Cytokine-Conditioned Dendritic Cells Induce Humoral Tolerance to Protein Therapy in Mice

Gautam Sule,¹ Masataka Suzuki,¹ Kilian Guse,¹ Racel Cela,¹ John R. Rodgers,² and Brendan Lee^{1,3}

Abstract

A major obstacle in the genetic therapy of inherited metabolic disease is host immune responses to the therapeutic protein. This is best exemplified by inhibitor formation in the protein therapy for hemophilia A. An approach to overcoming this is induction of immunological tolerance to the therapeutic protein. Tolerogenic dendritic cells (DCtols) have been reported to induce tolerance. In addition, cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)- β_1 are known to induce tolerance. To model protein therapy, we used ovalbumin (OVA) as antigen in BALB/c mice and their transgenic derivative, DO11.10 mice. In this study we show that adoptive transfer of antigen-pulsed dendritic cells (DCs) treated with a combination of IL-10 and TGF- β_1 can suppress the antibody response in mice. Adoptive transfer of cytokine-conditioned DCs in preimmunized mice results in reduction of antibody response in the mice. Furthermore, the effect is antigen specific, as the recipient mice were able to mount a potent antibody response to the control antigen. Last, we show that TGF- β_1 and IL-10-conditioned DCs are able to inhibit anti-FVIII antibody responses in FVIII knockout (KO) mice. Analysis of the contribution of IL-10 and TGF- β_1 to the DCtol phenotype shows that IL-10 treatment of DCs is sufficient for inducing OVA-specific tolerance in BALB/c mice, but we observed a requirement for treatment with both human TGF- β_1 and human IL-10 to significantly inhibit anti-FVIII antibody responses in FVIII KO mice. This paper demonstrates that autologous cell therapy for antigen-targeted immune suppression may be developed to facilitate long-term therapy.

Introduction

PROTEIN THERAPEUTICS are widely used to treat diverse disorders including infections, genetic deficiency, and cancer. Antibody responses to protein therapies represent important clinical obstacles as illustrated in patients with hemophilia A. The incidence of inhibitor formation is about 7% in all unselected hemophilia A patients, with the prevalence rising to 12–13% in those with mild to severe hemophilia. The only treatment options for such patients are escalating doses of factor VIII (FVIII) or induction of immune tolerance. Tolerance or partial tolerance can be induced by repeated infusions of high doses of the deficient protein, and in some cases this is followed by a combination of various nonspecific immunosuppressive regimens (Franchini *et al.*, 2008). However, this therapy is expensive, unsuccessful in up to 40% of cases, and rarely useful after relapse (Franchini *et al.*, 2008). Therefore, a method to control or suppress the

immune response in an antigen-specific manner would have obvious theoretical advantages for the long-term success of therapies requiring repeated and/or long-term protein administration.

Normally, when therapeutic proteins are administered intravenously, antigen presentation is expected to take place largely in the spleen (Andre *et al.*, 2009; Waters and Lillicrap, 2009). In the marginal zone of the spleen, antigen is endocytosed and processed by antigen-presenting cells (APCs). The APCs prime and activate naive T cells. Once activated, the T cells interact with B cells (Waters and Lillicrap, 2009). During the ensuing germinal center reaction, antigen-specific T and B cells costimulate each other, leading to B cell expansion and differentiation of B cells into terminally differentiated antibody-producing plasma cells, or into memory B cells. Once started, repeated administration of antigen in the form of therapeutic protein will drive additional rounds of B cell proliferation and increasingly higher titers of

¹Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030.

²Department of Pathology and Immunology, Baylor College of Medicine, Houston, TX 77030.

³Howard Hughes Medical Institute, Houston, TX 77030.

neutralizing antibodies (Lacroix-Desmazes *et al.*, 2008; Andre *et al.*, 2009; Waters and Lillicrap, 2009).

Dendritic cells (DCs) are a heterogeneous set of professional APCs that avidly take up, process, and present antigens to T cells. In some cases, however, DCs can be manipulated to induce tolerance (Thomson, 2010). Tolerogenic DCs (DCTols) are maturation-resistant, antigen-presenting DCs that do not produce inflammatory cytokines such as interferon (IFN)- γ , tumor necrosis factor (TNF)- α , and interleukin (IL)-12 (Rutella *et al.*, 2006; Morelli and Thomson, 2007; Ehser *et al.*, 2008; Thomson, 2010). DCTols induce tolerance by diverse mechanisms including anergy, production of tolerogenic cytokines, and development of regulatory T cells (Tregs) (Xiao *et al.*, 2006).

DCs have been reported to become tolerogenic after treatment with a variety of factors such as IL-10, transforming growth factor (TGF)- β_1 , vasoactive intestinal peptide, and vitamin D₃ (Rutella *et al.*, 2006; Ehser *et al.*, 2008; Torres-Aguilar *et al.*, 2010a; Su *et al.*, 2011). Numerous groups have reported that activated T cells, when exposed to TGF- β_1 , acquire suppressive functions, becoming Tregs (Chen *et al.*, 2003; Zheng *et al.*, 2008). The importance of TGF- β_1 in tolerance is illustrated by the excessive inflammatory response, with massive infiltration of lymphocytes and macrophages observed in many organs of TGF- β_1 -null mice (Kulkarni *et al.*, 1993). Another key mediator of tolerance is IL-10. DCs overexpressing IL-10 can suppress activated T cells and induce IL-10-producing Tregs *in vitro* (Fu *et al.*, 2008). DCs treated with TGF- β_1 and IL-10 are tolerogenic *in vitro* and able to suppress T cell proliferation (Torres-Aguilar *et al.*, 2010a,b). Sato and colleagues used TGF- β and IL-10-conditioned, and lipopolysaccharide (LPS)-matured DCs to prevent graft-versus-host disease (GVHD) and leukemia relapse in allogeneic bone marrow transplant mice (Sato *et al.*, 2003a,b). Taken together, these observations suggest that TGF- β_1 and IL-10 can play a vital role in the induction of tolerance. However, there is a paucity of literature regarding the use of DCs for the induction of tolerance to protein therapy (specifically toward FVIII). Qadura and colleagues reported that infusion of antigen-pulsed immature DCs modestly but not significantly reduced anti-FVIII antibody titers in hemophilia A mice (Qadura *et al.*, 2008). Consistent with this report, Ragni and colleagues reported that adoptive transfer of TGF- β_1 -treated and antigen-pulsed DCs in hemophilia A mice decreased anti-FVIII antibody titers in the mice, for up to 3 weeks (Ragni *et al.*, 2009), but did not report antigen-specific tolerance. Su and colleagues reported a decrease in anti-FVIII antibody titers when using DCs modified with foamy viral vector expressing human FVIII (Su *et al.*, 2011). In addition, they demonstrated adoptive transfer of T cells conferred antigen-specific tolerance to recipient mice. However, questions regarding the effectiveness of this strategy in various strains of mice, and individual contributions of the cytokines used to treat the DCs remain unanswered.

