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Gene Transfer of Ig-Fusion Proteins Into B Cells Prevents and Treats Autoimmune Diseases

Marco E. F. Melo,* Jiahua Qian,2* Moustapha El-Amine,2* Rajeev K. Agarwal,† Nadejda Soukhareva,* Yubin Kang,* and David W. Scott3*

Based on the tolerogenic properties of IgG carriers and B cell Ag presentation, we developed a retroviral-mediated gene expression approach for treatment of autoimmune conditions. In this study, we show that the IgG-Ag retroviral constructs, expressing myelin basic protein (MBP) or glutamic acid decarboxylase in B cells, can be used for the treatment of murine models for multiple sclerosis and diabetes. Transduction of syngeneic B cells with MBP-IgG leads to the amelioration of ongoing experimental allergic encephalomyelitis induced by the transfer of primed cells from PL×SJL F1 mice with ongoing disease and could be effective even after symptoms appeared. This effect is specific and does not involve bystander suppression because treatment with MBP-IgG does not affect disease induced after immunization with proteolipid protein immunodominant peptide plus MBP. Interestingly, if donor B cells are derived from gld mice (Fas ligand-negative), then tolerance is not induced with a model Ag although there was no evidence for Fas ligand-mediated deletion of target T cells. In spontaneous diabetes in nonobese diabetic mice, we were able to stop the ongoing autoimmune process by treatment at 7–10 wk with glutamic acid decarboxylase-IgG retrovirally transduced B cells, or attenuate it with B cells transduced with an insulin B chain (B9–23) epitope IgG fusion protein. Furthermore, IgG fusion protein gene therapy can also protect primed recipients from Ag-induced anaphylactic shock, and thus does not cause immune deviation. These results demonstrate proof of principle for future efforts to develop this approach in a clinical setting.


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Treats Autoimmune Diseases

Genic therapy holds great promise for the treatment of a variety of diseases, including immunologically mediated diseases. In such diseases, autoreactive T cells and/or autoantibodies directly or indirectly damage self-tissues, leading to clinical manifestations of autoimmunity. Although some degree of immunosuppression or immune deviation has become part of current treatment plans, approaches to achieve specific tolerance are desirable. Moreover, it has been reported that treatment modalities using immune deviation may have deleterious effects on the course of multiple sclerosis (MS) (1) and its animal model, experimental allergic encephalomyelitis (EAE) (2), possibly leading to anaphylactic shock. Thus, the ability to reintroduce tolerance in an already primed and “perturbed” immune system is a challenge.

Based on the tolerogenic properties of Ig carriers combined with the efficacy of B cell Ag presentation for unresponsiveness, we previously demonstrated the induction of tolerance using LPS-activated B cells retrovirally transduced with an IgG-Ag fusion protein (3,4). These transduced B cells can efficiently express multiple antigenic epitopes presented in a tolerogenic manner requiring only MHC on the B cells (4,5). This protocol led to epitope-specific protection not only in naive, but also in already primed, recipients (3,5,6). Furthermore, the clinical potential of this form of gene therapy was first demonstrated in an experimental model of uveitis, a cell-mediated autoimmune disease (6). In this report, we extend our novel gene transfer approach to the treatment of two additional, widely used Th1-mediated autoimmune disease models: EAE, an inducible model of MS, and a spontaneous diabetes in nonobese diabetic (NOD) mice. We also examine whether this approach would cause immune deviation toward an allergic phenotype in the recipient mice, and whether it could be effective after disease symptoms appeared.

Several retroviral constructs that display myelin basic protein (MBP), glutamic acid decarboxylase (GAD) or insulin (B9–23) at the N terminus of murine IgG1 H chain were generated and were used to transduce LPS-stimulated B cell blasts. These B cells were then injected into primed recipients. The results demonstrated that our gene therapy approach to induce expression of Ag-IgG fusion proteins not only protected mice from ongoing EAE and diabetes, but also prevented allergic reactions in sensitized mice. These findings expand the clinical potential of our approach to EAE and diabetes and reiterate that the risk of horror autotoxicus associated with Th2 immune deviation (2) might not occur after such therapy, providing further evidence of the power of this tolerogenic treatment.

