibody and not with the multitude of other antibodies that may be evoked by other antigens.

A monoclonal antibody (mAb) is a substantially homogeneous population of antibodies to a specific antigen. MAbs may be obtained by methods known to those skilled in the art. See, for example Kohler et al (1975); US patent 4,376,110; Ausubel
10 et al (1987-1999); Harlow et al (1988); and Colligan et al (1993), the contents of which references are incorporated entirely herein by reference. The mAbs of the present invention may be of any immunoglobulin class including IgG, IgM, IgE, IgA, and any subclass thereof. A hybridoma producing a mAb may be cultivated *in vitro* or *in vivo*. High titers of mAbs can be obtained in *in vivo* production where cells from the
15 individual hybridomas are injected intraperitoneally into pristine-primed Balb/c mice to produce ascites fluid containing high concentrations of the desired mAbs. MAbs of isotype IgM or IgG may be purified from such ascites fluids, or from culture supernatants, using column chromatography methods well known to those of skill in the art.

20 Chimeric antibodies are molecules, the different portions of which are derived from different animal species, such as those having a variable region derived from a murine mAb and a human immunoglobulin constant region. Antibodies that have variable region framework residues substantially from human antibody (termed an acceptor antibody) and complementarity determining regions substantially from a
25 mouse antibody (termed a donor antibody) are also referred to as humanized antibodies. Chimeric antibodies are primarily used to reduce immunogenicity in application and to increase yields in production, for example, where murine mAbs have higher yields from hybridomas but higher immunogenicity in humans, such that human/murine chimeric mAbs are used. Chimeric antibodies and methods for their production are known in the
30 art (Better et al, 1988; Cabilly et al, 1984; Harlow et al, 1988; Liu et al, 1987; Morrison et al, 1984; Boulianne et al, 1984; Neuberger et al, 1985; Sahagan et al, 1986; Sun et al, 1987; Cabilly et al; European Patent Applications 125023, 171496, 173494, 184187, 173494, PCT patent applications WO 86/01533, WO 97/02671, WO 90/07861, WO

92/22653 and US patents 5,693,762, 5,693,761, 5,585,089, 5,530,101 and 5,225,539). These references are hereby incorporated by reference.

Besides the conventional method of raising antibodies *in vivo*, antibodies can be generated *in vitro* using phage display technology. Such a production of recombinant antibodies is much faster compared to conventional antibody production and they can be generated against an enormous number of antigens. In contrast, in the conventional method, many antigens prove to be non-immunogenic or extremely toxic, and therefore cannot be used to generate antibodies in animals. Moreover, affinity maturation (i.e., increasing the affinity and specificity) of recombinant antibodies is simple and relatively fast. Finally, large numbers of different antibodies against a specific antigen can be generated in one selection procedure. To generate recombinant monoclonal antibodies one can use various methods all based on phage display libraries to generate a large pool of antibodies with different antigen recognition sites. Such a library can be made in several ways: One can generate a synthetic repertoire by cloning synthetic CDR3 regions in a pool of heavy chain germline genes and thus generating a large antibody repertoire, from which recombinant antibody fragments with various specificities can be selected. One can use the lymphocyte pool of humans as starting material for the construction of an antibody library. It is possible to construct naive repertoires of human IgM antibodies and thus create a human library of large diversity. This method has been widely used successfully to select a large number of antibodies against different antigens. Protocols for bacteriophage library construction and selection of recombinant antibodies are provided in the well-known reference text Current Protocols in Immunology, Colligan et al (Eds.), John Wiley & Sons, Inc. (1992-2000), Chapter 17, Section 17.1.

Phage display of human antibody fragments has proved to be an effective method to investigate *in vivo* antibody responses in autoimmune diseases. In this method, a patient's antibody repertoire is expressed fused to the coat protein of a phage vector that carries the encoded protein gene, with each phage carrying a single antibody specificity. Briefly, antibody V regions are amplified with regions of overlap, either to a separately amplified linker region, or to each other, in such a way that mixing the two V regions recreates a linker region joining the two V genes. A number of amplification cycles without the addition of external primers are first performed. These involve an initial annealing of the regions of overlap followed by an extension. In this way V_H

regions are joined to V_L to make the single-chain antibody fragment (scFv) that is, finally, cloned upstream the gene III coding for the minor coat protein g3 in a phagemid vector. Following E. coli transformation and infection by a helper phage, phage particles expressing a reactive antibody on their surface are produced. Antibodies specific to a given antigen can be isolated from phage antibody libraries by recursive cycles of binding on an immobilized antigen, washing, elution, and amplification by bacterial infection of bound phages. Finally, bacterial clones expressing single antibody specificity are characterized for the epitope recognized.

10 Antibodies against TGase II from Celiac Disease Patients

The inventors of the present invention have previously reported (Marzari et al., 2001, J. Immunol. 166: 4170-4176) the production and analysis of six phage antibody libraries from the peripheral and intestinal lymphocytes of three celiac disease (CD) patients. They were able to isolate antibodies to TGase II from all intestinal lymphocytes libraries but not from those obtained from peripheral lymphocytes. This is in contrast to antibodies against gliadin, which could be obtained from all libraries, indicating that the humoral response against TGase II occurs at the local level, whereas that against gliadin occurs both peripherally and centrally. On the basis of the features reported above, antibody scFvs selected from the three libraries were grouped according to the V_H family, the CDR3 sequence and the framework mutations. Clones with similar CDR3s with a limited number of mutations in the other parts of the molecules (CDR1, CDR2 and framework regions) were considered to be derived from the same ancestor and assigned to the same group. In general, V_H gene use was restricted to three (V_H 5, V_H 3, V_H 1) of the seven human antibody V_H families, with many of the V_H genes belonging to the V_H5 family (12/29 cases) with a preferential use of the DP73 segment (10/29 cases). Interestingly, this was the only V_H gene segment selected from all three libraries, indicating the possible preferential usage of this segment in the autoimmune response to TGase II. To determine how many different TGase II epitopes were recognized by the different selected scFvs, two approaches were taken. In the first, crossreactivity of the different scFvs to guinea pig TGase II by ELISA was determined, and it was found that only a fraction of the antibodies were able to recognize guinea pig TGase II with OD values comparable to those obtained with human TGase II, with negative scFvs giving OD values similar to negative controls. In the second approach,

an inhibition ELISA was carried out using couples of different scFv. After this, all clones could be grouped into two main association groups, termed epitope1 (Ep1) and epitope 2 (Ep2), with Ep1 recognized almost exclusively by those scFv belonging to the VH5 family. All scFvs recognizing Ep1 also recognized guinea pig TGase II (GP TGase II), whereas none of the others was able to do so, suggesting that Ep1 (defined as Ep1/GP+) is common to human and GP TGase II. More recently, the inventors have described the cloning of human TGase II in the pET28b expression vector. Although this cloning was effective, most of the TGase II synthesized in bacteria was present as insoluble inclusion bodies and only a reduced amount of functional enzyme could be extracted and purified. The inclusion body fraction can be solubilized by 4 M urea but neither the enzymatic activity nor the antigenic functionality is recovered by renaturing procedures. Also, the soluble fraction, treated with 4M urea is no longer recognized by CD sera and CD phage display antibodies to TGase II, strongly suggesting that the epitopes recognized by these antibodies are very sensitive to denaturation. These findings suggested investigating the epitope specificity of these antibodies with a rational approach in which PCR primers recognizing DNA sequences encoding amino acid sequences at the ends of each of the 4 main TGase II domains and the loop connecting the core domain to the C1 domain are used to amplify these domains. By using transglutaminase gene fragments, the inventors found that the epitope recognized by the cloned antibodies is located in the core region of the enzyme and this is identical to the epitope recognized by anti-transglutaminase antibodies found in the serum of celiac disease patients. This result outlines the possibility that a limited number of immunodominant epitopes are involved in the humoral immune response. These antibodies were also inspected for their inhibitory properties of the TGase II enzymatic activity. The human monoclonal antibodies displayed a dose-dependent inhibitory effect toward the catalytic activity of the enzyme *in vitro* and *in situ*. Preincubation of the enzyme with CaCl₂ did not affect the inhibition caused by human monoclonal antibodies.

30 Preferred Embodiments

One aspect of the present invention is directed to neutralizing antibodies and more generally to a molecule that comprises at least the CDR3 variable regions of SEQ ID NO: 9 or SEQ ID NO: 10 of a recombinant antibody which inhibits TGase II enzymatic

activity. Another aspect of the present invention is directed to recombinant antibody molecule comprising the CDR3 variable region of SEQ ID NO: 9 and also comprising SEQ ID NO: 2, the antibody molecule having specific binding affinity for TGase II and being capable of inhibiting the enzymatic activity of the TGase II enzyme.

- 5 A further aspect of the present invention is directed to a recombinant antibody molecule comprising the CDR3 variable region of SEQ ID NO: 10 and also comprising SEQ ID NO:6, the antibody molecule having specific binding affinity for TGase II and being capable of inhibiting the enzymatic activity of the TGase II enzyme

10 The molecule having the CDR3 variable regions of the antibody according to the present invention can be used in a method for inhibiting TGase II enzymatic activity.

A preferred embodiment of such antibodies/molecules, obtained from a phage display of human antibody library is the clone 2/A2, presented in **Table 2** with the unique V_H CDR3 and V_L CDR3 sequences given. All the obtained antibodies were
15 characterized for a series of genetic and biochemical features to identify the best ones to be used in further experiments. The results showed that the antibody RS1 (acronym of Renal Scarring 1) was the most preferable because of the following *inter alia*:

A. Sequence

1. Unique V_H/V_L cDNA sequence due to: a. CDR3 specific rearrangement b. high
20 number of mutations respect to the germline.

B. Structure

1. Optimal V_H/V_L pairing. It was found that the substitution of the V_L chain by random shuffling causes the loss of the antibody reactivity.

C. Reactivity

- 25 1. Very good inhibitory properties against human TGase II cross-linking enzymatic activity.
2. Very good affinity. The k_{on}/k_{off} estimated by plasmon resonance was in the range of 5 nM.
3. Crossreactivity to animal TGase II (Guinea pig, mouse), which is very important
30 to set up animal models.
4. Antigenic determinant located in the core enzymatic region.

D. Production

1. Good production level as soluble form at periplasmic level as well as in the culture supernatant.

E. Engineering

1. Suitable for further modification by adding human or animal antibody constant domains. The antibody maintains its reactivity.
2. Expression in vertebrate cells. The antibody is well expressed and functional *in vivo* when subcloned in a eukaryotic vector.

F. Antigenicity

1. It was demonstrated that the antibody did not elicit an antibody response when expressed *in vivo* in a mouse model for up to one year.

The nucleotide sequences of the V_L, linker and V_H domains of the preferred embodiment of the present invention are presented below.