In the present study we use a highly sensitive T cell receptor (TCR)-transgenic mouse model (DO11.10 mice) in which 50–70% of all naive peripheral CD4⁺ T cells express an ovalbumin (OVA)-specific TCR (Murphy *et al.*, 1990). This system has the advantage that a large fraction of OVA-specific T cells can be readily stimulated, providing a robust measure of these types of strategies for suppressing antibody

responses to potentially clinically relevant levels. DO11.0 mice have been used to monitor changes in CD4⁺ T cells in the context of tolerance induction toward antigenic proteins (Dobrzynski *et al.*, 2004; Nayak *et al.*, 2009; Skupsky *et al.*, 2010). Using DO11.10 and BALB/c mice, we show the efficacy of autologous transfer of cytokine-conditioned DCs in inhibiting antibody responses. In mice receiving TGF- β_1 and IL-10-conditioned, antigen-pulsed DCs, we observe a 10-fold decrease in antibody responses. This tolerance is robust to a second antigen challenge and is antigen specific. In addition, we compare the relative contributions of IL-10 and TGF- β_1 to the inhibition of antibody responses *in vivo*. This strategy also inhibits anti-FVIII antibody titers in FVIII knockout (KO) mice challenged with recombinant FVIII coadministered with an adjuvant.

Materials and Methods

Animal models

BALB/c, C.Cg-Tg 10Dlo/J (DO11.10; BALB/c background) mice and hemophilia A C57BL/6 exon 16 knockout mice (referred to as FVIII KO mice, C57BL/6 background) were purchased from Jackson Laboratory (Bar Harbor, ME). All the mice were housed under pathogen-free conditions. Food and water were provided *ad libitum*. Mice used for studies were between 4 and 12 weeks of age. Blood was collected retro-orbitally for analysis. Plasma was frozen immediately and stored at -80°C until analysis. All experimental procedures were conducted in accordance with institutional guidelines for animal care and use.

Generation of DCs

DC precursors were harvested from femurs and tibias, and cultured as described (Seiler *et al.*, 2007). DCs were cultured in complete medium (RPMI 1640 with 10% fetal bovine serum, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, penicillin [100 U/ml], and streptomycin [100 $\mu\text{g}/\text{ml}$]) supplemented with mouse granulocyte-macrophage colony-stimulating factor (GM-CSF, 20 ng/ml; ProSpec, East Brunswick, NJ) and mouse IL-4 (10 ng/ml; ProSpec) for DCs alone; human TGF- β_1 (hTGF- β_1 , 10 ng/ml; eBioscience, San Diego, CA) for DCs + TGF- β ; human IL-10 (hIL-10, 10 ng/ml; eBioscience) for DCs + IL-10; or TGF- β_1 and IL-10 for DCs + TGF- β + IL-10, for 6 days with medium change on every alternate day of culture. On day 7 of DC culture, OVA (grade V, 25 $\mu\text{g}/\text{ml}$; Sigma-Aldrich, St. Louis, MO) or 2 IU of recombinant FVIII {ADVATE [antihemophilic factor (recombinant), plasma/albumin-free method]; Baxter, Deerfield, IL} was added to the culture. The next day, DCs were washed twice and 1 million cells were resuspended in 200 μl of Hanks' balanced salt solution (Thermo Scientific/HyClone, Logan, UT).

Timeline for OVA challenge

One million DCs were injected via the tail vein on day -14 (2 weeks before OVA challenge) and day -7 (1 week before OVA challenge). On day 0, the mice were challenged intravenously with 25 μg of OVA and at week 5 they were challenged (second challenge) intravenously with 25 μg of OVA. The mice were bled retro-orbitally and the plasma obtained was used for antibody titer determination by ELISA.

For the preimmunization, mice were injected intravenously with 25 μ g of OVA on day -28 (4 weeks before OVA challenge) and on day -21 (3 weeks before OVA challenge). Blood samples were collected to measure antibody development after the second injection.

Timeline for FVIII challenge

One million DCs were injected via the tail vein on day -14 (2 weeks before FVIII challenge) and day -7 (1 week before FVIII challenge). On day 0, mice were challenged intraperitoneally with a 1:1 (v/v) emulsion of complete Freund's adjuvant (CFA) and FVIII (200 μ l of the mixture was injected with 6 IU of FVIII per mouse), and at week 5 they were challenged (second challenge) intravenously with 2 IU of recombinant FVIII. The mice were bled retro-orbitally and the plasma obtained was used for antibody titer determination by ELISA.

