Generation of Gene-Engineered Chimeric DNA Molecules for Specific Therapy of Autoimmune Diseases

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Abstract

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the development of self-reactive B and T cells and autoantibody production. In particular, double-stranded DNA-specific B cells play an important role in lupus progression, and their selective elimination is a reasonable approach for effective therapy of SLE. DNA-based vaccines aim at the induction of immune response against the vector-encoded antigen. Here, we are exploring, as a new DNA-based therapy of SLE, a chimeric DNA molecule encoding a DNA-mimotope peptide, and the Fv but not the immunogenic Fc fragment of an Fc γ RIIb-specific monoclonal antibody. This DNA construct was inserted in the expression vector pNut and used as a naked DNA vaccine in a mouse model of lupus. The chimeric DNA molecule can be expressed in eukaryotic cells and cross-links cell surface receptors on DNA-specific B cells, delivering an inhibitory intracellular signal. Intramuscular administration of the recombinant DNA molecule to lupus-prone MRL/*lpr* mice prevented increase in IgG anti-DNA antibodies and was associated with a low degree of proteinuria, modulation of cytokine profile, and suppression of lupus nephritis.

Introduction

SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) is a systemic autoimmune disease with complex pathogenesis and multiple organ involvement. This autoimmune syndrome is characterized by overproduction of autoantibodies, directed to a large spectrum of self-antigens, mostly to double-stranded DNA (dsDNA) and nucleosome antigens. These high-titer autoantibodies may persist in patient sera for 6–9 years before clinical manifestation, indicating that the breaking of tolerance to self-antigens is a starting point for lupus progression (Ehrenstein *et al.*, 1995; Kalsi *et al.*, 1996; Putterman, 2004).

Contemporary therapy for SLE includes corticosteroids as well as other immunosuppressive agents, which have a number of side effects (Dooley *et al.*, 1999; Toubi *et al.*, 2000; Kang *et al.*, 2003; Dorner, 2010). Immunosuppressive intervention often leads to severe imbalance of immune-regulatory mechanisms, manifesting as recurrent infections and malignant diseases (Monneaux and Muller, 2009). Therefore, development of new drugs for specific treatment of SLE is particularly important.

Self-reactive B cells play a key role in SLE pathogenesis. The different aspects of autoimmune B cell performance in-

clude antigen presentation, generation of antibody-secreting plasmacytes and memory B cells, and production of regulatory cytokines modulating the immune response. Hence, elimination of self-reactive B cells is a promising therapeutic approach against SLE (Shlomchik *et al.*, 1994; Chan *et al.*, 1999). A possible way for doing that is total B cell depletion, using pan-B cell-specific surface molecules. A number of relevant therapeutic monoclonal antibodies (mAbs) are currently under evaluation. Rituximab, a chimeric anti-CD20 monoclonal antibody, has been applied successfully for the treatment of autoimmune disorders such as rheumatoid arthritis and SLE (Anolik *et al.*, 2004, 2007; Edwards *et al.*, 2004; Eisenberg and Looney, 2005). Anti-CD20 mAbs target, bind, and induce Fc receptor-mediated and complement-mediated lysis of CD20-positive cells. However, CD20 is absent on early B cell precursors and plasma cells. Suppression of B cell activation and proliferation by epratuzumab (anti-CD22 mAb) and blocking of costimulatory receptors by Lympho-Stat-B (an anti-B cell-activating factor [BAFF] mAb) have also been introduced in clinical practice, but long-term therapy data are still missing (Baker *et al.*, 2003; Dorner *et al.*, 2006; Auyeung-Kim *et al.*, 2009).

Another possibility for B cell-targeted therapy is the induction of B cell apoptosis by B cell receptor (BCR)-mediated

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signaling, or by blocking key factors for B cell survival. The co-cross-linking of BCR with Fc γ RIIb (the IgG-inhibitory receptor) negatively regulates BCR signaling and inhibits BCR-induced cellular proliferation and other downstream biological responses through immunoreceptor tyrosine-based inhibition motifs (ITIMs) in the cytoplasmic tail (Ravetch and Lanier, 2000; Tarasenko *et al.*, 2007; Venkatesh *et al.*, 2009). Moreover, in contrast to CD20, Fc γ RIIb is expressed on plasma cells and may control their activity as well (Xiang *et al.*, 2007). Thus, Fc γ RIIb is an attractive target for downregulation of autoimmunity.

Whereas the administration of self-antigens or self-peptides may trigger tolerogenic mechanisms and delay the progression of autoimmune diseases in autoimmune experimental models (Pugliese, 2003; Miller *et al.*, 2007; Stoll *et al.*, 2007), the immunization of nonautoimmune mice with the dsDNA-mimicking peptide DWEYSVWLSN induced production of anti-dsDNA IgG antibodies (Putterman and Diamond, 1998; Putterman *et al.*, 2000).

Several murine strains spontaneously developing SLE-like syndrome have been used as animal models of autoimmunity. Among them, lupus-prone MRL/*lpr* mice develop autoimmune symptoms with characteristics close to human SLE with multiple organ involvement: the presence of autoantibodies to various nuclear components, massive T cell lymphadenopathy, splenomegaly, mononuclear cell infiltration of the kidneys, immune complex deposition, and nephritis. This lupus-prone strain has been used successfully to investigate the effects of B cell depletion and the precise role of self-reactive B lymphocytes as antigen-presenting cells in SLE (Chan and Shlomchik, 1998; Takeuchi *et al.*, 1998; Ahuja *et al.*, 2007; Dong *et al.*, 2007).