Materials and Methods

Replication-defective retroviral constructs and gene-transfer protocols

Retroviral constructs inserted with MBP-IgG, GAD-IgG, and B9-23-IgG and control retroviral constructs, p1-102-IgG and OVA-IgG. Molecular cloning of these retroviral vectors was similar to those described previously (3,4,6). Briefly, for MBP-IgG, a mouse MBP cDNA encoding exons 1, 2,
3, 4, 5, and 7 was amplified by PCR from pPHP45 plasmid, a gift of Dr. L. Hudson (National Institutes of Health, Bethesda, MD). The PCR product was ligated into pGEM-T vector and the fidelity of DNA polymerase was confirmed by DNA sequencing. Subsequently, mouse MBP cDNA was subcloned into the BSSK-IgG plasmid, a murine IgG1 H chain plasmid containing NorI and XhoI cloning sites at the N terminus of the IgG1 H chain (a gift of Dr. R. Tisch, University of North Carolina, Chapel Hill, NC). The BSSK-IgG-OVA and BSSK-IgG-GAD constructs were similarly engineered. dsDNA encoding insulin B9-23 was generated by annealing single-strand complementary synthetic nucleotides, and then inserted at NorIjXhoI sites in BSSK-IgG plasmid. BSSK-IgG-MBP, BSSK- IgG-OVA, BSSK-IgG-B9-23, and BSSK-IgG-GAD were later digested with Sall to extract the IgG-Ag cDNA. These IgG cDNA fragments were then cloned into a Sall-digested MAGE retroviral vector (3). Successful clones with correct orientation were used to transfect packaging cell lines. The expression of IgG-Ag fusion protein was driven by the β-actin promoter/enhancer, and a neomycin selection marker was included in the MAGE vector and driven by viral long-terminal repeat.

The GPE86 packaging cell line was transfected with MAGE-IgG-con- structs described above, and then selected under neomycin (G418, 0.6 mg/ml in active form), as described previously (3, 4). High titer clones (10^10–10^11 neomycin-resistant NIH 3T3 CFU/ml) free from replication competent retroviral particles were stored in liquid nitrogen for later use in gene therapy experiments. The virus producer cell lines used for the experiments described in this paper were named GPE-MBP9, GPE-GAD, GPE-OVA, GPE-p1-102, and GPE-InslunB9-23.

Gene transfer protocols. The splenic lymphocytes for gene therapy were performed as previously described (3–5). Briefly, splenic B cells were stimulated with 20–50 μg/ml bacterial LPS (Escherichia coli O55:B5; Sigma-Aldrich, St. Louis, MO) overnight, and recultured (4 × 10^6 cells/ml) in viral packaging cells (GPE.MBP, GPE-GAD, GPE-OVA, or GPE.InslunB9-23) in the presence of 6 μg/ml polybrene and LPS for an additional 24 h. The virally infected cells were washed and injected i.p. into syngeneic mice at designated intervals after the transfer of primed cells, except as noted.

