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A Novel Pathway That Regulates Inflammatory Disease in the Respiratory Tract

Naiqian Niu,²* Marc K. Le Goff,²* Fangyong Li,⁎ Marina Rahman,⁎ Robert J. Homer,† and Lauren Cohn³*" 

In animals with acute airway inflammation followed by repeated exposure to inhaled Ag, inflammation wanes over time and thus limits the study of chronic airway inflammatory diseases such as asthma. We developed a model of airway inflammation and inhalational tolerance to investigate regulatory pathways in the respiratory tract. We show that Th1- and Th2-induced airway inflammation followed by repeated exposure to inhaled Ag leads to a state of immunosuppression. Challenge of these animals with a marked population of TCR transgenic effector Th1 or Th2 cells results in a striking inhibition of inflammation and effector Th cells. In Th2 models, airway hyperresponsiveness, mucus, and eosinophilia are reduced. The inhibitory effects observed are Ag nonspecific, can be induced in lymphocyte-deficient mice, and are associated with a population of TGF-β1-expressing macrophages. Induction of this pathway may offer potent localized treatment of chronic T cell-mediated respiratory illnesses and provide insights into the development of such diseases. The Journal of Immunology, 2007, 178: 3846–3855.

Immune regulation is a critical function of mucosal surfaces that continuously interact with environmental Ags. In the respiratory tract this function is paramount, because inflammation can compromise respiration. The mucociliary escalator, macrophage scavengers, and the epithelium form a first line of defense to prevent the development of immune responses to inhaled agents. Once innocuous Ags interact with immune components, active immunity limits inflammation. Inhaled tolerance (IT) has been demonstrated in naive animals that are repeatedly exposed to inhaled proteins (1). IT is Ag-specific and mediated by regulatory T cells (Treg) that produce TGF-β and/or IL-10 (1–3). In IT, Treg cells control the development of effector T cell responses in the uninflamed respiratory tract. During acute and chronic inflammatory respiratory diseases, regulatory controls must be active so that sensitization to environmental Ags does not occur. In addition, mechanisms that resolve inflammatory responses in the respiratory tract must always be functional. The anti-inflammatory pathways that control T cell responses during airway inflammation have not been defined. Although failures in Treg cells have been associated with the development of chronic airway disease (4–6), defects in other pathways may also play a role in the development and/or persistence of inflammation in lung diseases.

Allergic airway inflammation and airway hyperresponsiveness (AHR) wane over a period of weeks in Ag sensitized mice exposed repeatedly to inhaled Ag (7–10). This is commonly discussed as a reason why murine models do not develop fully the features of chronic asthma (11). Some have presumed that IT was induced by these long-term exposures to inhaled Ag, but a specific cell population and cytokine(s) mediating these effects was never identified (8–10). Furthermore, the proinflammatory environment present during airway inflammation should preclude the development of IT due to its dependence on immature dendritic cells (12).

We developed a model to investigate the mechanisms whereby long-term exposure to inhaled Ag during airway inflammation inhibits lung disease. We conditioned mice by inducing airway inflammation and administering inhaled Ag repeatedly over a period of weeks. To define the mechanisms of immunosuppression, we challenged mice with a traceable population of indicator CD4 T cells. We show that airway inflammation with repeated exposure to inhaled Ag suppresses CD4 T cells and their effector responses in the respiratory tract. We call this effect airway inflammation-related inhibition of disease (AIRID). AIRID is a potent regulatory pathway in the respiratory tract that can be induced in Th1 and Th2 inflammatory environments and inhibits in an Ag-nonspecific manner. AIRID does not require host lymphocytes and is associated with a marked expansion of TGF-β1-expressing lung macrophages. This novel regulatory pathway may be essential to inhibit the development of chronic inflammatory airway diseases and may be activated to treat T cell-mediated lung diseases.

Materials and Methods

Mice

OT-II mice, which are transgenic for the TCR-recognizing OVA peptide 323–339 (pOVA323–339), were bred in our facilities and backcrossed to B6.PL-Thy1aCy (The Jackson Laboratory). OT-II Thy1.2, OT-II Thy1.1, and OT-II Thy1.1/1.2 mice were used for the generation of Th1 and Th2 cells. DO11.10 mice on a BALB/c background were bred in our facility. C57BL/6, BALB/c, RAG1−/−, and IL-10−/− mice 6–8 wk of age were used as recipient mice. (The Jackson Laboratory or National Cancer Institute). All experimental protocols were approved by the Yale Animal Care and Use Committee (New Haven, CT).

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Abbreviations used in this paper: IT, inhaled tolerance; AHR, airway hyperresponsiveness; AIRID, airway inflammation-related inhibition of disease; BAL, bronchoalveolar lavage; HDM, house dust mite; KLH, keyhole limpet hemocyanin; inh-KLH, inhaled KLH; inh-OVA, inhaled OVA; PAS, periodic acid–Schiff; MCh, methacholine; MSC, myeloid suppressor cell; pOVA, OVA peptide 323–339; Tg, transgenic; Treg, regulatory T cell.

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FIGURE 1. Protocol for the induction of AIRID. OT-II Th1 or Th2 cells (1.0–2.5 × 10⁶) were transferred into C57BL/6 mice. Inh-OVA was administered as shown by gray ovals; each oval represents one 20-min exposure on 1 day. Mice were sacrificed on days 10 and 25. This is the conditioning phase. In both groups of mice there were peribronchial and perivasculary inflammatory infiltrates. Th1 cells led to neutrophilic inflammation, whereas Th2 cells stimulated eosinophilia. Th2 cells stimulated mucous metaplasia. On day 25, there was reduced inflammation. To test whether there was dominant suppression, on day 35 mice were challenged with a population of OT-II Th1 or Th2 indicator cells (1.0–2.5 × 10⁶) and exposed to inh-OVA for 7 days (in a 9-day period). Mice were sacrificed 24 h after the last OVA exposure. Lung histopathology was photographed at ×100 and ×400 original magnification.

**Generation of Th1 or Th2 cells**

To generate Th1 or Th2 cells from OT-II or DO11.10 mice, CD4 T cells were isolated by negative selection as previously described (14) using mAbs to CD8, class II MHC I–A, and anti-Ig-coated magnetic beads (Qiagen). For CD25 depletion experiments, anti-CD25 (clone PC61; Ref/15) was added to the Ab mixture. To generate keyhole limpet hemocyanin (KLH)– or house dust mite (HDM)–specific Th cells from nontransgenic mice, B6.PL-Thy1a/Cy mice (The Jackson Laboratory) were injected s.c. with 100 μg of KLH (Sigma-Aldrich) in CFA for Th1 cells or i.p. with 100 μg of KLH or 50 μg of HDM (Greer Laboratories) in 2.0 mg of alum (Imject; Sigma-Aldrich) for Th2 cells. Seven days after immunization, spleens and local draining lymph nodes were harvested and CD4 T cells were isolated by negative selection as described above. Syngeneic APCs were prepared by negative selection using mAb to CD4, anti-CD8, and anti-Thy1 and treated with rabbit complement. APCs were mitomycin-C treated. To generate Th1 or Th2-like cells from OT-II or DO11.10 mice, CD4 T cells were stimulated with pOVA323–339 (15 μg/ml), IL-12 (10 ng/ml) (Wyeth), and anti-IL-4 at an inhibitory concentration. All cultures were set up in flasks containing a 1:2 (OT-II or DO11.10) or 1:1 (KLH or HDM) ratio of CD4 T cells to APCs at concentrations of 2.5 × 10⁶ to 1 × 10⁷ CD4 T cells/ml and maintained for 4 days.

**Th cell transfer and respiratory exposure**

To induce lung inflammation 1.0–2.5 × 10⁶ cells were injected i.v. into recipient mice and 1 day after the transfer of cells the mice were challenged with inhaled 1% OVA (inh-OVA) in PBS as previously described (14) for 20 min daily for a total of 7 days over a period of 9 days (4 consecutive days exposed, 2 days rested, 3 consecutive days exposed). Ten days later the mice were exposed to inh-OVA on two consecutive days a week for 2 wk. On day 35, mice received the challenge cells, a second i.v. transfer of 1.0–2.5 × 10⁶ Th1 or Th2 cells followed by 7 days of inh-OVA (Fig. 1). For KLH–specific Th cells, KLH (25 μg) was administered intranasally every other day for 10 days. For HDM-induced inflammation, HDM (25 μg/mouse) was given daily intranasally 5 days/wk for 3 wk followed by one intranasal dose in wk 5 and three intranasal doses (day 1, 4, and 7) after challenge with HDM Th2 cells.

**Bronchoalveolar lavage (BAL) and lung digestion**

BAL was performed by cannulation of the trachea and lavage with 1 ml of PBS. Isolation of lung leukocytes was performed after BAL and perfusion of blood from lungs. Lung tissue was minced and digested with collagenase type IV at 150 U/ml (Worthington Biochemical) and DNase at 10 U/ml (Sigma) for 1 h at 37°C and passed through a wire mesh to dissociate cells. BAL and lung cells were counted using a hemocytometer. Cytospin preparations of BAL and lung cells were stained with Diff-Quik (Baxter Healthcare), and differentials were performed on 200 cells based on morphology and staining characteristics. Lung data are presented because the total number of granulocytes (neutrophils or eosinophils) and TCR Tg cells isolated from the lung makes up a vast majority of these cells in the respiratory tract (compared with BAL). Lung cells were pooled in a group and purified in lymphocyte separation medium (MP Biomedical) and 1 × 10⁶ lung cells/ml were cultured with 2 × 10⁶ APC/ml with pOVA323–339 (15 μg/ml) in 24-well plates. Culture supernatants were collected after 24 or 48 h.

**Cytokine assays**

At the time of transfer, an aliquot of Th1 or Th2-like cells was retained for restimulation. A total of 2.5 × 10⁶ CD4 T cells/ml, 5.0 × 10⁷/ml freshly isolated APCs, and pOVA323–339 (15 μg/ml) or KLH (50 μg/ml) were cultured and supernatants were collected at 24 h. IFN-γ, IL-4, and IL-5 levels from cell supernatants were determined by ELISA (Endogen). Th2 cell populations used in these experiments typically produced IL-4 (4–20 ng/ml), IL-5 (2–12 ng/ml), and IFN-γ (⩽150 ng/ml). The Th1 cell populations used produced IFN-γ (250–1200 ng/ml) but IL-4 and IL-5 were undetectable. Cytokines (IFN-γ, TGF-β1, IL-10, and IL-13) from BAL fluids were assayed using the respective ELISA kits (R&D Systems). For measuring total levels of TGF-β1 in supernatant or BAL fluids, 100 μl of supernatant was incubated with 20 μg of 1 N HCl for 10 min at room temperature. Then, the acidified samples were neutralized by adding 18 μg of 1.2 N NaOH/0.5 M HEPES and assayed immediately.

**FACS analysis**

At the time of transfer, FACS (BD Biosciences) analysis was performed on Th1 or Th2 cell preparations to determine the purity of the transferred cell populations. Cells were stained with anti-CD4 or anti-CD5 for OT-II TCR Tg cells, anti-Thy1.1 and/or anti-Vβ5 (BD Biosciences) for DO11.10 TCR Tg cells, the anti-clonotypic Ab KJ1.26 (16). Transferred cells were
uniformly >95% CD4 and TCR transgenic (Tg). At the time of sacrifice, FACS was performed on BAL lung and spleen specimens to identify the transferred cells (including transfer no. 1 and transfer no. 2) using combinations of Abs to CD4, Vβ5, Vα2, Thy1.1, Thy1.2, or KJ1.26, depending on the phenotype of the transferred cell populations. In Figs. 2–4 the total number of lung TCR Tg cells from conditioning (transfer no. 1) and challenge (transfer no. 2) is shown, as the Thy1 marker was the same on both populations of cells. In other figures, the conditioning and challenge populations had different Thy1 surface markers (Thy1.1 or Thy1.1/1.2) and the number of TCR Tg cells from the challenge is depicted. In all experiments tested, cells derived from transfer no. 1 were ≤30%.

**Lung histology**

Lungs were prepared for histology by perfusing the animal via the right ventricle with 20 ml of PBS. Lungs were then inflated with 1.0 ml of fixative instilled through a tracheostomy tube. Samples for paraffin sectioning were formalin fixed, sectioned in the coronal plane at 5 μm, and stained with H&E, trichrome, or periodic acid–Schiff (PAS). A histological mucus index was calculated using PAS-stained lung sections as previously described (14). This index is equivalent to the linear percentage of epithelium positive for mucus and was calculated for each mouse lung, and then the mean of the histological mucus index was calculated for each experimental condition.
group. Periodate-lysine-paraformaldehyde-fixed, OCT-embedded lung tissue was used for immunofluorescent staining. Anti-TGF-β1 (Santa Cruz Biotechnology), goat anti-rabbit Alexa Fluor 568 (Molecular Probes), anti-CD5 (BD Biosciences), anti-CD4 (BD Biosciences), anti-CD45 (BD Biosciences), F4/80 (Caltag Laboratories), anti-CD11b (eBioscience), and anti-CD11c (eBioscience) were used as previously described (14). In control mice, the specificity of anti-TGF-β1 was confirmed using a blocking peptide (Santa Cruz Biotechnology) that consistently inhibited TGF-β1 staining. To quantify positive staining cells in lung sections, five contiguous photographs were taken of each lung section at 100 (original magnification) and scored for positive cells per microscopic field by two different individuals blinded to the experimental group.

Lung physiology

Airway responsiveness to five doses of inhaled methacholine (MCh) (4–33 mg/ml) was determined. Mice were anesthetized (50 mg/kg pentobarbital and 1.8 g/kg urethane) and intubated with a 20-gauge stainless steel catheter through which they were ventilated (Harvard Apparatus) at a tidal volume of 9 ml/g and 150 breaths/min after paralysis with 0.5 mg/kg pancuronium bromide. Mice were placed in a volume displacement body plethysmograph (Penn Century) and exposed to 10 l of saline (baseline) or MCh delivered via the tracheostomy tube by an in-line nebulizer. Continuous measurements of airway pressure and thoracic flow were obtained using a computerized data acquisition system (Buxco Electronics) and pulmonary resistance was computed (17). The mean baseline and the peak pulmonary resistance after each dose were used for statistical analysis.

Statistical analysis

In all figures statistical significance was determined by Student’s t test using the Bonferroni correction for multiple comparisons. Lung physiology was analyzed using repeated measures ANOVA.

Results

Inhaled Ag administered during Th1 and Th2-induced airway inflammation induces immunosuppression

We have previously shown that Th1 or Th2 cells adoptively transferred into syngeneic recipient mice and exposed to inhaled Ag over 10 days cause marked airway inflammation (14). In multiple experiments, repeated exposure to inh-OVA over a period of >2 wk led to a decline in airway inflammation and a reduction in TCR

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FIGURE 4. AIRID is induced by HDM. Mice were conditioned with HDM given intranasally 5 days per week for 3 wk followed by intranasal HDM once in week 5 or by OT-II Th2 cells and inh-OVA as shown. On day 35 mice were challenged with either HDM-specific Th2 Thy1.1 cells and inh-HDM or OT-II Th2 and inh-OVA (inflam, inflammation; inh-agn, inhaled Ag). On Day 45, mice were sacrificed. From lung cell suspensions, eosinophils were enumerated on cytopsins, and TCR Tg cells from the challenge were assessed by FACS analysis of Thy1.1+ cells. Mean eosinophil and TCR Tg cell counts are shown (± SEM; n = 4–6 mice per group). Data are representative of three experiments. *, p ≤ 0.005.

FIGURE 5. Inhaled Ag administered during airway inflammation inhibits T cell responses better than inhaled Ag in uninflamed airways. Mice were conditioned with OT-II Th1 (A) or OT-II Th2 (B) cells or no cells and inh-OVA (IT) as shown (inflam, inflammation; inh-agn, inhaled Ag; Uncond, unconditioned). Mice were challenged with Th1 or Th2 cells and inh-OVA on day 35, and on day 45 the mice were sacrificed. Neutrophils or eosinophils were counted on cytopsins of lung cell suspensions. TCR Tg cells were assessed in lung cell suspensions by FACS of Thy1.1+ cells. Total TCR Tg cells are shown (conditioning plus challenge). Mean cell counts are shown (± SEM; n = 4 mice per group). BAL cytokines were assessed by ELISA. Data is representative of three experiments. *, p ≤ 0.005 compared with unconditioned group and compared with naive OVA-conditioned group; †, p ≤ 0.02; #, p ≤ 0.05 compared with unconditioned group; ‡ p, NS compared with IT group.
Tg cells (not shown). To study how inhaled Ag, when administered during ongoing airway inflammation, caused a reduction in inflammation over time, we induced airway inflammation in mice and repeatedly exposed them to inh-OVA as shown in Fig. 1. Mice received either OT-II Th1 or Th2 cells followed by inh-OVA over 10 days. Mice that received Th1 cells had a marked neutrophilic inflammatory response whereas OT-II Th2 cells stimulated airway eosinophilic inflammation (Fig. 1), and these responses were similar to our past observations using DO11.10 TCR Tg cells in BALB/c mice (14). Mice were then exposed to inh-OVA twice weekly for 2 wk, during which the inflammation was still present but reduced in intensity (Fig. 1, panel labeled Day 25). We will refer to the induction of inflammation followed by inhaled Ag exposure as Th1 or Th2 “conditioning.” We hypothesized that during the conditioning period an inhibitory effect was induced. Then, to test whether these animals exhibited dominant suppression of an effector CD4 T cell population, on day 35 we transferred a new population of effector CD4 Th1 or Th2 indicator cells into the mice and recruited/activated these cells in the respiratory tract with inh-OVA over 10 days. This will be referred to as Th1 or Th2 “challenge.” We chose to use effector cells as our challenge population because effector cells are likely to be the cells inhibited during prolonged Ag exposure, and in treating inflammatory lung diseases the target of our therapy would be preexisting effector/memory CD4 T cells. In most of the experiments the TCR Tg cells used in conditioning and challenge expressed different Thy1 surface markers and, thus, each population of indicator cells could be enumerated separately.

FIGURE 6. Th1 inflammation and inh-OVA inhibits Th2-induced disease and vice versa. A, Mice were conditioned with OT-II Th1 cells and inh-OVA, as shown (inflam, inflammation; inh-agn, inhaled Ag). On day 35 mice were challenged with OT-II Th1 or Th2 cells and inh-OVA. B, Mice were conditioned with OT-II Th2 cells and inh-OVA. On day 35 mice were challenged with OT-II Th2 or Th1 indicator cells and inh-OVA. On day 45 mice were sacrificed. TCR Tg cells were assessed in lung leukocyte suspensions by FACS of Thy1.1+ cells from the challenge. Mean total leukocytes isolated from the lung and TCR Tg cell counts are shown (± SEM; n = 3–5 mice per group). †, p < 0.02; #, p < 0.05 compared with unconditioned group.

FIGURE 7. AIRID is induced in lymphocyte deficient mice. RAG1−/− or WT mice were conditioned with OT-II Th1 (A) or OT-II Th2 (B) cells and inh-OVA (inflam, inflammation; inh-agn, inhaled Ag). C, WT mice were conditioned with Th2 cells that were nondepleted (Non-D) or CD25-depleted (CD25-D) before in vitro stimulation. On day 35 the mice were challenged with OT-II Th1 or Th2 cells and inh-OVA. On day 45 the mice were sacrificed. From lung cell suspensions neutrophils or eosinophils were counted on cytospins and TCR Tg cells from the challenge were assessed by FACS analysis of Thy1.1+ cells. Mean neutrophil or eosinophil and TCR Tg cell counts are shown (± SEM; n = 4–5 mice per group). *, p < 0.005; †, p < 0.02; #, p < 0.05 compared with unconditioned group.
In the presence of Th1 or Th2 conditioning, challenge with Th1 or Th2 cells results in markedly reduced inflammation and fewer TCR Tg Th1 or Th2 cells in the respiratory tract when compared with Th1 or Th2 challenge of unconditioned mice (Fig. 2). In Th1-conditioned mice, neutrophilic infiltration in the lung (Fig. 2, A and C) and BAL (not shown) was minimal, as was IFN-γ, unlike in the unconditioned mice where neutrophils made up 20–30% of BAL cells and IFN-γ levels were high. Eosinophils in the lung and BAL and IL-13 in BAL were markedly reduced in Th2-conditioned mice compared with unconditioned mice (Fig. 2, B and C). In both Th1- and Th2-conditioned mice, lung histopathology showed significantly less inflammation than in unconditioned mice (Fig. 2C). In none of the conditioned animals was there evidence of a Th1 to Th2 shift to explain the inhibitory effects observed, such that on day 45 IFN-γ was not detected in the BAL in any of the Th2-conditioned mice and IL-13 was not detected in Th1-conditioned mice (not shown). Th2-conditioned mice did not exhibit evidence of subepithelial fibrosis by histopathological assessment or Sircol assay for collagen content (not shown). Th1- and Th2-conditioned mice that were not challenged had minimal inflammation in the respiratory tract with few TCR Tg cells (Fig. 2, A and B). Aerosolized Ag alone did not lead to significant airway inflammation in any of these experiments (Fig. 2, A and B).

To confirm that the inhibitory effect observed was not unique to C57BL/6 mice and to test airway physiologic responses, we performed Th2 experiments in BALB/c mice. We conditioned BALB/c mice with DO11.10 Th2 cells followed by repeated exposure to inh-OVA. We then challenged mice with DO11.10 Th2 cells. Upon challenge, Th2-conditioned mice compared with unconditioned mice exhibited marked inhibition of inflammation with reduced eosinophilia and airway mucus staining and diminished airway responsiveness to inhaled MCh (Fig. 3).

In challenged Th1- and Th2-conditioned mice, comparable low numbers of TCR Tg cells were identified in the spleens and TCR Tg cells were insignificant in the blood, mesenteric lymph nodes, spleen, and liver (not shown). This argues against the inhibitory effect being a mere defect in recruitment, because recently activated transferred TCR Tg cells accumulate predominantly in the spleen. TCR Tg cells still present after the conditioning phase (from transfer no. 1) contributed a minority of the total recovered TCR Tg cells after the challenge on day 45 (≤30% in 10 experiments analyzed). Furthermore, inflammation alone is not inhibitory to a subsequent T cell response, because neither CD4 Th1 nor Th2 TCR Tg cells were inhibited in other models of airway inflammation that were free of inhaled Ag (not shown). These studies show that long-term intermittent exposure to inhaled Ag during active airway inflammation leads to a state of dominant suppression. We call this effect airway inflammation-related inhibition of disease or AIRID.

**Inflammation generated by inhaled HDM inhibits HDM-specific T cell responses**

If AIRID is a physiologically relevant phenomenon, then it should be inducible by repeated exposure to inhaled Ags other than OVA and by an inflammatory response generated in situ rather than induced by transferred TCR Tg cells. We used HDM allergen given intranasally because it stimulates Th2 priming and airway inflammation in contrast to intranasal OVA, which leads to IT (18). We induced robust eosinophilic inflammation after 3 wk of intranasal administration of HDM and then repeated the intranasal exposure to HDM to complete the conditioning regimen (Fig. 4). We challenged mice with non-Tg HDM-specific Th2 Thy1.1+ cells to determine whether HDM-specific T cells were inhibited. The challenge of unconditioned mice led to striking lung inflammation with high numbers of eosinophils and Thy1.1+ CD4 T cells (Fig. 4). In HDM-conditioned mice there was a marked reduction in lung eosinophils and Thy1.1+ cells. These studies show that AIRID can be induced during an inflammatory response generated in the host and is not a unique response to inh-OVA because it can be induced by other inhaled Ags.

### Table I. Cytokine production by in vitro stimulated lung lymphocytes

<table>
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<th>Conditioning</th>
<th>Inflam</th>
<th>Inh-agn</th>
<th>Challenge</th>
<th>Cytokine (ng/ml)</th>
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* Lung lymphocytes were isolated from unconditioned or conditioned mice on day 45. FACS analysis was performed to determine the number of Thy1.1+ CD4+ cells present in each group. Cells (1 × 10⁶/ml) were cultured with APC and pOVA for 24–48 hours. IFN-γ or IL-13 in the culture supernatant was determined by ELISA. Cytokines were normalized for 10⁶ CD4+ Thy1.1+ cells/ml. The data are representative of two experiments.

Inflam, inflammation; Inh-agn, inhaled Ag; –, no conditioning.
Inhaled Ag administered to mice with airway inflammation inhibits more effectively than when administered to uninflamed mice

Past studies show that IT is induced in naive mice exposed to inhaled Ag over a period of weeks, leading to the development of Ag-specific limitation in T cell responses (1, 3, 19, 20). We hypothesized that IT would be acting by a mechanism different from AIRID, because previous studies indicate that tolerance does not develop during active inflammation (21). To investigate these differences, naive mice exposed to inh-OVA (IT) and Th1- or Th2-conditioned mice were challenged with Th1 or Th2 cells and inh-OVA. Inhibition of lung inflammation, neutrophils, and IFN-γ was more effective in Th1-conditioned mice compared with naive OVA-exposed mice (Fig. 5A). Lung inflammation, eosinophils, and IL-13 were more markedly reduced in Th2-conditioned mice compared with naive OVA-exposed mice (Fig. 5B). These data show that inhaled Ag administered during airway inflammation is more effective in reducing CD4 effector Th cells, their cytokines, and airway inflammation than Ag administered in uninflamed mice. Thus, AIRID results in more potent inhibition of effector CD4 T cells compared with IT.

Th1-conditioning reduces Th2 cell effects and vice versa

To determine whether the inhibitory effect observed in AIRID depends on the type of inflammatory response in which it was generated, we conditioned mice with Th1 or Th2 cells and inh-OVA and challenged them with Th2 or Th1 cells, respectively. Th1-conditioned mice that were challenged with Th2 cells had a marked reduction in both total lung inflammatory cells and TCR Tg cells, and this effect was comparable to the reductions observed in Th1-conditioned mice challenged with Th1 cells (Fig. 6A). Likewise, Th2-conditioned mice challenged with Th1 cells exhibited a reduction in lung inflammation and in TCR Tg cells (Fig. 6B). These experiments show cross-inhibition of Th1 and Th2 cells in AIRID and suggest that dominant suppression in AIRID is independent of the type of inflammatory response in which it is induced.

T cell inhibition is not mediated by endogenous T or B cells

To define whether the regulatory cells in AIRID are derived from a lymphocyte population resident in the recipient mice, we asked whether we could induce inhibition in RAG1−/− mice that lacked T cell and B cells. In Th1-challenged, Th1-conditioned RAG1−/− and wild-type (WT) mice there was a reduction in TCR Tg cells and neutrophils in the respiratory tract compared with unconditioned mice (Fig. 7A). In Th2-challenged, Th2-conditioned RAG1−/− and WT mice, both TCR Tg cells and eosinophils were inhibited in the lung compared with unconditioned mice (Fig. 7B). In RAG1−/− mice there were more TCR Tg cells in the respiratory tract compared with unconditioned mice (Fig. 7C). In Th2-challenged, Th2-conditioned RAG1−/− mice, both TCR Tg cells and eosinophils were inhibited in the lung compared with unconditioned mice (Fig. 7D). In Th2-challenged, Th2-conditioned RAG1−/− mice there were more TCR Tg cells in the respiratory tract compared with unconditioned mice (Fig. 7E). In Th1-challenged, Th2-conditioned RAG1−/− mice there were more TCR Tg cells in the respiratory tract compared with unconditioned mice (Fig. 7F).
tract of both the conditioned and unconditioned mice compared with WT mice. This likely reflects the presence of T cell proliferative signals active in T cell-deficient mice, such as those that cause homozygotic proliferation (22). In studies using TCR αβ−/− recipient mice that lack αβ T cells, there was also marked inhibition of inflammation in conditioned vs unconditioned groups (data not shown). Thus, these studies show that endogenous T and B lymphocytes are not required for AIRID.

To rule out the possibility that AIRID is mediated by preexisting CD25+ Treg cells derived from TCR Tg cells transferred in the conditioning phase, we generated Th2 cells from a population of CD25-deleted OT-II CD4 T cells. Mice were then conditioned with these CD25-deleted or nondepleted Th2 cells and inh-OVA and challenged with Th2 cells. In mice conditioned with CD25-depleted compared with nondepleted Th2 cells, there was comparable potent inhibition of inflammation (Fig. 7C). Furthermore, there was no difference in FoxP3 expression in whole lung RNA and RNA from lung leukocytes of Th1- or Th2-conditioned mice compared with unconditioned mice (data not shown). These data further support a Treg-independent mechanism of inhibition in AIRID.

T cell inhibition in AIRID is Ag-non-specific

To determine whether T cell inhibition in AIRID is Ag-specific, Th1 or Th2 OVA-conditioned mice were, as before, challenged with KLH-specific Th1 or Th2 cells. Upon challenge with KLH-specific Th1 cells, Th1 OVA-conditioned mice showed marked inhibition of inflammation and neutrophils (not shown) and a marked reduction in Th1 cells compared with unconditioned mice (Fig. 8A). KLH-specific Th2-challenged Th2 OVA-conditioned mice had a dramatic reduction in Th2 cells and eosinophils (not shown) when compared with unconditioned mice. The addition of inh-OVA to inhaled KLH (inh-KLH) during the challenge phase did not result in additional inhibition of inflammation in Th1 or Th2 experiments. Unconditioned mice that were challenged with KLH-specific Th1 or Th2 cells and inh-OVA did not have any evidence of Th cell recruitment to the airway, confirming the Ag specificity of the KLH-specific cell population. These data indicate that mice conditioned with Th1 or Th2 cells and inh-OVA can suppress KLH-specific responses in the respiratory tract; thus, AIRID is Ag nonspecific.

Lung lymphocytes in AIRID are functional

To determine whether AIRID renders lymphocytes unresponsive as a mechanism of T cell inhibition, we isolated lymphocytes from Th1- or Th2-conditioned lungs on day 45 and activated them in vitro with APC and pOVA (323–339). Lung cells from Th1- and Th2-conditioned mice contained fewer CD4 and Thy1.1+ cells as a percentage of total cells compared with unconditioned mice, as expected (Table I). When normalized for CD4+Thy1.1+ cells to reflect the OVA-responsive cells, IFN-γ and IL-13 levels were comparable or increased in the conditioned groups compared with the unconditioned groups. These data show that in AIRID the lymphocytes that persist in the respiratory tract can respond to OVA; thus, the regulatory effect is not due to anergy of the transferred T cells.

Increased macrophage TGF-β1 expression in AIRID

We measured anti-inflammatory cytokines produced in vivo in AIRID. IL-10 was not detected in any BAL samples of mice with AIRID. Quantitative real-time PCR showed that IL-10 mRNA in whole lung was very low in both Th1- and Th2-conditioned mice, and this was comparable to the levels observed in unconditioned mice (not shown). Although these results suggested that IL-10 was not a mediator in AIRID, we tested to determine whether IL-10 produced in recipient mice could mediate AIRID using IL-10−/− mice. IL-10−/− and WT mice were conditioned with OT-II Th2 cells and inh-OVA, and on day 35 the mice were challenged with OT-II Th2 cells (Fig. 9). After challenge, Th2-conditioned IL-10−/− mice exhibited inhibition of eosinophilia and TCR Tg cells, and the inhibitory effect was comparable in WT mice. Thus, in the absence of endogenous IL-10 there was effective inhibition of Th2 cells. Taken together, these data indicate that IL-10 is not likely to be a mediator of AIRID.

The anti-inflammatory cytokine TGF-β1 was increased in the BAL fluid of both Th1- and Th2-conditioned mice compared with naive mice on day 34 after the mice were conditioned and just before challenge (Th1-conditioned, 150 ± 6 pg/ml vs undetectable; Th2-conditioned, 188 ± 8 pg/ml vs undetectable; p < 0.01). After the challenge, on day 45 TGF-β1 was higher in Th1-challenged, Th1-conditioned mice compared with unconditioned mice (Fig. 10A). TGF-β1 levels were also higher in Th2 conditioned mice on day 38, 3 days into the Th2 challenge (Fig. 10B; 60 ± 21 pg/ml vs 14 ± 6 pg/ml, p = 0.06). On day 45, TGF-β1 levels were comparable in Th2-conditioned and unconditioned mice. Bioactive TGF-β was a small fraction of total TGF-β (not shown) in the BAL fluid of both conditioned and unconditioned mice. Because TGF-β1 is active in its membrane-bound form, we examined lung sections immunostained with anti-TGF-β1. There was a dramatic increase in the number of bright TGF-β1-staining cells on day 45 in Th1- or Th2-conditioned WT and RAG1−/− mice vs unconditioned mice (Table II). In conditioned mice, TGF-β1-positive cells were most prominent in areas of inflammation in
peribronchial regions and in the bronchovascular bundles (Fig. 10D). In unconditioned mice, TGF-β1 immunostaining was much weaker (Fig. 10C).

Lung TGF-β1-positive cells had the morphologic features of monocytes/macrophages and a majority costained for CD45, F4/80, CD11b, and CD11c, whereas a minority of TGF-β1-positive cells costained for class II MHC (Table II). This phenotype is consistent with lung/alveolar macrophages (23). TGF-β1 did not colocalize with CD5 or CD4, excluding TGF-β1 expression on the transferred or resident T cells. These data show that TGF-β1-expressing cells are macrophages in both conditioned and unconditioned mice and, strikingly, in AIRID there is an extensive increase in their number and in the intensity of TGF-β1 staining.

Discussion

These studies reveal a novel regulatory pathway in the respiratory tract that provides a potent anti-inflammatory signal for T cells and limits inflammatory airway disease. AIRID is induced during airway inflammation by repeated exposure to inhaled Ag over a period of weeks and limits effector CD4 T cell responses. Different inflammatory environments (Th1 and Th2) support the induction of AIRID, the effects are Ag nonspecific, and host lymphocytes are not required. AIRID contrasts with IT, a well-established regulatory pathway in the lung, because IT is mediated by Treg and inhibits the generation of Ag-specific immune responses. AIRID is a pathway that can limit local inflammatory responses.

These are not the first studies to investigate a regulatory pathway stimulated during airway inflammation by repeated inhaled Ag exposure. This effect has hampered chronic studies of allergic airway disease in OVA-immunized and aerosol-challenged rats whose airway inflammation and AHR declined during repeated inhaled Ag exposure (10) and in models using immunized and repeatedly challenged mice (7–9). Because these effects led to reduced Th effector function, a tolerance mechanism was proposed, although the pathway by which this occurred was never fully characterized. Hurst and colleagues (24) specifically investigated the effects of Th1- and Th2-like airway inflammation followed by repeated exposure to inh-OVA. Mice were then challenged by OVA immunization and the end point was serum IgE level. Th2 inflammation and inh-OVA led to increased OVA-specific IgE, whereas Th1 inflammation had no effect on this parameter. The authors concluded that immune regulation could not be induced during airway inflammation. An investigation into the lung-specific effects may have revealed a regulatory effect.

Pulmonary macrophages appear to play a role in the anti-inflammatory effect observed in AIRID. This finding is supported by studies in RAG−/− mice showing that a nonlymphocyte is responsible for T cell inhibition. Furthermore, in AIRID TGF-β1-expressing macrophages are markedly increased. Alveolar macrophages are known to limit the development of effector CD4 Th cell responses as shown in studies in which their elimination led to enhanced pulmonary immune responses (25). The regulatory effects of macrophages observed previously were believed to be through their effects on dendritic cells and by their poor APC function (25–27). Myeloid lineage suppressor cells (MSCs) that express CD11b have been shown to potent inhibitors of antitumor immune responses and act by inhibiting T cell responses (28). MSCs are believed to act at the site of the tumor (28) and reminiscent of this theory is our observation of a population of intrapulmonary, TGF-β1-positive, F4/80-positive cells within the inflamed airways. MSCs have also been observed in other inflammatory conditions including graft-vs-host disease and in the lymphoid tissues of mice with bacterial and parasitic infections (29–31). MSCs have been shown to exert their suppressive effects through production of NO, prostaglandins, inhibitory cytokines, and reactive oxygen intermediates (28). AIRID may be mediated by such immunosuppressive biochemicals or inhibition may be an effect of TGF-β1. We have been unable to definitively assess whether TGF-β1 mediates AIRID, because Ab inhibition studies with different anti-TGF-β1 Abs did not provide effective inhibition of TGF-β bioactivity in the BAL and receptor blockers did not effectively inhibit TGF-β1 signaling in the lung (not shown). TGF-β1-deficient mice die young from inflammatory disease (32) and cannot be used as recipients in these studies. Given the complexity of inhibiting TGF-β1 in vivo in these studies, mice with cell-specific deletion of TGF-β1 are essential for defining whether TGF-β1 mediates AIRID.

We hypothesize that the induction of AIRID requires two signals. The first is the inflammatory signal. The essential components of this signal may be the activation of certain cells such as macrophages. The second signal is delivered by the inhaled Ag. This step appears to require multiple exposures and a period of weeks for the full induction of AIRID. The inhaled Ag may transform the activated macrophage population into an inhibitory subset that is marked by the expression of TGF-β1.

Inhaled Ag is required to induce AIRID, yet the effect is Ag nonspecific. One possible explanation for this discrepancy is that AIRID is induced by nonprotein moieties in the inhaled particulate such as endotoxin or other pathogen-associated molecular patterns. The commercial OVA used in these studies contains moderate levels of LPS (33). Despite this potential link between AIRID and pathogen-associated molecular patterns, AIRID is not reminiscent of endotoxin tolerance. Short-term, repeated exposure to endotoxin blunts the production of inflammatory cytokines by macrophages, but this form of “tolerance” resolves within 8 days (34). We are currently investigating the different components of inhaled particulates that can stimulate AIRID.

The existence of regulatory pathways that control immune responses during lung inflammation should be expected. There must be mechanisms in place to inhibit ongoing T cell responses and, during inflammation, to limit the effects of environmental Ag exposure. We may find that we can exploit AIRID as a treatment for chronic inflammatory lung diseases. AIRID is a potentially powerful tool because the effects are Ag nonspecific, thus allowing treatment of lung diseases of unknown Ag specificity such as sarcoidosis and diseases of known specificity such as allergic asthma for which an inhaled specific Ag might exacerbate symptoms. In disease, the activation of AIRID offers us a potential new pathway that may limit disease-specific T cell responses in the respiratory tract. We will also investigate what role AIRID could play in the development and/or persistence of chronic inflammatory diseases in the respiratory tract. It has been proposed that immune regulation is defective in asthma (35), a disease that is associated with airway infiltration of allergen-specific Th2 cells. The pulmonary capability to limit ongoing airway inflammation may be a crucial step in protection from chronic airway diseases. As we define the precise mechanisms of inhibition in AIRID, we will move toward understanding how such a pathway influences lung health and disease.

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Disclosures

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