Flow cytometric analysis

Cells were washed twice with stain buffer (BD Biosciences, Palo Alto, CA) and then blocked with purified rat anti-mouse CD16/CD32 (mouse BD Fc block; BD Biosciences) for 15 min on ice. After blocking the cells were washed twice with stain buffer and stained with specific antibodies for 30 min on ice. For analysis, the following antibodies were used: phycoerythrin (PE)-cyanine 7 (Cy7)-conjugated anti-mouse CD62L (Biolegend, San Diego, CA), PE-conjugated hamster anti-mouse CD69 (BD Biosciences), PE-conjugated anti-mouse/rat Foxp3 (eBioscience), R-PE-conjugated IgG (Sigma-Aldrich), Alexa Fluor 647-conjugated ovalbumin (Invitrogen, Carlsbad, CA), allophycocyanin (APC)-conjugated anti-mouse CD357 (GITR; eBioscience), and anti-human/mouse CD44 APC and anti-mouse CD3 APC-eFluor 780 (eBioscience). After staining, the cells were washed three times with stain buffer and subjected to flow cytometric analysis. Samples were analyzed with a BD FACSArray bioanalyzer (BD Biosciences) and data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Peripheral blood analysis

Red blood cells (RBCs) were lysed in 100 μ l of whole blood, using RBC lysis buffer (10 $\times$; Biolegend). After lysis, samples were washed with stain buffer twice and stained with CD3, CD69, CD62L, and CD44 for analysis.

Splenocyte culture assay

Splenocytes were collected from each group of mice (Suzuki *et al.*, 2011). One million splenocytes were washed with stain buffer and used for immediate *ex vivo* analysis by flow cytometry. Two million splenocytes were cultured with OVA (5 μ g/ml) for 2 and 5 days in duplicate and then washed with stain buffer twice and stained with CD3, CD69, CD62L, and CD44 for analysis.

ELISA

Rabbit anti-OVA at 10 μ g/ml (Sigma-Aldrich; for human serum albumin [HSA] ELISA, goat anti-HSA at 10 μ g/ml [Bethyl Labs, Montgomery, TX]; for FVIII ELISA, a matched-pair antibody set for ELISA of human FVIII [hFVIII] was

used at 10 μ g/ml [Affinity Biologicals, Ancaster, ON, Canada]), diluted in coating buffer, was used to coat the wells of 96-well plate. The plate was allowed to incubate overnight at 4°C. Plates were washed with phosphate-buffered saline (PBS)-Tween 20 (0.05%) (PBS-T) solution, and blocking buffer (5% milk in PBS-T) was added to the wells. The plates were then incubated at room temperature for 2 hr. Each plate was washed and OVA at 20 μ g/ml (for HSA ELISA, HSA at 20 μ g/ml; for FVIII ELISA, FVIII at 10 IU/ml) was added to the wells, followed by incubation overnight at 4°C. Plates were washed with PBS-T solution. To titer total IgG, plasma samples were diluted in dilution buffer (2% milk in PBS-T), added to the plates, and incubated overnight at 4°C. Wells were washed with PBS-T, and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Promega, Madison, WI) was added at a 1:3000 dilution in PBS-T. Tetramethylbenzidine (100 μ l; Sigma-Aldrich) substrate was added to each well for detection, and the reaction was stopped with 50 μ l of 2 N sulfuric acid. Plates were read at 450 nm in a microplate spectrophotometer. A standard curve was included in every plate and antibody titer was calculated by comparison with the standard curve.

TGF- β reporter assay

Transformed mink lung cells (TMLCs) stably transfected with a plasminogen activator inhibitor (PAI)-1-luciferase reporter gene (generously provided by D.B. Rifkin, New York University, New York, NY) were used. This bioassay is based on the ability of TGF- β to upregulate PAI-1 expression (Abe *et al.*, 1994). TMLCs were cultured in complete medium and were treated with IL-10 (10 ng/ml) and/or TGF- β_1 (10 ng/ml). Twenty-four hours later cells were assayed for luciferase activity. Luciferase activity was assayed with the Dual-Luciferase reporter assay system (Promega) as per the manufacturer's instructions.

Statistical analysis

Antibody titers were log transformed and all data are expressed as means $\pm$ standard deviation of the mean of the transformed data. One-way analysis of variance was used to compare groups.

Results

DCs conditioned with hTGF- β_1 and hIL-10 inhibit antibody formation and responses to antigenic challenge

In vitro effects of treating or conditioning DCs with IL-10 and TGF- β_1 have been described by Torres-Aguilar and colleagues; and Sato and colleagues have described the beneficial effects of cytokine-conditioned DCs in preventing graft-versus-host disease (GVHD) (Sato *et al.*, 2003a,b; Torres-Aguilar *et al.*, 2010a). To test whether conditioned DCs could decrease antibody responses *in vivo*, DCs generated *in vitro* were cultured with hIL-10 and hTGF- β_1 , pulsed with 25 μ g of OVA, and intravenously injected into DO11.10 mice. Recipient mice received two doses of the DCs on days -14 and -7. The groups included mice that received DCs pulsed with OVA alone (DCs-only group), mice that received DCs conditioned with hTGF- β_1 and hIL-10 and pulsed with OVA (DCs+TGF- β +IL-10 group), and mice that received

PBS injections (OVA-only group). The mice were challenged on day 0 and week 5 as shown in Fig. 1A. The antibody response was measured during two sequential periods after the primary and secondary antigen challenge. The OVA-only group received OVA injections only and the naive mice received PBS injections only. Total anti-OVA IgG immune re-

sponses were measured by ELISA at weeks 1 and 4 after the OVA injections. The DCs+TGF- β +IL-10-treated mice had a significantly lower OVA-specific antibody response at week 1 and at week 4 ($p < 0.01$ vs. DCs-only group; Fig. 1B).

Mice pretreated with conditioned DCs developed lower antibody responses than mice pretreated with control DCs. To test whether the reduction in antibody responses was due to inhibition by cytokine-conditioned DCs and not because the cytokine-conditioned DCs are poor immunogens, we challenged the mice with OVA. Even after a second OVA challenge (25 μ g), we observed a 10-fold reduction in OVA-specific antibody response at weeks 6 and 9 (1 and 4 weeks after challenge) versus mice treated with DCs alone (Fig. 1C). In addition, antibody responses in mice pretreated with conditioned DCs were even lower than in mice pretreated with PBS alone. This suggests that conditioned DCs functioned as tolerogens rather than as weak vaccines. Therefore, we conclude that pretreatment of mice with DCs pulsed with antigen and conditioned with TGF- β and IL-10 persistently suppressed antibody responses, even in the face of repeated challenge with a strongly immunogenic antigen.