RT-PCR and methods to ascertain the efficiency of gene therapy. In preliminary experiments, detection of MBP, GAD, or OVA transcripts in transduced cell lines or splenic tissues harvested from the mice receiving the transduced gene was used to verify that B cells were expressing the respective transgene by RT-PCR. Primers were designed to amplify MBP-, GAD- or OVA-specific sequences. One microgram of total RNA from splenic tissue was reverse transcribed (two rounds) with avian myeloblastosis virus reverse transcriptase, dNTPs, and oligo(dt) and random hexamer primers (Invitrogen cDNA cycle kit; San Diego, CA). The resultant cDNA was amplified using 2′ and 3′ primers (5′-primer/T7 DNA polymerase promoter; Roche Molecular Biochemicals, Palo Alto, CA). PCR conditions were 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C for 30 cycles. Amplified DNA products were loaded onto 1% agarose gels. β-Actin RT-PCR using commercially available primers (Stratagene, La Jolla, CA) was similarly performed to confirm the integrity of RNA samples and served as a loading control. Note that we have previously demonstrated that the construct and primers used to transduce B cells persisted for at least 4–8 mo based on the PCR signal and tolerance experiments (Refs. 4 and 6 and R. K. Agarwal and R. Caspi, unpublished observations). The efficiency of transfection was estimated to be 3–5% in our original experiments (3). The expression of Ag-IgG in the serum of mice receiving gene therapy was further verified using a modified 5-iodo-4-hydroxy-3-nitrophenylacetil (NIP)-binding ELISA to detect the ability of the IgG to bind to NIP hapten, as described previously (3, 4). However, the amount of NIP-binding activity did not correlate with the degree of tolerance induction or clinical efficacy, as we reported earlier (3, 4, 6). In other studies, CFSE-labeled B cell blasts actually proliferated and were detectable for at least 30 days (M. Litzinger and D. W. Scott, manuscript in preparation).

Immunological challenge and methods

Antigens. Bovine myelin basic protein (bMBP) was purchased from Sigma-Aldrich. Peptides MBP-Ac1-17 (MASQKRPSQRSKYLATA), MBP60-80 (HTRTHYGLSPQKSOHGRQT), and myelin oligodendro- cyte glycoprotein 35–55 (MEVGYWRSFPRVNYLHRNGK) were syn- thesized at the Molecular Biology Core of the Holland Laboratory (Rock- ville, MD). Proteolipid protein (PLP) 139-151 (HCLGKWLHPKDKF) was synthesized by Sigma-Genosys (The Woodlands, TX). In additional experiments, the bacteriophage Ac1 immunodominant peptide, p12–26, was used for immunization as described earlier (3, 4).

Mice. Female PL/J, SJL, F1 (PLSJL), B10.PL, C57BL/6, and NOD mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 5–6 wk of age; glzd (Fas ligand (FasL))– mice on a BALB/c background and control BALB/c mice were generously provided by Dr. W. Davidson, Holland Laboratory. All animals were housed in pathogen-free, microisolator cages at the animal facilities of the Holland Laboratory. All animal procedures were approved by the Animal Care and Use Review Committee at the Holland Laboratory of the American Red Cross.

Treatment for tolerance induction. In the EAE experiments, tolerance was induced with a single i.p. injection of 1–2 × 10^5 gene-transferred LPS-stimulated spleen or purified B cells diluted in 0.5 ml of PBS. Except as noted, injections of tolerogenic B cells were done within 6 h after the i.v. injection of MBP-reactive lymph node (LN) T cells, which had been re- stimulated in vitro with MBP, to transfer disease. Recipients were analyzed for disease, expression of recombinant retrovirus, as well as immune tol- erance to MBP for 4–6 wk after EAE induction. In NOD mice, tolerogenic treatment was performed at three different time points in separate exper- iments (starting at 7, 10, and 14 wk of age), using 1 × 10^5 LPS-stimulated spleen cells/mouse. To confirm these results, in one replicate experiment, groups of NOD mice were started on the tolerogenic gene therapy protocol at 7 or 14 wk of age. Groups of 5–10 mice were used in each replicate experiment.

