

Human alveolar macrophages induce functional inactivation in antigen-specific CD4 T cells

Robin L. Blumenthal, PhD, Dianne E. Campbell, MD, PhD, Paul Hwang, MD, Rosemarie H. DeKruyff, PhD, Lorry R. Frankel, MD, and Dale T. Umetsu, MD, PhD
Stanford, Calif

Background: Alveolar macrophages (AMCs) are the most abundant phagocytic cells in the lung, but they present antigen poorly to T cells.

Objectives: The objectives of our studies were to more clearly define the mechanisms by which AMCs present antigen to T cells and to determine whether AMCs actively inhibit T-cell activation.

Methods: We studied purified human CD4 T cells and compared the capacity of allogeneic AMCs and peripheral blood monocytes to induce T-cell proliferation and cytokine production.

Results: We previously demonstrated that human AMCs fail to upregulate expression of B7-1 and B7-2 on stimulation with IFN- γ . We now demonstrate that AMCs actively induce T-cell unresponsiveness (functional inactivation) in an antigen-specific manner and reduce the capacity of CD4 T cells to respond on secondary stimulation. The induction of unresponsiveness was reversed by the addition of CD28 costimulation or IL-2. However, interruption of Fas/Fas ligand interactions or of B7/CTLA-4 interactions did not prevent unresponsiveness, indicating that neither CTLA-4 triggering nor Fas-induced apoptosis was involved in the induction of T-cell unresponsiveness.

Conclusions: These studies indicate that AMCs actively tolerate CD4 T cells in an antigen-specific fashion. We propose that AMCs mediate a form of immune privilege in the lungs that effectively limits immune responses in the pulmonary compartment but has little effect on systemic immunity. (*J Allergy Clin Immunol* 2001;107:258-64.)

Key words: Alveolar macrophages, tolerance, T cells, immune privilege, CD86, CD28

The respiratory mucosa interfaces broadly with the environment and, as such, is exposed continuously to a wide variety of environmental antigens. These antigens include innocuous, nonreplicating antigens, to which

Abbreviations used

AMC: Alveolar macrophage
APC: Antigen-presenting cell
cpm: Counts per minute
FasL: Fas ligand
NO: Nitric oxide
PBM: Peripheral blood monocyte

indiscriminate, overzealous host immune responses could be detrimental, causing injury to the lung and interfering with gas exchange. Therefore mechanisms specific to the respiratory mucosa exist to limit immune responses and prevent mucosal damage. These mechanisms may include processes that reduce airway inflammation and enhance the development of tolerance to antigen exposure. Some of these mechanisms include rapid clearance of inspired antigen, induction of the development of regulatory/suppressor cells, limitation of costimulatory signals, or induction of functional inactivation in CD4 T cells.¹⁻⁷ Although it is clear that dendritic cells are rapidly recruited to the respiratory tract in the face of inflammatory signals to initiate immune responses in the lung,⁸ the role of other resident antigen-presenting cells (APCs) in downregulating local immune responses has not been clearly delineated.

Alveolar macrophages (AMCs), which are derived from blood monocytes, are the most abundant phagocytic cells in the lung. However, numerous studies indicate that they do not present antigen effectively to T cells.^{4,9,10} These studies suggest that AMCs might function to limit rather than initiate immune responses at the pulmonary mucosal surface. AMCs actively phagocytize foreign materials that reach the lung, and mucociliary processes then rapidly remove AMCs from the lung.¹¹⁻¹³ In addition, we previously demonstrated that AMCs fail to upregulate expression of the costimulatory molecules B7-1 (CD80) and B7-2 (CD86) on stimulation with IFN- γ ,⁴ suggesting that AMCs limit T-cell responses in the lung by activating T cells in the absence of costimulatory signals. An important role of AMCs in reducing respiratory inflammation is supported further by studies demonstrating that elimination of AMCs from the lungs, for example, with liposome-encapsulated dichloromethylenediphosphonate, leads to a signifi-

From the Divisions of Immunology and Allergy and Critical Care Medicine, Department of Pediatrics, Stanford University.

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Reprint requests: Dale T. Umetsu, MD, PhD, Department of Pediatrics, Room G309, Stanford University, Stanford, CA 94305-5208.