Clone 2/A2 V_L nucleotide sequence (SEQ ID NO: 1)

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1  GATATGTGTG TGACCCAGTC TCCTTCACCC CTGTCTGCAT CTGTAGGAGA CAGAGTCACC ATCACTTGCC 70
71  GGGCCAGTCA GAGTATTAGT AGGTGGTTGG CCTGGTATCA GCAGAAACCA GGGAAAGCCC CTAAGCTCCT 140
141 GATTTATAAG GCGTCTAGTT TACAAAGTGG GGTCCCATCA AGGTTTCAGCG GCAGTGGATC TGGGACAGAC 210
211 TTCCTCTCA CCATCAGCAG CCTGCAGCCT GATGATTTTG CAACTTATTA CTGCCTACAC TATAATAGTT 280
281 ATTCTCCGCG GTACACTTTT GGCAGGGGGA CCAAAGTGGA TATCAAA 327

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Clone 2/A2 V_L-corresponding amino acid sequence (SEQ ID NO: 2)

Asp Ile Val Leu Thr Gln Ser Pro Ser Thr Leu Ser Ala Ser Val Gly

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Ser Ile Ser Arg Trp

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile

Tyr Lys Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro

Asp Asp Phe Ala Thr Tyr Tyr Cys Leu His Tyr Asn Ser Tyr Ser Pro

5 Arg Tyr Thr Phe Gly Arg Gly Thr Lys Val Asp Ile Lys

Clone 2/A2 nucleotide sequence of Linker (SEQ ID NO: 3):

10 1 TCCGGAGGGT CGACCATAAC TTCGTATAAT GTATACTATA CGAAGTTATC CTCGAGCGGT ACC 63

Clone 2/A2 corresponding amino-acid sequence of Linker (SEQ ID NO: 4):

15 Ser Gly Gly Ser Thr Ile Thr Ser Tyr Asn Val Tyr Tyr Thr Lys Leu

Ser Ser Ser Gly Thr

20 **Clone 2/A2 nucleotide sequence of V_H (SEQ ID NO: 5):**

1 CAGGTCCAGC TTGTGCAGTC TGGAGCAGAG GTGAAAAAGC CCGGGGAGTC TCTGAAGATC TCCTGTAAGG 70

25 71 GTTCTGGATA CAGGTTTACC AGCTACTGGA TCGGCTGGGT GCGCCAGATG CCCGGGAAAG GCCTGGAGTG 140

141 GATGGGGATC ATCTATCCTG GTGACTCTGA TCCAGATAC AGCCCGTCCT TCCAAGGCCA GGTCAACATC 210

211 TCAGCCGACA GGTCCAGCAG CACCGCCTAC CTGCAGTGGG GCAGCCTGAA GGCCTCGGAC ACCGCCATGT 280

30 281 ATTACTGTGC GAGACCATCA GTGATCGATA CGACGGACGC TTTTGATATC TGGGGCCAAG GGACCCTGGT 350

351 CACCGTCTCC 360

35 **Clone 2/A2 corresponding amino acid sequence of V_H (SEQ ID NO: 6):**

Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Lys Lys Pro Gly Glu

Ser Leu Lys Ile Ser Cys Lys Gly Ser Gly Tyr Arg Phe Thr Ser Tyr

40 Trp Ile Gly Trp Val Arg Gln Met Pro Gly Lys Gly Leu Glu Trp Met

Gly Ile Ile Tyr Pro Gly Asp Ser Asp Pro Arg Tyr Ser Pro Ser Phe

Gln Gly Gln Val Thr Ile Ser Ala Asp Arg Ser Ser Ser Thr Ala Tyr

Leu Gln Trp Ser Ser Leu Lys Ala Ser Asp Thr Ala Met Tyr Tyr Cys

5

Ala Arg Pro Ser Val Ile Asp Thr Thr Asp Ala Phe Asp Ile Trp Gly

Gln Gly Thr Leu Val Thr Val Ser

10 **Clone 2/A2 corresponding amino acid sequence of V_H CDR3 (SEQ ID NO: 9):**

Pro Ser Val Ile Asp Thr Thr Asp Ala Phe Asp Ile

Clone 2/A2 corresponding amino acid sequence of V_L CDR3 (SEQ ID NO: 10):

Leu His Tyr Asn Ser Tyr Ser Pro Arg Tyr Thr

15

The antibody of the invention is preferably of human origin, i.e. it is entirely derived from an antibody repertoire obtained from human serum. These antibodies have both framework and antigen complementary regions (CDR) of human origin, unlike humanized antibodies where only the framework is of human origin, while the CDR are of murine origin.

In one embodiment, the recombinant antibody of the invention comprises a V_L chain consisting of an amino acid sequence preferably corresponding to SEQ ID NO: 2 that could be covalently linked to a V_H chain which preferably corresponds to the amino acid sequence of SEQ ID NO: 6. In another embodiment, the recombinant antibody comprises at least one of the polypeptide sequences having SEQ ID NO: 2 and SEQ ID NO: 6. In a preferred embodiment, said recombinant antibody comprises V_L and V_H regions having SEQ ID NO: 2 and SEQ ID NO: 6, and optionally a linker comprising SEQ ID NO: 4.

In another aspect, the present invention provides pharmaceutical compositions comprising said molecules. The pharmaceutical compositions according to the present invention is similar to those used for passive immunization of humans with other antibodies. Typically, the molecules of the present invention will be suspended in a sterile saline solution for therapeutic uses. The pharmaceutical compositions may alternatively be formulated to control release of active ingredient (molecule comprising

the antigen binding portion of an antibody) or to prolong its presence in a patient's system. Numerous suitable drug delivery systems are known and include, e.g., implantable drug release systems, hydrogels, hydroxymethylcellulose, microcapsules, liposomes, microemulsions, microspheres, and the like. Controlled release preparations
5 can be prepared through the use of polymers to complex or adsorb the molecule according to the present invention. For example, biocompatible polymers include matrices of poly (ethylene-co-vinyl acetate) and matrices of a polyanhydride copolymer of a stearic acid dimer and sebacic acid (Sherwood et al, 1992). The rate of release molecule according to the present invention, i.e., of an antibody or antibody fragment,
10 from such a matrix depends upon the molecular weight of the molecule, the amount of the molecule within the matrix, and the size of dispersed particles (Saltzman et al., 1989 and Sherwood et al., 1992). Other solid dosage forms are described in Ansel et al., 1990 and Gennaro, 1990.

The pharmaceutical composition of this invention may be administered by any
15 suitable means, such as orally, intranasally, subcutaneously, intramuscularly, intravenously, intra-arterially, intralesionally or parenterally. Ordinarily, intravenous (i.v.) or parenteral administration will be preferred.

It will be apparent to those of ordinary skill in the art that the therapeutically effective amount of the molecule according to the present invention will depend, *inter*
20 *alia* upon the administration schedule, the unit dose of molecule administered, whether the molecule is administered in combination with other therapeutic agents, the immune status and health of the patient, the therapeutic activity of the molecule administered and the judgment of the treating physician. As used herein, a "therapeutically effective amount" refers to the amount of a molecule required to alleviate one or more symptoms
25 associated with a disorder being treated over a period of time.

Although an appropriate dosage of a molecule of the invention varies depending on the administration route, age, body weight, sex, or conditions of the patient, and should be determined by the physician in the end, in the case of oral administration, the daily dosage can generally be between about 0.01-200 mg, preferably about 0.01-10 mg,
30 more preferably about 0.1-10 mg, per kg body weight. In the case of parenteral administration, the daily dosage can generally be between about 0.001-100 mg, preferably about 0.001-1 mg, more preferably about 0.01-1 mg, per kg body weight. The daily dosage can be administered, for example in regimens typical of 1-4 individual

administration daily. The molecules of the present invention may be administered in one dose or periodically, for several days, weeks, months, years or indefinitely the dose may be daily, as described, bi-weekly or even weekly, as required. Various considerations in arriving at an effective amount are described, e.g., in Goodman and Gilman's: The Pharmacological Bases of Therapeutics, 8th ed., Pergamon Press, 1990; and Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Co., Easton, Pa., 1990.

The molecule of the present invention as an active ingredient is dissolved, dispersed or admixed in an excipient that is pharmaceutically acceptable and compatible with the active ingredient as is well known. Suitable excipients are, for example, water, saline, phosphate buffered saline (PBS), dextrose, glycerol, ethanol, or the like and combinations thereof. Other suitable carriers are well known to those in the art. (See, for example, Ansel et al., 1990 and Gennaro, 1990). In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents.

The present invention also provides for a nucleic acid molecule, which contains a nucleotide sequence encoding the molecule having the antigen-binding portion of an antibody that inhibits the enzymatic activity of TGase II and a host cell transformed with this nucleic acid molecule. This nucleic acid molecule comprises SEQ ID NO: 1 and SEQ ID NO: 5 and may also comprise the linker sequence SEQ ID NO: 3. Furthermore, also within the scope of the present invention is a nucleic acid molecule containing a nucleotide sequence having at least 90% sequence identity, preferably about 95%, and more preferably about 97% identity to the above encoding nucleotide sequence as would well understood by those of skill in the art.

The invention also provides nucleic acids that hybridize under high stringency conditions to polynucleotides comprising SEQ ID NO: 1 and SEQ ID NO: 5 or the complement thereof. As used herein, highly stringent conditions are those which are tolerant of up to about 5-20% sequence divergence, preferably about 5-10%. Without limitation, examples of highly stringent (-10°C below the calculated T_m of the hybrid) conditions use a wash solution of 0.1 X SSC (standard saline citrate) and 0.5% SDS at the appropriate T_i below the calculated T_m of the hybrid. The ultimate stringency of the conditions is primarily due to the washing conditions, particularly if the hybridization conditions used are those which allow less stable hybrids to form along

with stable hybrids. The wash conditions at higher stringency then remove the less stable hybrids. A common hybridization condition that can be used with the highly stringent to moderately stringent wash conditions described above is hybridization in a solution of 6 X SSC (or 6 X SSPE), 5 X Denhardt's reagent, 0.5% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA at an appropriate incubation temperature T_i .
 5 *See generally* Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2d edition, Cold Spring Harbor Press (1989) for suitable high stringency conditions.

Stringency conditions are a function of the temperature used in the hybridization experiment and washes, the molarity of the monovalent cations in the hybridization solution and in the wash solution(s) and the percentage of formamide in the hybridization solution. In general, sensitivity by hybridization with a probe is affected by the amount and specific activity of the probe, the amount of the target nucleic acid, the detectability of the label, the rate of hybridization, and the duration of the hybridization. The hybridization rate is maximized at a T_i (incubation temperature) of 20-25°C below T_m for DNA:DNA hybrids and 10-15°C below T_m for DNA:RNA hybrids. It is also maximized by an ionic strength of about 1.5M Na^+ . The rate is directly proportional to duplex length and inversely proportional to the degree of mismatching.
 10
 15

Specificity in hybridization, however, is a function of the difference in stability between the desired hybrid and "background" hybrids. Hybrid stability is a function of duplex length, base composition, ionic strength, mismatching, and destabilizing agents (if any).
 20

The T_m of a perfect hybrid may be estimated for DNA:DNA hybrids using the equation of Meinkoth *et al* (1984), as

$$25 \quad T_m = 81.5^\circ\text{C} + 16.6 (\log M) + 0.41 (\%GC) - 0.61 (\% \text{ form}) - 500/L$$

and for DNA:RNA hybrids, as

$$T_m = 79.8^\circ\text{C} + 18.5 (\log M) + 0.58 (\%GC) - 11.8 (\%GC)^2 - 0.56(\% \text{ form}) - 820/L$$

where M, molarity of monovalent cations, 0.01-0.4 M NaCl,

%GC, percentage of G and C nucleotides in DNA, 30%-75%,
 30

% form, percentage formamide in hybridization solution, and

L, length hybrid in base pairs.

T_m is reduced by 0.5-1.5°C (an average of 1°C can be used for ease of calculation) for each 1% mismatching.

The T_m may also be determined experimentally. As increasing length of the hybrid (L) in the above equations increases the T_m and enhances stability, the full-length rat gene
5 sequence can be used as the probe.

Filter hybridization is typically carried out at 68°C, and at high ionic strength (e.g., 5 - 6 X SSC), which is non-stringent, and followed by one or more washes of increasing stringency, the last one being of the ultimately desired high stringency. The equations for T_m can be used to estimate the appropriate T_i for the final wash, or the
10 T_m of the perfect duplex can be determined experimentally and T_i then adjusted accordingly.

The present invention also relates to a vector comprising the nucleic acid molecule of the present invention. The vector of the present invention may be, e.g., a plasmid, cosmid, virus, bacteriophage or another vector used e.g. conventionally in
15 genetic engineering, and may comprise further genes such as marker genes which allow for the selection of said vector in a suitable host cell and under suitable conditions.

Furthermore, the vector of the present invention may, in addition to the nucleic acid sequences of the invention, comprise expression control elements, allowing proper expression of the coding regions in suitable hosts. Such control elements are known to
20 the artisan and may include a promoter, a splice cassette, translation initiation codon, translation and insertion site for introducing an insert into the vector.

Preferably, the nucleic acid molecule of the invention is operatively linked to said expression control sequences allowing expression in eukaryotic or prokaryotic cells.

Control elements ensuring expression in eukaryotic or prokaryotic cells are well known
25 to those skilled in the art. As mentioned herein above, they usually comprise regulatory sequences ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript.

Methods for construction of nucleic acid molecules according to the present invention, for construction of vectors comprising said nucleic acid molecules, for
30 introduction of said vectors into appropriately chosen host cells, for causing or achieving the expression are well-known in the art (see, e.g., Sambrook et al., 1989; Ausubel et al., 1994).