Induction of EAE. To efficiently induce EAE with increased disease inci- dence, we adopted a combination of passive and active EAE induction protocols, because the incidence of EAE in our colony was low under standard immunization (M. Melo, unpublished observations). Briefly, 8–12-wk-old female NOD mice were injected with 100 μg of bMBP emulsi- fied in an equal volume of CFA (Bacto-Difco, West Molesey, Surrey, U.K.) at the base of the tail. After 15–30 days, LN T cells were harvested and cultured with bMBP (or MBP Ac1-17) and IL-2. In most experiments, spleen cells from the same primed animals were re- moved and used as sources of tolerogenic B cells, as described above, to accurately mimic a clinical situation. After 4–5 days, recipient mice were boosted with 200 μg of bMBP plus 100 μg of Ac1-17 emulsified in CFA. Pertussis toxin (List Biological Laboratories, Campbell, CA; 200 ng) was given i.p. in 0.3 ml at the time of immunization and again one day later. Groups of 5–10 mice were monitored for disease daily and EAE scored on a standard basis as follows: I = flaccid tail; II = partial paralysis of one or two limbs or flaccid tail with ataxia; III = total paralysis of two hind limbs; IV = quadriparalysis; moribund, V = death. Paralyzed mice were afforded easier access to food and water. In the experiments testing specificity and bystander suppression, disease was induced with a combination of bMBP emulsified with PLP-p139–151 or with the PLP peptide alone.

Lymphocyte proliferation and lymphokine measurements. To assay T cell responsiveness, animals receiving passive EAE transfer and subse- quent active immunization were euthanized at 12 days after s.c. immuni- zation with bMBP/CFA, and the LN and spleen were removed. Cells were cultured in X-Vivo medium (Life Technologies, Grand Island, NY) sup- plemented with 2 × 10^-3 2-ME (Sigma-Aldrich) and stimulated with 3, 10, or 30 μM concentrations of MBP or peptides. Proliferation was assayed by the addition of 1 μCi/well of [3H]thymidine (ICN Pharmaceuticals, Irvine, CA) for the last 18–48 h of a 3- to 4-day culture. The results were expressed as the stimulation index (cpm with Ag/cpm with medium alone). For cytokine measurements, LN or spleen cells were cultured with MBP or MBP peptides for 24–36 h in a 24-well plate. Cell sup- ernatants were collected and assayed for IFN-γ, IL-2 and IL-4 using standard capture ELISA as described elsewhere (4, 6).

Evaluation of disease in NOD mice

For treatment of NOD mice, B cell blasts from age- and sex-matched syngeneic donors were transduced to express IgG-GAD or IgG-Insulin B9-23, and then injected into 7-, 10-, or 14-wk-old female NOD recipients. Serum glucose levels were measured by standard methods in blood taken from the retro-orbital plexus. Mice were also evaluated daily for clinical signs of diabetes (e.g., weight loss) and mortality rate.

Statistical analysis

The Student t test (for paralysis scores and glucose levels) was used to evaluate significance for each time point. The statistical differences be- tween cumulative incidence and incidence density of the groups was also calculated. Values of p < 0.05 were considered significant, whereas p < 0.07 was considered clinically significant. (see Ref. 7 for discussion). De- tails for a given set of experiments are presented later in the legends and text.
Results

Injection of LPS blasts transduced with MBP-IgG construct prevents and treats ongoing EAE

Induction of specific tolerance to ameliorate autoimmune disease is desirable, especially if it can be achieved after the appearance of overt symptoms. One approach to induce tolerance is the delivery and presentation of the self Ag on an Ig carrier (3, 8, 9). We previously demonstrated that gene therapy via retroviral expression of IgG-fusion protein leads to tolerogenic B cell presentation of the endogenously produced autoantigen (4). To extend our gene therapy approach in animal models of autoimmune diseases, we developed a retroviral construct-encoding MBP autoantigen at the N terminus of murine IgG1 H chain for the treatment of EAE, an inducible animal model of MS. LPS-stimulated B cell blasts transduced with MBP-IgG construct were injected into susceptible mice to determine the efficacy of this approach on the course of ongoing EAE.