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cant increase in pulmonary immune responses to antigens encountered in the respiratory tract.¹³

The purpose of our studies was to more clearly define the mechanisms by which AMCs present antigen to T cells and limit pulmonary inflammation and antigen-specific immune responses in the normal lung. We demonstrated that AMCs actively induce T-cell unresponsiveness in an antigen-specific fashion, strongly suggesting that AMCs represent a key cell type in the lung that is responsible for regulating immune responses locally in the form of immune privilege.

METHODS

Study population

Healthy volunteers with no history of smoking, asthma, allergic disease or other respiratory disorders were recruited. The volunteers underwent flexible bronchoscopy and gave a sample of blood for peripheral blood monocyte (PBM) purification. A group of unrelated, healthy, nonatopic individuals gave peripheral blood from which purified T cells were used in an allogeneic in vitro culture. The volunteers included 11 male and 7 female subjects, with a mean age of 20 years. Serum IgE levels from all volunteers were within normal ranges (<25 IU/mL), and RAST tests results showed no response to a panel of 13 allergens. Experimental protocols were approved by the Stanford University Administrative Panel on Human Subjects in Medical Research, and all subjects gave informed consent.

mAbs

mAbs used in this study, anti-CD3 OKT3, anti-CD28, anti-IL-10 (used by permission of J. Abrams), and control rat IgG HB129, have been previously described.¹⁴ Anti-CTLA-4-blocking mAb (a kind gift from Dr Peter Linsley, Bristol-Meyers-Squibb, Seattle, Wash) was used at 1 µg/mL. Anti-TGF-β₁ was used at 40 µg/mL (PharMingen, San Diego, Calif). Fas Ig (a kind gift from Dr Shyr-Te Ju, Boston University, Boston, Mass) was used to block Fas-Fas ligand (FasL) interactions at a concentration up to 0.5 µg/mL.

Cytokines

Recombinant human IL-2 (Biosource International, Camarillo, Calif; specific activity 5×10^6 U/mL) was added to some cultures at a concentration of between 1 and 100 U/mL.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed as previously described,⁴ under controlled conditions based on the recommendation of National Heart, Lung, and Blood Institute Workshop on the Investigative Use of Fiberoptic Bronchoscopy and Bronchoalveolar Lavage in Asthmatics.¹⁵ The bronchoalveolar lavage cells were then counted; AMCs typically comprised approximately 85% to 95% of lavage fluid. The macrophages were further purified by layering over Percoll (specific gravity 1.130g/mL; Pharmacia, Uppsala, Sweden) and centrifuging at 4°C, 2000 rpm (833g). The resulting cell population was greater than 98% AMCs by morphology.

Mononuclear cell isolation

PBMCs were isolated as previously described.⁴ Monocytes were enriched from peripheral blood by adherence to plastic, as previously described.⁴

Preparation of highly purified, CD8-depleted T cells

Plastic-nonadherent cells (primarily lymphocytes) were collect-

ed and resuspended in buffer (RPMI-1640 containing 10% human AB⁺ serum) and then purified by nylon wool column separation. Cells were incubated with L243 mAb (anti-class II MHC), OKM1 (anti-human Mac 1), and OKT8 (anti-human CD8) on ice for 45 minutes and then treated with baby rabbit complement at 37°C for 30 minutes to remove CD8 T cells, B cells, monocytes, and activated class II-expressing T cells. These cells were highly depleted of APC activity such that they failed to respond to antigen or concanavalin A (5 µg/mL) in the absence of added APCs.

Statistical analysis

Data were analyzed with the Student *t* test.

RESULTS

We compared the capacity of AMCs and PBMs obtained from the same individual to present antigen to purified CD4 T cells. Peripheral blood CD4 T cells were extensively purified such that they failed to proliferate in response to the mitogen concanavalin A in the absence of added APCs, indicating that no functional APCs were present. These highly purified CD4 T cells proliferated vigorously when PBMs presented antigen but not when AMCs presented antigen⁴ (data not shown). The magnitude of proliferative response induced by AMCs in CD4 T cells was less than 15% of that induced by PBMs in all experiments. The reduced capacity of AMCs to induce T-cell proliferation, however, was not due to nonspecific suppressive effects because the AMCs did not inhibit T-cell proliferation when both PBMs and AMCs, mixed 1:1, were used as APCs. Supernatants from these cultures with either AMCs or PBMs did not inhibit T-cell proliferation of cultures containing allogeneic PBMCs (mixed lymphocyte reaction) (data not shown), indicating that the decreased proliferation observed with AMCs was likely due to cell-cell interaction and not to soluble factors.