By means of protein engineering, we have constructed a number of chimeric molecules consisting of self-epitope-containing peptides (dsDNA-mimicking peptides or parts of the histone H1 peptide molecule) and a rat anti-mouse Fc γ RIIb-specific monoclonal antibody. When administered to lupus-prone MRL/*lpr* mice these chimeras could target pathological B lymphocytes specific to dsDNA or H1 and co-cross-link the BCR and Fc γ RIIb receptors on these B cells. Treatment of lupus mice with the chimeric molecules reduced the levels of anti-H1 and anti-dsDNA IgG antibodies and proteinuria, and prolonged the overall survival (Tchorbanov *et al.*, 2007; Mihaylova *et al.*, 2008, 2009; Nikolova *et al.*, 2009). However, after 6–8 weeks of administration of anti-rat immunoglobulin, antibodies appeared in all chimera-injected animals, thus restricting prolonged immunization (Mihaylova *et al.*, 2008).

The Fc fragment is the most immunogenic part of the immunoglobulin molecule. Numerous approaches for eliminating the Fc effects of therapeutic monoclonal antibodies have been used, such as mAb humanization and application of Fab fragments alone (Hwang and Foote, 2005; Presta, 2006). In the present study, we created a chimeric gene-engineered DNA molecule (a combination of coupled DNA fragments) encoding, respectively: a single-chain variable fragment (scFv) of an Fc γ RIIb-specific monoclonal antibody and a dsDNA-like peptide as a B cell epitope. The resulting bifunctional molecule co-cross-links the dsDNA-specific immunoglobulin B cell receptors with inhibitory Fc γ RIIb receptors. Moreover, the expressed fusion protein lacks the immunogenic Fc fragment, resulting in a lower anti-chimeric

immune response as compared with the formerly used native protein construct. We immunized lupus-prone MRL/*lpr* mice with the naked DNA construct, aiming to inhibit the dsDNA-specific autoreactive B lymphocytes and to suppress autoimmune symptoms in the treated mice.

Materials and Methods

Genetic work

V_L and V_H genes of scFv specific for mouse CR1/2 and CD16/32 have been described and submitted to GenBank (GenBank ID: AF220555, AF214704, FJ888350, and FJ888349). Antibody fragments generated from these sequences were shown to bind target receptors with high specificity (Prechl *et al.*, 1999; Szekeres *et al.*, 2010). Regions encoding scFv were transferred into the eukaryotic expression vector pNut, using *Bam*HI and *Not*I restriction sites (after the insertion of a linker containing a *Bam*HI site into the bacterial expression vector pET). The resulting vectors (pNUT-7G6scFv and pNUT-2.4G2scFv) thus contained the scFv genes fused to a secretion signal peptide at the 5' end and a stop codon and bovine poly(A) region at the 3' end, under the control of a metallothionein promoter. To add a mimotopic peptide sequence to the N terminus of these scFv molecules, we synthesized genes encoding the DWEYSVWLSN peptide with CATG overhangs to allow introduction into the *Nco*I site. Clones containing the DWEYSVWLSN-coding insert in the correct orientation were identified by PCR screening. The final constructs encoded the following protein regions: signal peptide–mimotopic peptide–scFv [DWEYSVWLSN G₃ AF220555/FJ888350 (G₄S)₃ AF214704/FJ888349]. Both vectors, containing chimeric molecules (DNA-like peptide–7G6scFv or DNA-like peptide–2.4G2scFv), were amplified in a prokaryotic competent culture, isolated, and purified (Pure-Yield plasmid maxiprep system; Promega, Madison, WI).

For the identification of correct construct size, enzyme restriction (*Bam*HI and *Eco*52I; Fermentas/Thermo Fisher Scientific, Waltham, MA) of the vectors was performed. The restriction mixtures were subjected to 1.2% agarose gel electrophoresis (Bio-Rad, Hercules, CA).

Antibodies

Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Sigma-Aldrich, Taufkirchen, Germany) was used for histological staining.

Mice

MRL/*lpr* mice were purchased from Charles River Laboratories (Saint Germain sur l'Arbresle, France) and bred in our animal facility under specific pathogen-free (SPF) conditions. Four-week-old female animals were used for the experiments. The manipulations were approved by the National Animal Care Commission in accordance with EU regulations.

BSA–DNA-like peptide conjugation

The DNA mimotopic peptide Ac-DWEYSVWLSN-Ahx-K-NH₂ was purchased from CASLO Laboratory (Lyngby, Denmark). Covalent coupling of the peptides to bovine serum albumin (BSA; Sigma-Aldrich) was performed by the

1-ethyl-3-(3'-dimethylaminopropyl) carbodiimide·HCl (EDC; Fluka, Buchs, Switzerland) cross-linking technique, using a spacer (Ahx-K-NH₂) at the C end of the peptides (Bauminger and Wilchek, 1980). BSA and the peptide (20-fold molar excess) were mixed and conjugation was initiated by the inclusion of EDC at 60-fold molar excess over BSA. The reaction mixture was stirred overnight at 4°C, dialyzed against phosphate-buffered saline (PBS), and concentrated by ultrafiltration up to 1.0 mg/ml.

Immunization schedule

Groups of 4-week-old female MRL/*lpr* mice (5 to 10 animals each) were immunized intramuscularly in the hind quadriceps with 50 µg of plasmid DNA encoding one of the hybrid molecules: sc7G6-DNA peptide or sc2.4G2-DNA peptide, or with 50 µg of empty plasmid (pNut), all diluted in 0.1 ml of sterile PBS.