In preliminary experiments, i.v. injection of $8 \times 10^6$ MBP-IgG transduced B cells 7 days before disease induction was sufficient to protect PL×SJL mice from EAE (data not shown). In another Ag model system, p12–26 of λ repressor protein, we were able to induce tolerance with as low as $2 \times 10^6$ peptide-IgG expressing B cells (M. El-Amine and D. W. Scott, unpublished data). Because our goal is to arrest or reverse ongoing disease in a simulated clinical setting, B cell blasts from syngeneic bMBP-primed animals (exhibiting disease symptoms) were used to deliver the gene therapy. Injection of LPS B cell blasts expressing the murine MBP-fusion protein significantly protected PL×SJL (four experiments) and C57BL/6 mice (one experiment) from EAE even when the tolerogenic treatment was initiated after mice had received cells from MBP/CFA-immunized mice. Fig. 1 is a representative experiment in PL×SJL mice that received LN T cells from donors priamed 15 days earlier with MBP in CFA. As expected, the transfer of the MBP-primed LN T cells induced EAE and 100% mortality in all control mice receiving LPS blasts transduced with OVA-IgG construct. However, in contrast, both the average scores (Fig. 1), the prevalence (40%), and mortality rate (0%) were drastically diminished in the experimental group receiving MBP-IgG-transduced LPS blasts.

To test the efficacy of treatment for ongoing EAE, treatment was initiated after disease symptoms were apparent in the recipients of primed T cells. The results are shown in Fig. 2. In this experiment, we used LN and spleen cells from PL×SJL F1 mice with ongoing EAE to transfer disease to syngeneic recipients ($5 \times 10^6$ cells/mouse). Recipient mice were further boosted with MBP/CFA plus pertussis toxin 4 days later. Ten days after active immunization (which corresponds to day 3 after disease onset in Fig. 2), each mouse received $10^7$ primed B cell blasts transduced with either a control construct (OVA-IgG) or the experimental construct (MBP-IgG). At the time of the tolerogenic treatment, ~60% of the animals in each group were already showing signs of EAE (Fig. 2B). The donors of these tolerogenic and control B cell blasts came from mice primed in a similar way as the recipient animals. That is, we were treating ongoing disease with splenic B cells from animals that were already symptomatic. The experiment was carefully designed so that each subgroup of mice had the same number of sick animals and the same average score. Disease was monitored daily. Fig. 2A shows the average daily scores of a group of animals in which EAE was induced with MBP. Note that the difference between the average score of the control subgroup (GPE.OVA-treated) vs the experimental subgroup (GPE.MBP/9-treated) was not significant up to day 8. At day 9, the p value was 0.001 and it remained significantly different ($p < 0.05$) at all subsequent time points, except for day 32. It is also remarkable that at the end of the experiment, there was no animal showing signs of EAE in the experimental group (Fig. 2B). Note that the disease prevalence in control, mock-construct-treated mice is reduced slightly due to spontaneous remission in a subset of mice.

Despite the marked protection in vivo, there was only a modest inhibition of T cell proliferation and a marginal decrease in IL-2 production by lymphoid cells from treated mice in vitro (Fig. 3). There was also no effect on IFN-γ production by spleen or LN cells in any group (data not shown; M. Melo, R. Agarwal, and D. Scott, manuscript in preparation).

Mechanisms involved in tolerance via gene therapy

To investigate possible mechanisms of our gene therapy approach, several approaches tested the role of suppression, as well as FasL, in tolerance via this gene therapy protocol. First, we coimmunized one group of animals with bMBP/PLPp139 and another group with PLPp139 alone to test for specificity of tolerance and bystander suppression. Primed cells were transferred to syngeneic recipients, which then received MBP-IgG-transduced B cells. The results in Fig. 4 demonstrate that the gene therapy with MBP-IgG is specific in that no significant effect on PLP-induced disease is observed...
with PLP peptide induction (Fig. 4A), or with a mixture of PLP 139–151 plus MBP (Fig. 4B). Importantly, these results also suggest that bystander suppression is not involved in tolerance in this model, although this may require further study.