The inability of AMCs to induce T-cell proliferation was not because AMCs were simply inert but rather reflected an active process that inhibited the capacity of the T cells to subsequently proliferate to antigen. Thus CD4 T cells cultured with AMCs for 6 days did not proliferate (1164 counts per minute [cpm] vs 24,014 cpm in control cultures; Table I) and proliferated poorly when subsequently stimulated with PBMs from the same donor (12,914 cpm vs 115,882 cpm in control cultures; Table I). However, the CD4 T cells cultured with AMCs were still viable because they proliferated vigorously when stimulated with monocytes from a third-party donor, donor C (66,570 cpm). This demonstrated that the T-cell unresponsiveness induced by AMCs was antigen specific and due to an active process.

The unresponsive T cells produced very limited quantities of cytokines when stimulated. Supernatants produced in control cultures of CD4 T cells with PBMs produced high levels of IFN-γ and IL-10 and low levels of IL-4. In contrast, T cells cultured with AMCs produced much lower levels of IFN-γ, IL-10, and IL-4 (Fig 1, A). Production by the unresponsive T cells of TGF-β, a cytokine associated with regulatory activity, was also examined, but only minimal levels were observed, and no

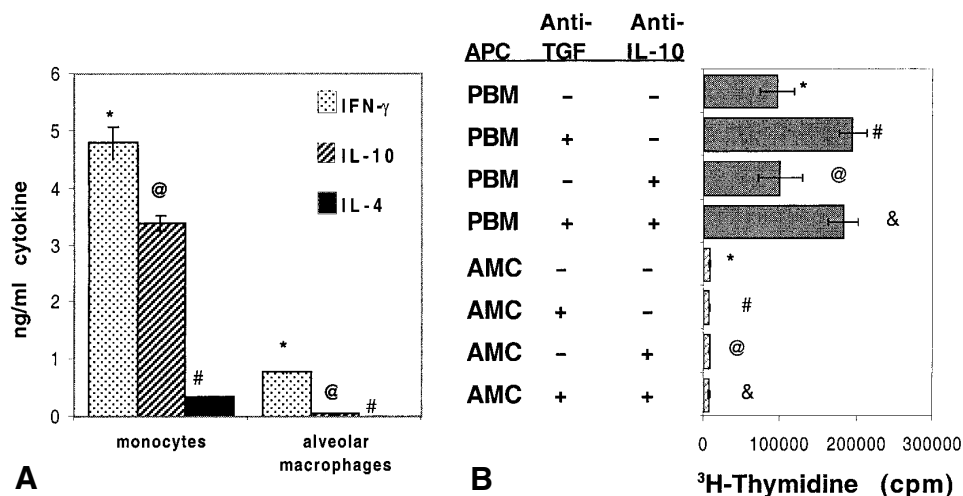


FIG 1. A, T cells cultured with AMCs produce reduced levels of cytokines. CD4 T cells (1×10^6 cells/mL) were cultured in complete media with either allogeneic PBMs (0.5×10^6 cells/mL) or AMCs (0.5×10^6 cells/mL). After 6 days in culture, CD4 T cells were harvested by gentle aspiration off of culture plates and were restimulated with PMA ($10 \mu\text{g/mL}$) and PHA ($1 \mu\text{g/mL}$). The supernatants were harvested after 24 hours and analyzed by ELISA for IFN- γ , IL-10, and IL-4, as previously described.¹⁴ * $P < .03$, @ $P < .02$, # $P < .003$. These results are representative of 4 experiments. **B**, The reduced capacity of AMCs to induce CD4 T-cell proliferation is not due to production of TGF- β or IL-10. Purified CD4 T cells (1×10^5 cells/well) were cultured with allogeneic PBMs (0.5×10^5 cells/well) or with allogeneic AMCs (0.5×10^5 cells/well) and either anti-TGF- β mAb, anti-IL-10 mAb, or both anti-TGF- β mAb and anti-IL-10 mAb. Results are given as cpm of ^3H -thymidine $\pm$ SD incorporated during the last 18 hours of culture. * $P < .03$, @ $P < .001$, # $P < .003$, & $P < .002$. Results are representative of 3 separate experiments.