Control animals were injected with PBS only. The second and third immunizations were performed at 14-day intervals, in accordance with the same scheme. Another 14 days later, members of the group injected with pNut-2.4G2-DNA peptide were separated into two equal subgroups. The first one was boosted twice at 14-day intervals with BSA-DNA peptide molecule (25 µg per mouse), and the second group was injected with PBS only. The animals from all groups were bled weekly and serum samples were kept frozen at -70°C.

Detection of urine albumin levels

Once per week, urine was collected directly from the urethra, and the urine protein content was measured with CombiScreen strips (Analyticon Biotechnologies, Lichtenfels, Germany) and scored semiquantitatively as 0, none; 1, 30–100; 2, 100–300; 3, 300–500; or 4, >500 mg dl⁻¹.

Cytokine quantification

Interleukin (IL)-4, IL-10, and interferon (IFN)-γ levels were measured in mouse sera, using ELISA sets (Bender MedSystems, Vienna, Austria).

Anti-DNA antibody detection

Anti-DNA antibodies were detected by ELISA as previously described (Tchorbanov *et al.*, 2007). Briefly, 96-well MaxiSorp immunoplates (Nunc, Roskilde, Denmark) were coated with methylated bovine serum albumin (10 µg/ml PBS; from Calbiochem, Darmstadt, Germany) followed by incubation with S1 nuclease-treated calf thymus DNA (Sigma-Aldrich) at a concentration of 2.5 µg per ml PBS overnight at 4°C. The plates were then blocked with 1.0% gelatin, incubated with serum dilutions, and later detected with peroxidase-conjugated anti-mouse IgG antibody (BD Biosciences, San Jose, CA). The samples were developed with an ABTS solution [2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)] (Sigma-Aldrich) and read at 405 nm. For anti-DNA antibody determination a standard mouse anti-dsDNA antibody, 10F10, was used.

Flow cytometry

Spleen cell suspensions from MRL/*lpr* mice were prepared by grinding cells through sterile cell mesh; erythro-

cytes were lysed with a hypotonic ammonium chloride solution. The cells were incubated with biotinylated anti-CD19 antibody followed by streptavidin-phycoerythrin (PE) (Sigma-Aldrich). To study T cell presence splenocytes were stained with an anti-mouse CD3-FITC antibody (BD Biosciences). Each incubation step was performed for 30 min at 4°C. Last, the cells were washed twice and kept at 4°C. Ten thousands cells were analyzed from each sample with a BD LSR II flow cytometer using Diva 6.1.1. software (BD Biosciences).

Immunofluorescence and histology

Kidneys from sacrificed experimental animals were removed for immunofluorescence and histopathological analysis. One kidney from each treated mouse was frozen and sectioned. The cryostat sections were incubated with fluorescein-conjugated goat anti-mouse IgG, and the immune complex depositions in renal glomeruli were examined with a fluorescence microscope (Carl Zeiss, Jena, Germany). The second kidney of the animals was fixed with buffered formalin, embedded in a paraffin block, and stained with hematoxylin-eosin according to a standard technique for histopathological examination. Quantitation of kidney pathology was scored: 3 (excellent renal preservation) to 0 (poor renal preservation).

Statistical analysis

The values in figures correspond to means ± SD. The unpaired Student *t* test was used to determine differences between each set of two groups. The two-tailed Mann-Whitney *U* test was used when appropriate. *p* < 0.05 was considered statistically significant.

Results

Construction of recombinant DNA molecules

The DNA-like peptide-7G6scFv and DNA-like peptide-2.4G2scFv genetic constructs were engineered in the eukaryotic expression vector pNut, possessing a viral promoter for foreign protein expression (Fig. 1). After amplification in prokaryotic culture and purification, the chimeric DNA molecules were analyzed by enzyme restriction (*Bam*HI and *Eco*52I) and the correct size of the constructs was demonstrated by agarose electrophoresis (data not shown).

Anti-dsDNA IgG antibodies in MRL/*lpr* mice are significantly decreased after treatment with chimeric DNA molecules

The *in vivo* effects of the DNA chimeras were studied in young lupus-prone MRL/*lpr* mice without autoimmune symptoms. Groups of animals were immunized with naked plasmid carrying DNA encoding the above-described antibody chimeras: DNA-like peptide-7G6scFv or DNA-like peptide-2.4G2scFv. A subgroup of animals immunized with the chimeric DNA-like peptide-2.4G2scFv molecule was prime-boosted with a BSA-DNA-like peptide conjugate. Empty plasmid or PBS was used for immunization of control animals.

The presence of serum anti-dsDNA autoantibodies is an important symptom of lupus development in MRL/*lpr* mice.

FIG. 1. Genetic construction of recombinant DNA molecules: an overview. (For details, see Materials and Methods.)

The administration of DNA-like peptide-2.4G2scFv to MRL/*lpr* mice resulted in a decrease in IgG anti-DNA antibody levels as compared with the PBS-treated group (Fig. 2). Significantly lower levels of anti-dsDNA antibodies were detected in the sera of DNA chimera-treated mice starting from week 7 of age until the end of the observation period. The prime-boost with a protein conjugate (BSA-DNA-like peptide) did not improve the results observed without protein immunization. Lupus-prone mice treated with DNA-like peptide-7G6scFv or empty plasmid pNut exhibited significantly higher levels of anti-dsDNA antibodies after week 15 of age.

