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Original Article

Prevention of tracheal high-dose tolerance induction by granulocyte–macrophage colony stimulating factor-dependent restoration of antigen-presenting cell function

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Abstract

The intrusion of airborne allergens into airways elicits eosinophilic inflammation, as represented by bronchial asthma. It has been shown that excessive amounts of allergen in murine trachea lead to an unexpected evasion of deleterious eosinophilic inflammation by inducing T cell tolerance. In the present study, the mechanisms of tracheal high-dose tolerance are examined with regard to accessory cell functions and the effects of pro-inflammatory cytokines on tolerance. Antigen-induced tracheal eosinophilia was suppressed on instillation of high doses of antigen into the trachea, while concurrent instillation of granulocyte–macrophage colony stimulating factor (GM-CSF) with the antigen restored the diminished responses. The restoration of eosinophilic infiltration by GM-CSF occurred in parallel with an increase in interleukin (IL)-4 production by $CD4^+$ T cells from the mediastinal lymph nodes. This was found to reflect the empowerment of antigen-presenting cells by GM-CSF, because the impaired ability of Ia^+ cells from the tolerant mice to stimulate IL-4-producing T cells is restored by GM-CSF administration. The prevention of tolerance by up-regulating accessory cell functions is a feature unique to GM-CSF, because another pro-inflammatory cytokine, IL-1β, failed to empower antigen-presenting cells. Thus, besides the induction of transforming growth factor-β-secreting $CD4^+$ T cells, high-dose tolerance in the trachea includes an impairment of the accessory cell functions that support IL-4 production from T cells, which was reversed by GM-CSF. This report is the first demonstration that GM-CSF breaks the T cell tolerance of IL-4-producing T helper cells.

Key words: bronchial asthma, eosinophilia, T cell tolerance.

Introduction

The respiratory tract, a representative site of mucosal tissue, is repeatedly exposed to a broad array of airborne foreign allergens.[1] Inhalation of allergens evokes deleterious immune and inflammatory responses in the airway, as in bronchial asthma.[2] The characteristic features of bronchial asthma include airway eosinophilia and elevated serum IgE levels, both of which are reported to be orchestrated by T helper (Th)2 cells.[3–9] The suppression of Th2-dominated immune responses in this setting could be a potential target of immunotherapy for bronchial asthma.

Several distinct experimental approaches have been taken to control airway eosinophilia by manipulating Th2 functions. For example, another $CD4^+$ T cell subset, the Th1 cells, exerts inhibitory effects on Th2 cells through the secretion of cytokines, such as γ-interferon (IFN-γ).[10] This subset is likely to be induced on infection with intracytoplasmic pathogens[11–17] and exposure to *Mycobacterium tuberculosis* is reported to inhibit Th2-mediated eosinophilia by inducing contra-acting Th1 cells.[18,19] Transforming growth factor (TGF)-β, with properties that

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inhibit a wide variety of inflammatory responses, could be another candidate for ameliorating immune-mediated disorders.[20] The TGF-β-secreting $CD4^+$ T cells are induced by antigen feeding and in turn inhibit tracheal eosinophilia.[21] Similar TGF-β–secreting $CD4^+$ T cells have been found to be induced in response to high doses of antigens in the trachea and the inhibition of tracheal eosinophils occurrs in parallel with the inhibition of the IL-4-producing ability of mediastinal lymph node (LN) T cells.[22]

Some pro-inflammatory cytokines are known to potentiate eosinophilic inflammation. Granulocyte–macrophage colony stimulating factor (GM-CSF) has pronounced effects on eosinophilia by extending the survival and promoting maturation and activation.[23–25] Interleukin (IL)-1 enhances eosinophil migration and infiltration by enhancing adhesion molecules on endothelial cells or by the secretion of other cytokines.[26–34]

We have reported previously that high doses of antigen in the trachea induce Th2 cell tolerance, which we referred to as tracheal high-dose tolerance.[22] The tolerance was found to be mediated by TGF-β secreted from $CD4^+$ T cells, while the roles of antigen-presenting cells (APC) and pro-inflammatory cytokines remained unexplored. In the present study, we examined the mechanisms of tracheal high-dose tolerance with regard to accessory cell functions and the effects of pro-inflammatory cytokines on the tolerance. We found that, as well as the induction of TGF-β-secreting $CD4^+$ T cells, the high-dose tolerance in the trachea includes an impairment of accessory cell functions that support IL-4 production from T cells, which was reversed by GM-CSF.

METHODS

Animals and immunization

BALB/c mice were bred in our animal facility and were used at 5–10 weeks of age. These animals were primed intraperitoneally with 10 μg ovalbumin (OVA; Sigma Chemical Co., St Louis, MO, USA) precipitated with 4 mg aluminum hydroxide (alum) in 200 μL phosphate-buffered saline three times at weekly intervals. Seven days after the last immunization, the mice were challenged with intratracheally administered OVA, as described later. BALB/c mice transgenic (tg) for T cell receptor (TCR) specific for $OVA_{323–339}$ and $I\text{-}A^{d35}$ were primed intraperitoneally with 10 μg OVA in alum and the spleen was used as a source of $CD4^+$ T cells after 7 days.

Antigen-induced eosinophilic infiltration in the trachea

The methods have been detailed previously.[21] In brief, 1 week after the last intraperitoneal immunization with OVA/alum, sensitized mice were lightly anesthetized with pentobarbital (Abbott Laboratories, North Chicago, IL, USA), and graded doses of OVA were instilled directly into the surgically exposed trachea. After 2 days, the excised trachea was fixed in 10% formalin and frozen in optimal cutting temperature (OCT) compound (Miles Laboratories, Naperville, IL, USA). Cryosections (7 μm thick) from the frozen tissue were stained with Diff-Quik (International Reagents Corporation, Kobe, Japan). The numbers of eosinophils infiltrating into the submucosal tissue of the trachea were determined by light microscopy. The perimeter of the basement membrane of the trachea was measured with a microcomputer imaging device (MCID) image analyzer (Imaging Research Inc., St Catherines, Ontario, Canada). For every trachea, six to eight sections were used for counting, each separated from the adjacent one by over 70 μm. Results were converted to the number of eosinophils per 1 mm section of basement membrane. Each point represents the mean ± SEM for four to seven mice from one experiment. Results are representative of two or three independent experiments.

In vitro culture and assay for IL-4 and IL-5

BALB/c mice primed intraperitoneally with OVA/alum three times were challenged intratracheally with 10 μg OVA or 500 μg OVA either alone or with 10 ng GM-CSF (R&D Systems, Minneapolis, MN, USA) or IL-1β (R&D systems). After 7 days, 1×10^5 mediastinal LN cells were cultured with 100 μg/mL OVA in 96-well plates for 2 days. Otherwise, Ia^+ cells as antigen-presenting cells (2×10^4/well) and $CD4^+$ T cells (2×10^4/well) were cocultured under the same culture conditions. Ia^+ cells were prepared by means of anti-Ia beads (Miltenyi Biotec, Gladbach, Germany) and a magnetic activated cell sorter (MACS; Miltenyi Biotec GmbH) from mediastinal LN of BALB/c mice that received an intratracheal administration of 10 μg OVA or 500 μg OVA either alone or with 10 ng GM-CSF 7 days before. The $CD4^+$ T cells were prepared from spleen of anti-OVA tg mice primed with OVA/alum 7 days before using anti-CD4 beads and MACS. The concentrations of IL-4 and IL-5 in the culture supernatants were determined by enzyme-linked immunosorbent assay (ELISA) with paired anti-IL-4 and -IL-5 monoclonal antibodies, respectively (Pharmingen,

San Diego, CA, USA) and tetramethylbenzidine reagent (Kierkegaard and Perry, Gaithersburg, MD, USA) according to the manufacturer's recommendations. Standard recombinant mouse IL-4 and IL-5 was purchased from Genzyme (Genzyme Co., Cambridge, MA, USA). The purity of Ia^+ or $CD4^+$ cells was always > 95% by reanalyses. The detection level of IL-4 or IL-5 was > 20 pg/mL.

Assay of transforming growth factor-β

Anti-ovalbumin tg mice were treated intratracheally with 10 μg OVA or 500 μg OVA either alone or with 10 ng GM-CSF. After 7 days, 1×10^5 mediastinal LN cells were cultured with or without 100 μg/mL OVA in 96-well round-bottomed plates for 3 days. Levels of TGF-β were determined as described previously.[21] In brief, culture supernatants were applied to 96-well microtiter plates coated with chicken antihuman TGF-β1 antibody (5 μg/mL; R&D Systems). Bound TGF-β was detected by monoclonal anti-TGF-β (1 μg/mL) (Genzyme), followed by peroxidase-labeled goat antimouse IgG (1 μg/mL; Kierkegaard and Perry) and tetramethylbenzidine reagent (Kierkegaard and Perry). Optical densities were determined at 450 nm and converted to concentrations (ng/mL) according to the standard curve obtained with titrated concentrations of human recombinant TGF-β1 (R&D Systems). The minimum detection level of TGF-β was > 50 pg/mL.

Statistics

Statistical significance was determined by Student's *t*-test.

RESULTS

Granulocyte–macrophage colony stimulating factor and IL-1β augmented tracheal eosinophilias induced by high, but not low, doses of antigen

We have previously shown that tracheal eosinophilia evoked by OVA is regulated in an antigen dose-dependent manner; 10 μg OVA in the trachea elicits the maximal eosinophilic responses, while higher or lower doses of OVA induces reduced levels of eosinophilia (Fig. 1).[21] We examined whether the lack of optimal eosinophilic responses induced by high or low doses of OVA could be reversed by pro-inflammatory cytokines. In our preliminary experiments with graded doses of GM-CSF or IL-1, 10 ng of cytokines showed maximal effects (data not shown). When 10 ng GM-CSF was given intratracheally together with graded doses of OVA, it exerted no enhancing effects on the eosinophilia induced by low or optimal doses of OVA (approximately 10 μg; Fig. 1a). In contrast, GM-CSF reversed the low eosinophilic responses evoked by higher doses of OVA to levels comparable to those induced by optimal antigen doses. Similar observations were obtained when another

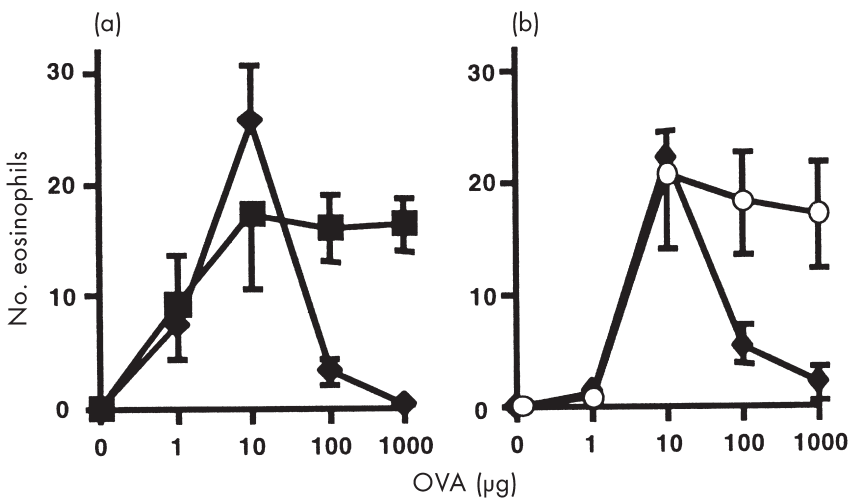

Fig. 1 Granulocyte–macrophage colony stimulating factor (GM-CSF)- and interleukin (IL)-1β-augmented tracheal eosinophilia induced by high, but not low, doses of antigen. BALB/c mice immunized intraperitoneally with ovalbumin (OVA)/alum three times were given an intratracheal administration of titrated doses of OVA either alone (◆) or with 10 ng GM-CSF (■) or 10 ng IL-1β (○). The numbers of eosinophils infiltrating in the trachea were determined as described in Methods. Each point is the mean ± SEM for four to seven mice from one experiment. Results are representative of two or three independent experiments.

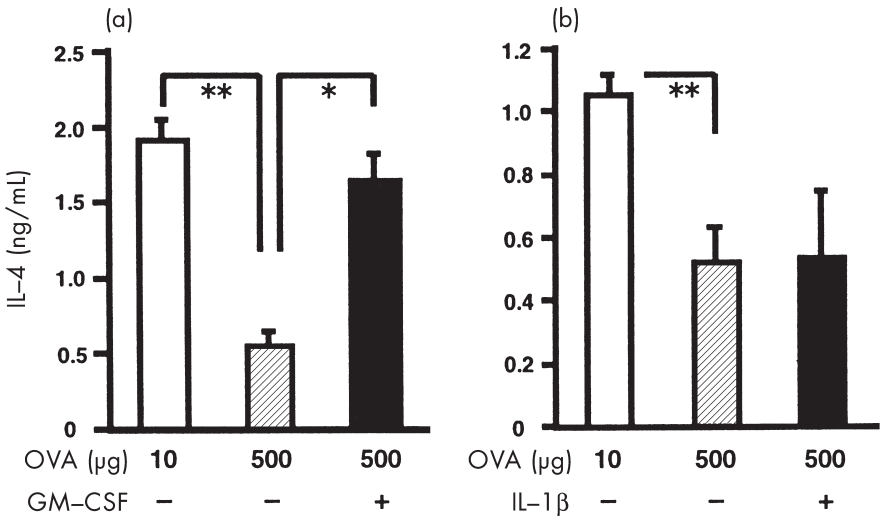

Fig. 2 Granulocyte–macrophage colony stimulating factor (GM-CSF), but not interleukin (IL)-1β, restored IL-4 production by mediastinal lymph node (LN) cells from high-dose tolerant mice. BALB/c mice were primed intraperitoneally with ovalbumin (OVA)/alum three times and then challenged intratracheally with 10 μg OVA, or 500 μg OVA either alone or with 1 ng GM-CSF (a) or IL-1β (b). After 7 days, mediastinal LN cells were cultured *in vitro* with 100 μg/mL OVA for 48 h. Culture supernatants were assayed for IL-4 by enzyme-linked immunosorbent assay. The failure of mediastinal LN cells from high-dose tolerant mice to produce IL-4 was restored by intratracheal administration of GM-CSF (a), but not by IL-1β (b). The results of *in vitro* cultures are given as the mean ± SEM of four wells. The data shown are representative of two independent experiments. (*$P < 0.002$; **$P < 0.0001$).

pro-inflammatory cytokine, IL-1β, was given; eosinophilia elicited by only high doses of OVA was increased by IL-1β (Fig. 1b). These results indicate that the impairment in eosinophilia induced by high, but not low, doses of OVA could be circumvented by the pro-inflammatory cytokines.

Granulocyte–macrophage colony stimulating factor, but not IL-1β, restored IL-4 production by mediastinal LN cells from high-dose tolerant mice

We have reported previously that the low levels of tracheal eosinophilia elicited by a high dose of OVA in the trachea are attributable to the tolerance of $CD4^+$ Th2 cells, which we referred to as tracheal high-dose tolerance.[22] Because restoration of the eosinophilic responses by pro-inflammatory cytokines may reflect the up-regulation of Th2 cells in regional LN cells, we examined whether simultaneous intratracheal instillation of GM-CSF or IL-1β with high doses of OVA increased the IL-4 production from mediastinal LN cells. Interleukin-4 production from mediastinal LN cells from mice receiving high doses of antigen was impaired in comparison to production in mice receiving optimal doses of OVA (Fig. 2a,b; $P < 0.0001$). The down-regulated IL-4 production from high-dose tolerant mice was not reversed by intratracheal instillation of IL-1β (Fig. 2b), whereas another pro-inflammatory cytokine, GM-CSF, augmented the IL-4 production to levels comparable to those produced by the optimal antigen doses (Fig. 2a). These results indicate that two pro-inflammatory cytokines up-regulate the eosinophilia induced by high doses of antigen through distinct mechanisms; GM-CSF enhanced eosinophilia by restoring the impaired Th2 function, while IL-1β did so by other mechanisms.

Restoration of impaired APC function from high-dose tolerant mice by GM-CSF

We have reported that tracheal high-dose tolerance is due to the induction of TGF-β-secreting $CD4^+$ T cells, which inhibit Th2 cells.[22] Here, we tried to determine whether the impaired ability of APC to activate Th2 cells was another possible mechanism of high-dose tolerance and, if so, whether the restoration of eosinophilia by GM-CSF was in parallel with the improved ability of APC. $CD4^+$ T cells prepared from OVA/alum-primed anti-OVA TCR tg mice were cocultured with Ia^+ cells of mediastinal LN, and the levels of IL-4 and IL-5 in culture supernatants were determined by ELISA. In comparison with Ia^+ cells from animals receiving optimal doses of

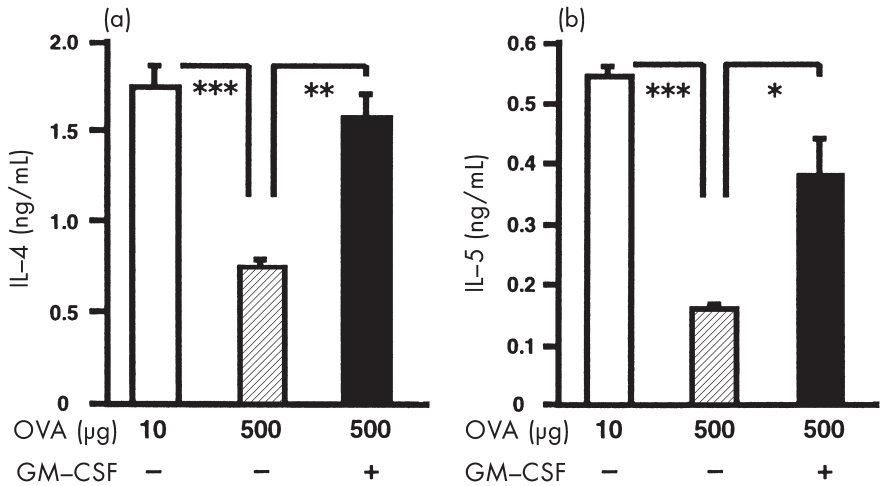

Fig. 3 Restoration of impaired antigen-presenting cell function from high-dose tolerant mice by granulocyte–macrophage colony stimulating factor (GM-CSF). BALB/c mice were instilled intratracheally with 10 μg or 500 μg ovalbumin (OVA), or 500 μg OVA plus GM-CSF. After 7 days, Ia^+ cells were separated from their mediastinal lymph nodes. The mitomycin C (MMC)-treated Ia^+ cells were cultured with 100 μg/mL OVA and $CD4^+$ splenic T cells from anti-OVA T cell receptor transgenic mice that had been primed with OVA/alum 7 days before. After 2 days, culture supernatants were assayed for IL-4 (a) and IL-5 (b) by enzyme-linked immunosorbent assay. Low efficacy of the Ia^+ cells from high-dose tolerant mice to support T helper cell 2 cytokine production was restored by intratracheal administration of GM-CSF. The results of *in vitro* cultures were given as the mean ± SEM of four wells. The data shown are representative of two independent experiments. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.002$.

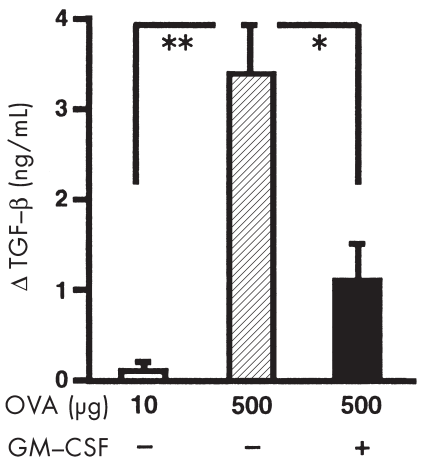

Fig. 4 Inhibition of transforming growth factor (TGF)-β production from high-dose tolerant lymph node (LN) cells. Anti-ovalbumin (OVA) transgenic mice were treated intratracheally with 10 μg OVA, or 500 μg OVA either alone or with 10 ng granulocyte–macrophage colony stimulating factor (GM-CSF). After 7 days, 1×10^5 mediastinal LN cells were cultured with or without 100 μg/mL OVA in 96-well round-bottomed plates for 3 days. Levels of TGF-β were determined by enzyme-linked immunosorbent assay. The results of *in vitro* cultures were given as the mean ± SEM of four to six wells after TGF-β levels secreted in an antigen-non-specific fashion were subtracted. The data shown are representative of three independent experiments. $^*P < 0.01$; and $^{**}P < 0.0001$.

OVA, those receiving high doses of OVA had an impaired ability to stimulate CD4 T cells to produce IL-4 (Fig. 3a, middle bar; $P < 0.002$) or IL-5 (Fig. 3b, middle bar; $P < 0.002$). Interestingly, the deteriorated ability of APC was overcome by GM-CSF; when GM-CSF was concomitantly given intratracheally together with high doses of OVA, the impaired function of APC from mediastinal LN was fully restored to the optimal levels, as judged by the ability to induce IL-4 (Fig. 3a, right bar) and IL-5 (Fig. 3b, right bar) secretion from T cells. These results revealed a strict relationship between the ability of APC to induce Th2 cytokine secretion and the extent of tracheal eosinophilia and showed that GM-CSF, which restored eosinophilia by high doses of OVA, also empowered APC to stimulate Th2 cells.

Inhibition of TGF-β production from high-dose tolerant LN cells

We examined effects of GM-CSF on TGF-β production from high-dose tolerant LN cells. As we have described previously,[22] the mediastinal LN cells from anti-OVA TCR tg mice receiving high doses of OVA (500 μg) produce TGF-β in response to *in vitro* antigen challenge ($P < 0.05$), whereas the concomitant intratracheal

treatment with OVA and GM-CSF abolishes production of TGF-β (Fig. 4). Thus, the restoration of the Th2 function was in parallel with the abolition of TGF-β production.

Discussion

The down-regulation of harmful immune responses to inhaled antigens is a possible approach to immunotherapy for bronchial asthma. Two components that play crucial roles in the pathogenesis of bronchial asthma are elevated IgE levels and eosinophilic inflammation in the airways, both of which are orchestrated by cytokines elaborated by Th2 cells.[3–9] Selective abrogation of the Th2 cell-mediated responses specific for allergen, with sparing of other immune functions, averts such adverse effects as the deterioration of host defenses, which is inevitable when antigen-non-specific immunosuppressants are used.

We have demonstrated previously that high doses of antigen in the trachea inhibit eosinophilic inflammation by down-regulating the IL-4 production of $CD4^+$ T cells.[22] The impairment of IL-4 production is mediated by TGF-β secreted from another $CD4^+$ T cell subset.[22] In the present study, we examined the mechanisms of the tracheal high-dose tolerance with regard to accessory cell functions and the effects of pro-inflammatory cytokines on this tolerance. The concurrent intratracheal instillation of GM-CSF or IL-1β with high doses of antigen restored the tracheal eosinophilia (Fig. 1), while augmentation of eosinophilia by GM-CSF, but not by IL-1β, was accompanied by a parallel increase in IL-4 production in CD4 T cells from the mediastinal lymph nodes (Fig. 2). The prevention of tolerance reflected the empowerment of APC by GM-CSF (Fig. 3). Thus, exposure of the airway to high doses of antigen led to the inhibition of the antigen-presenting ability, with consequent failure of T cells to produce IL-4 and IL-5, and scant eosinophilic inflammation.

Granulocyte–macrophage colony stimulating factor and IL-1β enhanced the tracheal eosinophilia induced by high doses of OVA (Fig. 1). Both of these cytokines have stimulatory effects on eosinophils. Interleukin-1 facilitates eosinophil adhesion to and transmigration through endothelial cells by up-regulating adhesion molecules.[26–30] Interleukin-1β also promotes eosinophil recruitment by inducing the secretion from endothelial cells of other cytokines, including GM-CSF, eotaxin or monocyte chemoattractant protein (MCP)-4.[31–33] Direct injection of IL-1β has been reported to induce eosinophil accumulation *in vivo*.[34] Granulocyte–macrophage colony stimulating factor has other pronounced effects on eosinophils by extending their survival and promoting their maturation and activation.[23–25] Additional novel mechanisms were noted for GM-CSF in the present experiments. Granulocyte–macrophage colony stimulating factor, but not IL-1β, restored the function of IL-4-producing T cells. (Fig. 2). The prevention of tolerance was ascribed to the up-regulation of APC functions, which otherwise remain down-regulated by high doses of antigen (Fig. 3). Roles of IL-1β in the prevention of tolerance[36–39] or enhancement of dendritic cell function[40–42] have been reported, although IL-1β neither prevented the tolerance nor restored the APC functions in the present study. These results imply that GM-CSF plays a critical role in the induction and prevention of tracheal high-dose tolerance, which is not compensated by IL-1β.

Granulocyte–macrophage colony stimulating factor, in addition to its effects on eosinophils, plays an essential role in the activation and maturation of antigen-presenting cells or dendritic cells.[43,44] In peripheral tissues, immature dendritic cells, as sentinel APC, capture antigens with poor antigen presentation, while they acquire a potent capability to present antigens to T cells in lymphoid tissues.[45–50] Immature dendritic cells, such as skin Langerhans cells or airway dendritic cells, are deficient in the expression of costimulatory molecules.[51–54] Stimulation of T cells by such costimulatory molecule-deficient APC results in the unresponsiveness of T cells, namely anergy.[55,56] The gain of stimulatory and the loss of tolerogenic properties of APC is accompanied by the up-regulation of costimulatory molecules by cytokines such as GM-CSF.[43,53] These observations are well in accord with those of ours in which GM-CSF broke the tolerance induction. Our experiments demonstrated the novel finding that enhancement of APC with GM-CSF blocked the suppressive effects of TGF-β-secreting T cells on Th2-mediated inflammation. The inhibitory effects of the TGF-β-secreting T cells were no longer observed after restoration of the APC function by addition of GM-CSF (Fig. 3). We did not clarify the precise mechanisms underlying the reversal of the inhibitory T cells. One possible mechanism is that APC that remained unactivated, due to a lack of GM-CSF, may have preferentially induced TGF-β-secreting T cells at the expense of IL-4- and IL-5-producing cells. Another possibility is that activated APC stimulated IL-4- and IL-5-producing T cells more efficiently than TGF-β-producing T cells and the former outnumber the latter, with a resultant restoration of

IL-4 production. Regardless of the underlying mechanisms, we found that APC that were not activated in a proper manner could down-regulate the immune responses by inducing suppressor T cells in addition to inducing T cell anergy.

In summary, we report in the present paper that the impaired function of APC is another cause of tracheal high-dose tolerance. We believe that this is the first report that reveals the prevention of tolerance by the empowerment of APC, which otherwise induce suppressor T cells.

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