Next, we took advantage of the observation that LPS-activated B cells can express FasL (10) and gld mice lack functional Fasl, to test whether Fas-FasL interactions were important in tolerance in this model. Because gld mice on an SJL background were not available, we used the λ p12-26 immunodominant peptide and a p12-26-IgG vector to test for tolerance in BALB/c congenic mice. B cells from gld or +/+ mice were stimulated with LPS and then transduced with a p12-26-IgG construct used in our earlier experiments. We verified that FasL was up-regulated in normal B cells but not in gld B cells (M. El-Amine, unpublished observations). The results in Fig. 5 indicate that tolerance to p12-26 was induced with transduced wild-type B, but not with gld B, cells. These data suggest that up-regulation of FasL on target B cells by LPS (10) and Fas-FasL interactions are required for effective tolerance in this model. However, these data do not imply a deletion mechanism for tolerance because studies of the fate of TCR transgenic mice do not show a loss of specific T cells, and lck-driven Bcl-2 transgenic mice are not resistant to tolerance induction (M. Littzinger and D. W. Scott, manuscript in preparation; R. K. Agarwal and R. Caspi, unpublished observations) as would be expected if deletion via apoptosis was involved.

Injection of LPS blasts transduced with GAD-IgG or insulin-B9-23-IgG construct treats ongoing diabetes in NOD mice

Previously, we demonstrated that our gene therapy approach was effective in an experimental model of uveitis, experimental autoimmune uveoretinitis (6). Because both EAE and experimental autoimmune uveoretinitis involve deliberate immunization with pathogenic Ags or peptides, we extended our model to a spontaneous autoimmune disease, and therefore tested the efficacy of gene therapy in the NOD model of diabetes. NOD mice develop an immune response to GAD and peri-insulitis by 3–7 wk of age. Approximately 85% of female NOD mice will then develop overt diabetes by 4–5 mo of age. Therefore, we initiated the treatment in these NOD mice at three different time points: 7, 10, and 14 wk of age. We used two tolerogenic constructs coding for IgG-fusion proteins that contain either full-length GAD or an immunodominant insulin peptide (Insulin-B9–23). As shown in Fig. 6,
gene therapy using both constructs protected mice from disease, especially when treatment was initiated at 7 wk. For example, when glucose levels were measured at week 18 just before fatalities occurred, the difference between the controls and the GAD-IgG treated mice was highly significant (p < 0.02). The difference in disease incidence between GAD-IgG and control irrelevant construct (α p1–102-IgG) was clinically significant (p < 0.07; cf Ref. 7). When treatment was initiated at 10 wk, there was still protection both in terms of diabetes incidence and prolonged survival (data not shown). However, if treatment is delayed until 14 wk of life, there was no protection, presumably due to the extent of permanent islet damage that has occurred before the initiation of treatment.

IgG fusion protein gene transfer does not cause immune deviation toward an allergic phenotype: effect of gene therapy on anaphylaxis

Oral, i.v., and intranasal administration of soluble autoantigens or their peptides have been reported to ameliorate autoimmune diseases through a mechanism involving immune deviation toward Th2. However, this kind of “split tolerance” has recently been reported to increase the risk of an allergic response to the tolerogen (2). To determine whether the mechanism of our gene therapy for tolerance involved immune deviation that would carry an increased risk for anaphylaxis, we examined the effect of our approach on anaphylaxis to MBP in B10.PL mice to test efficacy in a related EAE-susceptible strain. After passive transfer of the MBP-reactive cells, mice were boosted with MBP/CFA and after 20 days, the animals were injected with 100 µg of bMBP i.v. As shown in Fig. 7, the anaphylactic shock response was observed in all control animals (OVA-IgG) but overall scores and incidence of fatal anaphylaxis were reduced in the experimental group (MBP-IgG); pathological examination confirmed the diagnosis of shock in the lungs of mice dying after the i.v. challenge (data not shown). Ten minutes after i.v. challenge, all animals in the control group had anaphylaxis, but three-eighths of the animals in the MBP-IgG-treated group did not have any signs of shock. Furthermore, after 1 h, fatal outcome occurred in five-eighths of the animals in the control group but only in two-eighths of the experimental mice. Thus, these results suggested that our gene transfer protocol did not cause immune deviation toward an allergic shock, but actually ameliorated anaphylaxis to MBP in contrast to peptide therapy.