Administration of DNA-like peptide-2.4G2scFv chimera prevents cell infiltration at the level of the kidneys and the development of glomerulonephritis

Anti-dsDNA IgG plays an important role in the development of autoimmune glomerulonephritis by the formation of immune complex depositions followed by kidney damage. Differences in albuminuria and kidney histology observed in the treated groups were related to the levels of anti-dsDNA antibodies. MRL/*lpr* mice treated with DNA-like peptide-2.4G2scFv had lower albuminuria levels during the treatment in comparison with PBS-injected control mice (Fig. 3). Additional immunization with BSA-DNA-like peptide also resulted in lower proteinuria after week 21 of treatment, compared with the PBS-treated group. Mice immunized with the other two control DNA constructs maintained relatively higher proteinuria levels as compared with PBS-injected controls, which progressively increased.

Developed glomerulonephritis and mononuclear cell infiltration were typical findings in lupus-prone MRL/*lpr* mice. As shown in Fig. 4 (top) the administration of DNA-like peptide-2.4G2scFv reduced renal pathology and preserved kidney structure (score, 3), whereas PBS-treated controls presented massive mononuclear cell accumulations surrounding the blood vessel structure (score, 1). Mice

FIG. 2. Treatment of MRL/*lpr* mice with chimeric DNA molecule suppresses anti-dsDNA antibody production. Groups of mice (5 to 10 animals per group) were treated with 50 μ g of plasmid DNA encoding sc2.4G2-DNA peptide, or with empty plasmid (pNut). Control animals were injected with PBS only. Half of the group injected with pNut-2.4G2-DNA peptide was boosted twice at 14-day intervals with BSA-DNA peptide molecule (25 μ g per mouse); the second half was injected with PBS only. Administration of chimeric DNA molecule prevented the appearance of high titers of anti-dsDNA IgG antibodies in lupus-prone MRL/*lpr* mice. All samples were analyzed in triplicate and average values were used for analysis. Means $\pm$ SEM were calculated for each group; *p* values were calculated by Mann-Whitney *U* test (**p* < 0.05; ***p* < 0.01; ****p* < 0.002), in comparison with PBS-treated controls. PBS, phosphate-buffered saline.

prime-boosted with a BSA-DNA-like peptide or immunized with DNA-like peptide-7G6scFv also had preserved kidney histology even though some infiltrates were present. Reduced organ pathology corresponded to the lower proteinuria levels scored in treated mice.

IgG-containing immune complexes were detected by immunofluorescence. At the end of the observation, the DNA-like peptide-2.4G2scFv-treated MRL/*lpr* mice (whether boosted or not with BSA-DNA like peptide) had fewer IgG depositions than mice treated with the alternative DNA construct (Fig. 4, bottom). As expected, PBS-injected animals exhibited massive mesangial glomerular depositions of IgG-containing immune complexes.

Treatment with chimeric DNA molecule decreases the size of lymphoid organs in MRL/*lpr* mice

After the end of experiment (age, 27 weeks) all PBS-treated mice had enlarged spleens and lymph nodes, compared with DNA chimera-treated animals. It was proven by fluorescence-activated cell-sorting (FACS) analyses that the enlargement of lymphoid organs in MRL/*lpr* mice is due primarily to the accumulation of large numbers of T cells (data not shown).

Cytokine measures

MRL/*lpr* mice present spontaneous and rapid development of SLE symptoms, which makes them an attractive

FIG. 3. Proteinuria levels in MRL/*lpr* mice after treatment with chimeric DNA molecules. Groups of mice (5 to 10 animals per group) were treated with 50 μ g of plasmid DNA encoding sc2.4G2-DNA peptide, or with empty plasmid (pNut). Control animals were injected with PBS only. Half of the group injected with pNut-2.4G2-DNA peptide was boosted twice at 14-day intervals with BSA-DNA peptide molecule (25 μ g per mouse); the second half was injected with PBS only. Immunization with chimeric DNA molecule prevented the rise in proteinuria in lupus-prone MRL/*lpr* mice. Means $\pm$ SEM were calculated for each group; *p* values were calculated by Mann-Whitney *U* test (**p* < 0.05; ***p* < 0.01; ****p* < 0.002), in comparison with PBS-treated controls.

model of autoimmune diseases. To investigate the effect of DNA chimera treatment on disease progression we determined serum levels of several cytokines. Helper T cell type 1 (Th1) cytokines, including IFN- γ , are known to play an important role in MRL/*lpr* disease. Although IFN- γ is one of the major mediators of several autoimmune disorders, the regulatory cytokine IL-10 has also been implicated in MRL/*lpr* pathology. High levels of IFN- γ and IL-10 in mouse serum correlate with disease severity (Bobe *et al.*, 2006).

As expected, serum IFN- γ and IL-10 levels were increased in PBS-injected MRL/*lpr* mice (up to 1200 pg/ml and up to 490 pg/ml, respectively), whereas administration of chimeric DNA molecules significantly reduced the levels of these cytokines (Fig. 5).

In addition, the group of animals treated with DNA-like chimera exhibited higher serum IL-4 levels (up to 170 pg/ml) that increased during the observation period, as compared with the groups of mice injected with PBS or with empty vector. Therefore, IL-4 could be involved in the down-modulation of autoimmune response in MRL/*lpr* mice.

Discussion

The loss of tolerance to self-molecules has been identified as the trigger of autoimmune pathways. A number of specific and safe self-antigens or peptides have been explored as immune therapy of various autoimmune diseases including SLE, multiple sclerosis (MS), and rheumatoid arthritis in animal models or in clinical trials in humans. Therefore, using self-peptides appears to be a promising therapeutic

FIG. 4. Administration of chimeric DNA molecules preserves renal histology and restricts immune complex deposition in the kidneys of MRL/*lpr* mice. *Top:* (A–D) Immunofluorescence analysis of IgG deposition in the glomeruli of MRL/*lpr* mice treated with PBS (A), sc7G6-DNA peptide (B), sc2.4G2-DNA peptide (C), and sc2.4G2-DNA peptide followed by prime-boost with BSA-DNA peptide molecule (D). The kidney sections from at least five mice were stained with FITC-labeled goat anti-mouse IgG. Data are representative of at least five experiments. Original magnification, $\times 400$. *Bottom:* (E–H) Hematoxylin-eosin staining of kidney sections from at least five MRL/*lpr* mice treated with PBS (E), sc7G6-DNA peptide (F), sc2.4G2-DNA peptide (G), and sc2.4G2-DNA peptide followed by prime-boost with BSA-DNA peptide molecule (H). Data are representative of at least five experiments. Original magnification, $\times 250$.

approach. The administration of autoantigens to delay or prevent disease progression is a disease-specific pathogenic approach, which may avoid the side effects of current nonspecific corticosteroid and immunosuppressive therapy. However, despite a number of data about successfully induced self-tolerance in animal experimental models, the translation of this therapy into clinical practice is not yet available (Miller *et al.*, 2007).

The administration of soluble peptide in high doses results in the generation of regulatory cells and the inhibition of specific autoimmune responses. In T cell-dependent autoimmune diseases such as MS and type 1 diabetes, peptide treatment limits the costimulatory effects of antigen-presenting cells to autoreactive CD4⁺ T cells and results in

FIG. 5. Reduction of serum IL-10 and IFN- γ in sc2.4G2-DNA-treated MRL/*lpr* mice. Serum levels of IFN- γ (**A**), IL-4 (**B**), and IL-10 (**C**) in MRL/*lpr* mice treated as described in Fig. 2 were measured by sandwich ELISA. (**A**) IFN- γ (** p < 0.01, PBS vs. sc2.4G2-DNA peptide + BSA-DNA; *** p < 0.002, PBS vs. sc2.4G2-DNA peptide); (**B**) IL-4 (** p < 0.002, PBS vs. sc2.4G2-DNA peptide and PBS vs. sc2.4G2-DNA peptide + BSA-DNA); (**C**) IL-10 (** p < 0.002, PBS vs. sc2.4G2-DNA peptide and PBS vs. sc2.4G2-DNA peptide + BSA-DNA). Data are represented as means $\pm$ SEM of 5–10 mice per group; p values were calculated in comparison with PBS-treated controls, using the unpaired Student t test.

decreased IL-2 and IFN- γ production (Nepom, 2002; Martinez *et al.*, 2003; Miller *et al.*, 2007; Stoll *et al.*, 2007). Immunization with autoantigen-derived peptides has also proved beneficial in B cell-mediated autoimmune diseases, such as SLE, in which B cells are major antigen-presenting

cells (APCs) and produce antibodies against self-structures (Kaliyaperumal *et al.*, 1999; Marino *et al.*, 2000). Promising results were obtained in treating both BALB/c and severe compromised immune-deficient (SCID) mice with the DNA-mimotope peptide DWEYS. The administration of this peptide prevented pathological complex depositions and renal pathology development in animals injected with anti-dsDNA antibodies (Gaynor *et al.*, 1997).

In general, gene therapy induces antibody immune responses to the encoded protein or peptide. Immunization with DNA vectors encoding self-peptides, followed by prime-boosting with the same soluble peptide, results in specific suppression of autoreactive T cells, which in turn leads to cell anergy. The possible mechanism is promotion of autoreactive CD4⁺ T cell proliferation after the first immunization, and activation-induced cell death (AICD) after the second one (Ferrera *et al.*, 2007; Miller *et al.*, 2007).

MRL/*lpr* mice develop symptoms similar to the immunological abnormalities observed in human SLE. This mouse strain is suitable for testing newly generated therapeutic proteins or genetic molecules aiming at specific elimination of autoreactive B cells (Chan and Shlomchik, 1998; Takeuchi *et al.*, 1998; Ahuja *et al.*, 2007; Dong *et al.*, 2007).

We have previously established an experimental model consisting of DNA vaccination of MRL/*lpr* mice with a DNA-like peptide-coding vector, followed by prime-boosting with a BSA-DNA-like peptide protein conjugate. Using this immunization protocol we could not induce effective tolerance. Chang and colleagues obtained similar results with a DNA vaccine encoding a preproinsulin in nonobese diabetic (NOD) mice. This vaccine was rendered tolerogenic only when administered together with a vector encoding an inhibitory costimulatory chimeric protein (Chang *et al.*, 2005).

In the present study, we engineered a chimeric DNA molecule, encoding simultaneously the heavy and light chains of an inhibitory Fc γ II receptor-specific antibody and the DNA-like peptide DWEYSVWLSN. As vector a pNut plasmid with enhanced expression in eukaryotic cells was used, as a naked DNA vaccine.

By chemical coupling of the dsDNA-mimicking peptide alone or in combination with another peptide, to a rat anti-mouse Fc γ RIIb monoclonal antibody, we have constructed chimeric protein molecules binding specifically to disease-associated B lymphocytes. Using this targeting device, we have already successfully suppressed dsDNA-specific B cells and prevented the appearance of autoantibodies in the intact MRL/*lpr* autoimmune mouse model (Tchorbanov *et al.*, 2007; Mihaylova *et al.*, 2009). The precise mechanism of signal transduction after co-cross-linking of the BCR and Fc γ RIIb receptors is well known (Brown *et al.*, 2004; Carter *et al.*, 2004). However, the development of anti-rat immunoglobulin antibodies after 8 weeks of treatment limits the long-term administration of this chimera to lupus-prone mice.

By means of protein engineering, we have constructed a number of chimeric molecules consisting of self-epitope-containing peptides (dsDNA-mimicking peptides or parts of the histone H1 peptide molecule) and a rat anti-mouse Fc γ RIIb-specific monoclonal antibody.

There are various approaches to avoid this effect, including protein and genetic processing eliminating the immunogenic “foreign” and nonfunctional compounds (Hwang

and Foote, 2005; Presta, 2006). The genetic construct used in the present study lacks the sequence encoding the Fc portion of the rat antibody. There is no experimental approach to demonstrate the direct action of plasmid DNA molecules on autoreactive B cells. When this mode of immunization is used *in vivo*, the expressing system is a muscle cell according to the conventional method of DNA administration. Therefore, the expressed fusion protein targeting DNA-like peptide to the corresponding protein-specific B cells was expected to be less immunogenic than the previously used protein chimera.

Indeed, we observed stable suppression of dsDNA-specific B cells during the experiment. The groups of MRL/*lpr* mice treated with DNA-like peptide-2.4G2scFv had low levels of anti-dsDNA IgG antibodies and proteinuria as compared with the control groups. In addition, animals treated with the chimeric DNA molecule were protected from pathological immune complex deposition and demonstrated intact kidney histology. Prime-boosting with a conjugated BSA-DNA-like peptide did not improve further the clinical symptoms, meaning that antigen targeting by an expressed single-chain antibody could reach the maximal beneficial effect.

FcγRIIb has a prominent role in B cell control and could be a useful tool for the regulation of B cell-induced autoimmune pathology (Venkatesh *et al.*, 2009; Horton *et al.*, 2011). The coengagement of inhibitory FcγRIIb with BCR, using the chimeric DNA-like peptide-2.4G2scFv molecule, down-regulated dsDNA-specific B cells and prevented the progression of lupus in MRL/*lpr* mice.

The pathogenic role of cytokines has been studied in many autoimmune diseases. SLE is characterized by an imbalanced immune response reflected in the abnormal levels of some regulatory cytokines. Yet, what remains unclear is the exact combination of cytokines involved in disease progression, as cytokine levels and balance in lupus pathogenesis differ from those in healthy control subjects, and vary considerably at the different stages of the disease (O'Shea *et al.*, 2001).

In healthy individuals, IFN-γ-producing regulatory T cells suppress pathogenic Th2 cells and autoreactive B cells (Peng *et al.*, 1997). Although significantly higher IFN-γ levels are detected in patients with SLE and in lupus-prone mice, IFN-γ-dependent B and T cell regulation in MRL/*lpr* mice is defective, leading to the generation of pathogenic autoantibodies and self-specific T cells. It has been demonstrated that the increased production of IFN-γ is the main reason for the development of lupus symptoms in MRL/*lpr* mice, and that the administration of IFN-γ accelerates disease development in both mice and humans (Bobe *et al.*, 2006). Animals with defective expression of IFN-γ or IFN-γ receptor did not develop severe lupus symptoms (Haas *et al.*, 1997; Schwartz *et al.*, 1998). Our results show high IFN-γ levels in PBS-treated MRL/*lpr* mice and suppression of IFN-γ production in animals immunized with chimeric DNA molecules.

The precise role of the regulatory cytokine IL-10 in the pathogenesis of SLE remains uncertain. It seems that IL-10 overexpression contributes to the abnormal production of autoantibodies and disease progression in mice and humans. Patients with SLE and untreated MRL/*lpr* mice produce large amounts of IL-10, and treatment of patients with active disease with anti-IL-10 antibodies suppresses lupus symptoms (Bobe *et al.*, 2006). In our hands, IL-10

levels were abnormally high in PBS-treated MRL/*lpr* mice, and decreased significantly after administration of chimeric DNA molecule.

MRL/*lpr* mice exhibit reduced levels of IL-4, resulting in an increased ratio of IFN-γ to IL-4. In contrast to animals lacking IFN-γ, IL-4-deficient mice develop autoantibodies and renal damage (Peng *et al.*, 1997). The Th1/Th2 dysregulation in MRL/*lpr* mice is a mediator of progressive autoimmune symptoms. Our results support the statement that IFN-γ and IL-4 play opposing but dispensable roles in the development of lupus-associated hypergammaglobulinemia and autoantibody production.

DNA-based therapy represents a new strategy for specific antigen delivery and autoimmune disease treatment. The administration of chimeric DNA molecule strongly suppresses the ongoing autoimmune disease in lupus-prone MRL/*lpr* mice: cytokine and antibody production, kidney mononuclear cell infiltration, glomerular immune-complex deposition, and tissue destruction. Using such a novel therapy, it is possible to downregulate selectively the activity of lupus-associated B lymphocytes and to restrict progression of spontaneous autoimmune disease.

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Author Disclosure Statement

No competing financial interests exist.

References

- Ahuja, A., Shupe, J., Dunn, R., *et al.* (2007). Depletion of B cells in murine lupus: Efficacy and resistance. *J. Immunol.* 179, 3351–3361.
- Anolik, J.H., Barnard, J., Cappione, A., *et al.* (2004). Rituximab improves peripheral B cell abnormalities in human systemic lupus erythematosus. *Arthritis Rheum.* 50, 3580–3590.
- Anolik, J.H., Barnard, J., Owen, T., *et al.* (2007). Delayed memory B cell recovery in peripheral blood and lymphoid tissue in systemic lupus erythematosus after B cell depletion therapy. *Arthritis Rheum.* 56, 3044–3056.
- Auyeung-Kim, D.J., Devalaraja, M.N., Migone, T.S., *et al.* (2009). Developmental and peri-postnatal study in cynomolgus monkeys with belimumab, a monoclonal antibody directed against B-lymphocyte stimulator. *Reprod. Toxicol.* 28, 443–455.
- Baker, K.P., Edwards, B.M., Main, S.H., *et al.* (2003). Generation and characterization of LymphoStat-B, a human monoclonal antibody that antagonizes the bioactivities of B lymphocyte stimulator. *Arthritis Rheum.* 48, 3253–3265.
- Bauminger, S., and Wilchek, M. (1980). The use of carbodiimides in the preparation of immunizing conjugates. *Methods Enzymol.* 70, 151–159.
- Bobe, P., Bonardelle, D., Benihoud, K., *et al.* (2006). Arsenic trioxide: A promising novel therapeutic agent for lymphoproliferative and autoimmune syndromes in MRL/*lpr* mice. *Blood* 108, 3967–3975.
- Brown, K., Blair, D., Reid, S., *et al.* (2004). FcγRIIb-mediated negative regulation of BCR signalling is associated with the recruitment of the MAPkinase-phosphatase, Pac-1, and the 3'-inositol phosphatase, PTEN. *Cell. Signal.* 16, 71–80.

- Carter, N., and Harnett, M. (2004). Dissection of the signalling mechanisms underlying Fc γ RIIB-mediated apoptosis of mature B-cells. *Biochem. Soc. Trans.* 32, 973–975.
- Chan, O., and Shlomchik, M. (1998). A new role for B cells in systemic autoimmunity: B cells promote spontaneous T cell activation in MRL-*lpr/lpr* mice. *J. Immunol.* 160, 51–59.
- Chan, O., Hannum, L.G., Haberman, A.M., *et al.* (1999). A novel mouse with B cells but lacking serum antibody reveals an antibody-independent role for B cells in murine lupus. *J. Exp. Med.* 189, 1639–1648.
- Chang, Y., Yap, S., Ge, X., *et al.* (2005). DNA vaccination with an insulin construct and a chimeric protein binding to both CTLA4 and CD40 ameliorates type 1 diabetes in NOD mice. *Gene Ther.* 12, 1679–1685.
- Dong, J., Wang, Q.-X., Zhou, C.-Y., *et al.* (2007). Activation of the STAT1 signaling pathway in lupus nephritis in MRL/*lpr* mice. *Lupus* 6, 101–109.
- Dooley, M.A., Cosio, F.G., Nachman, P.H., *et al.* (1999). Mycophenolate mofetil therapy in lupus nephritis: Clinical observations. *J. Am. Soc. Nephrol.* 10, 833–839.
- Dorner, T. (2010). Therapy: Hydroxychloroquine in SLE: Old drug, new perspectives. *Nat. Rev. Rheumatol.* 6, 10–11.
- Dorner, T., Kaufmann, J., Wegener, W., *et al.* (2006). Initial clinical trial of epratuzumab (humanized anti-CD22 antibody) for immunotherapy of systemic lupus erythematosus. *Arthritis Res. Ther.* 8, R74.
- Edwards, J.C., Szczepanski, L., Szechinski, J., *et al.* (2004). Efficacy of B-cell-targeted therapy with rituximab in patients with rheumatoid arthritis. *N. Engl. J. Med.* 350, 2572–2581.
- Ehrenstein, M., Katz, D., Griffiths, M., *et al.* (1995). Human IgG anti-DNA antibodies deposit in kidneys and induce proteinuria in SCID mice. *Kidney Int.* 48, 705–711.
- Eisenberg, R., and Looney, R.J. (2005). The therapeutic potential of anti-CD20: “What do B-cells do?” *Clin. Immunol.* 117, 207–213.
- Ferrera, F., Hahn, B., Rizzi, M., *et al.* (2007). Protection against renal disease in (NZB $\times$ NZW) F_1 lupus-prone mice after somatic B cell gene vaccination with anti-DNA immunoglobulin consensus peptide. *Arthritis Rheum.* 56, 1945–1953.
- Gaynor, B., Putterman, C., Valadon, P., *et al.* (1997). Peptide inhibition of glomerular deposition of an anti-DNA antibody. *Proc. Natl. Acad. Sci. U.S.A.* 94, 1955–1960.
- Haas, C., Ryffel, B., and Le, H.M. (1997). IFN- γ is essential for the development of autoimmune glomerulonephritis in MRL/*lpr* mice. *J. Immunol.* 158, 5484–5491.
- Horton, H., Chu, S., Ortiz, E., *et al.* (2011). Antibody-mediated coengagement of Fc γ RIIb and B cell receptor complex suppresses humoral immunity in systemic lupus erythematosus. *J. Immunol.* 186, 4223–4233.
- Hwang, W.Y., and Foote, J. (2005). Immunogenicity of engineered antibodies. *Methods* 36, 3–10.
- Kaliyaperumal, A., Michaels, M.A., and Datta, S.K. (1999). Antigen-specific therapy of murine lupus nephritis using nucleosomal peptides: Tolerance spreading impairs pathogenic function of autoimmune T and B cells. *J. Immunol.* 162, 5775–5783.
- Kalsi, J.K., Martin, A.C., Hirabayashi, Y., *et al.* (1996). Functional and modelling studies of the binding of human monoclonal anti-DNA antibodies to DNA. *Mol. Immunol.* 33, 471–483.
- Kang, I., and Park, S.H. (2003). Infectious complications in SLE after immunosuppressive therapies. *Curr. Opin. Rheumatol.* 15, 528–534.
- Marino, M., Ruvo, M., De Falco, S., and Fassina, G. (2000). Prevention of systemic lupus erythematosus in MRL/*lpr* mice by administration of an immunoglobulin-binding peptide. *Nat. Biotechnol.* 18, 735–739.
- Martinez, N.R., Augstein, P., Moustakas, A.K., *et al.* (2003). Disabling an integral CTL epitope allows suppression of autoimmune diabetes by intranasal proinsulin peptide. *J. Clin. Invest.* 111, 1365–1371.
- Mihaylova, N., Voynova, E., Tchorbanov, A., *et al.* (2008). Selective silencing of disease-associated B-lymphocytes by chimeric molecules targeting their Fc γ IIb receptor. *Int. Immunol.* 20, 165–175.
- Mihaylova, N., Voynova, E., Tchorbanov, A., *et al.* (2009). Simultaneous engagement of Fc γ IIb and CD22 inhibitory receptors suppresses the activity of targeted autoreactive B cells. *Mol. Immun.* 47, 123–130.
- Miller, S.D., Turley, D.M., and Podojil, J.R. (2007). Antigen-specific tolerance strategies for the prevention and treatment of autoimmune disease. *Nat. Rev.* 7, 665–677.
- Monneaux, F., and Muller, S. (2009). Molecular therapies for systemic lupus erythematosus: Clinical trials and future prospects. *Arthritis Res. Ther.* 11, 234.
- Nepom, G.T. (2002). Therapy of autoimmune diseases: Clinical trials and new biologics. *Curr. Opin. Immunol.* 14, 812–815.
- Nikolova, K., Tchorbanov, A., Djoumerska-Alexieva, I., *et al.* (2009). Intravenous immunoglobulin up-regulates the expression of the inhibitory Fc γ IIb receptor on B cells. *Immunol. Cell Biol.* 87, 529–533.
- O’Shea, J., Ma, A., and Lipski, P. (2001). Cytokines and autoimmunity. *Nat. Rev.* 2, 37–45.
- Peng, S., Moslehi, J., and Craft, J. (1997). Roles of interferon- γ and interleukin-4 in murine lupus. *J. Clin. Invest.* 8, 1936–1946.
- Prechl, J., Tchorbanov, A., Horvath, A., *et al.* (1999). Targeting of influenza epitopes to murine CR1/CR2 using single-chain antibodies. *Immunopharmacology* 42, 159–165.
- Presta, L.G. (2006). Engineering of therapeutic antibodies to minimize immunogenicity and optimize function. *Adv. Drug Deliv. Rev.* 58, 640–656.
- Pugliese, A. (2003). Peptide-based treatment for autoimmune diseases: Learning how to handle a double-edged sword. *J. Clin. Invest.* 111, 1280–1282.
- Putterman, C. (2004). New approaches to the renal pathogenicity of anti-DNA antibodies in systemic lupus erythematosus. *Autoimmun. Rev.* 3, 7–11.
- Putterman, C., and Diamond, B. (1998). Immunization with a peptide surrogate for dsDNA induces autoantibody production and renal immunoglobulin deposition. *J. Exp. Med.* 188, 29–38.
- Putterman, C., Deocharan, B., and Diamond, B. (2000). Molecular analysis of the autoantibody response in peptide-induced autoimmunity. *J. Immunol.* 164, 2542–2549.
- Ravetch, J.V., and Lanier, L.L. (2000). Immune inhibitory receptors. *Science* 290, 84–89.
- Schwartz, A., Wada, T., Kinoshita, K., *et al.* (1998). IFN- γ receptor signaling is essential for the initiation, acceleration, and destruction of autoimmune kidney disease in MRL-Fas/*lpr* mice. *J. Immunol.* 161, 494–503.
- Shlomchik, M.J., Madaio, M.P., Ni, D., *et al.* (1994). The role of B cells in *lpr/lpr*-induced autoimmunity. *J. Exp. Med.* 180, 1295–1306.
- Stoll, M.L., Price, K.D., Silvin, C.J., *et al.* (2007). Immunization with peptides derived from the idiotype region of lupus-associated autoantibodies delays the development of lupus nephritis in the (SWR $\times$ NZW) F_1 murine model. *J. Autoimmun.* 29, 30–37.

- Szekeres, Z., Herbath, M., Angyal, A., *et al.* (2010). Modulation of immune response by combined targeting of complement receptors and low-affinity Fc γ receptors. *Immunol. Lett.* 130, 66–73.
- Takeuchi, K., Inada, M., Miyashima, S., *et al.* (1998). A new strategy for treatment of autoimmune diseases in chimeric resistant MRL/*lpr* mice. *Blood* 12, 4616–4623.
- Tarasenko, T., Dean, J.A., and Boland, S. (2007). Fc γ RIIb as a modulator of autoimmune disease susceptibility. *Autoimmunity* 40, 409–417.
- Tchorbanov, A., Voynova, E., Mihaylova, N., *et al.* (2007). Selective silencing of DNA-specific B lymphocytes delays lupus activity in MRL/*lpr* mice. *Eur. J. Immunol.* 37, 3587–3596.
- Toubi, E., Rosner, I., Rozenbaum, M., *et al.* (2000). The benefit of combining hydroxychloroquine with quinacrine in the treatment of SLE patients. *Lupus* 9, 92–95.
- Venkatesh, J., Kawabata, D., Kim, S., *et al.* (2009). Selective regulation of autoreactive B cells by Fc γ RIIb. *J. Autoimmun.* 32, 149–157.
- Xiang, Z., Cutler, A., Brownlie, R., *et al.* (2007). Fc γ RIIb controls bone marrow plasma cell persistence and apoptosis. *Nat. Immunol.* 8, 419–429.

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