The invention also provides for conservative amino acid variants of the antibody of the invention. Variants according to the invention also may be made that conserve the overall molecular structure of the encoded proteins. Given the properties of the individual amino acids comprising the disclosed protein products, some rational
5 substitutions will be recognized by the skilled worker. Amino acid substitutions, *i.e.* "conservative substitutions," may be made, for instance, on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved.

For example: (a) nonpolar (hydrophobic) amino acids include alanine, leucine,
10 isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; (b) polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; (c) positively charged (basic) amino acids include arginine, lysine, and histidine; and (d) negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Substitutions typically may be made within groups (a)-(d). In addition,
15 glycine and proline may be substituted for one another based on their ability to disrupt α -helices. Similarly, certain amino acids, such as alanine, cysteine, leucine, methionine, glutamic acid, glutamine, histidine and lysine are more commonly found in α helices, while valine, isoleucine, phenylalanine, tyrosine, tryptophan and threonine are more commonly found in β -pleated sheets. Glycine, serine, aspartic acid, asparagine, and
20 proline are commonly found in turns. Some preferred substitutions may be made among the following groups: (i) S and T; (ii) P and G; and (iii) A, V, L and I. Given the known genetic code, and recombinant and synthetic DNA techniques, the skilled scientist readily can construct DNAs encoding the conservative amino acid variants.

The invention also provides that the antibody of the invention may comprise a sequence
25 having preferably 90% identity, more preferably 95% identity, and even more preferably 98% or 99% identity to SEQ ID NO: 2 or 6, provided that this antibody has the same or substantially the same biological activity as the antibody of the invention.

As used herein, "sequence identity" between two polypeptide sequences indicates the percentage of amino acids that are identical between the sequences.
30 "Sequence similarity" indicates the percentage of amino acids that either are identical or that represent conservative amino acid substitutions.

Having now generally described the invention, the same will be more readily understood through reference to the following examples, which are provided by way of illustration and are not intended to be limiting of the present invention.

5

EXAMPLES

Materials and Methods

(i) **Plasmids and bacteria.** DH5aF' (F'/endA1 hsdR17 ($rK^- mK^+$) supE44 thi-1 recA1 gyrA (Nal^r) relA1 D (lacZYA-argF)U169 deoR (F80dlacD(lacZ)M15)) was used for
10 phage propagation, HB2151 (K12, ara Δ (lac-pro), thi/F' proA⁺B⁺, lacI^qZ Δ M15) was used to make soluble scFv. pDAN5 (Sblattero and Bradbury, 2000, Nat. Biotechnol. 18:75) was used for scFv display.

(ii) **Lymphocyte RNA preparation and library construction.** Total RNA was prepared as previously described (Chomczynski and Sacchi, 1987, Anal. Biochem. 162:156)
15 from 10 ml of PBL or IBL from three previously untreated CD adult patients with high titres of anti- α gliadin, anti-human TGase II and endomysial antibodies. All the patients had HLA-DQ2 histocompatibility antigens. PBL were purified by Ficoll Hypaque (Pharmacia) while IBL were treated directly. cDNA was synthesized using random hexamers and SuperScript II Reverse Transcriptase (Gibco BRL). Immunoglobulin V-
20 regions were amplified using specific V region primers (Sblattero and Bradbury, 1998, Immunotechnology 3:271) and assembled into scFv as reported in Krebber et al., 1997, J. Immunol. Methods, 201:35 before cloning into pDAN5.

(iii) **Antigens.** Purified α -gliadin was prepared as previously described (Bernardin et al., 1967, J. Biol. Chem. 242:445). Guinea pig TGase II (gp TGase II), BSA and
25 lysozyme were purchased from Sigma. Human TGase II (h TGase II) cDNA was obtained by amplifying cDNA from an intestinal biopsy with specific primers (Gentile et al., 1991, J. Biol. Chem. 266:478) and cloning into pTrcHis (Invitrogen). The TGase II was extracted as soluble cytoplasm fraction and purified by Ni-NTA chromatography (Qiagen).

(iv) **Selection and testing of phage antibodies.** Rescue of phagemid particles was as described in Marks et al., 1991, J. Mol. Biol. 222:581. Panning was performed by
30 adding phages diluted to 2% non-fat milk in PBS (MPBS) to immunotubes (Nunc)

coated with purified human TGase II and α -gliadin (10 μ g/ml), washing 20 times with PBS, 0.1% Tween20 (PBST) and 20 times with PBS, followed by elution with 1 ml of *E. coli* cells at 0.5 OD₆₀₀ for 30' at 37° C and overnight growth after addition of ampicillin, helper phage and kanamycin. The panning procedure was repeated up to
5 three times. After selection, 48 individual clones from each selection were screened for reactivity to their respective antigens as well as irrelevant antigens (BSA and lysozyme) by microtiter plate ELISA (Marks et al., 1991, J. Mol. Biol. 222:581).

(v) **Preparation and testing of soluble scFv.** Phagemids from individual colonies were infected into HB2151, grown to OD₆₀₀ 0.2, induced with 1 mM, isopropyl- β -D-thiogalactopyranoside (IPTG) and further grown overnight at 28°C. scFv were used
10 directly as supernatants of induced bacterial cultures. ELISA was performed with soluble scFv essentially as described for phage particles. Culture supernatants containing scFv were serially diluted with MPBS, added to microtiter plate wells and detected with a mAb recognizing the SV5 tag (Hanke T. et al., 1992, J. Gen. Virol. 73:653) found at the scFv C terminus, and an HRP conjugated secondary anti mouse Ig
15 antiserum (Dako) and 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB) as substrates. Competitive ELISA was performed as conventional ELISA, except that 100 μ l of individual scFv, diluted 1:10 with MPBS, were mixed with equal volumes of serially diluted CD sera positive for TGase II.

(vi) **Immunofluorescence.** Immunofluorescence was performed on histological sections of human umbilical cord prepared according to standard techniques. ScFvs from bacterial culture supernatant were added to the sections, incubated for 30' at room temperature in a moist chamber, followed by mAb SV5 and fluorescein isothiocyanate (FITC) labeled anti mouse IgG (Dako). Double labeling was performed by adding CD
20 sera, diluted 1:200, to the same section and revealed with tetramethylrhodamine B isothiocyanate (TRITC) labeled anti human Ig antiserum (Dako).

EXAMPLE 1: Library construction

The scFv libraries were constructed from either PBL or IBL from three
30 untreated CD adult patients with high anti α -gliadin and human transglutaminase antibody titers, the latter determined both by anti-endomysium antibodies assayed on human umbilical cord sections, as well as human TGase II ELISA (Sblattero et al.,

2000, Am. J. Gastroenterol. 95:1253). The biopsy materials were obtained from patients undergoing intestinal biopsy to confirm their diagnoses.

V_H and V_L chains were amplified from PBL (peripheral blood lymphocyte) and IBL (intestinal biopsy lymphocyte) cDNA by PCR using a set of oligonucleotides that
5 recognize all human V genes (Sblattero and Bradbury, 1998, Immunotechnology 3:271). For the V_H chains the 3' primer was specific for IgA antibodies.

V_H and V_L amplicates were assembled by PCR (Krebber et al., 1997, J. Immunol. Methods, 201:35) and cloned into the phagemid vector pDAN5 (Sblattero et al., 2000, Am. J. Gastroenterol. 95:1253) to obtain the primary libraries. The six libraries ranged
10 in size from of 5×10^6 to 5×10^7 , and twenty clones picked at random from each were shown to contain full-length scFv with different *Bst*NI restriction pattern, confirming the diversity and integrity of the libraries. The libraries are reported according to the donor patient, and given reference numbers 2, 3 and 4.

15 EXAMPLE 2: Library selection

Antibodies were affinity selected using purified cloned human TGase II and α -gliadin. According to this method, V genes derived from a patient's lymphocytes are used to express a patient's antibody repertoire fused to the coat protein of a filamentous phage vector. Each phage carries single antibody specificity and can be selected by
20 subjecting the phage library to recursive rounds of binding, washing and elution on the target antigen. After every cycle of selection the eluted phages were reamplified for the next cycle and tested by ELISA against the antigen used for the selection. When the polyclonal signal of the eluted phages was positive by ELISA, the selection was considered concluded, and 48 individual clones were analyzed from each selection.
25 This was adopted to avoid overgrowth of deleted or well-expressed positive clones during the cycles of amplification. Clones positive on the antigen used for the selection (TGase II and α -gliadin) and negative on control antigens (BSA and lysozyme) were analysed by *Bst*NI fingerprinting and sequencing to determine the number of different clones, and these results are reported in **Table 1**. Interestingly, after only one cycle of
30 selection all three IBL libraries showed a variable number of positive clones to human TGase II, whereas no positive clones to TGase II were registered after three cycles of selection using the PBL libraries. To further control this result, the antigenic quality of the cloned human TGase II was assessed using a large naïve library described in

(Sblattero and Bradbury, 2000, Nat. Biothechnol. 18:75). This was prepared from V_{μ} , V_{λ} and V_{κ} genes with an estimated diversity of 7×10^7 different V_H and V_L genes recombined to a final estimated diversity of 10^{11} . After two cycles of selections 12 different clones to TGase II were identified, confirming that the failure of the PBL
 5 selections was not due to the low efficiency of the system in isolating rare scFv but rather to the lack of such antibodies in the libraries. In contrast, different antibodies against α -gliadin were obtained from all six CD libraries, as well as the naïve library after two rounds of selection. The total number of positives and different positives clones is reported in **Table 1**.

10

Table 1. Antibodies to TGase II or α -gliadin in CD patients

Patient	Source ^A	Tissue transglutaminase II				α -gliadin			
		Round ^B	Tested	Positives	Different clones ^C	Round ^B	Tested	Positives	Different clones ^C
2	PBL	3	48	0	0	2	48	14	4
2	IBL	1	48	19	15	2	48	9	2
3	PBL	3	48	0	0	2	48	21	10
3	IBL	1	48	23	19	2	48	16	4
4	PBL	3	48	0	0	2	48	10	2
4	IBL	1	48	10	6	2	48	8	2
-	Naïve	2	48	16	12	2	48	29	12

^A Antibody libraries from peripheral blood (PBL) or intestinal biopsy (IBL) lymphocytes of three CD patients (2, 3, 4). ^B Rounds of affinity selection. ^C Number of
 15 different clones determined by *Bst*NI fingerprinting and sequencing.

EXAMPLE 3: V family usage and somatic mutations

The V genes from the different anti TGase II scFv clones were sequenced and the V_H and V_L families as well as the gene segments used were assessed by screening
 20 against the VBASE database (**Table 2**, columns 2 and 5). The amino acid sequence of the complementarity determining regions (CDR3) are reported in **Table 2** (columns 3 and 6), and the numbers of silent (S) mutations leading to base substitution or amino acid replacement (R), for V_H and V_L , determined by comparing the V sequence to the

closest germline sequence, are reported in columns 4 and 7. On the basis of the features reported above, scFvs selected from the three libraries (2, 3 and 4) were grouped according to the V_H family, the CDR3 sequence and the framework mutations. Clones with similar CDR3s with a limited number of mutations in the other parts of the molecules (CDR1, CDR2 and framework regions) were considered to be derived from the same ancestor and assigned to the same group, but given different reference numbers (column 1). In general, V_H gene use was restricted to three (V_H 5, V_H 3, V_H 1) of the seven human antibody V_H families (for a complete classification see VBASE), with many of the V_H genes belonging to the VH5 family (12/29 cases) with a preferential use of the DP73 segment (10/29 cases). Interestingly, this was the only V_H gene segment selected from all three libraries, indicating the possible preferential usage of this segment in the autoimmune response to TGase II. This is in contrast to the V_L sequences, which appeared to be completely random, with V_K and V_λ chains belonging to many of the ten V_λ and six V_K families being found. The overall ratio of replacement over silent mutations of both V_H and V_L domains (Table 2, columns 4 and 7) was always greater than 1 suggesting that the mutations were the consequence of antigen-driven selections. A big difference between the scFvs containing DP73 and those containing other V_H segments is that the DP73 segments appeared to be able to pair with any light chains, whereas the others appeared to pair only to specific light chains. This is particularly striking in group 3/D where a series of somatically mutated VKI/DPK9 genes are coupled exclusively to VH1/DP10 segments. As a general rule, most of the selected scFv of the three libraries show the same feature. Since during antibody library construction V_H and V_L regions are randomly assembled, it is likely that these co-selected V_H/V_L pairs represent cases where heavy and light chain both need to be present for binding to occur, and hence, may mirror the *in vivo* pairing, whereas those scFv containing DP73, appear to be able to pair with any light chain, and so presumably have most of the binding activity located in the heavy chain. These results are in contrast to those obtained when similar selections were carried out on a naïve library made from peripheral blood lymphocytes from 40 different healthy donors (Sblattero and Bradbury, 2000, Nat. Biotechnol. 18:75), in which of 12 scFvs selected, only two VH5 gene segments were found, and both of these were derived from the COS-25 gene. The remaining ten scFvs had 1 VH2, 4 VH3 and 5 VH6, VH genes, while the V_L genes were randomly distributed among four different families.