IL-10 conditioning of DCs is sufficient to suppress antibody responses

To ascertain the individual contributions of IL-10 and TGF- β_1 and to delineate whether their effects were synergistic, we treated DO11.10 mice with DCs conditioned either with a combination of TGF- β_1 and IL-10 or with TGF- β_1 or IL-10 separately. The mice were treated according to the timeline shown in Fig. 1A. The various groups included mice that received DCs alone (DCs group), mice that received DCs conditioned with hTGF- β_1 and hIL-10 (DCs+TGF- β +IL-10 group), mice that received DCs conditioned with hIL-10 (DCs+IL-10 group), mice that received DCs conditioned with hTGF- β_1 (DCs+TGF- β group), mice that received OVA injections only (OVA-only group), and naive mice that received PBS injections only (naive group). As expected, DCs conditioned with both cytokines reduced antibody titers at week 1, week 3 ($p < 0.05$ vs. DCs group; Fig. 2B), and week 5 ($p < 0.01$ vs. DCs group; Fig. 2C) after the first OVA injection and at week 9 (4 weeks after a second OVA challenge; $p < 0.05$ vs. DCs group; Fig. 2D). Unexpectedly, IL-10-conditioned DCs suppressed antibody responses to the same extent: There was a 10-fold reduction in titers at week 3 ($p < 0.05$ vs. DCs group; Fig. 2B), week 5 ($p < 0.01$ vs. DCs group; Fig. 2C) after the first OVA injection, and week 9 (4 weeks after a second OVA challenge; $p < 0.05$ vs. DCs group; Fig. 2D). Moreover, DCs conditioned with hTGF- β_1 alone had no suppressive effect at any time point tested (Fig. 2A–D). We confirmed the bioactivity of our hTGF- β_1 preparation, using a TGF- β reporter cell line (as a control, we used TGF- β reporter cells that were not treated with TGF- β_1 ; Fig. 2E). These results indicate that IL-10-conditioned DCs, but not TGF- β_1 -conditioned DCs, suppress antibody responses to OVA in DO11.10 mice. Because DO11.10 mice are transgenic mice, we decided to repeat this experiment in pure wild-type mice. Similar results were obtained in the BALB/c mice (Fig. 3A–E). These results supported the hypothesis that the IL-10 conditioning of DCs was sufficient to generate tolerogenic DCs in mouse strains on the BALB/c background.

FIG. 1. TGF- β_1 and IL-10-conditioned DCs inhibit antibody response in DO11.10 mice. **(A)** Timeline: DO11.10 mice received DCs pulsed with antigen (OVA) but under various conditioning regimens (control DCs group, hTGF- β +hIL-10-treated DCs+TGF- β +IL-10 group, hTGF- β_1 -treated DCs+TGF- β group, and hIL-10-treated DCs+IL-10 group). On days -14 and -7, 1×10^6 cytokine-conditioned and OVA-pulsed DCs were injected intravenously into mice via the tail vein. On day 0 the mice were injected intravenously with 25 μ g of OVA and at week 5 they were challenged intravenously with 25 μ g of OVA. Mice were grouped on the basis of DC treatment. Control mice received OVA injections (OVA only) and naive mice received PBS injections. **(B)** Antibody toward OVA was measured at weeks 1 and 4, by ELISA (DO11.10 mice, $n=5$; data representative of two experiments; one-way analysis of variance [ANOVA] was performed, with comparison against DCs-only group; error bars represent the SD). **(C)** After the second OVA challenge, antibody toward OVA was measured at week 6 and week 9 (1 and 4 weeks after the second OVA injection) (DO11.10 mice, $n=5$; data representative of two experiments; one-way ANOVA was performed, with comparison against DCs-only group; error bars represent the SD).

FIG. 2. IL-10 treatment is sufficient to condition DCs to inhibit the antibody response in DO11.10 mice. DO11.10 mice were treated as indicated in the timeline (Fig. 1A), with the addition of individual cytokine-conditioned DC groups (hTGF- β alone, hIL-10 alone) to the existing groups. The groups of mice were as follows: control DCs group, DCs + TGF- β + IL-10 group, DCs + TGF- β group, DCs + IL-10 group, OVA-only group, and naive group. Antibody responses at week 1 (A), week 3 (B), week 5 (C), and week 9 (D) (week 4 after the second challenge) were measured by ELISA (DO11.10 mice, $n=5-10$; data representative of two experiments; one-way ANOVA was performed, with comparison against DCs-only mice; error bars represent the SD). (E) TGF- β_1 reporter cells were treated with IL-10 (10 ng/ml) and/or TGF- β_1 (10 ng/ml). Twenty-four hours later cells were assayed for luciferase activity ($n=2$; one-way ANOVA was performed, with comparison against control; error bars represent the SD).

TGF- β_1 and IL-10-conditioned DCs reduce antibody responses in preimmunized BALB/c mice and act in an antigen-specific manner

We tested whether the cytokine-conditioned DCs could suppress established immune responses. This would be an important preclinical model of clinical scenarios in humans who have established responses to therapeutic proteins. Mice were preimmunized intravenously with 25 μ g of OVA 2 weeks (day -28) and 1 week (day -21) before DC injections as shown in the timeline (Fig. 4A). Mice that received OVA injections had a significantly higher antibody response than did naive mice (Fig. 4B). The mice next received two doses of antigen-pulsed and cytokine-conditioned DCs on days -14 and -7 (Fig. 4A). In our experiments we did not observe any differences in the inhibition of antibody response in mice pretreated with TGF- β and IL-10-conditioned DCs compared with mice pretreated with IL-10-conditioned DCs. Hence, we decided to use TGF- β and IL-10-conditioned DCs rather than DCs treated with IL-10 alone, as the focus of the experiment was not on the role of individual cytokines, but rather on whether cytokine-treated DCs could inhibit established im-

mune responses. The groups were as follows: mice that received DCs alone (DCs group), mice that received DCs conditioned with hTGF- β_1 and hIL-10 (DCs + TGF- β + IL-10 group), mice that received OVA injections only (OVA-only group), and the naive mice, which received PBS injections only (naive group). Antibody responses were measured after primary and secondary antigen challenges. Mice that received cytokine-conditioned and antigen-pulsed DCs exhibited a modestly lower antibody titer at weeks 3 and 5 (Fig. 4C and D) and a 5-fold reduction in antibody titer ($p<0.05$) at week 9 (4 weeks after the second challenge) versus mice that received DCs only (Fig. 4E). Thus, cytokine-conditioned DCs can also reduce antibody titers in preimmunized animals.

It is possible that conditioned DCs might be globally immunosuppressive. To exclude this hypothesis, BALB/c mice were injected twice with 25 μ g of human serum albumin (HSA) at week 9 (4 weeks after the second OVA challenge). Immunization induced HSA-specific antibodies within 1 week of the second HSA injection in both groups receiving DCs ($p<0.05$ vs. naive group), but there was no significant difference between titers in mice receiving DCs alone versus those receiving cytokine-conditioned, antigen-pulsed DCs

FIG. 3. IL-10-conditioned DCs inhibit antibody responses in BALB/c mice. BALB/c mice were treated as indicated in the timeline (described in Fig. 1A) with the addition of individual cytokine-conditioned DC groups (hIL-10 alone and hTGF- β alone) to the existing groups. Antibody responses at week 3 (**A** and **C**) and week 5 (**B** and **D**) were measured by ELISA (BALB/c mice, $n=5$; data representative of two experiments; one-way ANOVA was performed, with comparison against DCs-only mice; error bars represent the SD). At week 5 mice were challenged with 25 μ g of OVA. (**E**) Antibody responses at weeks 6, 9, and 11 (weeks 1, 3, and 5 after the second challenge) were measured by ELISA (BALB/c mice, $n=3-5$; one-way ANOVA was performed, with comparison against DCs-only mice; error bars represent the SD).

(Fig. 4F). In addition, suppression by IL-10-conditioned DCs was similarly antigen specific in that responses to HSA were not inhibited (Fig. 4G). These results demonstrate that cytokine-conditioned, antigen-pulsed DCs suppress antibody response in an antigen-specific manner.

TGF- β_1 and/or IL-10-conditioned DCs do not reduce activated T cells and decrease effector T cells in mice

Mechanisms by which DCs induce tolerance include Treg generation, apoptosis of activated T cells, and inhibition of T cell activation (Xiao *et al.*, 2006; Torres-Aguilar *et al.*, 2010a). In addition, antibody development toward therapeutic proteins such as FVIII is known to be T cell dependent (Qian *et al.*, 2000). To dissect the mechanism by which the cytokine-conditioned DCs inhibited anti-OVA responses and possible effects on T cells, we assayed peripheral blood and splenocyte cultures from BALB/c mice (treated as shown in Fig. 1A) for expression of markers CD62L and CD44 (Anderson *et al.*, 2003).

Peripheral blood analysis for the relative frequency of effector memory T cells (CD3⁺CD62L⁺CD44⁺), memory T cells (CD3⁺CD62L⁺CD44⁺), and naive T cells (CD3⁺

CD44^{low}CD62L⁺) (Anderson *et al.*, 2003) at week 3 (Supplementary Fig. S1A; supplementary data are available online at <http://www.liebertpub.com/hum>) and week 5 (Supplementary Fig. S1B) showed reduction (not significant) in the effector T cell populations in mice receiving cytokine-conditioned DCs versus mice receiving control DCs, but at levels similar to those in OVA-only mice. Blood-borne antigens are filtered by the spleen, which provides the ideal environment for initiation and amplification of the immune responses (Lacroix-Desmazes *et al.*, 2008; Andre *et al.*, 2009). Mice were killed 1 week after the second OVA challenge (week 6), and splenocytes were analyzed *ex vivo*. In addition to subtyping T cells, we also assayed T cells for their activation status. Mice that received DCs treated with hTGF- β_1 and/or hIL-10 alone (DCs+TGF- β +IL-10 and DCs+IL-10) had significantly lower numbers of activated T cells (CD3⁺CD69⁺, DCs+TGF- β +IL-10 [$p<0.001$], DCs+IL-10 [$p<0.001$], vs. DCs group) (Supplementary Fig. S1D) (Sprent and Surh, 2002) and had reduced CD3⁺CD69⁺CD25⁺ cells compared with mice that received control DCs ($p<0.001$, DCs+IL-10 group vs. DCs group) (Supplementary Fig. S1D). In addition, mice treated with cytokine-conditioned DCs had

FIG. 4. TGF- β_1 and IL-10-conditioned DCs reduce antibody response in preimmunized mice. **(A)** Timeline: BALB/c mice were preimmunized 2 weeks (day -28) and 1 week (day -21) before DC injection, with 25 μ g of OVA. Antibody development after the second OVA injection was measured. On days -14 and -7, 1×10^6 cytokine-conditioned (control and TGF- β + IL-10 treated) and OVA-pulsed DCs were intravenously injected into the mice via the tail vein. On day 0 the mice were injected intravenously with 25 μ g of OVA and at week 5 they were challenged intravenously with 25 μ g of OVA. Mice were grouped on the basis of DC treatment. Control mice received OVA injections only (OVA-only group) and naive mice received PBS injections. The groups were as follows: DCs, DCs + TGF- β + IL-10, OVA only, and naive. **(B)** Antibody responses at week 1 after priming or preimmunization with OVA by ELISA (BALB/c mice, $n=4-6$). Antibody responses at week 3 **(C)**, week 5 **(D)**, and week 9 **(E)** (week 4 after the second challenge) were measured by ELISA (BALB/c mice, $n=4-6$; one-way ANOVA was performed, with comparison against DCs-only mice). **(F)** At week 9, the mice were given two doses of human serum albumin (HSA, intravenous; injections were spaced 1 week apart). One week later antibody responses toward HSA were measured (BALB/c mice, $n=3$ or 4; one-way ANOVA was performed; error bars represent the SD). **(G)** BALB/c mice were treated as indicated in the timeline in Fig. 1A, and at week 9 (4 weeks after the OVA challenge) mice were given two doses of human serum albumin (HSA, intravenous). The groups were as follows: DCs, DCs + TGF- β + IL-10, DCs + IL-10, and naive. One week later antibody responses toward HSA were measured (BALB/c mice, $n=3$ or 4; one-way ANOVA was performed, with comparison against naive mice; error bars represent the SD).

fewer effector T cells compared with mice receiving control DCs (Supplementary Fig. S1C). Next, we decided to test the effect of antigen-specific restimulation on splenocytes. Mice that received DCs treated with hIL-10 and hTGF- β_1 had significantly lower numbers of activated T cells (CD3⁺CD69⁺, $p < 0.001$, vs. DCs group) (Supplementary Fig. S1F) and both the cytokine-conditioned DC-treated groups had reduced CD3⁺CD69⁺CD25⁺ cells compared with the mice that received control DCs (DCs+TGF- β +IL-10 [$p < 0.001$], DCs+IL-10 [$p < 0.001$], vs. DCs group) (Supplementary Fig. S1F) in *ex vivo* 3-day splenocyte culture. In addition, mice treated with cytokine-conditioned DCs (DCs+TGF- β +IL-10 and DCs+IL-10) had fewer effector T cells compared with mice receiving control DCs (Supplementary Fig. S1E). However, in mice treated with OVA only, the phenotypic analysis of T cells did not vary significantly from that of the cytokine-conditioned DCs group. In cultures expanded *ex vivo* in the presence of OVA, we observed no difference in the Treg population (CD3⁺CD25⁺GITR⁺FOXP3⁺ cells; Supplementary Fig. S2) or in apoptosis (Supplementary Fig. S3). Although the reduced effector T cell frequency and decreased number of activated T cells in the mice receiving cytokine-conditioned DCs as compared with mice in the DCs-only group imply that conditioned-DCs lead to a lower frequency of OVA-specific T cells *in vivo*, the peripheral blood and splenocyte analysis revealed that the numbers of effector T cells and activated T cells in the cytokine-conditioned DCs treated mice were similar to those of the OVA-only mice. These results suggest that the reduction in effector T cell population and activation status of T cells, at least in the spleen and peripheral blood, might not be the mechanism responsible for tolerance induction.

TGF- β_1 and IL-10-conditioned DCs inhibit antibody responses against FVIII

To test whether cytokine-treated DCs can inhibit antibody responses to a therapeutic antigen such as FVIII, we intravenously injected autologous primary bone marrow-derived DCs from FVIII KO mice into FVIII KO recipient mice according to the timeline shown in Fig. 5A. One week after the DC injection, we challenged recipient mice with recombinant FVIII emulsified with CFA. The mice were monitored for 5 weeks with regular bleeds. At week 5, after the FVIII-CFA challenge, the mice were challenged with recombinant FVIII (2 IU per mouse, intravenous) and monitored for 4 weeks. Mice that received TGF- β and IL-10-conditioned DCs had lower antibody responses at week 3 ($p < 0.05$ vs. DCs group) and week 5 (Fig. 5B and C), and a 4-fold reduction in antibody titer ($p < 0.05$) at week 9 (4 weeks after the second challenge) versus mice that received DCs only (Fig. 5D). We also tested whether IL-10-conditioning of DCs was sufficient to inhibit anti-FVIII responses. In contrast to OVA, we observed only a modest reduction in titers (not significant) in mice that received IL-10-conditioned DCs versus mice that received DCs only (Fig. 5F). Hence, in the context of FVIII, treatment with both TGF- β and hIL-10 was needed for maximal tolerogenic effect.

Discussion

Dendritic cells that induce tolerance rather than immunity would be useful tools in transplantation and autoimmunity

(Rutella *et al.*, 2006; Thomson 2010). They might play a physiological role in suppressing autoimmunity or a pathophysiological role in antitumor immunity. For example, Sato and colleagues reported that DCs conditioned with TGF- β_1 and IL-10 reduced allograft rejection *in vivo* (Sato *et al.*, 2003a,b). In addition, DCs treated with IL-10 are able to generate adaptive type 1 T-regulatory (Tr1) cells, which are FOXP3⁺ and GITR⁺ (Jonuleit and Schmitt, 2003; Torres-Aguilar *et al.*, 2010a) and DCs conditioned with hTGF- β_1 and hIL-10 suppressed memory T cell responses in type 1 diabetes patient cells *in vitro* (Torres-Aguilar *et al.*, 2010b). However, the mechanisms of action of tolerogenic DCs are poorly understood and many technical obstacles lie between the concept and its clinical application. Moreover, most studies of DCtols have targeted T cell responses, and there have been limited efforts to develop this approach in the context of protein therapy, in which case neutralizing antibodies are often the limiting factor.

