Specific depletion of autoreactive B lymphocytes by a recombinant fusion protein in vitro and in vivo

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Abstract

Antigen-specific B cells are key players in many autoimmune diseases through the production of autoreactive antibodies that can cause severe tissue damage and malfunction. We have designed and expressed a fusion protein, referred to as MOG–Fc, composed of the extracellular Ig-like domain of human myelin oligodendrocyte glycoprotein (MOG) and the CH2 and CH3 domains of the human IgG1 heavy chain. The dimerized fusion protein was capable of mediating cytotoxicity against a MOG-reactive hybridoma line in vitro. Likewise, MOG–Fc significantly reduced the number of circulating MOG-reactive B cells in an anti-MOG Ig heavy chain knock-in mouse model. Our study shows that autoantigen-reactive B lymphocytes can be efficiently and selectively eliminated by an autoantigen Fcγ1 fusion protein in vitro as well as in vivo. Such fusion proteins may provide a platform for the development of highly selective therapeutic approaches.

Introduction

Autoimmune diseases represent an increasing clinical burden in developed countries. The pathogenesis of autoimmune diseases is poorly understood. Consequently, targeted therapeutic reagents are very rare and treatments normally consist of systemic immunosuppressive drugs. In the case of multiple sclerosis (MS), the inflammatory destruction of white matter in the central nervous system (CNS), as is apparent from the typical plaques of demyelination in affected patients, is supposed to result from autoimmune attacks specifically directed towards components of the myelin sheath of axons (1). This assumption is based on findings made in experimental autoimmune encephalomyelitis (EAE), an induced autoimmune, inflammatory disease in experimental animals commonly accepted to recapitulate relevant immunological and pathological events of MS. Experimental findings in both MS and EAE collectively demonstrate a crucial role for autoantibodies in demyelination (2,3). B cell responses and clonal B cell expansion have been demonstrated within the cerebrospinal fluid of MS patients (4–6). In particular, the autoimmune response against the CNS autoantigen myelin oligodendrocyte glycoprotein (MOG) generates autoantibodies with a strong demyelinating potential in primates and rodents (7).

Clinically effective immunotherapeutic approaches to B cell-mediated autoimmune diseases and MS include plasmapheresis (8). For treatment of malignant B cell diseases, antibodies targeting B cells via the CD20 antigen (9) have been shown to be very effective in B cell elimination. However, such approaches lack selectivity. While plasmapheresis induces a short-term reduction of all Ig, usually followed by a rebound in antibody production (9,10), treatment with pan-B cell antibodies leads to loss of B lymphocytes regardless of specificity, thereby inducing long-term humoral immunodeficiency.

Our aim was the design of a protein with the potential to reduce antigen-specific antibody titers while, at the same time, depleting autoreactive B cells as the root cause of pathogenic autoantibodies. We have chosen MOG as a model target self-antigen for the investigation of this therapeutic strategy. Unlike the vast majority of other autoantigens, which are expressed in thymus and periphery, MOG is found exclusively in the CNS (7,11,12). Therefore, MOG-specific clones are not subject to clonal deletion or peripheral selection, making MOG a highly...
potent immunogen. Thus, it is feasible to engineer mice transgenic for a functional MOG-specific BCR. In order to examine both in vivo efficacy as well as pathological side-effects, we employed the anti-MOG transgenic TH mouse (13). This model system possesses high numbers of autoreactive, fully functional B cells as well as high MOG-specific autoantibody titers. For this reason, TH mice are a unique tool to study the effect of a recombinant protein and also clinical complications of antigen-specific therapy. These may comprise renal dysfunction induced by immune complex deposition, systemic anaphylaxis by triggering of mast cells, systemic inflammatory reactions and induction of MOG-specific immunopathology.

Here, we report that a dimerized fusion protein (MOG–Fc) consisting of the extracellular domain of MOG at the N-terminus and the hinge–CH2–CH3 region of human IgG1 at the C-terminus can be functionally expressed in CHO cells, and binds to both MOG-specific B cells as well as FcγR+ immune effector cells. MOG–Fc induced the specific elimination of autoreactive B cells in vitro and in vivo in the absence of clinical side-effects. To our knowledge, this is the first report demonstrating antigen-specific depletion of autoreactive B cells through a bispecific Fcγ fusion protein.