Table 2. V family usage and somatic mutations of anti antibodies
Summary of the features of 40 scFv selected against TGase II mutation

Library/ref. No. ^A	V _H family/ V _H segment ^B	V _H CDR3 ^C	SEQ ID No:	V _H R/S ^D	V _L CDR3 ^C	SEQ ID No:	V _L R/S ^D
2/A 1	VH5/DP73	PSVPDATDAFDI	7	4/3	QQYNSYSPGYT	8	4/3
2/A 2	VH5/DP73	PSVIDTTDAFDI	9	2/2	LHYNSYSPRYT	10	3/3
2/A 3	VH5/DP73	PSVIDTTDAFDI	9	2/2	QHYNYSLSMYT	11	2/5
2/B	VH5/DP73	PKDYDSGSSLVY	12	6/2	QKYNSYSPGYT	13	-
2/C 1	VH5/DP73	QAYCSGGSCYRGGAFDI	14	4/1	SSYTSSSTVYV	15	-
2/C 2	VH5/DP73	QAYCSGGSCYRGGAFDI	14	5/2	QQSYSTPRT	16	1/-
2/C 3	VH5/DP73	QGYCSGGSCYRGGAFDI	17	1/1	QQSYSTPRT	16	1/-
2/D 1	VH3/DP51	DSPKGMVDV	18	-/1	AAWDDSRRGV	19	-
2/D 2	VH3/DP51	DSPKGMVDV	18	3/2	AAWDDSRRGV	19	5/1
2/E	VH1/DP88	RLSDGYKHYYFDY	20	6/1	QQSYSPPT	21	3/4
2/F	VH1/DP88	SLVMLQTLLEDY	22	6/-	QQSYSTLKT	23	-/2
3/A	VH5/DP73	PYYDSTGPFYD	24	-	QHYNRYSPGYT	25	4/2
3/B	VH5/DP73	IRSDSSGWYRQAFDI	26	6/1	QQYNNWPLT	27	6/5
3/C 1	VH3/DP33	DYGDYDYMHV	28	6/2	QQYVGSPWT	29	11/8
3/C 2	VH3/DP33	DYGDYDYMDV	30	6/2	QQSYSSPPT	31	1/7
3/C 3	VH3/DP33	DYGDYDYMDV	30	8/3	QQYGSSPT	32	1/1
3/D 1	VH1/DP10	RVAAPGHFYFDY	33	7/6	QQTYSLPRT	34	5/1
3/D 2	VH1/DP10	RVAAPGHFYFDY	33	7/6	QQYNSFPLT	35	3/1
3/D 3	VH1/DP10	RVAAPGHFYFDY	33	7/6	QQSYNTLWT	36	5/1
3/D 4	VH1/DP10	RVAAPGHFYFDY	33	5/4	QQSYSTPRT	37	2/-
3/D 5	VH1/DP10	RVAAPGHFYFDY	33	5/4	QTDLQSSSD	38	-
3/D 6	VH1/DP10	RVAAAGHFYFDY	39	4/3	QQSYSTLWT	40	10/5
3/E 1	VH1/DP75	RFKFGNYNYGMVD	41	9/4	QQSYTIPRT	42	1/4
3/E 2	VH1/DP75	RFKFGNYNYGMVD	41	10/3	QQSYTIPRT	42	3/2

4/A	VH5/COS24	PRTYDRADVFN	43	7/1	QRYNSYSPVYT	44	2/2
4/B	VH5/COS24	PNHFDSSGPFY	45	1/1	QQYNSYSPGYS	46	5/5
4/C	VH5/DP73	PVEMATSSGPGAFDI	47	10/-	QHYNSYRLSWT	48	-/3
4/D	VH3/DP49	DRLSSSWSFAN	49	7/5	QQAYSLPLT	50	12/7
4/E	VH3/DP54	GGGGYDFWSGYYRLAFDI	51	2/4	SSYTSSSTLYV	52	-/3

^A ScFvs classified by library (2,3,4), V_H group (A-F) and reference numbers (1-6) of individual selected clones. ^B V_H and V_L family and gene segment. ^C Sequence and length of CDR3. ^D Number of mutations with respect to the germ line. R: replacement mutation; S: silent mutation.

EXAMPLE 4: Reactivity of soluble scFv to human TGase II and endomysial antigen by immunofluorescence

Soluble scFv were derived from each of the IBL clones recognizing and reactivity to human TGase II by ELISA, identical to that shown by phage antibody, was confirmed. The scFv were also tested in immunofluorescence for their reactivity to human umbilical cord histological sections (the classic anti-endomysial staining). The results were compared to the fluorescence pattern obtained with a human serum positive for endomysium. In almost all cases, the scFv gave an immunofluorescence pattern identical to those of CD patients, and in those cases where the scFv did not stain umbilical cord sections, the ELISA signals tended to be low, suggesting that either affinities or expression levels were low. In Table 3, column 4, the subjective evaluation of the fluorescence intensity is reported. When a selected number of scFv were used in double-labeling experiments, the two images overlapped perfectly

Table 3. Reactivity pattern of anti TGase II scFv

Lib./ref. number ^A	Gp TGase II recognition ^B	H TGase II epitope ^C	Umb. cord immunofl. ^D
2/A 1	+	1	3
2/A 2	+	1	4
2/A 3	+	1	0
2/B	+	1	4
2/C 1	+	1	nd
2/C 2	+	1	0

2/C 3	+	1	3
2/D 1	-	2	4
2/D 2	-	2	4
2/E	-	x	4
2/F	-	2	4
3/A	+	1	3
3/B	+	1	0
3/C 1	-	2	4
3/C 2	-	2	4
3/C 3	-	2	2
3/D 1	-	2	0
3/D 2	-	2	4
3/D 3	-	2	nd
3/D 4	-	2	2
3/D 5	-	2	nd
3/D 6	-	2	4
3/E 1	-	x	4
3/E 2	-	x	nd
4/A	+	1	1
4/B	+	1	2
4/C	+	1	4
4/D	-	2	4
4/E	+	1	1

^A ScFv classifications as described in Table 2. ^B Reactivity to guinea pig TGase II.

^C Epitope recognized on human TGase II. X: not assigned. ^D Subjective evaluation of the immunofluorescence intensity on umbilical cord histological sections (0-4).

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EXAMPLE 5: Competition with patient sera

The correspondence of the scFv to the *in vivo* antibody repertoire was further confirmed by competitive ELISA. Patient sera positive for TGase II were serially diluted from 1: 50 to 1:400, mixed with scFv diluted 1:10, added to a microplate coated with h TGase II or BSA as negative control and tested for scFv binding. Five scFv

representative of the different groups were tested, and all showed a reduction in the ELISA signals due to the competition with patient sera. The extent of the reduction ranged from 10% to 80%, depending on the CD patient serum and probably reflecting a difference in the titer of the specific serum antibodies competing for the binding. No inhibition was observed using control serum from a healthy donor. In **Figure 1** a typical response of two antibodies with sera from a CD patient and a healthy donor is reported. The conclusion was that the patient sera contained antibodies recognizing the same antigenic determinants recognized by the scFv or, at least, reacting to the same antigenic area.

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EXAMPLE 6: Mapping the antigenic determinants of anti TGase II clones

To determine how many different TGase II epitopes were recognized by the different selected scFvs, two approaches were taken. In the first, crossreactivity of the different scFvs to guinea pig TGase II by ELISA was determined, and it was found (Table 3, column 2) that only a fraction of the antibodies were able to recognize guinea pig TGase II with OD values comparable to those obtained with human TGase II, with negative scFvs giving OD values similar to negative controls. In the second approach, an inhibition ELISA was carried out in which the binding of different phage scFvs to human TGase II coated to a plate (detected using a peroxidase labeled anti-phage monoclonal) was tested in the presence of different soluble scFvs. Negative controls were the same phages without competitor scFv, and positive controls the inhibition of binding by soluble scFv corresponding to that displayed by the phage. In the first experiment, a single, well-expressed scFv belonging to group 2/A (see below) was tested against all phage antibodies. When a reduction in OD of at least 50% with respect to the control was registered, the scFv and the phage were considered as recognizing the same antigenic region. After the first experiment a second scFv (group 2/D, see below) was chosen among those clones not inhibited by the first scFv and tested. After this, almost all clones could be grouped into two main association groups, termed epitope1 (Ep1) and epitope 2 (Ep2) (Table 3, column 3). Only 3 (10%) of the clones could not be assigned to either of these groups and are reported in Table 3 as "x". Clones recognizing Ep1 could be isolated from all three libraries (groups 2/A, 2/B, 2/C, 3/A, 3/B, 4/A, 4/B, 4/C) and the level of inhibition caused by the 2/A scFv was almost total, with a drop of the OD value to the level of positive controls, suggesting that all these

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scFvs recognized the same antigenic determinant. While scFvs recognizing Ep2 were also selected from all libraries, the level of inhibition (caused by scFv 2/D) was far more variable, ranging from 50% to 100%, suggesting that a larger antigenic region is involved, with partial inhibition being caused more by steric hindrance than competition for the same epitope. Ep1 was recognized almost exclusively by those scFv belonging to the VH5 family. The only exception was 4/E, with two segments (VH3/DP54 and VλIII/DP10) not present in any other scFv. All scFvs recognizing Ep1 also recognized guinea pig TGase II, whereas none of the others was able to do so, suggesting that Ep1 is common to human and guinea pig TGase II.

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EXAMPLE 7: An *in vivo* model for renal fibrosis

Male Wistar rats (weighing 300±30g at the start of the experiment) are used in this study. They are allowed to acclimatize to their environment for one week. Rats are assigned to undergo renal mass reduction (RMR) by 5/6 nephrectomy or sham operation, under anesthesia with intraperitoneal injection of pentobarbital (35mg/kg body weight). RMR is performed by ligation of 2 of 3 major branches of the left renal artery and right nephrectomy in the same session. Sham rats undergo exposure of the kidneys and removal of the peri-renal fat. After 24 hours recovery the rats are assigned to one of the following groups:

- 1) Group I: RMR rats, intraperitoneally (i.p.) administered with anti- TGase II antibody (RS1), 2.5 mg/kg on alternate days starting 24 hours post surgery.
- 2) Group II: RMR rats, i.p. administered with control antibody, started 24 hours post surgery.
- 3) Group III: age matched, sham operated rats served as the controls.

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All animals are allowed free access to a standard diet and water ad libitum. Every week, systolic blood pressure is measured by tail cuff manometry and urine samples are collected individually in metabolic cages for determination of total protein and creatinine excretion. Protein concentration in urine is determined by a colorimetric method using pyrogallol-red molybdate complex (cobas integra 700, Roche). Body weight is also measured. At sacrifice (10 weeks after RMR) blood is withdrawn from abdominal aorta for determination of creatinine and TGase II concentrations. Serum

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creatinine is measured with a Hitachi model 747 autoanalyzer, using the kinetic Jaffe method. Samples of kidney are fixed in 10% formal saline and processed into paraffin wax. Numbers of crescents are counted in 100 consecutive glomeruli in each section. Further assessment of scarring is performed by ranking the intensity of Masson's trichrome staining in glomeruli and interstitium.

EXAMPLE 8: An *in vivo* model for GVHD

The effect of anti- TGase II antibody (RS1) on skin collagen content in the murine chronic type of GVHD is evaluated. To induce chronic GVHD, spleen cells from B10.D2 mice are injected i.v. into BALB/c mice, which received 600r .sup.60 Cobalt (Claman, et al., 1985, J. Invest. Dermatol., Vol. 84, p. 246). Three days before spleen cell transplantation and through all the experiment, mice are injected daily (i.p.) with 1.5 mg/mouse/day of anti- TGase II antibody (RS1). After cell transfer, mice are maintained in laminar flow hoods and received tetracycline water (250 mg/L) for 2 weeks. The control BALB/c mice, which are similarly irradiated and given BALB/c spleen cells, are treated similarly. Every few days, the body weight is recorded, and at 45 and 52 days after transplantation, breast skin samples are taken for collagen content determination and for histology.