Discussion

The data presented in this manuscript demonstrate that B cells, infected via our gene therapy protocol to express autoantigen-IgG fusion proteins, can induce tolerance for clinical efficacy in EAE even after symptoms appear, and can prevent spontaneous diabetes in NOD mice. Moreover, immune deviation was not observed as in other tolerogenic therapies. Thus, this protocol is safe, effective, and without untoward side effects in modulating the symptoms of murine autoimmune diseases.

Because one of the most tolerogenic APCs is the B cell, and one of the most tolerogenic carriers is IgG (11–14), our laboratory has developed protocols to gene transfer IgG-fusion proteins into B lymphocytes that are then used to express self IgG-fusion protein in vivo. Previously, we found that in primed mice, the expression of a model Ag in B cells as part of an IgG scaffold down-regulated both the Th1 and Th2 arms of the immunologic response (3, 6). We also found that MHC expression by the Ag-presenting B cells is required for tolerance (5), and that the IgG scaffold is important for down-regulation of the secondary immune response and maintenance of tolerance (4). Recently, the clinical potential of this therapeutic modality was shown in a mouse model of uveitis (6), a Th1-mediated disease that targets the retina. In this study, we extend our approach to two other autoimmune diseases, EAE and diabetes in mice (both Th1-mediated diseases) and to one murine model of anaphylactic shock (a Th2 disease). Thus, we demonstrate the general applicability of our approach and extend it to additional mouse strains.
A myriad of tolerogenic protocols designed to turn off pathological immune responses have been reported in the last decade (8, 15–29). These involve i.v. (18), intrathymic (19), i.p. (20), or mucosal (15–17, 21) administration of proteins and peptides, altered peptide ligands (22), and also i.v. injection of fixed APCs coupled with protein Ags (28, 29). Our goal was not to compare these tolerogenic therapies with our ex vivo gene therapy approach. Rather, we simply wished to establish its efficacy and safety, and begin to explore its mechanisms. Miller et al. (28) have previously proposed that specific peripheral immune tolerance induced by the i.v. administration of chemically-fixed splenocytes could be due to the induction of Ag-specific regulatory T cells. However, it has also been proposed that i.v. injection of MBP-coupled splenocytes induces tolerance by either anergy or deletion (29). In our model, we have obtained no data suggesting the involvement of regulatory cells (6) or of bystander suppression or a Th1→Th2 shift (3, 6). Thus, improvement in EAE symptoms correlated with reduced LN proliferation, a small decrease in IL-2 production, and no change in IFN-γ, IL-4, or IL-10 production (M. Melo, manuscript in preparation). This dissociation of the effect on disease and immunologic responses in vitro is not totally unexpected because modest effects on in vitro parameters of responsiveness were previously reported (6).

Interestingly, we found that B cells from gld mice were not tolerogenic in this protocol, suggesting that expression of FasL (as well as MHC class II) was required on the tolerogenic B cell APC. However, further data suggest that FasL-mediated deletion of target T cells is not involved because tolerance is equally effective in Bcl-2 transgenics (R. K. Agarwal, unpublished observations) and no loss of TCR transgenic T cells occurs (Litzinger and D. W. Scott, manuscript in preparation) in this gene therapy approach to tolerance.

Because of feasibility, injection, feeding, or nasal administration of autoantigens are very convenient ways to induce systemic tolerance. However, the therapeutic potential of each one of these approaches has not yet been proven in clinical practice (23, 24). Furthermore, in animal models, tolerance induction for ongoing immune responses or immune deviation is not only difficult to achieve in all strains and ages, but also can have deleterious consequences (2, 25). Altered peptide ligands are elegant and have potential, but these require knowledge of the precise autoantigenic epitope for a given MHC haplotype, and therefore cannot be used in every patient. Alternative protocols are clearly needed to stop
ongoing pathological autoreactivity by sustaining tolerance in the largest number of patients without causing side effects.