The aim of this study was to compare the efficacy of adoptive transfer of autologous DCs cultured with hTGF- β_1 and/or hIL-10 in mice receiving a model protein antigen. Specifically, we demonstrate that hTGF- β_1 and/or hIL-10-conditioned DCs decreased the antibody response toward OVA in BALB/c mice and their transgenic derivative, DO11.10 mice, expressing a class II MHC-restricted OVA-specific transgenic TCR. The magnitude of this effect was about 10-fold, compared with mice pretreated with untreated DCs. This could be explained by the hypothesis that DCtols merely make poor vaccines. However, antibody responses in mice pretreated with DCtols were up to 100-fold lower compared with PBS controls, showing that they functioned as tolerogens rather than as weak vaccines. In addition, we validated the application of this strategy toward a therapeutic antigen by inhibiting antibody responses toward FVIII in FVIII KO mice treated with recombinant FVIII. In our studies, "immature DCs" generated with GM-CSF and IL-4 were themselves variably tolerogenic, in agreement with reported observations (Qadura *et al.*, 2008), suggesting that the effect of cytokines might be to "stabilize" the tolerogenic state of immature DCs. DCtols pulsed with OVA suppressed previously induced immunity to OVA but failed to suppress immune responses to an unrelated antigen, HSA. The fold change in antibody responses to HSA was no more than 0.26 different (not significant) between the control DC-treated mice and cytokine-conditioned DC-treated mice, compared with a 2- to 15-fold change in the antibody responses toward OVA. These results suggest that hTGF- β_1 and/or hIL-10-conditioned DCtols can induce antigen-specific active suppression of both new and ongoing antibody responses.

Three groups have used DCs to modulate immune responses to FVIII, demonstrating reduction in antibodies toward FVIII (Qadura *et al.*, 2008; Ragni *et al.*, 2009; Su *et al.*, 2011). Our results also showed reduction of anti-FVIII antibodies, similar to those reported by other groups. In addition, we were able to inhibit antibodies toward FVIII after challenging the mice with an adjuvant and subsequent rechallenge with FVIII. Furthermore, we obtained similar results with a different antigen (OVA), verified the effect was antigen specific, and validated this across different strains of mice (BALB/c and DO11.10). Last, we demonstrate the strain- and antigen-specific requirement for TGF- β and IL-10

FIG. 5. TGF- β_1 and IL-10-conditioned DCs inhibit antibody response in FVIII KO mice. **(A)** Timeline: FVIII KO mice received DCs pulsed with antigen (recombinant FVIII) but different conditioning regimens (control DCs group, hTGF- β + hIL-10-conditioned DCs group). On days -14 and -7, 1×10^6 cytokine-conditioned and FVIII-pulsed DCs were intravenously injected into mice via the tail vein. On day 0 the mice were injected intraperitoneally with CFA-FVIII emulsion and at week 5 they were challenged intravenously with 2 IU of FVIII. Mice were grouped on the basis of DC treatment. Control mice received FVIII injections (FVIII-only group) and naive mice received PBS injections. Antibody toward FVIII was measured at week 3 **(B)** and week 5 **(C)** by ELISA (FVIII KO mice, $n=10-12$; one-way ANOVA was performed, with comparison against the DCs-only group; error bars represent the SD). **(D)** Antibody toward FVIII was measured at week 9 (4 weeks after FVIII challenge) (FVIII KO mice, $n=3-5$; one-way ANOVA was performed, with comparison against the DCs-only group; error bars represent the SD). **(E)** FVIII KO mice were treated as indicated previously in the timeline, with the only change being conditioning regimens of the DCs (control DCs group and hIL-10-conditioned DCs group). Control mice received FVIII injections only (FVIII-only group) and naive mice received PBS injections. Antibody toward FVIII was measured at weeks 3 and 5 by ELISA (FVIII KO mice, $n=3-5$; one-way ANOVA was performed, with comparison against the DCs-only group; error bars represent the SD).

to generate tolerance. In the case of FVIII and C57BL/6 mice (FVIII KO mice) both TGF- β and IL-10 were required to inhibit antibody responses, but IL-10 alone was sufficient to significantly inhibit antibody responses against OVA in BALB/c mice. Thus our results, along with the reports by Qadura and colleagues, Su and colleagues, and Ragni and colleagues, suggest that DCs can be used as effective tools to inhibit immune responses toward a therapeutic protein (Qadura *et al.*, 2008; Ragni *et al.*, 2009; Su *et al.*, 2011).

Several mechanisms might contribute to suppression by cytokine-conditioned DCs. TGF- β_1 and/or IL-10-conditioned DCs have reduced maturation markers and are poor antigen presenters (Sato *et al.*, 2002; Steinbrink *et al.*, 2002; Torres-

Aguilar *et al.*, 2010a; Manicassamy and Pulendran, 2011). Moreover, both TGF- β_1 and IL-10 are reported to induce Tregs (Wan and Flavell, 2006), which suppress helper T cells indirectly and without antigen specificity by secreting suppressive cytokines, and antigen specifically through direct engagement of conventional DCs. Antigen-specific direct effects of Tregs on DCs and/or B cells remain plausible although we failed to detect a significant change in the population of CD3⁺CD25⁺FOXP3⁺GITR⁺ Tregs. Thus, classical Tregs might not be involved in suppression in our *in vivo* model. Further studies involving adoptive transfer of the T cells would be needed to elucidate the complete role of Tregs in our model. In the report by Torres-Aguilar and colleagues,

DCs conditioned with TGF- β_1 and IL-10 produced IL-10 and TGF- β (Torres-Aguilar *et al.*, 2010a). Hence, it is possible the DCs in our studies are capable of secreting IL-10 and TGF- β . DCs secreting IL-10 and/or TGF- β_1 are known to generate Tr1 and Th3 T-regulatory cells that inhibit T cells by secretion of IL-10 or TGF- β , respectively (Mills, 2004; Qadura *et al.*, 2008). In addition, Tr1 regulatory cells are able to suppress antibody responses toward allergens and protect mice from allergic asthma (Ahangarani *et al.*, 2009). Consistent with this, we observed reduced frequencies of the effector T cell population in the peripheral blood and splenocytes of mice that received cytokine-conditioned DCs when compared with mice that received DCs only. Additional flow cytometric analysis of splenocytes revealed a decrease in activated T cells (CD3⁺CD69⁺ T cells and CD3⁺CD69⁺CD25⁺ T cells) in the cytokine-conditioned DCs group. Consistent with this interpretation, splenocyte cultures from tolerized animals generated fewer activated T cells and effector T cells in response to OVA *in vitro* when compared with mice that received DCs only. Because B cells are the primary APCs within the germinal center, and T and B cells cooperate for mutual costimulation in that reaction, even modest suppression of antigen-specific helper T and/or B cells might exert the large synergistic suppression of antibody titers observed. However, there was no significant difference in the frequency of activated T cells and effector T cells between the OVA-only mice and the mice pretreated with conditioned DCs. This suggests that the inhibition of T cell activation and reduction of effector T cells are not the mechanism by which the tolerogenic DCs act in our system and that further studies need to be carried out to elucidate their mode of action and the mechanism responsible for tolerance induction.