Skin biopsies are hydrolyzed for 22 h at 110°C with 6N HCl. Nitrogen is determined after Kjeldahl digestion by the spectrophotometric procedure, using an autoanalyzer. The collagen-unique amino acid hydroxyproline is determined, as described by Dabev and Struck (Biochem. Med., Vol. 5, p. 17, 1971). Mice skin samples are collected into phosphate buffered saline (PBS) and fixed overnight in 4% paraformaldehyde in PBS at 4°C. Serial 5 mm sections are prepared after the samples has been dehydrated in graded ethanol solutions, cleared in chloroform and embedded in Paraplast. The sections are deparafinized in xylene, rehydrated through a graded series of graded ethanol solutions, rinsed in distilled water (5 min), and incubated in 2xSSC at 70°C for 30 min. The sections are then rinsed in distilled water and treated with pronase (0.125 mg/ml in 50 mM Tris-HCl, 5 mM EDTA, pH 7.5) for 10 min. After digestion, slides are rinsed in distilled water, postfixed in 10% formalin in PBS, blocked in 0.2% glycine, rinsed in distilled water, rapidly dehydrated through graded ethanol solutions, air-dried for several hours, and stained by hematoxylin-eosin.

EXAMPLE 9: Construction of miniantibodies for the *in vivo* study of human autoimmune diseases in animal models**1. Introduction**

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Autoimmunity is an important cause of disease in humans, it is estimated to affect at least 3% to 5% of the human population and depends on a failure of the mechanisms normally responsible for maintaining self-tolerance (for a review see Marrack et al., 2001). Although many factors causing these diseases, including the genes that may predispose to autoimmunity, have been identified, the etiology of most autoimmune diseases remains obscure. It is well known that the susceptibility to many autoimmune diseases is associated to specific MHC alleles (Wucherpfennig, 2001), but this is thought to be only the first step of a multifactorial process, since not all subjects with a matched MHC develop a particular disease. Much interest has focused on the analysis of the immune factors leading to the tissue lesions. In some cases the cellular immune response stimulated by lymphokines seems to play a major role, whereas in others the humoral antibody response is deemed prevalent. Functional genomics may offer a solution to these problems by using biological systems which allow the massive interaction between an autoimmune patient's cloned antibody repertoire and individual antigens. One of these systems is phage display, a technique which involves the coupling of phenotype to genotype in a selectable format. It has been extensively used in molecular biology to study protein-protein interactions and one of the most successful applications of phage display has been the isolation of monoclonal antibodies to purified antigens (Marks et al., 1991; Sblattero and Bradbury, 2000; Bradbury et al., 2003b; Bradbury et al., 2003a). Antibodies specific to a given antigen can be isolated from phage antibody libraries by recursive cycles of binding to an immobilized antigen, washing, elution, and bacterial infection and amplification of bound phages. Finally, bacterial clones expressing single antibody specificity are characterized for the epitope recognized. In addition to libraries from naive or immunized sources, phage antibody libraries have also been made from patients suffering from autoimmune diseases. This work has been most extensively carried out with thyroid disease (McIntosh et al., 1996), systemic lupus erythematosus (Roben et al., 1996), paraneoplastic encephalomyelitis (Graus et al., 1998), myasthenia gravis (Graus et al., 1997) and type 1 diabetes mellitus

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(Jury et al., 2001). The co-inventors of the instant invention have described the antibody response in Celiac disease (CD) (Marzari et al., 2001). This is a genetic illness strongly linked to HLA DQ2, in which other genetic factors are also thought to be important. It is characterized by flattening of the intestinal mucosa and malabsorption.

5 The pathogenesis is precipitated by dietary exposure to wheat gluten and similar proteins in rye, barley and possibly oats (for a review see Goggins and Kelleher, 1994), with gliadins specific antigenic determinants found in glutens (Wieser, 1996), playing a prominent role. The disease is characterized by the presence of specific antibodies recognizing gliadins, food proteins and an endomysial autoantigen, identified as being

10 tissue transglutaminase (TGase II) (Dieterich et al., 1997). The co-inventors of the instant invention recently made and selected phage antibody libraries from CD patient lymphocytes and were able to isolate single-chain antibody fragments (scFv) to TGase II from all intestinal lymphocyte libraries but not from any peripheral lymphocyte libraries, indicating that the site of synthesis of these antibodies is the intestinal mucosa.

15 IgA antibodies from several different patients recognized the same TGase II epitopes and by ELISA competition experiments it was demonstrated that the number of epitopic regions recognized was restricted to two, distinguished by the ability to recognize guinea pig (GP) TGase II and mouse TGase II, and the over-representation of genes from the VH5 antibody family.

20 Below, the production of a series of miniantibody constructs is described in which a human anti-TGase II scFv is combined with antibody constant Fc regions from human, rat and mouse, and are used to produce antibodies by *in vivo* gene expression. The purpose is to provide the scFv with effector domains so to allow the *in vivo* studies of the pathogenetic properties of cloned autoimmune antibody fragments.

25

2. Materials and Methods

2.1 BACTERIAL STRAINS AND ENZYMES

The strain used was dh5af (f/enda1 hsdR17 (rk⁻ mk⁺) supe44 thi-1 recA1 gyra (nal^r)
 30 rela1 d (laczya-argf)u169 deor (f80dlacd(lacz)m15)). This strain was used for the cloning of pdan5, put-sec and derivatives, pcdna3 and ptrchisb. Molecular biology enzymes were purchased from New England Biolabs, Promega or Life Technologies.

2.2 ANTIGENS

Human TGase II was cloned in pTrcHisB as described (Sblattero et al., 2000). Mouse TGase II gene was obtained by amplifying cDNA from an intestinal specimen with specific primers and cloned as PstI-EcoRI fragment in pTrcHisB. Protein purification
5 was performed as described in Sblattero et al., 2002.

2.3 RNA EXTRACTION AND CDNA SYNTHESIS

Peripheral blood lymphocytes from a healthy donor and spleen lymphocytes from mouse and rat were separated by density gradient centrifugation on Ficoll Hypaque
10 (Pharmacia). Total RNA was then prepared as described (Chomczynski and Sacchi, 1987). cDNA was prepared using SuperScript II Reverse Transcriptase (Gibco BRL) with random hexamers.

2.4 PUT-SEC modification

15 PUT-SEC (Li et al., 1997) plasmid vector was modified as follows: BspEI site was exchanged with BssHII by inverse polymerase chain reaction (PCR) using the primers PUT-ApaLI and PUT-BssHII reported as A and B in Tab. I. The human IgG1 CH3 human gene was amplified by PCR with the primers HuGCH3-s and HuGCH3-a which introduces the SV5 tag sequence for mAb SV5 recognition (Hanke et al., 1992)
20 and SpeI, EcoRI and PvuI sites at the 3' end. PCR fragment was cloned as BssHII - Pvu I in the PUT-SEC vector.

2.5 CLONING OF F_C DOMAINS

The CH3 (the last C-terminal domain of the antibody heavy chain) Fc domains were
25 PCR amplified from lymphocyte cDNA by using the primers MoG-CH3-s and MoG-CH3-a for mouse IgG, RaGCH3-s and RaGCH3-a for rat IgG. The CH2-CH3 domains (the region spanning from the flexible antibody region to the C-terminus) were PCR amplified by using the sense primers HuACH2-s for human IgA, HuGCH2-s for human IgG, MoGCH2-s for mouse IgG, RaGCH2-s for rat IgG. All the antisense primers were
30 the same of the CH3 amplicates. All the amplicates were purified from the PCR mix and cloned in PUT-SEC/HuGCH3/SV5 vector replacing the resident Fc domain by cutting with BssHII and SpeI and ligation. The series of vectors was designated PUT/SV5.

2.6 CLONING OF SCFV

The cloning of individual scFv was performed by PCR of phagemid pDAN5 clones 2.8 and 3.7 using a mix of primers sense (Tab.1 n. 12-15) and antisense (Tab.1 n. 16-17) designed for the amplification of all scFv from pDAN5 libraries. Following PCR, the amplicates were purified, cut with ApaLI and BssHII and ligated in the series of vectors PUT/SV5 cut with the same enzymes.

2.7 CLONING IN PCDNA3

All the PUT/SV5 vectors with either scFv 2.8 TGase II or scFv 3.7 TGase II were cut with EcoRI and HindIII and ligated into the vector pCDNA3 (Invitrogen) cut with the same enzymes.

2.8 HEK 293T transfection and selection

The human kidney derived HEK 293T cell line was cultured in D-MEM medium (GIBCO) supplemented with 10% FCS. Cells were harvested by shaking and plated in a 24 well microtiter plate (2×10^5 cells per well). For transient transfection, after 24 h, 1 μ g of purified plasmid DNA resuspended in 50 μ l of D-MEM without FCS and 2 μ l of Lipofectamine 2000 (Invitrogen) in 50 μ l of D-MEM were mixed, left at RT for 20 min and added to each well of cultured cells. The cells were grown for further 24/48 h and the supernatant inspected for miniantibody production. Stable cell clones secreting miniantibodies were obtained by treating the cells in the same way as for the transient transfection, diluting the cells 1:10 with fresh medium after 24 h from transfection and adding 400 μ g/ml antibiotic G418 (Gibco-BRL) for the selection of neomycin resistant cells. After 10 days of culture, the G418 concentration was reduced to 200 μ g/ml.

2.9 ELISA

ELISA was performed by coating ELISA plates with purified human or mouse recombinant TGase II at 10 μ g/ml for 15 h at 4° C. Wells were blocked with 2% non-fat milk in PBS (MPBS). The primary antibodies were the supernatants of cultured HEK 293T cells diluted 1:1 with 4% MPBS or sera of mice injected with plasmid DNA diluted 1:50 with 2%MPBS. Secondary antibodies used were mAb SV5 (Hanke et al., 1992) recognizing the SV5 tag found at the miniantibody C-terminus and goat anti

human, mouse and rat IgG or IgA conjugated with peroxidase. The secondary antibodies were used as following: a) mAb SV5 diluted 1:2000 with 2% MPBS, followed by goat anti-mouse Ig conjugated with HRP (Dako) diluted 1:1000, b) goat anti human, mouse and rat IgG or IgA conjugated with peroxidase (Dako) diluted 5 1:1000. All the immunocomplexes were revealed with TMB (Pierce) and read at O.D.₄₅₀.

2.10 TGASE II INHIBITION ASSAY

ELISA plate wells were adsorbed with 20 µg/ml purified gliadin for 2 h at 37° C and 10 washed twice with PBS. To each well 100 µl of a solution of Biopentilamine (Pierce) 0.2 mM, 0.25µg of purified clone mouse TGase II in CaCl₂ 5 mM, NaCl 150 mM, Tris 50 mM pH 7.5 with increasing amount of purified miniantibody ranging from 0 to 0.2 µg were added. After 1 h incubation at 37° C, the wells were washed three times with PBS plus 1% Tween20 and three times with PBS. 100 µl of a solution of streptavidin 15 conjugated with alkaline phosphatase (Pierce) in PBS 2% BSA were added to each well and incubated for 1 h at RT. After extensive washing, the TGase II activity, based of crosslinking of gliadin and Biopentilamine was revealed by adding 100 µl of pNPP (Sigma) and read at O.D.₄₀₅.

20 2.11 Western blotting

Sodium dodecyl sulphate poliacrylamide gel electrophoresis (SDS PAGE) was performed according to standard techniques. Cell culture supernatants containing miniantibody fractions were separated by SDS PAGE and transferred onto nitrocellulose (Amersham) by semi dry blotting using the Pharmacia Multiphor II. The 25 membrane was blocked using 2% MPBS for 1 hour at room temperature. mAb SV5 was used as primary antibody. After 2 h incubation at RT and extensive washing with PBS plus 0.1% Tween20, the nitrocellulose was subsequently incubated with anti-mouse IgG goat antibodies diluted 1:1000 conjugated with alkaline phosphatase (Dako) and revealed by the chromogenic substrate BCIP (5-bromo-4-chloro-3-indolyl - 30 phosphate) and NBT (nitro blue tetrazolium).