Because our major goal is to treat ongoing disease, we used as B cell donors mice that had been primed earlier and that had already shown signs of EAE. Indeed, in most experiments, we transduced primed B cells (as sources of tolerogenic APC) from the same MBP-primed animal that served as a donor for LN T cells to transfer EAE. This was to simulate the clinical situation in which autologous B cells from the patient to be treated would be the ideal delivering cells for the gene therapy. In this study, we showed that even using a potent encephalitogenic induction protocol, disease arrest was still possible. The experiment in Fig. 1 shows that PL×SJL mice can be protected after induction with cells from donors actively immunized 15 days earlier. In other experiments, we could protect mice from EAE transferred with T cells from mice primed for 30 days (30), during which time epitope spreading could have occurred. The experiment shown in Fig. 2 shows that we can actually arrest ongoing disease. After inducing disease with cells from ongoing EAE and waiting until 60% of the recipients show signs of disease, we can still significantly reduce disease scores (Fig. 2A) and prevalence (Fig. 2B). This provides evidence that protection can be achieved after the pathogenic T cells have migrated to the local sites of the lesions in the CNS. Although protection was achieved in this case, it is not possible to conclude that we are affecting determinant spreading (26). Further studies are needed to evaluate the response to minor Ags. The ability to track both the responding T cells with MBP-specific T cells, as well as to define the precise location of the tolerogenic B cells using CFSE labeling, will aid in understanding the mechanisms involved in this tolerogenic therapy.

In mice, EAE is more easily induced in strains such as SJL, PL, and B10.PL; however, it is now recognized that most, if not all, “resistant” strains have the machinery for mounting a pathogenic immune response to myelin Ags. Induction in EAE-resistant mice requires in vitro stimulation of myelin-reactive T cells to break their in vivo unresponsiveness to MBP/CFA immunization. Such in vitro manipulations provide another opportunity to test the efficiency of new therapeutic modalities to stop the effector phase of EAE in strains with different immunodominant epitopes. Using the IgG-MBP construct, we tested the gene therapy on EAE induced in B6 mice with MBPp60-80. Our data confirm the efficacy of this approach also in this strain (data not shown).

In NOD mice, B cell blasts transduced to express IgG-GAD or IgG-Insulin B9-23 were able to delay diabetes onset even when administered into 7- to 10-wk-old mice, a time when there is already evidence of peri-insulitis. Furthermore, in these studies, tolerogenic B cells came from similarly aged donors. This indicates that primed NOD B cells can be made to be tolerogenic. After 14 wk, no protective effect was observed. This could be explained by the speed in which the insulitis occurs in female NOD mice. In humans, the prediabetic stage can last for years, and it is postulated that during this period, insulitis is already taking place. Probably because of the small amount of tissue composing the mouse pancreas, the prediabetic stage lasts for only a few weeks in NOD mice. Because insulitis can be detected as early as 3–4 wk of age, by week 14, the inflammatory process could have already consumed all the islets in these mice. Hopefully, we may be able to achieve therapeutic effects with this protocol at later stages of disease when combined with islet transplantation.

The GAD-IgG retroviral construct we used was more effective than the insulin peptide-IgG construct. We suggest that this may reflect the cascade of responses to different epitopes that occurs in NOD animals. Thus, it has been postulated that the autoimmune cascade of determinant spreading in NOD mice starts with GAD (26, 27). This would explain the efficacy of this construct, because we are blocking the spreading of the response upstream in the cascade, although we cannot eliminate expression levels as an explanation.

In summary, our findings confirm the results in previous publications showing that this gene therapy approach induces tolerance at both the Th1 and Th2 levels. Because there were no signs of immune deviation, we conclude that our gene therapy does not increase (and actually can decrease) the risk of anaphylaxis in these disease models. The fact that the in vivo protection from disease was consistently demonstrated in different disease models and MHC haplotypes suggests potential general usefulness of this gene therapy approach in the diverse genetic background of the human population. This, together with our earlier data in the uveitis model (6), provides proof of principle for using this approach in a clinical setting.

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