TABLE I. AMCs induce antigen-specific unresponsiveness in CD4 T lymphocytes

Primary stimulus	cpm $\pm$ SD	Secondary stimulus	cpm $\pm$ SD
Alveolar macrophages _{donor B}	1,164 $\pm$ 80*	None	367 $\pm$ 41
		Monocytes _{donor B}	12,914 $\pm$ 2,235†
		Monocytes _{donor C}	66,570 $\pm$ 16,956‡
Monocytes _{donor B}	24,014 $\pm$ 4,568*	None	271 $\pm$ 33
		Monocytes _{donor B}	115,882 $\pm$ 12,147‡
		Monocytes _{donor C}	73,511 $\pm$ 6,453‡

Purified CD4 T cells (from donor A) were cultured with allogeneic AMCs or PBMs (both from donor B) for 6 days. Proliferation in this primary culture was assessed by measuring the incorporation of ^3H -thymidine during the last 18 hours of the culture period (column 2). T cells from parallel cultures were harvested, washed, and restimulated with PBMs from donor B or with third-party PBMs (donor C). Proliferation in the secondary culture was assessed after 2 days (for donor B cultures) or 5 days (for Donor C cultures) by measuring the incorporation of ^3H -thymidine during the last 18 hours of the culture period (column 4). Results are representative of 3 separate experiments.

* $P < .006$.

† $P < .005$.

‡ $P < .08$.

significant differences between the AMC and PBM cultures were observed (data not shown). The reduced capacity of AMCs to induce CD4 T-cell proliferation was not due to production by the AMCs of regulatory cytokines such as TGF- β or IL-10. Thus culture of CD4 T cells and AMCs with neutralizing anti-TGF- β mAb, anti-IL-10 mAb, or with both mAbs had no effect on the proliferation of CD4 T cells stimulated with AMCs (Fig 1, B). The addition of anti-TGF- β mAb increased proliferation of T cells stimulated with PBMs, suggesting that this cytokine plays a role in regulating monocyte-induced T-cell proliferation.

The reduced capacity of AMCs to induce CD4 T-cell proliferation was not due to the induction of T-cell apop-

tosis or CTLA-4 induced inactivation. The induction of apoptosis by the interaction of the Fas protein with its ligand is well recognized,¹⁶ and AMCs express FasL,¹⁷ which could induce T-cell apoptosis. Addition of a Fas Ig fusion protein, at concentrations known to inhibit Fas-FasL interactions,¹⁸ had no effect on the proliferation when either AMCs or PBMs were used as the APCs (Fig 2, A). CTLA-4, which has a higher affinity for B7 molecules than CD28 and is expressed by activated T cells, produces a predominantly negative costimulatory signal to T cells.^{19,20} Because AMCs express low levels of B7-2, which is not upregulated on macrophage activation,⁴ we added a blocking anti-CTLA-4 mAb to cultures of AMCs and CD4 T cells. This did not reverse the reduced

proliferative response (Fig 2, B), indicating that neither Fas-induced apoptosis nor CTLA-4-induced suppression was involved in reducing T-cell proliferation by AMCs.

The reduced capacity of AMCs to induce T-cell proliferation was not due to the release of other suppressive molecules such as nitric oxide (NO)²¹ because addition of an inhibitor of inducible NO synthase, NG-monomethyl-L-arginine acetate,²² to our cultures had no effect on proliferation induced by AMCs in CD4 T cells (data not shown). This strongly suggested that production of NO did not inhibit proliferation in our cultures and was consistent with our observations that suppression was antigen specific.

The reduced T-cell proliferation induced by AMCs was reversed by the addition of IL-2 to the cultures (Fig 3, A), indicating that the effect was mediated in part by inhibition of or failure to stimulate T-cell IL-2 production. Addition of IL-2 has been shown previously to reverse T-cell clonal anergy²³ and restore proliferation in our cultures to levels observed with PBMs and CD4 T cells. Moreover, CD4 T cells cultured with AMCs plus IL-2 proliferated vigorously when restimulated with PBMs from the donor of the AMCs, indicating that unresponsiveness was prevented by the presence of IL-2 (data not shown). Production of IL-2 in T cells is induced by costimulation through CD28, and the addition of anti-CD28 mAb to cultures of AMCs and CD4 T cells restored proliferation to the levels observed in the T-cell/PBM cultures (Fig 3, B). Because AMCs express very low levels of B7-2 (CD86) on their surface to trigger CD28,⁴ these results strongly suggest that AMCs provide inadequate costimulatory signals due to deficient expression of B7-2. Furthermore, CD4 T cells cultured with AMCs plus anti-CD28 mAb proliferated vigorously when restimulated with PBMs from the donor of the AMCs and produced high levels of IFN- γ , indicating that when adequate costimulation was provided, unresponsiveness was abrogated (data not shown). These observations indicate that AMCs induce in T cells a form of functional inactivation that is reversed by CD28 costimulation or IL-2.

DISCUSSION

The mechanisms that prevent and limit immune responses against nonreplicating antigens inhaled into the nonatopic lung are poorly understood. Our results indicate that AMCs, the most abundant phagocytic cell in the lung, participate in this inhibitory process by down-regulating T-cell responses in the pulmonary environment. Thus AMCs induced antigen-specific unresponsiveness in highly purified CD4 T cells in a manner that was independent of soluble suppressive factors such as TGF- β , IL-10, or NO. T cells stimulated by AMCs produced only small amounts of cytokines (IFN- γ , IL-4, and IL-10) and were thus anergic or functionally inactivated. The process was active and selective in that only antigen-specific T cells did not respond on secondary stimulation. The failure of AMCs to induce T-cell proliferation

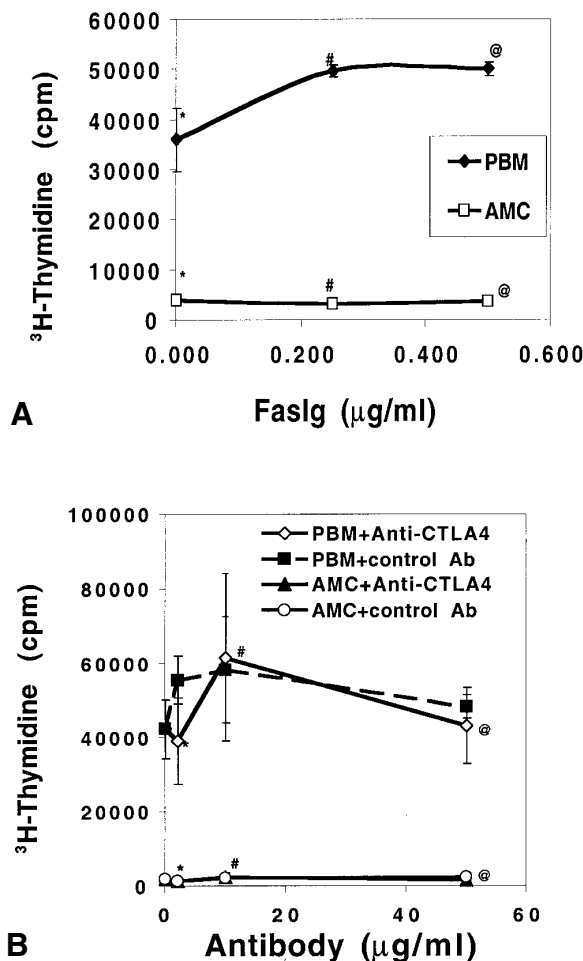


FIG 2. A, The reduced capacity of AMCs to induce CD4 T-cell proliferation is not due to T-cell apoptosis. Purified CD4 T cells (1×10^5 cells/well) were cultured with allogeneic PBMs (0.5×10^5 cells/well) or with allogeneic AMCs (0.5×10^5 cells/well) with or without Fas lg. Results are given as cpm of ^{3} H-thymidine $\pm$ SD incorporated during the last 18 hours of culture. * $P < .01$, # $P < .002$, @ $P < .004$. Results are representative of 4 separate experiments. **B,** The reduced capacity of AMCs to induce CD4 T-cell proliferation is not through CTLA-4-induced suppression. Purified CD4 T cells (1×10^5 cells/well) were cultured with allogeneic PBMs (0.5×10^5 cells/well) or with allogeneic AMCs (0.5×10^5 cells/well) and with or without an inhibitory anti-CTLA-4 mAb. Results are given as cpm of ^{3} H-thymidine $\pm$ SD incorporated during the last 18 hours of culture. * $P < .03$, # $P < .05$, @ $P < .02$. Results are representative of 3 separate experiments.

did not involve Fas-mediated apoptosis or inhibitory CTLA-4-associated pathways. Furthermore, the unresponsiveness induced in CD4 T cells by AMCs was rescued by the addition of recombinant IL-2 and by the addition of anti-CD28 mAb, indicating that unresponsiveness developed because AMCs provided inadequate T-cell costimulatory signals, presumably due to an inability to upregulate expression of B7-2.⁴

The role of AMCs in regulating immune responses appears to be distinct from that of pulmonary dendritic cells, which have been identified in lung parenchymal tissue and pulmonary lymph nodes and which rapidly

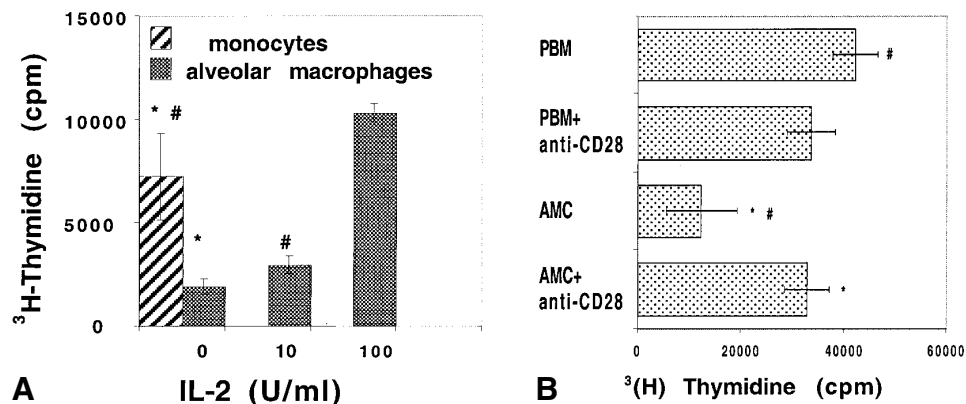


FIG 3. A, AMCs induce anergy in allogeneic CD4 T cells. AMCs (0.5×10^5 cells/well) or PBMs (0.5×10^5 cells/well) were used to stimulate allogeneic CD4 T cells (1×10^5 cells/well) in complete medium for 6 days. Human IL-2 ranging from 10 to 100 U/mL was added to the cultures at day 0. Cells were pulsed with ^3H -thymidine during the last 18 hours of the culture period. * $P < .01$, # $P < .03$. Results are representative of 2 separate experiments. **B,** Anti-CD28 mAb reverses functional inactivation of T cells by AMCs. AMCs (0.5×10^5 cells/well) or PBMs (0.5×10^5 cells/well) were used to stimulate autologous CD4 T cells (1×10^5 cells/well) in complete medium for 6 days. Anti-CD3 mAb (10 ng/mL), which cross-links the T-cell receptor and mimics antigen stimulation, was used instead of allogeneic APCs because anti-CD3 mAb binds all T cells, as does anti-CD28 mAb. Anti-CD28 mAb (1 $\mu\text{g/mL}$) was added to some cultures at day 0 as indicated. Anti-CD3 mAb stimulation alone or anti-CD28 mAb stimulation alone induced limited responses (<1000 cpm). Cells were pulsed with ^3H -thymidine during the last 18 hours of the culture period. * $P < .02$, # $P < .002$. Results are representative of 3 separate experiments.

increase in number in airway mucosa in response to inflammatory signals (eg, produced by bacteria and viruses).^{1,8} In response to such replicating pathogens, or in the setting of allergic inflammation, dendritic cells contribute to adaptive immunity by phagocytizing antigen, migrating to lymphoid organs, and potentially inducing systemic immunity, in processes that require high expression of B7 and CD40 molecules.^{24,25} Thus the pulmonary network of B7⁺ dendritic cells plays an important role in presenting antigen and inducing systemic immunity or systemic unresponsiveness to inhaled pathogens or proteins.^{26,27}

In contrast to dendritic cells, AMCs appear to be involved primarily in downmodulating local but not systemic immune responses. AMCs do not migrate to regional lymphoid organs but instead function in an environment restricted to the conducting airways and alveoli of the lung. Therefore AMCs function solely in the pulmonary compartment by inhibiting antigen-specific T-cell responses, and they do so even in the face of systemic sensitization to the antigen. Thus AMCs may function to limit inflammation in the pulmonary compartment, despite systemic allergen sensitization that may cause inflammatory responses and symptoms in other organ systems. This may explain the observation that some individuals with systemic sensitization to specific allergens (characterized by circulating allergen-specific IgE) experience allergic inflammatory symptoms confined to the nose (allergic rhinitis) but are protected from the development of pulmonary symptoms (asthma). This regulatory function of AMCs appears to be absent in patients with

asthma and sarcoidosis^{28,29} and can be overwhelmed or bypassed by, for example, inflammation generated by bacterial or viral infection, which recruits and activates pulmonary dendritic cells. In such individuals, AMCs express elevated levels of B7-2 and are capable of inducing vigorous T-cell proliferative responses.^{28,30}

The distinct functions of AMCs and dendritic cells are reflected in their differences in expression of B7 molecules. Whereas dendritic cells express high levels of B7-2, AMCs express low levels of B7-2, and expression cannot be enhanced by stimulation with IFN- γ .⁴ The induction of systemic sensitization by pulmonary dendritic cells after respiratory exposure to antigen requires the expression of high levels of B7 molecules, and sensitization is inhibited by treatment with anti-B7-2 mAb.³¹ In contrast, the reduced expression of B7-2 by AMCs from normal individuals results in the induction of T-cell tolerance. This is very consistent with the well-established idea that activation of CD4 T cells requires 2 signals—T-cell receptor cross-linking and a costimulatory signal provided by the ligation by B7 molecules of CD28^{32,33}—and that T-cell receptor cross-linking without adequate costimulation is associated with the induction of T-cell unresponsiveness.^{34,35}

Our observation that unresponsiveness is induced by AMCs is the first example of a normal bone marrow-derived cell whose primary role is to induce tolerance through reduced T-cell costimulation. This process may be similar to unresponsiveness induced when B cells, immature dendritic cells, or tumor cells that express low levels of B7 molecules present antigen and induce toler-

ance.³⁶⁻³⁸ However, AMCs may be unique in that their primary purpose as APCs is the prevention of T-cell expansion and in that, because they remain confined to the pulmonary compartment, they affect only T cells that enter the lung and not T cells in lymphoid organs.

The compartmentalization in the induction of unresponsiveness by AMCs has similarities to immune privilege of the eye and testis. In the eye, tolerance is mediated in part by the production in the ocular microenvironment of high levels of TGF- β ,³⁹ whereas in the testis, immune privilege is mediated by constitutive expression of FasL.⁴⁰ In the lung, we demonstrated that neither of these mechanisms plays a significant role. Instead, immune privilege and reduction in lung inflammation are mediated by AMCs, which functionally incapacitate the T cell because of reduced expression of B7-2. We thus propose that human AMCs mediate a form of immune privilege that is specific for the lung.

Previous studies of AMCs in rodents suggested that AMCs inhibited CD4 T-cell proliferation through several mechanisms, including release of NO or PGE₂^{41,42} by murine AMCs, which inhibited T-cell activation in a noncognate fashion,^{6,21,43,44} and by inhibition of IL-2 signal transduction in T cells.²² We did not find this with human AMCs because human AMCs did not suppress bystander T cells, nor did addition of IL-2 reverse the inhibition of T cells by AMCs, and inhibition of NO synthase did not reverse the inability of AMCs to present antigen to T cells.

In summary, our studies demonstrate a specific role for, and the use of specific mechanisms by, AMCs in regulating pulmonary T-cell inflammatory responses. AMCs induced antigen-specific unresponsiveness in CD4 T cells, characterized by functional inactivation, due to antigen recognition in the absence of costimulation. The regulatory effects of AMCs are confined to T cells that enter the lung, in contrast to the effects of pulmonary dendritic cells or PBMs, which are involved in both antigen sensitization and tolerance induction, and which affect T cells in the entire immune system. Thus we propose that AMCs mediate a form of immune privilege that limits inflammation only in the pulmonary compartment.

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