2.12 Immunochemistry

Immunoperoxidase was performed on histological sections of mouse muscle prepared according standard techniques. Miniantibodies from HEK 293T culture supernatant were added to the sections, incubated for 30' at room temperature in a moist chamber, followed by biotinilated mAb SV5 and peroxidase conjugated Streptavidin (Pierce) and
5 DAB as substrate.

2.13 DNA VACCINATION

Four 8 week-old females C57BL/6J mice were injected with 50 µl of bupivacaine 0.50% in isotonic NaCl into quadricep. Five days later, the bupivacaine treated zones
10 were injected with 50 µg of purified pCDNA3 MoCH2-3 2.8 DNA in 50 µl PBS. Small volumes of blood were periodically sampled and analyzed for the presence of serum miniantibodies.

2.14 In situ PCR

15 Frozen mouse muscle tissue histological sections (5-10µm) fixed on SuperFrost slides, were rehydrated to nuclease-free water through graded fresh aqueous solution of ethanol (100%, 90%, 80%) then permeabilized in a 0.01% Triton-X 100/PBS solution for 2 min, and rinsed in PBS for 2 min. After permeabilization, tissues were treated with RNase-free DNase (Celbio, Milan, Italy) (50U) at 37°C overnight. Primers
20 VLPTL and VHPT2 (Sblattero and Bradbury, 2000) and 5 mM dUTP Cy3fluorescent nucleotides (Amersham Pharmacia) were used for direct labelling of the amplicon. The direct fluorescent in situ PCR was performed using the following cycle: 94°C, 30 s; 53°C, 60 s; 72°C, 60 s, repeated 15 times. After the PCR reaction the slides were washed twice with PBS for 5 min and then counter-stained with DAPI (Vectashield,
25 Burlingame CA) and directly observed under a fluorescent microscope (Olympus Optical, Shinjuku-ku, Tokyo, Japan). Negative controls were used for RT and IS-PCR, without either RT or primers.

3. Results

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Cloning strategy

In a previous paper by the co-inventors (Marzari et al., 2001) the isolation of human IgA scFvs to TGase II from phage display antibody libraries (obtained from intestinal

lymphocytes of CD patients) was described. One of these scFv, indicated as 2.8 TGase II, crossreactive to rodent TGase II, was used as reference antibody fragment to make the series of constructs reported in Fig. 2. Other than the addition of the antibody domains CH2 and CH3 of the species and antibody classes reported in the scheme, the scFvs were provided with an eukaryotic leader sequence at the N-terminus to allow the secretion of the construct when expressed in a mammalian cell system.

The gene of the scFv 2.8 TGase II was cloned into a modified version of PUT-SEC vector (Li et al., 1997), originally constructed to provide the scFv with a leader sequence for the secretion of cloned scFv, in which the BspEI site was mutated into a BssHII site. This step was required by the presence of a BspEI site in the linker sequence of the scFv 2.8 TGase II (Marzari et al., 2001) and was carried out by inverse PCR using the primers A and B of Table I. Also, the mouse CH3 domain was replaced by a human CH3 domain, PCR amplified from lymphocyte cDNA using primers 1 and 2 of Table 1. Primer 2 also adds the SV5 tag sequence for mAb recognition (Hanke et al., 1992) and SpeI, EcoRI and PvuI sites for further modifications.

The CH2 and CH3 regions of human IgG1 and IgA, mouse IgG, rat IgG2b were amplified by PCR from either peripheral (human) or splenic (mouse and rat) B lymphocytes using the primers reported in Tab.1. The set of oligonucleotide primers was designed to comprise the C-terminal portion of soluble antibodies spanning either the CH3 domain or the CH2-CH3 domains, and including the flexible hinge region which harbors the cysteines forming the interchain antibody disulfide bonds. All antibody domains were from the IgG class except the human IgA CH2-CH3 domain which was used as a positive control, since the phage display library used as source of the scFv was derived from intestinal lymphocytes IgA. The PCR product human CH3/SV5 was cloned into PUT-SEC by replacing the BssHII – SpeI insert, thus obtaining a series of vectors PUT/SV5 with a cassette: scFv/Fc domain/SV5 tag.

The cloning of scFv 2.8 TGase II in PUT/SV5 series was obtained by PCR amplification of the clone 2.8 TGase II phagemid DNA with a mix of primers sense and antisense for the amplification of all scFv. The amplificate was cut with ApaLI and BssHII and inserted in the PUT/SV5 series cut with the same enzymes.

The PUT/SV5 inserts were transferred to pCDNA3, using HindIII-EcoRI, in order to allow expression in the eukaryotic environment under the control of the CMV promoter. Subsequently, all the purified plasmid DNAs were transfected into HEK 293T cells and the secretion of the miniantibodies in the culture medium after 72 h was analyzed by ELISA on plates coated with either human or mouse TGase II. The results are reported in **Fig. 3**. All the miniantibodies were able to recognize both antigens with high values of O.D. ranging from 0.6 (2.8 HuGCH3 and 2.8 HuACH2) to 1.7 (2.8 MoGCH3). To verify whether the difference in the miniantibody reactivity was due to a different reactivity to the antigens, the ELISA was repeated using commercial secondary antisera specific for human, mouse and rat Ig subclasses. The results, depicted in **Fig. 4**, indicate that the constructs are specifically recognized and the overall reactivity to human TGase II is confirmed, since the 2.8 HuGCH3 and 2.8 HuACH2 construct O.D. are still at low levels. All the miniantibody concentrations in the supernatants were tested and comparable values were obtained.

Stable clones for all the constructs were obtained by growing the transfected cells in the selective agent for neomycin resistance. Individual clones were selected for the best ELISA reactivity and expanded for further experiments. The electrophoretic characteristics of the miniantibodies were analyzed by Western blotting under reducing and non reducing conditions and after pretreatment of the purified miniantibodies with glycosidase PNGaseF to assay the level of glycosylation. The results of the Western blotting of two miniantibodies are reported, as an example, in **Fig.5**. Bands of the predicted molecular weight were found in the samples treated with reducing agent and a slight increase was found in the electrophoretic mobility in the deglycosylated samples, indicating that the miniantibodies are glycosylated in HEK 239T cells. Under non-reducing and non-denaturing conditions, a high molecular weight band was obtained in both cases. In the case of the CH2 domain miniantibodies, this is explained by the interchain disulfide, the presence of this higher form with the CH3 domain miniantibodies, suggests the presence of structurally stable interaction between the CH3 domains.

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Immunological and biological activity of miniantibodies

The miniantibodies were inspected for their ability to recognize TGase II on histological sections. These experiments were undertaken in view of the possible use of the

miniantibodies in *in vivo* studies. The immunolabeling of the histological section of mouse muscle was performed, with clear recognition of the extracellular TGase II present at the muscular endomysium and perimysium.

For the same reasons, an assay on the inhibition of TGase II activity by miniantibodies was tried. It had already been shown that scFvs to TGase II isolated from the intestinal lymphocytes of CD patients inhibit the *in vitro* transamidation activity of TGase II (Esposito et al., 2002). **Fig. 6** shows that the incorporation of pentilaminebiotin by immobilized gliadin (a TGase II substrate) is catalyzed by mouse purified recombinant TGase II, and that this is inhibited by increasing amounts of miniantibody 2.8 IgG1CH2CH3. The molar inhibition closely mirrors the values previously described for the scFv (Esposito et al., 2002) in a similar assay.

Immunization with miniantibodies

The *in vivo* expression of selected miniantibodies was studied by using DNA vaccination protocols. According to this method of gene transfer, DNA is delivered directly to the muscle of the laboratory animal where it is internalized by the muscular fibers and expressed if an appropriate eukaryotic promoter is present. In this case, in order to evaluate the possibility of using miniantibodies for *in vivo* studies of the biological activities of autoimmune antibodies, an anti-TGase II scFv was used which did not cross-react with rodent TGase II. The purpose was to monitor the serum level of an antibody not sequestered by TGase II at the tissue level, which may have occurred, had an antibody recognizing mouse TGase II been used. The 3.7 scFv (recognizing Human TGase II) was cloned in the vector cassette by ApaLI-BssHII replacement of the resident 2.8 scFv. 50 µg of purified 3.7 MoGCH2 DNA were injected into the quadriceps of four C7BL/6J mice. The mice were periodically examined for the presence of reactive miniantibodies to TGase II at serum levels by ELISA. As outlined in **Fig.7**, detectable levels of miniantibodies to TGase II were registered for blood samples taken up to 40 days after the injection. Mouse sera were also investigated for the presence of antibodies raised against the miniantibody molecule by ELISA, adsorbing purified 3.7 MoIgGCH2-3 on plastic wells. No evidence of such a response was given by the results, which were all negative at 40 days. At the end of the experiments, the mice were sacrificed and the hindquarter muscle examined by *in situ*

PCR for the presence of miniantibody DNA. The results showed a positive labeling for scattered muscular fibers, indicating the continued presence of the plasmid DNA.

Discussion

5 Engineered antibodies are increasingly being used as therapeutic agents in numerous cases including oncology, autoimmunity, inflammation and infectious diseases (Borrebaeck and Carlsson, 2001). Combinatorial approaches have been applied to scFv isolated from phage display libraries, modifying the reactive V regions by fusion with a range of molecules to improve the antibody stability and avidity (Pack et al., 1993), to
10 alter the effector functions (Coloma and Morrison, 1997; Reff and Heard, 2001), to balance the pharmacokinetics (Batra et al., 2002), to facilitate the purification (Shan et al., 1999), or to combine different antibodies giving rise to bifunctional antibodies (Muller et al., 1998 ; Alt et al., 1999; Kriangkum et al., 2001). In the case of the present work, the fusion of human autoimmune scFv to the Fc domains of different species had
15 the goal of using such constructs for expression *in vivo* in an autoimmune animal model. The most critical passage of this approach was the preservation of the antibody reactivity after fusion with Fc domains from other species. As demonstrated by ELISA, all the chimeric constructs were shown to recognize the TGase II antigen. Without being bound by theory, it is likely that miniantibodies have a higher avidity for the antigen
20 due to scFv dimerization, as previously shown (Pack et al., 1993). This is further attested by the Western blotting analysis in non denaturing conditions with a high molecular weight band seemingly corresponding to the dimeric form for both the constructs obtained fusing the scFv to CH2-CH3 and the CH3 domains, as well. The binding activity of the constructs with the scFv 2.8, crossreactive to rodent TGase II,
25 was also preserved to mouse TGase II tested in ELISA as well as in an immunochemical assay on histological section of mouse muscle, as was the ability to inhibit crosslinking activity of TGase II. As far as the *in vivo* expression of miniantibodies is concerned, the injection of plasmid DNA was tried. This approach derives from extensive studies on DNA vaccination. According to this method, naked
30 plasmid DNA, coding for an antigenic protein, undergoes either intramuscular injection (Danko et al., 1997) (Hong et al., 2002) or gun-mediated inoculation (Yoshida et al., 2000), resulting in transfection of cells *in vivo* and subsequent expression of the vector-encoded antigen, which results in the induction of cellular and humoral responses

(Tighe et al., 1998). In a similar vein, injection of DNA coding for an antitumoral scFv has also been carried out (Nicolet et al., 1995). Although other studies have demonstrated both cellular and humoral responses against human scFv in mice (Prasad et al., 1997) with a reduction of the therapeutic potential, this feature can also be exploited to generate anti-idiotypic responses against scFv derived from mouse lymphomas (Benvenuti et al., 2000) (Benvenuti and Burrone, 2001). In these experiments, the effectiveness of the anti-idiotypic response was enhanced by fusing the cloned antibody expressed by the tumoral cells with an additional CH3 antigenic region, as originally suggested by Syrengelas et al. (Syrengelas et al., 1996). In the present study, since a human scFv xenogenic for mice was used, the host immune response was minimized by using the scFv fused to a CH2-CH3 syngenic mouse Fc region. The outcomes of the experiments have confirmed this approach, with a detectable production of miniantibodies in the serum for at least 40 days and a peak of production after 20 days. The reactivity of the serum miniantibodies in ELISA together with the apparent lack of humoral response against the miniantibody molecule, led to the conclusion that the human scFv-mouse Fc fusion miniantibodies are poorly immunogenic in the mouse under the experimental conditions used. In conclusion, the results indicate that chimeric proteins generated by fusion of human scFv to human, murine and rat Fc regions are effectively produced and secreted by cultured cells; the polypeptides dimerize, forming disulfide bridges, so increasing the valence of the miniantibody; the miniantibodies retain the antigen recognition both in ELISA and immunohistology, the inhibitory properties of the scFv are preserved and, upon intramuscular injection of the plasmid, the ELISA antibody titer is still detectable after 40 days, suggesting the absence of an immune response by the host when a syngenic Fc fragment is present in the construct. For these reasons the injection in experimental animals of plasmid DNA coding for miniantibodies constructed from autoimmune scFv could be a valuable tool to investigate the pathogenetic role of the humoral autoimmune response.