Methods

Construction of autoantigen × Fc fusion protein MOG–Fc

Total RNA was isolated from human IgG1 (HD69)-transfected CHO cells (14) using the Qiagen RNeasy RNA Extraction Kit according to the manufacturer’s suggestions. Complementary DNA synthesis was performed using oligo(dT) primers via a standard protocol. In order to obtain cDNA coding for the Fc domain of the human IgG1 antibody backbone, the following primers were used: primer IgG1-Fc 5’-GTGTCGACTCATTTACCCGGAGACAGGG-3’; primer IgG1-Fc 3’-GTGTCGACTCTTTACCCGGAGACAGGG-3’. Primers were designed to amplify the 699 bases coding for the Fc part of the human IgG1 backbone, while introducing BspEI and SacI restriction sites at the 5’ and 3’ ends of the amplified fragment respectively. Amplification was performed according to standard PCR protocols. The final product, containing the flanking restriction sites, was 711 bp in length. Blunt-ended PCR products generated by Pfu DNA polymerase were ligated into pCR-Script vector (211188; Stratagene, La Jolla, CA) according to the manufacturer’s protocol. Plasmids were transformed into competent Escherichia coli strain XL-1 Blue, and plasmid DNA was isolated according to manufacturer’s instructions (Plasmid Maxi Kit; Qiagen, Hilden, Germany).

Isolation of RNA from MOG-transfected fibroblasts and cDNA synthesis

Total RNA was isolated from MOG-transfected fibroblasts (kindly provided by C. Lintoning, MPI Neurobiology, Martinsried) as above. cDNA was obtained through reverse transcription. In order to obtain cDNA coding for the extracellular domain of the human MOG protein (MOG-Ex), the following primers were used: primer MOG-Ex 5’-TAGAATTCTAGGCAAGCTTATCGAGACCC-3’; primer MOG-Ex 3’-CATCCGGATCCAGGGCTCACCCAGTAGA-3’. Primers were designed to amplify the first 462 bases of the coding region for the leader sequence and extracellular domain of the human MOG protein. The primers contained EcoRI and BspEI restriction sites at the 5’ and 3’ ends of the amplified fragment respectively, yielding a final PCR product of 474 bp in length. In order to obtain the desired construct, expression vector pEF-dhfr was subjected to restriction with EcoRI and SacI. The linearized vector was gel-extracted (Qiagen). Equally, MOG-Ex was partially digested with BsaWI and EcoRI, and the corresponding 474-bp fragment was isolated via gel extraction. BsaWI restriction was chosen due to this enzyme’s insensitivity to dam-methylation at the original BspEI restriction site. Ligation of isolated DNA fragments was performed with T4 DNA ligase and ligation products were used to transform E. coli XL-1 Blue as described above. Following analytical restriction enzyme digestion, appropriate clones were sequenced (SequServe, Munich, Germany).

Stable transfection of CHO cells and protein purification

DHFR-deficient CHO cells were plated at 3 × 10^6/well in six-well tissue culture plates and incubated at 37°C overnight. DNA (3 μg) was pipetted in sterile Eppendorf tubes, supplemented with 100 μl MEM-α medium (Gibco/BRL, Karlsruhe, Germany) and 10 μl SuperFect transfection reagent (Qiagen), and incubated for 10 min at room temperature. Aliquots of 600 μl of MEM-α medium were added and the reaction mixture was transferred to CHO cells. Following 2 h incubation at 37°C, the supernatant was aspirated, cells were washed once with PBS and 2 ml MEM-α medium (10% FCS, HT-supplement 1:100) was added to each well. Transfection efficiency was determined to be 10% via standard β-galactosidase control transfection. After 24 h at 37°C, transfected cells were transferred to 10-ml cell culture bottles (Nuncclone; Nalge Nunc, Neerijse, Belgium) and selected for expression of the dhfr gene via growth in non-supplemented MEM-α medium plus 10% dialyzed FCS. Following two passages of confluent cells at 1:5 splitting ratios, transfectants were further selected by addition of 20 nM methotrexate (MTX) to the selection medium. Cells were passaged 3 times, whereafter MTX concentration was increased to 100 nM. Following a further three passages, MTX was added to a final concentration of 500 nM. Stably transfected CHO cells were transferred to 500-ml roller bottles (Nalge Nunc) in MEM-α, 500 nM MTX and 2.5% dialyzed FCS. Supernatant was harvested and recombinant protein was purified using a one-step purification procedure via Protein A-affinity chromatography (HiTrap Protein A column; Pharmacia, Freiburg, Germany). Protein was eluted with 20 mM citrate, pH 3, using a linear gradient. Fusion protein yield amounted to ~10 mg/l. Protein was >95% pure as assessed by Coomassie blue staining of SDS gels.

SDS–PAGE and Western blot analysis of MOG–Fc

MOG–Fc fusion protein was analyzed via SDS–PAGE and Western blotting using standard procedures. MOG–Fc was detected with the murine anti-MOG mAb 8.18-C5 (kindly provided by C. Lintoning); bound anti-MOG mAb was detected via alkaline phosphatase-conjugated goat anti-mouse IgG antibody (A-2429; Sigma, St Louis, MO).
Whole lymphocytes from transgenic knock-in mice were prepared from spleens as described elsewhere (12). Cells were incubated with fusion protein and bound MOG–Fc was detected with goat anti-human IgG–FITC antibody (67-217; ICN Biomedicals, Orangeburg, New York, NY).

In order to allow binding to low-affinity Fc receptors, incubation with MOG–Fc in Fig. 2(B and C) was performed with 10 times the concentration of protein as in Fig. 2(A) and for a period of 45 min at room temperature as opposed to 20 min on ice. The subsequent incubation with anti-Mac-1, anti-CD5 or biotinylated 8.18-C5 antibodies was performed on ice for 20 min. The upper panels of Fig. 2(B and C) show incubations on ice alone without prior incubation with MOG–Fc at room temperature.

**In vitro cytotoxicity of MOG–Fc**

The 8.18-C5 hybridoma cell line (kindly provided by C. Lington) was adapted to serum-free medium (Hybridoma SFM; Gibco). Cells were passaged 1:5 every third day and cultured in 100% serum-free medium for a period of 4–5 months. Therefore, MOG reactivity in the hybridoma pool was assessed by FACS analysis for surface binding of biotinylated MOG protein. More than 90% of adapted cells showed cell-surface expression of murine IgG1 and did bind recombinant MOG. The FACS-based assay was performed using freshly isolated human peripheral blood mononuclear cells (PBMC) as effector cells. Effector cells and 8.18-C5 target cells were incubated overnight at 37°C/5% CO₂ at an E:T ratio of 10:1 and serial dilutions of fusion protein added in a constant volume of 20 μl to 180 μl cell suspension. Cells were stained with FITC-labeled goat anti-mouse IgG antibody (12064D; PharMingen, San Diego, CA) and propidium iodide (PI). The live target cell population was measured as a percentage of the whole cell population analyzed. Unspecific background was measured in the absence of protein. Cytotoxicity was calculated as: 100 × [1 – (live target cells in sample/live target cells in control)]. Background staining of human PBMC incubated alone was subtracted. Values >100% were due to slight proliferation of PBMC. To verify that reduction in the number of live target cells was due to cell lysis as opposed to a growth inhibitory effect, target and effector cells were incubated with the optimal dose of MOG–Fc (10 μg/ml) as described above, and PI⁺ dead target cells measured by FACS and expressed as percentage of total target cells detected. Cell lysis was calculated as: 100 × (dead target cells/total number of target cells). Heat-inactivated serum was used in all media. Error bars represent SD from triplicates.

**Ex vivo elimination of B cells by MOG–Fc**

Splenocytes from TH mice (SJL/J background) were prepared. Single-cell suspensions were incubated with MOG–Fc (10 μg/ml) for 16 h at 37°C/5% CO₂ in DMEM/10% FCS in 5 ml cell culture polystyrene vials (Becton Dickinson, Franklin Lakes, NY) at a density of 4 × 10⁶ cells/ml. Heat-inactivated serum was used in all media. Lymphocyte analysis was carried out by FACS using antibodies against MoM², IgD, Thy1 and CD19 (all BD PharMingen). All tests were carried out in triplicate.

**In vivo depletion of adoptively transferred, MOG-specific B cells in wild-type mice**

Spleen cells of TH mice were stimulated with lipopolysaccharide (LPS, 10 mg/ml; Sigma) for 3 days. Non-B cells were then separated through incubation with biotinylated anti-CD43 antibodies followed by sorting with streptavidin-coupled magnetic beads (Dynabeads M-280; Dynal, Oslo, Norway). Fifteen mice were injected i.v. with 16 × 10⁶ purified B cells. The next 3 days each group of five mice received a daily i.p. injection of either 100 μg recombinant MOG–Fc protein or 100 μg human IgG1 antibody or PBS respectively. Three days after the last i.p. injection peripheral blood was taken from each mouse, and the levels of transferred B cells as well as of the anti-MOG antibody titer were determined in FACS and ELISA experiments respectively. Up to this time point, 6 days after the B cell transfer, most LPS-stimulated TH B cells express low levels of MOG-binding, surface IgG, detected by double staining with biotinylated MOG, followed by streptavidin–phycoerythrin (PE) and with goat anti-mouse IgG (Fc specific) (BD PharMingen). MOG-specific antibodies were detected in ELISA assays as follows: microtiter plates coated with recombinant MOG protein at a concentration of 10 μg/ml were incubated with individual sera at three different dilutions (1:10, 1:100 or 1:1000) and bound antibodies were detected with alkaline phosphatase-labeled goat anti-mouse IgM or goat anti-mouse IgG antibodies (BD PharMingen). Values for IgM correspond to the 1:100 dilution and values for IgG correspond to the 1:1000 dilution. The levels of serum MOG–Fc protein were determined in ELISA by coating plates with

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**Fig. 1.** Design, expression, purification and characterization of MOG–Fc. (A) Design of MOG–Fc. The constant C H3 and C H2 domains of human IgG1 (Hu IgG1) (open boxes), including the hinge region with two disulfide bonds, were fused to the extracellular domain of human MOG (Hu MOG) (filled boxes). N, N termini; C, C termini. MOG–Fc was expressed in CHO cells and affinity purified by Protein A–Sepharose chromatography. (B) SDS–PAGE analysis of purified MOG–Fc in the absence (lane 1) and presence (lane 2) of the reducing agent dithiothreitol. (C) Western blot analysis of MOG–Fc in the absence (lane 1) and presence (lane 2) of dithiothreitol. After transfer of MOG–Fc from SDS gel to a nitrocellulose filter, MOG–Fc was detected by the MOG-specific mAb 8.18-C5 and an alkaline phosphatase-conjugated secondary goat anti-mouse antibody. The position of mol. wt standards (in kDa) is shown on the right.
Depletion of autoreactive B cells

In these mice (designated TH), the endogenous heavy chain J region has been replaced by the rearranged 8.18-C5 anti-MOG VDJ segment using knock-in technology. As a result, almost all B cells of transgenic mice express the MOG-specific heavy chain in combination with endogenous light chains (13).

FACS analysis revealed binding of recombinant MOG–Fc in >90% of all IgM+ splenic B cells from the TH knock-in mice (Fig. 2A, lower panel), but not in B cells from control littermate mice (Fig. 2A, upper panel). Approximately one-third of the IgM+ transgenic B cells bound MOG–Fc at high density, while the remaining two thirds bound MOG–Fc with low density (Fig. 2A, lower panel). This binding pattern differs from the binding of biotinylated, bacterially produced recombinant rat MOG (13). Recombinant MOG bound only to one-third of B cells, while the other two-thirds of TH B cells failed to bind significant doses of the protein. This difference likely reflects an intrinsically higher affinity of TH B cell receptors to MOG–Fc, which, in contrast to the bacterially generated recombinant MOG, is dimeric, properly folded, N-glycosylated and has not undergone biotinylation. In both cases, the broad distribution of MOG-binding patterns reflects the fact that the B cell repertoire is only fixed for the transgenic heavy chain, while endogenous light chains are randomly associated. TH splenocytes were not stained with the secondary anti-Fc antibody alone (data not shown).

Using wild-type splenocytes, binding of MOG–Fc was restricted to the Mac-1$^{\text{high}}$ (CD11b) population (Fig. 2B, lower panel), composed of macrophages and myeloid CD8+ dendritic cells (15). No binding of MOG–Fc to CD5+ T cells could be detected (Fig. 2C, lower panel), thus underscoring the selectivity for FcyRI+ cells. 8.18-C5+ cells in the lower quadrants of Fig. 2(B and C) most probably represented B cells binding the protein through their inhibitory, low-affinity Fc receptor FcyRII-B1. In contrast to the upper panel, stainings in the lower quadrants of Fig. 2(B and C) were performed at room temperature (see Methods). Binding of the human Fc portion of MOG–Fc to murine FcyR was additionally confirmed in the murine monocyte/macrophage line p388.D1 (data not shown).

**Results**

**Design, expression and characterization of MOG–Fc**

We fused the N-terminal 154 amino acids of the extracellular domain of human pro-MOG to the C-terminal 233 amino acids of human IgG1 encompassing the hinge, C$_{\text{H}}$2 and C$_{\text{H}}$3 domains, as shown in Fig. 1. An expression vector for MOG–Fc was used for stable transfection of CHO cells. MOG–Fc was purified from cell culture supernatant applying a single Protein A-affinity chromatography step. All of the MOG–Fc fusion protein purified from culture supernatant appeared to be in a dimerized form, as shown by SDS–PAGE analysis in the absence and presence of reducing agent (Fig. 1B), and by Western blotting using an anti-MOG mAb (Fig. 1C). The identity of the MOG–Fc protein was confirmed in an ELISA using the mouse anti-MOG mAb 8.18-C5 for capturing and an alkaline phosphatase-conjugated anti-human-IgG antibody for subsequent detection (not shown). SDS–PAGE, Western blotting and ELISA data suggest that CHO cells produced MOG–Fc of the correct size, quaternary structure and antigenicity.

**Cell binding activities of MOG–Fc**

Binding of MOG–Fc to MOG-reactive B cells was investigated using splenocytes from anti-MOG Ig transgenic mice (Fig. 2A).
In order to test whether the potential cross-linking of target and effector cells by MOG–Fc could eliminate MOG-reactive target cells, we developed an in vitro FACS-based cytotoxicity assay that is independent of isolated primary mouse splenocytes from transgenic animals. The assay employed the murine 8.18-C5 hybridoma line as target cells and human PBMC as effector cells. Under serum-free culture conditions, 8.18-C5 hybridoma cells, that normally secrete anti-MOG mAb, change to stable and sustained expression of cell surface-associated murine IgG, as shown in FACS analysis (Fig. 3A). The identity of the surface IgG1 molecules in these hybridoma cells was confirmed by the binding of recombinant MOG (Fig. 3B).

Co-incubation of human PBMC and 8.18-C5 target cells at a ratio of 10:1 in the presence of MOG–Fc resulted in a complete elimination of target cells as seen by the disappearance of PI−, anti-murine IgG+ cells (Fig. 4A). Target cell elimination depended on the presence of both PBMC and fusion protein.
was achieved at an optimal dose of 10 mg/ml as shown by cell lysis in our assay. Maximum efficacy of target cell lysis occurred at a concentration <100 ng/ml (Fig. 4B). A dose–response analysis showed that half-maximal blood.

ELISA. (B) FACS analysis of MOG-reactive B cells in peripheral IgG titers against MOG on day 6 post-transfer, as determined by ELISA. (A) Serum IgM and IgG1, white bars) or MOG–Fc (black bars) on days 1, 2 and 3 by i.p. injection. Five animals were analyzed per group. (A) Serum IgM and IgG titers against MOG on day 6 post-transfer, as determined by ELISA. (B) FACS analysis of MOG-reactive B cells in peripheral blood.

Depletion of autoreactive B cells

The number of anti-MOG B cells transferred into wild-type mice

Depletion of MOG-reactive B cells by MOG–Fc in wild-type mice. BL/6 mice were transferred i.v. with purified B cells from anti-MOG transgenic TH mice. Following transfer on day 0, animals were treated with PBS (black and white bars), isotype control (human IgG1, white bars) or MOG–Fc (black bars) on days 1, 2 and 3 by i.p. injection. Five animals were analyzed per group. (A) Serum IgM and IgG titers against MOG on day 6 post-transfer, as determined by ELISA. (B) FACS analysis of MOG-reactive B cells in peripheral blood. (Fig. 4B). A dose–response analysis showed that half-maximal efficacy of target cell lysis occurred at a concentration <100 ng/ml MOG–Fc (Fig. 4C). Complete elimination of target cells was achieved at an optimal dose of 10 μg/ml, while further increases in protein concentration up to 100 μg/ml resulted in a decrease in specific toxicity (data not shown). The assay was repeated 6 times. Maximum efficacy was dependent on PBMC donor and ranged from complete elimination (>98% specific lysis in three independent experiments) to 44% at optimal dose; anti-mouse IgG1 staining was not inhibited by the presence of the fusion protein (data not shown).

The specificity of MOG–Fc-mediated cell lysis in our assay was examined by using both unrelated, EpCAM antigen-specific human IgG1 and a mouse hybridoma line expressing unrelated IgG on its surface (Fig. 4D and E). The recombinant human control IgG1 (14), that can bind to human effector cells instead of resting, B cells permitted us to assess the effect of MOG–Fc on surface Ig* blast cells in the presence of secreted anti-MOG antibodies, a situation more likely to reflect an ongoing autoimmune response in need of therapy. As shown in Fig. 6, at 6 days after B cell transfer, recipient mice treated with PBS or control human IgG1 antibody (14) consistently accumulate in their peripheral blood ~3% of MOG-binding, IgG+ B cells, as well as high titers of MOG-specific IgM and IgG. In contrast, mice treated with MOG–Fc display a strong, although not complete, depletion of both MOG-binding B cells and antibodies (Fig. 6). MOG–Fc was detectable in serum at an average concentration of 21 μg/ml 3 days after treatment, while serum titers were below detection limit by day 10 (not shown). Interestingly, no pathological side-effects were observed in any of the treated animals followed for 2 months post-treatment, even though both MOG–Fc and MOG-specific antibodies were present at high concentrations. Not surprisingly, we observed an antibody response against human Fc within 21 days after treatment in TH mice (not shown). However, this response was not accompanied by pathological features, even though TH mice are strongly predisposed towards MOG-induced immunopathology (13).

Cytotoxic activity of MOG–Fc ex vivo

In order to assess whether MOG–Fc can also eliminate normal B cells expressing MOG-reactive cell-surface Ig, splenocytes from anti-MOG transgenic TH mice were isolated and incubated with 10 μg/ml of the fusion protein. The endogenous FcγR-bearing cells served as effectors. FACS analysis documented a highly significant depletion of B cells, as defined by antibodies specific for the allotype-specific B cell marker IgMα, IgD and CD19 (Fig. 5A). In all three systems, B cell depletion reached ~70% (Fig. 5B). The 10% increase in the percentage of Thy1+ T lymphocytes mirrored the decrease in MOG-specific B cells, representing ~10% of total live cells in the samples (Fig. 5B).

Depletion of MOG-reactive B cells by MOG–Fc in wild-type mice

To examine a more natural situation, we analyzed the capacity of MOG–Fc to deplete defined numbers of MOG-specific B cells adoptively transferred from TH mice into syngenic wild-type mice. C57BL/6 mice were first injected i.v. with 1.6 × 10^7 LPS-stimulated, purified TH B cells and then, on days 1, 2 and 3 after transfer, individual groups of transferred mice received 100 μg of MOG–Fc i.p., control human IgG1 or PBS. The use of LPS-stimulated, instead of resting, B cells permitted us to assess the effect of MOG–Fc on surface Ig* blast cells in the presence of secreted anti-MOG antibodies, a situation more likely to reflect an ongoing autoimmune response in need of therapy. As shown in Fig. 6, at 6 days after B cell transfer, recipient mice treated with PBS or control human IgG1 antibody (14) consistently accumulate in their peripheral blood ~3% of MOG-binding, IgG+ B cells, as well as high titers of MOG-specific IgM and IgG. In contrast, mice treated with MOG–Fc display a strong, although not complete, depletion of both MOG-binding B cells and antibodies (Fig. 6). MOG–Fc was detectable in serum at an average concentration of 21 μg/ml 3 days after treatment, while serum titers were below detection limit by day 10 (not shown). Interestingly, no pathological side-effects were observed in any of the treated animals followed for 2 months post-treatment, even though both MOG–Fc and MOG-specific antibodies were present at high concentrations. Not surprisingly, we observed an antibody response against human Fc within 21 days after treatment in TH mice (not shown). However, this response was not accompanied by pathological features, even though TH mice are strongly predisposed towards MOG-induced immunopathology (13).

Discussion

Antibody-mediated autoimmune diseases have several options for treatment. These include immunosuppressive drugs, corticosteroids or plasmapheresis, which all act only transiently, or may lead to general immunodeficiency. In the search for a more specific approach, we attempted to selectively deplete autoantibody-producing B cells through targeted lysis by immune effector cells employing the bispecific fusion protein MOG–Fc. We intended to combine human MOG for the specific targeting of MOG-reactive B cells with an immune effector mechanism leading to an efficient and at the same
time well-tolerated elimination of target cells. We disregarded approaches such as bacterial toxin fusions because of their relatively small therapeutic window and high immunogenicity (16–18). Because of its high tolerability, we decided to employ the Fc part of human IgG1 instead. This Ig isotype is well suited for induction of antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity, and is validated for the efficient elimination of tumor cells by the marketed antibody therapeutics trastuzumab (Herceptin) (19) and rituximab (Rituxan) (20). Another potential advantage is the dimerization of the C2 and C3 domains in the human IgG heavy chain that increases the avidity of target cell binding by linking two MOG molecules. In addition, human Fcy1 can efficiently bind murine Fcy receptors (see Fig. 2B) (21,22), thus facilitating functional studies with the MOG–Fc protein in transgenic mice expressing anti-MOG antibodies (13).

The MOG–Fc protein was expressed and purified as a disulfide-linked dimer in CHO cells, and shown to bind to MOG-reactive B lymphocytes, B cell hybridoma cells and FcyR+ phagocytes. MOG–Fc treatment resulted in effective and specific effector cell-dependent lysis of MOG-specific B cells. To explore the cytotoxic potential of MOG–Fc on normal B cells in vivo, we studied the fusion protein in transgenic mice expressing anti-MOG antibodies (13).

