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*J Immunol* 2004; 172:178-185;
doi: 10.4049/jimmunol.172.1.178

http://www.jimmunol.org/content/172/1/178

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Tolerogenic APC Generate CD8\(^+\) T Regulatory Cells That Modulate Pulmonary Interstitial Fibrosis\(^1\)

Jie Zhang-Hoover and Joan Stein-Streilein\(^2\)

Transforming growth factor-\(\beta\)-treated Ag-pulsed APC mimic APC from the immune privileged eye, and provide signals that generate regulatory T (Tr) cells and mediate peripheral tolerance. We postulated that TGF-\(\beta\)-treated Ag-pulsed APC (tolerogenic APC (tol-APC)) might also orchestrate regulation of immune mediated pathogenesis in nonimmune privileged tissues such as the lung. We used an adoptive transfer model of autoimmune pulmonary interstitial fibrosis called hapten immune pulmonary interstitial fibrosis (ADT-HIPIF) in this study. Mice that received 2,4,6-trinitrobenzene sulfonic acid-sensitized cells and challenged (intratracheally) with the hapten developed pulmonary interstitial fibrosis. However, transfer (i.v.) of TGF-\(\beta\)-treated 2,4,6-trinitrobenzene sulfonic acid-pulsed bone marrow-derived APC (tol-APC) to experimental mice 1 day after intratracheal challenge reduced the collagen deposition in the interstitium of the lung that usually follows challenge. Furthermore, ADT-HIPIF mice that received tol-APC developed Ag-specific efferent CD8\(^+\) Tr cells. Adoptive transfer of the Tr cells to another set of presensitized mice mediated suppression of the efferent phase of Th1 immune response and the subsequent immune dependent pulmonary interstitial fibrosis. Thus, tol-APC induced efferent CD8\(^+\) Tr cells in immune mice, and the regulation of the immune response limited the development of autoimmune pulmonary fibrosis in sensitized and pulmonary-challenged mice. Because ADT-HIPIF shares etiological and pathological characteristics with a variety of human immune inflammatory conditions in the lung that eventuate into interstitial fibrosis, these studies provide insight into potential therapy to alter the course of pulmonary fibrosis in humans. The Journal of Immunology, 2004, 172: 178–185.

Antigen-presenting cells play a critical role in determining the outcome of immune responses. Moreover, it seems that the microenvironment in which APC capture Ags influences the function of APC. It is reported that APC that are exposed to immunosuppressive factors (TGF-\(\beta\), vasoactive intestinal peptide, \(\alpha\)-melanocyte stimulating hormone, and calcitonin gene-related peptide), such as those within the local environment of immune privileged sites, induce tolerance instead of immune inflammation when they present Ags (1–5). Tolerance-inducing mechanisms of the eye are well studied. Indigenous APC capture Ags that are injected into the anterior chamber of the eye, carry them through blood, and present them to T cells in the marginal zone of the spleen. Eye-derived APC by default provide tolerogenic signals (enhanced production of TGF-\(\beta\) and 1, IL-10, thrombospondin, and macrophage-inflammatory protein 2) during Ag presentation and induce the generation of efferent CD8\(^+\) T regulatory (Tr)\(^3\) cells with the collaboration of NKT cells and marginal zone B cells (1–3, 6).

Soluble factors such as TGF-\(\beta\) influence APC function by blocking up-regulation of costimulatory molecule CD40 on the APC, reducing their IL-12 production, and promoting IL-10, TGF-\(\beta\), and thrombospondin production (7–11). Subsequently, TGF-\(\beta\)-treated APC that are pulsed with Ag, in vitro or in vivo, prevent development of Th1 and Th2 inflammatory responses and instead generate Tr cells that mediate peripheral tolerance (12).

In this study, we report that TGF-\(\beta\)-treated Ag-pulsed bone marrow-derived APC are tolerogenic (tol-APC), mimic eye-derived APC (4, 13), and promote peripheral tolerance to Ags responsible for immune inflammation in the lung, a nonimmune privileged site. Currently, the only model for study of immune mechanisms that induce autoimmune pulmonary interstitial fibrosis is the hapten immune pulmonary interstitial fibrosis (HIPIF) model (14–16). A modification of HIPIF is the adoptive transfer (ADT) HIPIF model used in this study. Donor mice are sensitized by epicutaneous application hapten, 2,4,6-trinitrobenzene sulfonic acid (TNBS), and recipient mice are sensitized by adoptive transfer of spleen and draining lymph node cells collected from donor mice. The recipient mice are then challenged intratracheally (i.t.) 1 day later, and clinical symptoms are assessed 7–21 days later. Our previous publication indicates that the fibrosis in the recipient mice is detectable as early as day 7 and peaked at day 14 after i.t. challenge (15). Because the fibrosis is dependent on a chronic immune response against altered (hapten) self Ags developing in the lung and is long lasting, the ADT-HIPIF model represents autoimmune mediated fibrosis and has many similarities to human idiopathic pulmonary interstitial fibrosis and sarcoidosis (15, 17, 18). The elicitation of ADT-HIPIF requires the interactions of dendritic cells (DCs) and activated T cells (19, 20). After i.t. challenge, CD40\(^\text{bright}\) DCs also express MHC II\(^\text{high}\), interact with activated T cells, and initiate fibrogenesis.

\(^1\) This work is funded by National Institutes of Health Grants R01 EY11983 (to J.S.-S.), R01 EY13066 (to J.S.-S.), and F32 HL10148 (to J.Z.-H.), and the Schepens Eye Research Institute and Pulmonary and Critical Care Division, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02114.

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0022-1767/04/$02.00

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cells, enhance IL-12 and TNF-α production, and elicit a Th1-mediated immune response and subsequent development of fibrosis in the ADT-HIPIF lung (20). Ab-neutralizing TNF-α suppresses the development of fibrosis in the ADT-HIPIF lung (19). Although a Th2 immune response exists in the lung of some idiopathic pulmonary interstitial fibrosis patients and Th2 type of cytokine IL-4 is detected in some pulmonary interstitial fibrosis models (21–23), but not in the ADT-HIPIF, the involvement of the fibrogenic cytokine TNF-α is common in all these models. Furthermore, the characteristics of the Th1-mediated chronic immune response in the ADT-HIPIF lung resemble the manifestations of pulmonary sarcoidosis (24).

A similar model of hapten-immune mediated pathogenesis has been quite valuable in the study of immune mediated inflammation of the intestine (10, 25). Haptens or small reactive chemicals are ubiquitous within the urban and rural environments, thereby making it possible that autoimmune mediated pulmonary interstitial fibrosis could actually be induced by small reactive chemicals. It is important to note that idiopathic pulmonary interstitial fibrosis is of unknown etiology; thereby, the potential for the pathology being driven by a chronic autoimmune response is plausible.

Previous reports show that tolerogenic peritoneal exudate cells (PEC) are generated by treating thioglycollate-induced PEC with TGF-β and pulsing with Ag. Tolerogenic PEC induce the generation of CD8+ effector T cells in the spleen of presensitized mice (5). In this study, we show for the first time that similar treatment of bone marrow-derived APC induces tolerance in a model of ongoing immune mediated fibrogenic response in the lung (ADT-HIPIF).

Materials and Methods

Animals

Female BALB/c ByJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the Schepens Eye Research Institute Vivarium until they reached the desired weight (20–24 g) for the experiments. All animals were treated humanely in accordance with National Institutes of Health guidelines and the approval of the Schepens Animal Care and Use Committee.

Reagents

TNBS, 1-fluoro-2,4-dinitrobenzene (DNFB), picryl chloride (TCNB), oxazalone, controlled processed serum replacement factor 1, and tissue culture grade BSA were purchased from Sigma-Aldrich (St. Louis, MO). Porcine TGF-β2 were purchased from R&D Systems (Minneapolis, MN). Ascites of CD4- and CD8-depleting Ab (clones GK1.5 and 2.43, respectively) were generated in the laboratory. Baby rabbit complement was purified by passage through a 0.2 μm filter. Ascites of CD4- and CD8-depleting Ab (clones GK1.5 and 2.43, respectively) were generated in the laboratory. Baby rabbit complement was purified by passage through a 0.2 μm filter.

Generation of bone marrow-derived APC

APC were generated by culturing mouse bone marrow cells with L929 cell-conditioned medium for 6 days (26, 27). Mouse bone marrow cells were collected from femurs and cultured in growth medium (GM: DMEM supplemented with 10% horse serum, 10% controlled-processed serum replacement factor 1, vitamins, l-glutamine, sodium pyruvate, nonessential amino acid, penicillin/streptomycin, and 10% L929 cell-conditioned medium (source of CSF-1)) for 6 days in tissue culture plates or petri dishes. The majority of APC generated in petri dishes are nonadherent. After culturing, the adherent cells, loosely adherent cells, and nonadherent cells were collected by gentle scraping with a rubber policeman. Cell viability (checked by the trypan blue exclusion method) was greater than 90% in all experiments. Over 95% of the APC were F4/80 and CD11b positive by flow cytometry analysis.

Tol-APC treatment

Tol-APC were generated by treating bone marrow-derived APC overnight with TGF-β2 (5 ng/ml) in serum-free medium (SFM; RPMI 1640 medium, 10 mM HEPES, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.1% BSA, and 1% insulin transferrin-selenium supplement (Life Technologies, Grand Island, NY)) (1, 6). Phenotypes (MHC II, CD40, B7.2, and CD11c surface expression) of tol-APC and APC in GM and SFM were monitored in each experiment by flow cytometric analyses. Tol-APC were pulsed with 1 mM TNBS or 0.002% oxazalone and transferred (i.v. 5 × 10^7/100 μl HBSS/mouse) to recipient mice either 1 day before or 1 day after i.t. challenge in ADT-HIPIF mice.

Flow cytometry analyses

Cells in staining buffer (PBS, 1% BSA, 0.1% sodium azide) were incubated with blocking reagent (Fc blocker, 2 μg/10^7 cells; rat IgG, 20 μg/10^7 cells) and then PE anti-CD4 (3/23), PE anti-CD11c (HL3), PE anti-CD11b (M1/70,15), FITC anti-F4/80, biotin anti-MHC II (2C9), or biotin anti-B7.2 (GL1) (1 μg/10^7 cell), followed by FITC streptavidin. FITC rat IgG2b, PE rat IgG2a and 2b, PE hamster IgG, and rat IgG2a were used as isotype controls. All cells were incubated on ice for 20–30 min and washed twice before the next reagent was added. The samples were analyzed by flow cytometry (EPICS XL, Beckman Coulter, Miami, FL).

T cell enrichment and Ab plus complement treatment

T cells were enriched using T cell column that was packed in the laboratory using goat anti-mouse IgG-coated IMMUNAL beads (Biotexx Laboratories, Houston, TX). After enrichment, the percentages of T cells were monitored by CD4 plus CD8 staining and flow cytometric analyses. Together, CD41 and CD8 cells account for ~80% cells.

Cells (10^7/ml) were resuspended in complete medium (RPMI 1640, 10% FBS, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 μ/ml penicillin, 100 μg/ml streptomycin). Ab ascites (GK1.5 or 2.43) (1/500 dilution) were added into the cell suspensions that were then incubated for 40 min on ice. The cells were washed, and baby rabbit complement (1/10 dilution) was added into the cell suspensions before 30-min incubation at 37°C. In all the experiments, cells were treated with two rounds of Ab plus complement to ensure the depletion of either CD41 or CD81 T cells. The depletion of CD41 and CD81 T cells was confirmed by staining the cells with either CD4 (RM4-4) or CD8 (53-6-7) recognizing Ab and flow cytometry analyses.

Contact hypersensitivity (CH) ear-swelling assay

Mice were skin sensitized with hapten TNBS (3%, 100 μl/mouse) or DNFB (0.5%, 25 μl/mouse). Five to six days after skin sensitization, the ears of the mice were painted with either TNCB (1% in acetone, 20 μl/ear) or DNFB (0.05% in acetone, 20 μl/ear). The ear thickness was measured using an imaging micrometer (Mitutoyo, Paramus, NJ) before and 24 h after the ear challenge. The change (Δ) of ear swelling = ear thickness measured at 24 h after ear challenge − ear thickness measured before ear challenge.

Local adoptively transferred delayed-type hypersensitivity (LAT-DTH) assay

Effector cells (sensitized T cells, 5 × 10^5), regulatory cells (5 × 10^5), and TNBS-pulsed PEC (5 × 10^5) were resuspended in 10 μl HBSS and then injected intradermally into the ear pinnae of a naive mouse (1, 5). PEC were obtained from mice that received i.p. 2.5 ml thio glycollate (Difco, Detroit, MI) 3 days before and cultured in SFM overnight. The adherent cells were collected by vigorous pipeting and pulsed with TNBS (1 mM) for 20–30 min at 37°C in RPMI 1640 and washed with HBSS twice. The ear swelling was measured and calculated as CH ear-swelling assay.

Hydroxyproline assay

Changes in collagen deposition in the lung were measured by a colorimetric hydroxyproline assay (28). In brief, lungs recovered from the experimental mice were minced and hydrolyzed in 6 N HCl (2 ml/lung) for 16 h.
at 110°C. The samples were filtered through Whatman number 1 filter paper, diluted with H2O, neutralized with 10 N NaOH, and assessed spectrophotometrically. The amount of hydroxyproline in the lung was calculated according to the standard curve that was generated using a serial dilution of trans-4-hydroxy-L-proline (Sigma-Aldrich). The change (Δ) of lung hydroxyproline was calculated as experimental hydroxyproline – baseline hydroxyproline and compared among experimental groups. The baseline hydroxyproline was the mean value of hydroxyproline from five naïve mice.

**Lung histological examination**

Lungs from experimental and control mice were harvested 14 days after i.t. challenge, inflated, and fixed in 10% buffered Formalin. The processing and staining of the lung sections were done by Histoscientific Research Laboratories (Woodstock, VA). In brief, the fixed lungs were paraffin embedded, sectioned (4 μm) longitudinally through all five lobes. The sequential lung sections were stained with either H&E for cellular infiltration or Masson’s Trichrome for definitive identification of collagen. The digital images of stained lung sections were captured under microscopy (Nikon Eclipse E800, bright field, ×20 and ×200) using Spot digital camera (Diagnostic Instruments, Sterling Heights, MI). The results were processed on Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA).

**Statistical analyses**

ANOVA posthoc Scheffe’s test was used to evaluate the difference among experimental groups. Value of p ≤ 0.05 was considered significant. Each group contained five mice. The data are presented as mean ± SEM. An asterisk (*) indicates a statistically significant difference between two groups. Each result shown is a representative of two to three experiments.

**Results**

TGF-β2-treated TNBS-pulsed bone marrow-derived APC suppress ongoing HIPIF

Previously, it was shown that TGF-β2-treated, Ag-pulsed (tolerogenic) PEC given i.v. induced the generation of efferent Tr cells in the spleen that suppressed Ag-specific DTH responses and production of complement-fixing Abs (4, 29). We tested whether similar treatment of bone marrow-derived APC produced tolerogenic APC. Bone marrow-derived APC were generated in culture, as described in Materials and Methods. The surface markers and functions of the APC were monitored by flow cytometry analyses and for their ability to suppress immune responses in vivo. APC that were harvested directly after 6 days of culture in GM (GM-APC) expressed low levels of MHC II, CD40, B7.2, and CD11c, and high levels of F4/80 and CD11b on their surface (Fig 1a). Although the GM that we used had L929-conditioned medium that contained CSF-1 and was similar to the medium for macrophage generation, the APC from our cultures had some characteristics of DCs. After 6 days of growth in petri dishes, the majority of the APC were nonadherent. After LPS treatment, these APC down-regulated the chemokine receptor CCR6 and up-regulated the CCR7 as well as MHC II and CD40 expression (data not shown). Therefore, in this study, we call these bone marrow-derived cells APCs. The switch from GM to SFM (SFM-APC) increased CD40 surface expression on these APC. Addition of TGF-β2 (5 ng/ml) to the cultures with SFM (tol-APC) blocked the up-regulation of CD40 on the tol-APC surface (Fig 1b), but did not change the expression of F4/80 and CD11b (data not shown). The ability of tol-APC to suppress an immune response was tested in a CH ear-swelling assay. Adoptive transfer of TNBS-pulsed tol-APC, but not control APC (GM-APC or SFM-APC), suppressed the CH-induced ear swelling in TNBS-presensitized mice (data not shown).

The effect of tol-APC was tested in vivo in the ADT-HIPIF model. Tol-APC or control APC (GM-APC and SFM-APC) were adoptively transferred to ADT-HIPIF mice 1 day before i.t. challenge, and hydroxyproline deposition (a marker of fibrosis) in the lung was monitored at various times after i.t. challenge. Tol-APC, but not control APC (SFM-APC, GM-APC), induced suppression of fibrosis in the ADT-HIPIF mice (Fig. 1b). Even when tol-APC were transferred into adoptively sensitized recipient mice 1 day after i.t. challenge with TNBS, hydroxyproline deposition in the lungs was reduced (Fig. 2a). The suppression was Ag specific because tol-APC pulsed with an unrelated Ag, oxazolone, had no effect on the hydroxyproline deposition in TNBS-sensitized and challenged mice (Fig. 2a). To exclude the possibility that tol-APC simply delayed the development of fibrosis, hydroxyproline deposition was measured in another group of lung tissue samples that were harvested from mice 19 days after i.t. challenge (Fig. 2b).

**FIGURE 1.** Hydroxyproline analysis of lungs of ADT-HIPIF mice treated with tol-APC vs control APC. a, Single-parameter overlays show the phenotypes of mouse bone marrow-derived APC by flow cytometry analyses. APC were harvested from GM at day 6 of the culture and stained for CD40, MHC II, B7.2, CD11c, F4/80, and CD11b expression on their surface (solid line). The cursor is set based on the isotype control staining (hatched line), and the percentage of positive is indicated in each histogram. b, The top two-parameter histograms from flow cytometry analyses show the expression of MHC II and CD40 on tol-APC (TGF-β2 treated) and control APC used in the experiment (GM, a medium that was used to grow APC from bone marrow cells; SFM, a medium that was used for TGF-β2 treatment). The percentage of positive in each quadrant is indicated above each histogram. The bottom graph shows the change (Δ) of lung hydroxyproline in experimental groups 14 days after i.t. challenge. Sensitized spleen and draining lymph node cells (3 × 10⁷/mouse) from donor mice were adoptively transferred to recipient mice 1 day before i.t. challenge. ADT APC, adoptive transfer of APC. The APC (i.v. 5 × 10⁷/mouse) that were harvested from GM or SFM or treated with TGF-β2 were adoptively transferred to the mice 1 day before i.t. challenge. The baseline hydroxyproline was 235.0 ± 17.6 µg/lung.
The level of suppression of fibrosis shown in day 19 lungs was similar to the level of suppression measured 12 days post-i.t. challenge. Furthermore, the suppression of immune mediated fibrosis in the ADT-HIPIF mice by tol-APC was confirmed by Masson’s Trichrome staining for collagen on lung samples that were harvested 14 days post-i.t. (Fig. 3). ADT-HIPIF lung had diffused fibrotic lesions in the interstitium, while tol-APC-treated (either 1 day before or 1 day after i.t.) ADT-HIPIF lung had inflammation and collagen deposition mainly around large airways and blood vessels that are similar to the lung of i.t. challenge-only mice (control group). Thus, tol-APC suppress ongoing fibrosis in the ADT-HIPIF mice presumably by interfering with the chronic autoimmune response that perpetuates the fibrogenic response.

**Tol-APC generate Tr cells in the ADT-HIPIF mice**

We postulated that the tol-APC-induced Ag-specific Tr cells that indirectly suppressed the development of fibrosis by regulating the Th1 autoimmune response needed to promote the fibrogenic response. To evaluate the postulate, we assessed both spleen and lung-draining lymph node (LdLN) cells from tol-APC-treated ADT-HIPIF mice for their ability to suppress CH. In brief, spleen cells ($5 \times 10^7$/mouse) and LdLN cells (one mouse equivalent) from either the ADT-HIPIF mice or the ADT-HIPIF mice that received tol-APC were collected 14 days after i.t. challenge and adoptively transferred (i.v.) to TNBS-presensitized mice. One day after the transfer, ears of the recipient mice were painted with the hapten. The ear swelling was measured before challenge and 24 h after challenge. The mice receiving either spleen or LdLN cells from tol-APC-treated ADT-HIPIF mice had reduced ear swelling compared with positive control mice or the mice that received spleen cells and LdLN cells from the untreated ADT-HIPIF mice (Fig. 4a). To assess the type of cell mediating the suppression in the CH ear-swelling assay, column-enriched T cells from the LdLN (one mouse equivalent) or spleen ($6 \times 10^6$/mouse) were adoptively transferred (i.v.) to hapten-sensitized mice 1 day before ear challenge. Ear thickness was measured before the painting and 24 h later. Enriched LdLN T cells from tol-APC-treated HIPIF mice, but not from untreated mice, suppressed the ear-swelling response in recipient mice (Fig. 4b). Enriched splenic T cells from tol-APC-treated ADT-HIPIF mice suppressed the ear-swelling CH response in TNBS-presensitized mice, but not DNFB-presensitized mice (Fig. 5a). Enriched T cells from either Ag-pulsed GM-APC or SFM-APC-treated HIPIF mice had no effect on the ear-swelling response. These data indicate that tol-APC induced the generation of Th cells in LdLN and spleens from the ADT-HIPIF mice. Furthermore, CD4$^+$ or CD8$^+$ cells were depleted from the enriched splenic T cells by treatment with complement and their specific Ab (GK1.5, 2.43, respectively), and the remaining cells were tested for their ability to suppress effector T cell function in a LAT-DTH assay. The enriched whole T cell ($5 \times 10^7$/mouse), CD8$^+$, or CD4$^+$ (equivalent to $5 \times 10^7$ enriched T cells/mouse) populations were mixed with effector T cells and TNBS-pulsed PEC and injected into the ear pinna of a naive mouse. Of the mice that received either CD8$^+$ or CD4$^+$ T cells, only the CD4$^+$ T cells suppressed the CH response, indicating that the Tr cell was CD8$^+$ (Fig. 5b). These data support the concept that adoptive transfer of tol-APC induces an Ag-specific CD8$^+$ effector Tr cell in the recipient-sensitized mice.

**Tr cells generated by tol-APC modulate pulmonary fibrosis**

Although above we show that the tol-APC induced a suppression of CH in the ADT-HIPIF model, it remains to be shown that the Tr cells generated also suppress the fibrogenic response. Thus, enriched Tr cells from tol-APC-treated ADT-HIPIF mice were transferred to a second set of ADT-HIPIF mice and assessed for fibrosis at various times post-i.t. challenge. As before, enriched splenic T cells ($3 \times 10^7$/mouse) from the variously APC-treated ADT-HIPIF mice as well as CD8-depleted T cells (equivalent to $3 \times 10^6$ enriched T cells/mouse) from tol-APC-treated ADT-HIPIF mice were harvested 14 days after i.t. challenge and transferred (i.v.) to another set of ADT-HIPIF mice 1 day before i.t. challenge. Splenic CD8$^+$ T cells from tol-APC-treated, but not T cells from any of the other type of Ag-pulsed APC-treated ADT-HIPIF mice suppressed the development of fibrosis in recipient mice (Fig. 6). Thus, tol-APC generate CD8$^+$ Tr cells that suppress the immune response and prevent the subsequent immune mediated fibrogenic response.

**Discussion**

Our results clearly demonstrate that tol-APC suppress ongoing Th1-mediated pathogenesis in the lung by generating Ag-specific CD8$^+$ Tr cells that suppress Th1 effector phase of immune responses in the lung. TGF-β is a critical factor for generating the tol-APC. TGF-β is a pleiotropic cytokine that has at least two fundamental roles in lung biology. One is that TGF-β enhances
extracellular matrix production and deposition (8). In several mouse models, TGF-β1 is demonstrated as a fibrogenic cytokine that promotes the development of fibrosis by enhancing fibroblasts to produce collagen (21, 30). This event seems to take place within the lung tissue itself. A second role of TGF-β is to regulate immune responses (8). TGF-β2-treated PEC are able to induce anterior chamber-associated immune deviation and alter the course of the experimental autoimmune uveitis (4, 5). We show in this

**FIGURE 3.** Masson’s Trichrome staining of lung sections from ADT-HIPIF mice treated with tol-APC. *Left panel*, Shows a representative lobe of lungs from various groups of mice with ×20 magnification under microscopy. *Right panel*, Shows the magnified view (×200) of the region that is selected by a rectangular and indicated by an arrow in the ×20 magnification view. Lungs were harvested at day 14 after i.t. challenge from experimental mice. The experimental groups are indicated on top of each picture: HIPIF, ADT-HIPIF mice; tol-APC day −1, ADT-HIPIF mice that received tol-APC 1 day before i.t. challenge; tol-APC day +1, ADT-HIPIF mice that received tol-APC 1 day after i.t. challenge; i.t. challenge-only, mice received naive cells and i.t. challenged with TNBS (control group for ADT-HIPIF mice). The blue color in the picture indicates the specific staining of collagen in the lung. Bronchial (B) and blood vessels (V) are marked in the pictures.
study that TGF-β2-treated Ag-pulsed bone marrow-derived APC, called tol-APC, suppress the development of pulmonary fibrosis by generating Tr cells in peripheral lymphoid organs and prevent chronic autoimmune inflammation in the lung. Although the difference in TGF-β function in the lung may be attributed to the TGF-β isoform, our data support the idea that the nano-environment in which TGF-β is functioning is crucial. That is to say, the TGF-β effects on APC and T cell function are in a protected nano-environment of a lymphoid organ and differ from the macro effect of the TGF-β delivered to fibroblasts by lung macrophages and other cells within the inflamed tissue.

TGF-β is not only crucial for the transformation of naive or immunogenic APC to being tolerogenic, but also is crucial to creating the immune privilege-like nano-environment in which the Tr cells are generated in the lymphoid organ. It is known that TGF-β2 treatment of PEC or macrophage hybridomas increases the production of TGF-β and thrombospondin-1 and decreases IL-12 and CD40 expression in resulting tolerogenic APC (11, 31). Thrombospondin-1 has the ability to bind and activate TGF-β, thus am-

FIGURE 4. Ear-swelling CH analysis of Tr cells in the LdLN of ADT-HIPIF mice treated with tol-APC. The change (Δ) of ear swelling in various groups of mice is indicated on the ordinate. Under the abscissa: CH response, mice were epicutaneously sensitized with TNBS (3%, 100 μl/mouse) and ear challenged with TNCB (1%, 20 μl/ear); ADT, either spleen or lung-draining lymph node (LdLN) cells (LdLNCs) from tol-APC-treated ADT-HIPIF mice or ADT-HIPIF mice without treatment were adoptively transferred to presensitized mice 1 day before their ear challenge. a, Sensitized mice received either spleen cells (5 × 10⁷/mouse) or LdLN cells (one mouse equivalent) that were harvested from tol-APC-treated ADT-HIPIF mice 14 days after i.t. challenge. The cells from ADT-HIPIF mice without treatment were used as control. b, T cells were enriched using T cell column from the LdLN cells of tol-APC-treated ADT-HIPIF mice or untreated ADT-HIPIF mice and transferred to recipient-presensitized mice. Ears of the recipient mice were challenged with TNCB 1 day after adoptive transfer.

FIGURE 5. Generation of CD8⁺ Tr cells in ADT-HIPIF mice treated with tol-APC. a, Ear swelling CH analysis of Tr cells in the spleens of ADT-HIPIF mice treated with tol-APC. The change (Δ) of ear swelling in various groups of mice is indicated on the ordinate. Under the abscissa: CH response, mice were epicutaneously sensitized with TNBS (3%, 100 μl/mouse) or DNFB (0.5%, 25 μl/mouse) and ear challenged with TNCB (1%, 20 μl/ear) or DNFB (0.05%, 20 μl/ear); ADT, enriched splenic T cells (6 × 10⁶/mouse) from tol-APC-treated HIPIF mice or control APC (GM-APC or SFM-APC) treated ADT-HIPIF mice were adoptively transferred (i.v.) to presensitized mice 1 day before their ear challenge. The ear thickness was measured 24 h after challenge. b, LAT-DTH analysis of CD8⁺ Tr cells in the spleen of ADT-HIPIF mice treated with tol-APC. The top panel shows flow cytometry analysis of percentage of CD4⁺ and CD8⁺ cells in enriched Tr cells before and after CD4 (GK1.5) or CD8 (2.43) depleting Ab and complement treatment. The percentage of positive in each quadrant is indicated above each histogram. The bottom figure shows the change (Δ) of ear swelling in various groups of mice in the LAT-DTH assay. The treatment for each group of mice is described under the abscissa. Using T cell column, Tr cells were enriched from spleen cells of ADT-HIPIF mice that were treated with tol-APC 14 days earlier. Enriched Tr cells were then treated with CD4 depleting Ab (GK1.5) or CD8 depleting Ab (2.43) plus complement to remove either CD4⁺ or CD8⁺ T cells. Tr cells (5 × 10⁵), GK 1.5 treated Tr cells, or 2.43 treated Tr cells (equivalent to 5 × 10⁵ T cells prior to depletion) were mixed with effector T cells (5 × 10⁶) and TNBS-pulsed PEC (5 × 10⁵) and injected intradermally into the ear pinnae of a naive mouse. PEC without Ag were used in the negative LAT-DTH group.
FIGURE 6. Hydroxyproline analysis of lungs from ADT-HIPIF mice that were adoptively transferred with Tr cell from tol-APC-treated ADT-HIPIF mice. The histogram shows the change (Δ) of lung hydroxyproline in each experimental group 14 days after i.t. challenge. T cells were enriched from spleen cells that were harvested from tol-APC-treated or control APC (GM-APC, SFM-APC)-treated ADT-HIPIF mice 14 days after i.t. challenge. CD8+ T cells were further depleted in splenic T cells from tol-APC-treated ADT-HIPIF mice by Ab (2.43) plus complement treatment. T cells (3 × 10⁶/mouse) and CD8+ T cells (equivalent to 3 × 10⁶ T cells/mouse) were adoptively transferred (i.v.) to another set of ADT-HIPIF mice 1 day before i.t. challenge. The baseline hydroxyproline was 201.9 ± 4.2 µg/lung.

ACKNOWLEDGMENTS

We appreciate the many helpful discussions with Drs. Takahiko Nakamura and Douglas E. Faunce (Schepps Eye Research Institute) and the critical evaluations of our work provided by Drs. Mark Exley and Steve Balk at the Beth Israel Care group. We thank Dr. Steven Shapiro for his critical reading of our manuscript. We are grateful to Marie Ortega for careful management of the Schepps Vivarium. We thank Jennifer Post, Jane Preotie, and Ania Terajewicz for providing technical assistance for this work.

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