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15

EXAMPLE 10: Evaluation of Neutralizing Anti TGMII Minibodies (mCH2CH3 2.8) Administered Once Daily in Unilateral Ureteral Obstruction (UUO) Operated mice

20

OBJECTIVE:

The aim of this study was to evaluate the effect of multiple Intraperitoneal administrations of neutralizing anti TGMII Minibodies (mCH2-CH3 2.8 mAb) on permanent Unilateral Ureteral Obstructed (UUO) in C57Bl mice.

25

MATERIALS AND METHODS:

Minibodies were purified from conditioned medium of transiently transfected 293T human embryonic kidney cells. Transient transfection was done using the CaPO₄ method with 20µg DNA per 10 cm plate. Conditioned medium was collected after 72 hrs; medium was replaced and collected after additional 48-72 hrs. Minibodies were purified by protein A sepharose followed by NiNTA column, Purity> 85 % by commassie blue on SDS-PAGE.

30

General Groups Design:

C57bl 20-25gr mice were used for the study as described below. Mice were treated once daily by intra-peritoneal injection beginning from 24hrs before the operation until 24 hours before termination, at 3 days post UUO operation according to the study design table.

Table 1: Study Group Design:

Group	Test Article	Dose (µg/mouse)	Volume (µl)	Administration Route/Schedule	Days of UUO	N=
A	Anti-TGMII- mAb	100	150	Once daily	3	8
B	PBS-Vehicle	-		Starting 24 h. pre-operation		7

Permanent Unilateral Ureter Obstruction (UUO) in mice:

Mice were anesthetized with I.P (4 ml/kg) injection of *Equithesine*. After anesthesia, the left ventrolateral abdominal region was shaved with an electric razor and scrubbed with alcohol solution. The mice were placed in dorsolateral recumbence, and an abdominal incision was performed lateral to *Linea Alba*, following an atraumatic intestine displacement to allow the exposure of the left ureter. The exposed ureter was then occluded using titanium micro clips (SLS-Clip, Vitalitec W 6060-1, USA). The clip was placed exactly at the lower left kidney pole level. The intestine was returned to its normal anatomic position. Thereafter, the abdominal muscles and skin were sutured in layers using 3-0 (or 4-0) silk. The animals were observed following the procedure up to the sacrificing.

Follow-up and clinical Observation:

Clinical signs were recorded 30 minutes after dose administration twice daily (a.m. and p.m.) until study termination. Observations included: morbidity / mortality, piloerection, salivation, change in gait, posture, body tonus, tremor and convulsion, as

well as the presence of bizarre behavior. Bodyweight and clinical sign were monitored and recorded daily throughout the study. Moribund animals, or animals showing severe pain, were humanely sacrificed.

5 Termination and tissue sampling:

At the termination point of the experiment, the animals were deeply anesthetized by Ketamine \ Xylazine, total blood was collected by heart puncturing and the animals were euthanized immediately after. Blood samples were collected in gel-containing test tubes, centrifuged, and separated serum samples were kept at 4°C until
10 analysis. Thereafter the kidneys of each individual mouse were harvested. Each kidney was weighed and cut longitudinally to 3 sections. The middle section was immediately placed in liquid nitrogen. The other 2 parts of each kidney were weighed and immersed in fixative for 24 hours. The fixed kidneys were then processed for paraffin embedding using an automatic tissue processor.

15

Paraffin block sectioning of kidneys:

The paraffin block was oriented so that longitudinal sections of the kidneys were prepared. Each block was subjected to exhaustive sectioning so that 6 sets of systematic uniform random sections separated by 350µm were collected per each
20 kidney. At least three sets of systematic sections were processed for collagen and total protein determination. All "leftover " sections were collected, deparafinized and used for Real Time -PCR analysis.

Fibrosis evaluation: Collagen staining (SFRG) and Real-Time PCR for collagen
25 expression were tested.

RESULTS AND DISCUSSION:**Clinical findings:**

Two mice were found dead during the study. One mouse from the mAb treated group died 45 min. post second injection and another mouse from the control PBS treated group died shortly after the UUO operation. Follow-up of the animals throughout the study did not show any morbidity or abnormal clinical findings, except for ~10% body weight loss, which is expected after UUO surgery.

10 **Table 2: Body-Weight Follow Up:**

Body Weight Follow-Up (gr.) - 90-23-05						
GROUP	Mouse Number	Study Days				
		1	2	3	4	5
mAb	1	23.93	23.33	22.78	22.73	22.74
	2	24	23.94	22.17	22.43	23.21
	3	25.89	25.09			
	4	23.82	23.12	21.01	21.05	21.49
	5	25.34	22.84	21.54	21.45	22.04
	6	24.27	23.79	21.38	22.53	22.61
	7	23.02	22.97	21.29	20.48	19.1
	8	23.58	22.85	21.15	21.07	21.06
	Average	23.99	23.26	21.62	21.68	21.75
PBS	9	22.42	22.15	20.85	21.51	21.33
	10	25.58	24.76	22.85	22.68	22.76
	11	21	20.86	19.46	20.12	17.51
	12	23.02	21.75			
	13	24.23	23.73	21.54	21.83	21.56
	14	22.31	22.11	20.23	20.12	19.87
	15	19.72	19.6	18.46	18.35	18.25
	Average	22.54	22.20	20.57	20.77	20.21

Collagen content measured by SRFG staining:

Relative collagen content values (mcg collagen per mg of total protein) after 3 days of UUO as detected in both obstructed (UUO) and contra lateral (CTR) kidneys are shown below.

Table 3: Relative collagen content

Treat	Kidney	N Obs	Mean	Std Dev
AB	UUO	7	12.62	1.38
	CTR	5	11.41	0.97
PBS	UUO	6	14.39	0.94
	CTR	6	12.06	2.05

Significant increase in the collagen content, as measured by SRFG staining (P-value<0.01) was observed in the left-ligated kidney as compared to the right CTRL kidneys in both tested groups. The left obstructed kidneys of the mAb treated mice showed a significant reduction (P value =0.0227) of 13% in collagen content, in comparison to the obstructed kidneys of the vehicle-PBS treated mice.

Collagen gene expression by Real-time PCR:

10

Table 5: Mean and standard deviation (Std Dev) of collagen expression levels [Ln(Collagen/S18-reference gene)]

Treatment	Kidney	N Obs	Mean	Std Dev
mAB	UUO	6*	-0.864	0.559
	CTR	7	-2.352	0.694
PBS	UUO	6	-0.321	0.302
	CTR	6	-2.696	0.483

* Animal #5 was excluded from the analysis due to inappropriate readings in both kidneys (>median+2std)

15

Real-time PCR measurements of collagen were compared to the S18 reference gene. Each of the measurements was repeated 3 times on the same day. All experiments have passed QC standards, i.e. the estimate of the slope of the collaboration curve was in the interval $[-4, -3]$, $R^2 > 0.99$, no extrapolation from the calibrator curve and no primer dimers. The reference gene S18 was not influenced by the treatment groups. The analysis of the results shows a significant increase in the collagen expression (P value < 0.01) in the ligated kidneys as compared to the CTRL, in both treatment groups. The obstructed kidney in the antibody treated group showed a significant reduction (P value $= 0.0021$) in collagen expression level ($\approx 50\%$) as compared to the PBS treated obstructed kidney.

Serum mAb levels:

Blood serum samples were collected at study termination, 24 hours after last treatment and were analyzed for mAb levels by ELISA using immobilized TGaseII. The average level of circulating mAb in the serum was $31.4 \mu\text{g/ml}$.

CONCLUSIONS:

This study was designed to test the effect of the mAb (mCH₂CH₃ 2.8) against TGaseII on the development of kidney fibrosis in UUO models. All mice which received an intraperitoneal injection of $100 \mu\text{g}$ mAb (once daily for 4 days) and were operated for UUO (kidneys obstructed for 3 days) appeared normal, did not show any abnormal clinical signs and pathological findings were not observed. Since the mAb were detected in the serum of these mice, it was concluded that the dose injected was safe and not toxic to mice according to study conditions.

As expected, the ureteral obstruction resulted in progressive kidney fibrosis, as suggested by the significant increase in all the fibrotic parameters measured in this study. The results of the study showed that the mAb against TGaseII is effective and can reduce kidney fibrosis following UUO as measured both by total collagen content and by collagen mRNA levels analysis. A significant positive effect ($p < 0.05$) of the mAb was observed in both parameters.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without undue experimentation and without departing from the generic concept, and, therefore, such adaptations and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

Citation of any document herein is not intended as an admission that such document is pertinent prior art, or considered material to the patentability of any claim of the present application. Any statement as to content or a date of any document is based on the information available to applicant at the time of filing and does not constitute an admission as to the correctness of such a statement.

CLAIMS

1. A recombinant antibody molecule comprising the CDR3 variable region of SEQ ID NO: 9 and also comprising the amino acid sequence of SEQ ID NO: 2, the antibody molecule having specific binding affinity for TGase II and being capable of inhibiting the enzymatic activity of the TGase II enzyme.
5
2. A recombinant antibody molecule comprising the CDR3 variable region of SEQ ID NO: 10 and also comprising the amino acid sequence of SEQ ID NO: 6, the antibody molecule having specific binding affinity for TGase II and being capable of inhibiting the enzymatic activity of the TGase II enzyme.
- 10 3. A recombinant antibody molecule having a V_L region comprising the amino acid sequence of SEQ ID NO: 2 and a V_H region comprising the amino acid sequence of SEQ ID NO: 6.
4. The antibody molecule according to claim 3, characterized in that it is in the form of single chain antibody fragment (scFv) comprising a V_L and a V_H regions covalently joined by a linker.
15
5. The antibody molecule according to claim 4, wherein the linker comprises the amino acid sequence of SEQ ID NO: 4.
6. The antibody molecule according to claim 5, further comprising one or more human IgG1 domains.
- 20 7. The antibody molecule of claim 6, wherein the one or more human IgG1 domains are hinge-CH₂ or hinge-CH₂ CH₃ domains.
8. An isolated nucleic acid molecule comprising the sequence of SEQ ID NO: 1 and the sequence of SEQ ID NO: 5 encoding the V_L and V_H regions of claim 3 respectively, or a nucleotide sequence hybridizing under high stringency conditions thereto.
25
9. The isolated nucleic acid molecule of claim 8, further comprising the sequence of SEQ ID NO: 3.
10. A pharmaceutical composition comprising as an active ingredient the antibody molecule according to any one of claims 1 to 7 and a pharmaceutically acceptable carrier, excipient, or auxiliary agent.
30

11. A vector comprising a nucleic acid molecule according to claims 8 or 9.
12. The vector according to claim 11, wherein the vector is an expression vector capable of expressing the recombinant antibody molecule of any one of claims 1 to 7.
- 5 13. A host cell transformed with the vector according to claim 12.
14. A method for treatment of fibrotic disease, scarring, fibrosis-related pathology, inflammatory disease or autoimmune disease comprising administering a therapeutically effective amount of the pharmaceutical composition according to claim 10 to a subject in need thereof.
- 10 15. The method according to claim 14, wherein the fibrotic disease is selected from: pulmonary fibrosis, liver fibrosis, cardiac fibrosis, kidney fibrosis, skin fibrosis and myelofibrosis.
- 15 16. The method according to claim 14, wherein the scarring is selected from: scleroderma, keloids and hypertrophic scars, ocular scarring, inflammatory bowel disease, macular degeneration, Grave's ophthalmopathy, drug induced ergotism and psoriasis.
- 20 17. The method according to claim 14, wherein the fibrosis-related pathology is selected from: glioblastoma in Li-Fraumeni syndrome, sporadic glioblastoma, myleoid leukemia, acute myelogenous leukemia, myelodysplastic syndrome, myeloproliferative syndrome, gynecological cancer, Kaposi's sarcoma, Hansen's disease and collagenous colitis.
18. The method according to claim 14, wherein the fibrosis-related pathology is selected from: ocular disease, a cardiovascular disease, atherosclerosis / restenosis and a neurological disease.
- 25 19. The method according to claim 15, wherein the pulmonary fibrosis is selected from: interstitial lung disease and fibrotic lung disease.
20. The method according to claim 16, wherein the ocular scarring is selected from: proliferative vitreoretinopathy (PVR) and scarring resulting from surgery to treat cataract or glaucoma.
- 30 21. The method according to claim 18, wherein the neurological disease is selected from: polyglutamine disease, spinobulbar muscular atrophy, dentatorubral-

pallidoluisian atrophy, spinocerebellar ataxias (SCAs) 1, 2, 3, 6, 7 and 17, Alzheimer's disease, Parkinson's disease, Huntington's disease, rubropallidal atrophy and spinocerebellar palsy.