The MOG–Fc protein was expressed and purified as a disulfide-linked dimer in CHO cells, and shown to bind to MOG-reactive B lymphocytes, B cell hybridoma cells and FcyR+ phagocytes. MOG–Fc treatment resulted in effective and specific effector cell-dependent lysis of MOG-specific B cells. To explore the cytotoxic potential of MOG–Fc on normal B cells in vivo, we studied the fusion protein in transgenic knock-in mice (TH mice). These mice possess large numbers of MOG-specific B cells (and high titers of anti-MOG antibodies) due to the site-directed targeting of a MOG-specific Ig Vh gene (13). MOG–Fc had the ability to deplete primary anti-MOG+ B cells from TH mice ex vivo and in vivo. Elimination of MOG-reactive B cells ex vivo was efficient (of the order of 70% within 16 h, Fig. 5), while in vivo it was lower, but still significant, among the population of highly MOG-reactive B cells in TH mice (from 45% in untreated animals to 28% after MOG–Fc treatment, not shown). This result deserves some comment. First, it should be noted that TH mice have an extremely high titer of circulating MOG-reactive antibodies (13), which interfere with the binding of MOG–Fc to MOG-specific B cells. Second, the large pool of continuously replenished, MOG-specific B lymphocytes in TH mice represents an exaggerated, large target cell population which may partly resist specific drug depletion in vivo. In contrast, the frequency of autoreactive B cells in human autoimmune disease is much lower (23,24). Under these conditions, the treatment of transferred mice with MOG–Fc resulted in efficient depletion of ~70% of all MOG-reactive B cells, as detectable 6 days after B cell transfer and 2 days after the last treatment (Fig. 6). At 4–6 days after transfer, most TH-derived B cells detected were actively switching, IgM+ IgM+ IgG+ B cell blasts (not shown). In MOG–Fc-treated mice, but not in control mice, serum titers of MOG-binding antibodies were reduced, despite the presence of actively secreting plasma cells, which cannot be targeted via antibody-dependent cellular cytotoxicity by MOG–Fc. Thus, the action of MOG–Fc on autoreactive B cells is not restricted to resting B cells (Fig. 5), but also includes the active elimination of activated B cells and the reduction of circulating antibodies (Fig. 6).

The ability of MOG–Fc to bind and eliminate autoreactive B cells even in the presence of high anti-MOG antibody titers in vivo demonstrate its utility for novel therapy of antibody-mediated autoimmune diseases. While bone marrow-resident plasma cells, which carry little if any Ig on their surface, cannot be eliminated by our approach, the introduction of recombinant antigen into circulation by the fusion protein could prove to be of additional therapeutic benefit. MOG–Fc could bind pathogenic autoantibodies and route them to clearance. Theoretically, the formation of such immune complexes might recall pathogenic pathways, such as glomerulonephritis. However, we have never observed any signs of clinical immune complex disease in TH mice monitored for >2 months following treatment with MOG–Fc. Anaphylactic reactions were never encountered either. Sustained elimination of MOG-reactive, resting, activated and possibly also memory B cells may, in the long run, lead to a depletion of MOG-secreting plasma cells as well.

The intact blood–brain barrier may limit the penetration of a therapeutic protein. However, active MS plaques go hand-in-hand with loss of blood–brain barrier integrity, exposing affected brain regions to the peripheral circulation. This could add to the selectivity of the protein. Furthermore, the pathological relevance of B cells in the CNS of MS subjects is still a matter of debate. Our own experiments with TH anti-MOG transgenic mice clearly demonstrate that exacerbated EAE in these animals is not accompanied by increased numbers of MOG-specific B cells in situ (unpublished observation) (7). Thus, peripheral production of anti-MOG Ig seems the most likely cause of EAE exacerbation induced by anti-MOG antibodies.

It has to be noted that MOG is a strictly CNS-specific autoantigen that is neither expressed in the thymus nor in the periphery, which sets it apart from other CNS constituents like myelin basic protein (7) or other target antigens of B cell-mediated autoimmune attack in indications like pemphigus vulgaris and myasthenia gravis (23,24). Thus, MOG-specific clones are not subject to clonal deletion or peripheral tolerance induction, making MOG a potent encephalitogen (13). Hence, we are aware of the long-term pathogenic potential of MOG-specific immune responses elicited by the acute treatment with MOG–Fc. It should be emphasized that MOG was chosen solely as a model antigen for the study of B cell-mediated autoimmune disease in the absence of an inflammatory context, as would be encountered in EAE or MS. In the case of T1,2-dominated and truly B cell-mediated autoimmune indications, the respective antigen is usually expressed in thymus and periphery, and participates in the generation of tolerance. In summary, the present data clearly demonstrate the potential of MOG–Fc-based compounds in the attenuation of autoreactive B cell responses in vivo.

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Abbreviations

CNS central nervous system
EAE experimental autoimmune encephalomyelitis
Depletion of autoreactive B cells

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