Studies have demonstrated the individual roles of IL-10 and TGF- β_1 in modulating properties of DCs *in vitro* (Sato *et al.*, 2003a; Torres-Aguilar *et al.*, 2010a). However, there have been limited studies reporting an *in vivo* comparison of TGF- β_1 and IL-10. In our experiments, hIL-10-conditioned DCs conferred tolerance toward OVA, to the same extent as the combination of IL-10 and demonstrably bioactive hTGF- β_1 in BALB/c mice. However, in FVIII KO mice (C57BL/6 background) and with FVIII as the antigen, IL-10-conditioned DCs tended to be less effective in inhibiting antibody responses. Moreover, hTGF- β_1 alone had little or no ability to inhibit anti-OVA antibody responses in BALB/c mice and with OVA as the antigen. These results appear to contrast those of Ragni and colleagues (2009), who reported that DCs generated with GM-CSF and TGF- β_1 suppressed antibody responses 10- to 20-fold compared with control DCs prepared with GM-CSF and IL-4. However, Ragni and colleagues used C57BL/6 mice and had used FVIII and OVA as their antigens, indicating a strain-specific requirement for TGF- β . Similar strain-specific requirements for IL-10 were demonstrated by Hoffman and colleagues, who found that IL-10 was necessary to prevent CD8⁺ T cell responses in C3H/HeJ mice but that a deficiency of IL-10 had no effect for tolerance induction in C57BL/6 mice (Hoffman *et al.*, 2011).

It is possible that TGF- β_1 behaves differently in our experimental system. For example, in contrast to Ragni and colleagues, we included IL-4 in the preparation of "DCtols" (Ragni *et al.*, 2009). Pillemer and colleagues reported that IL-4 compromises the effect of TGF- β_1 on T cells by inhibiting the

suppressive activity of TGF- β_1 -induced Tregs, by activating STAT6 (Pillemer *et al.*, 2009). Thus, it is possible that IL-4 inhibits TGF- β_1 (but not IL-10) signaling in DCs as well. Alternatively, TGF- β_1 may affect DCs from C57BL/6 and BALB/c strains differently, or TGF- β_1 may affect responses to FVIII and OVA differently. Another difference between the two studies lies in the different role of "control DCs" in the two experimental systems. It is well established that, in the absence of overt adjuvant (such as complete Freund's), C57BL/6 mice respond poorly when compared with BALB/c mice, to a number of soluble proteins, whether delivered by recombinant adenovirus (e.g., human erythropoietin [EPO; Tripathy *et al.*, 1996] and human α_1 -antitrypsin [AAT; Barr *et al.*, 1995]) or as a native protein (e.g., OVA [Hayglass and Stefura, 1991]; and human FVIII, serum albumin, and AAT [J.R. Rodgers, unpublished]). To resolve this issue mechanistically in mice will be important because the variation among mouse strains is likely to be mirrored in the human population.

Adoptive transfer of syngeneic DCs conditioned with TGF- β_1 and/or IL-10, and loaded with antigen, led to long-lasting and antigen-specific suppression of antibody responses, up to a factor of 100, in BALB/c and DO11.10 mice when compared with PBS-treated control mice. However, a 100-fold drop was still not enough to abrogate OVA-specific IgG. Similarly, there was a 0.5-log reduction in antibody responses toward FVIII in FVIII KO mice (C57BL/6 background) but again, the reduction did not ablate FVIII-specific IgG. This suggests that better understanding of the mechanisms, and further optimization of the technology, are needed to translate cytokine-conditioned DCtols from proven concept to clinical application. Methods used to generate the DCs, and to determine the dose and frequency of DC injections, are some of the factors that need to be optimized. At present there are few reports comparing the relative effectiveness of any of these factors. Molecules such as CTLA-4 immunoglobulin, rapamycin, and retinoic acid have been reported to augment the tolerogenic properties of DCs. They enhance the ability of DCs to promote a more active form of tolerance by the generation of Tregs (Zahorchak *et al.*, 2007; Zapata-Gonzalez *et al.*, 2007; Zhang *et al.*, 2010; Moghimi *et al.*, 2011). In addition, anti-CD20 antibody or rituximab treatment resulted in reduction of inhibitory antibodies (Franchini *et al.*, 2008; Zhang *et al.*, 2011). Our findings, along with the reports by Torres-Aguilar and colleagues and Sato and colleagues, indicate the translational potential of cytokine-conditioned DC therapy for the induction of tolerance (Sato *et al.*, 2003a,b; Torres-Aguilar *et al.*, 2010b). Last, the dramatic difference in efficacy of TGF- β_1 shown here, compared with that described by Ragni and colleagues, warns again against overreliance on any single animal model, but underscores that there is yet much to be learned by comparing disparate mouse models (Ragni *et al.*, 2009).

Acknowledgments

This work was supported by National Institutes of Health grants R01DK56787 and R01HL87836 to B. Lee.

Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Dr. Brendan Lee
Department of Human and Molecular Genetics
Baylor College of Medicine
One Baylor Plaza, Room R814
Mail Stop 225
Houston, TX 77030

E-mail: blee@bcm.tmc.edu

Received for publication December 16, 2011;
accepted after revision March 1, 2012.

Published online: April 2, 2012.