22. The method according to claim 14 for treatment of both acute and
5 chronic forms of fibrosis of organs.

1/6

Figure 1:

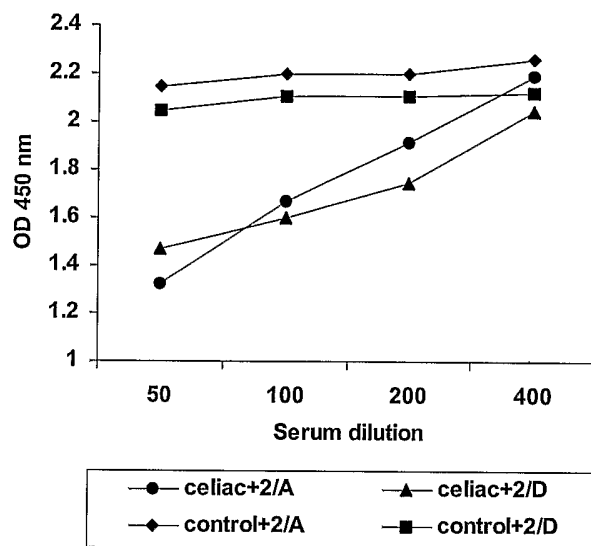


Figure 2:

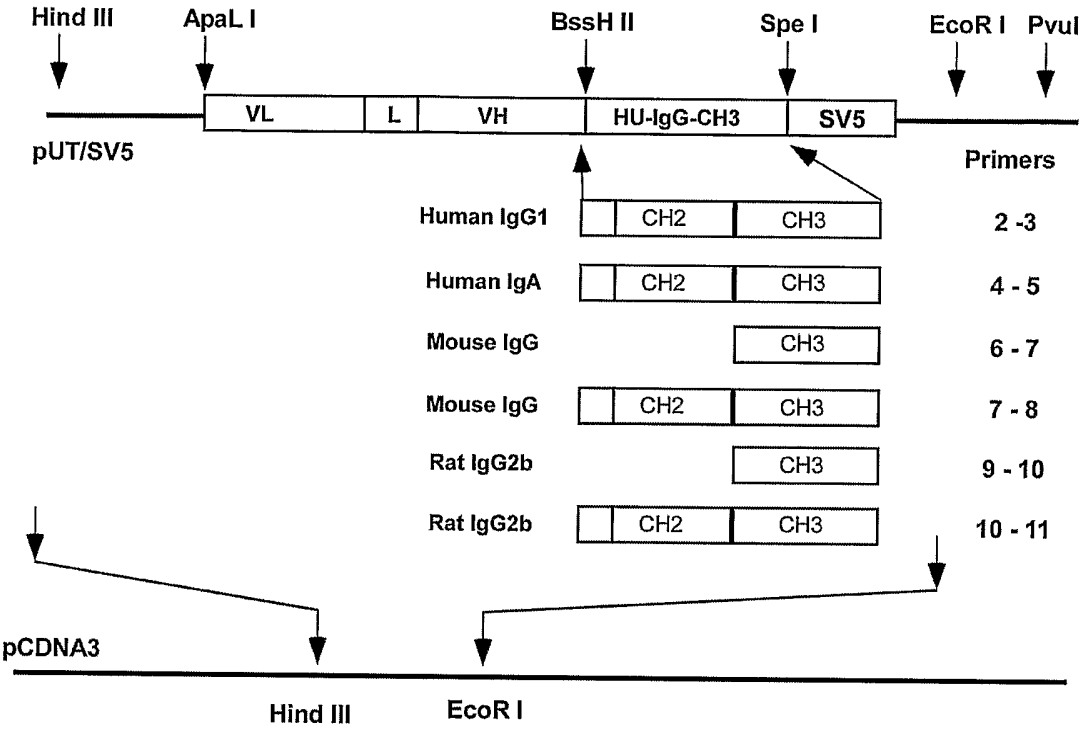
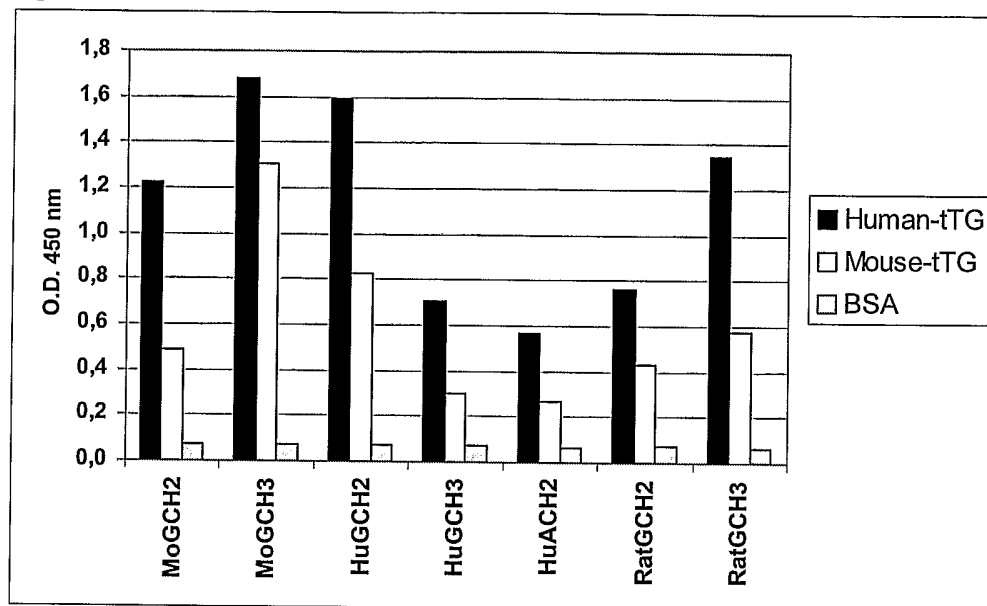


Figure 3:



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Figure 4A:

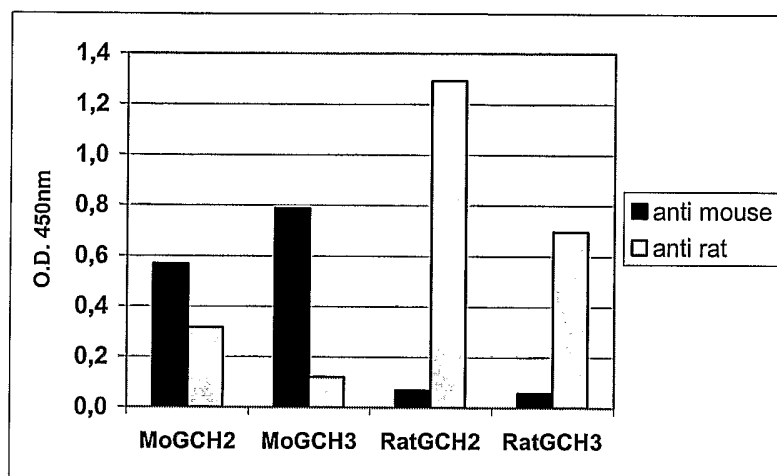
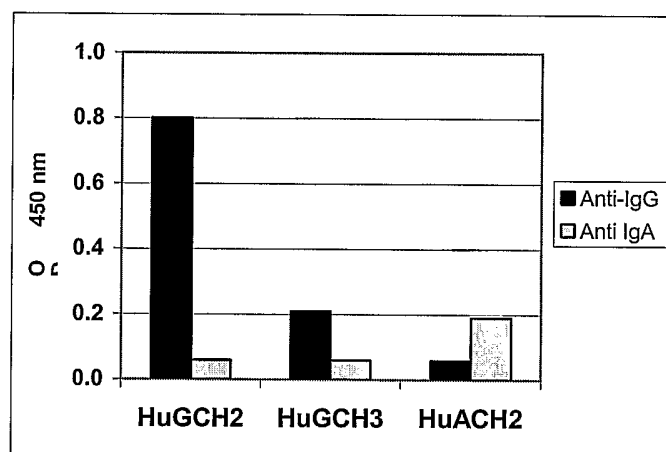


Figure 4B:



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Figure 5:

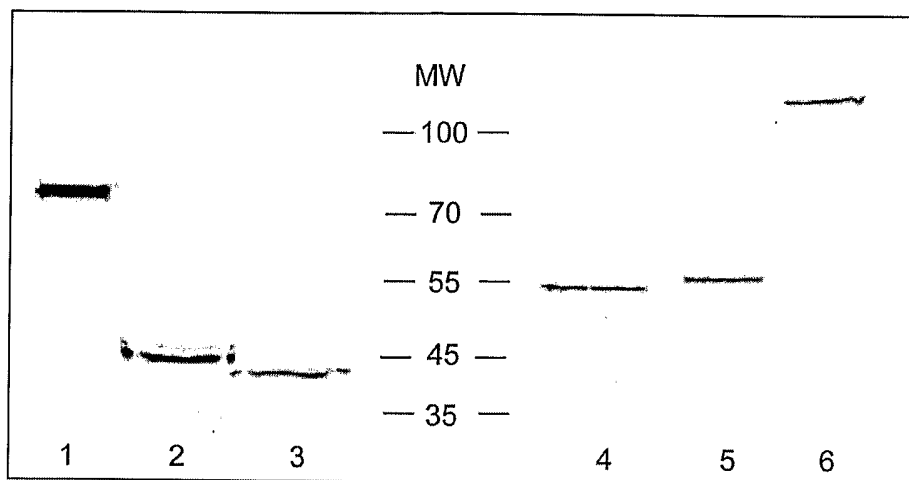
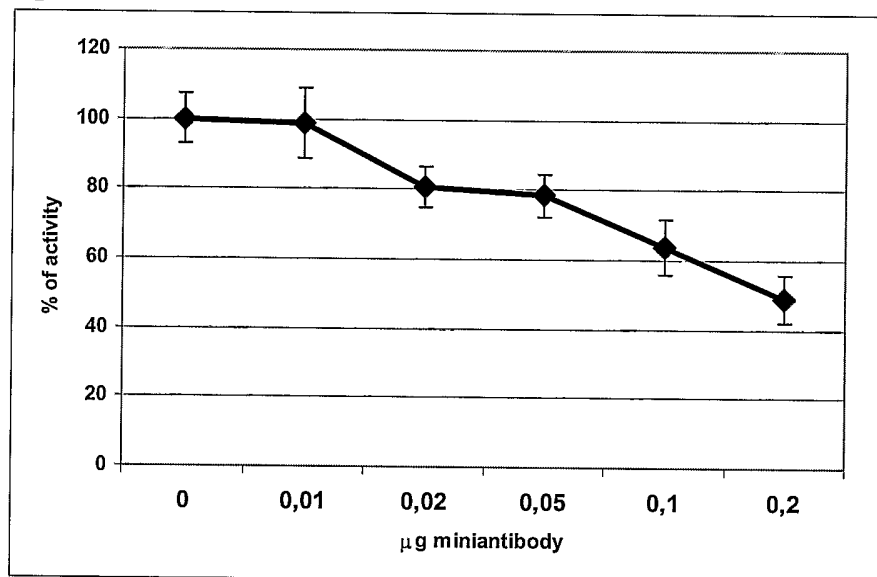


Figure 6:



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Figure 7:

