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Research Article

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Human Alveolar Macrophages Present Antigen Ineffectively due to Defective Expression of B7 Costimulatory Cell Surface Molecules

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Abstract

Alveolar macrophages, resident phagocytic cells in the lung that derive from peripheral blood monocytes, are paradoxically ineffective in presenting antigen to T cells. We found that antigen presentation by alveolar macrophages could be restored by the addition of anti-CD28 mAb to cultures of T cells and macrophages, indicating that costimulation by alveolar macrophages via the CD28 pathway was defective. In addition, we found that alveolar macrophages activated with IFN-γ failed to express B7-1 or B7-2 antigens, which normally ligate CD28 on T cells and provide a costimulatory signal required for the activation of T cells. These observations are the first to demonstrate the inability of a "professional" antigen-presenting cell type to effectively express the costimulatory molecules B7-1 and B7-2. Inasmuch as immune reactions within the lung are inevitably associated with inflammatory injury to pulmonary tissue, these observations suggest that reduced expression of B7-1 and B7-2 by alveolar macrophages may be advantageous, as a critical mechanism involved in the induction of peripheral tolerance to the abundance of antigens to which mucosal tissues are continuously exposed. (*J. Clin. Invest.* 1995. 95:1415–1421.)
Key words: alveolar macrophages • B7 • antigen presentation • CD28 • anergy

Introduction

Alveolar macrophages, resident phagocytic cells in the lung that derive from peripheral blood monocytes (1), are paradoxically ineffective in presenting antigen to T cells. Because the lung is a gas exchange organ, it is exposed to large quantities of contaminants and antigens suspended in the air, yet, despite expressing normal levels of class II MHC antigens (2, 3) and having normal bactericidal activity, alveolar macrophages have been shown to be defective in activating resting T lymphocytes and initiating inflammatory responses (3–6).

The purpose of these studies was to determine the reason for the failure of alveolar macrophages to effectively activate $CD4^+$ T cells. Activation of $CD4^+$ T cells requires at least two signaling events: one triggered by the ligation of the T cell receptor complex by antigen peptide/class II MHC on the antigen-presenting cells (APC);[1] the other signaling event generated by the ligation of costimulatory molecules on T cells (e.g., CD28) by counterreceptors (e.g., B7-2) on APC such as macrophages, B cells, and dendritic cells (7–11). We found that human alveolar macrophages were deficient at presenting the antigen tetanus toxoid (TT) to autologous $CD4^+$ T cells and were deficient at activating $CD4^+$ T cells with anti-CD3 mAb. Antigen presentation by alveolar macrophages in both cases (with TT and anti-CD3 mAb) was restored by the addition of anti-CD28 mAb, indicating that costimulation by alveolar macrophages via the CD28 pathway was defective. Moreover, we found that alveolar macrophages activated with IFN-γ failed to express B7-1 or B7-2 antigens, which normally ligate CD28 on T cells and provide a costimulatory signal required for the activation of T cells (9, 12, 13). These results indicated that the inability of alveolar macrophages to present antigen effectively to T cells was due to the failure of alveolar macrophages to express sufficient quantities of the B7-1 and B7-2 antigens which resulted in a failure to provide effective costimulatory signaling to T cells.

These observations were the first to demonstrate the failure of a "professional" APC type to effectively express the costimulatory molecules B7-1 and B7-2 and were thus unexpected. However, inasmuch as immune reactions within the lung are inevitably associated with inflammatory injury to pulmonary tissue, our observations indicate that reduced expression of B7-1 and B7-2 by APC in the lung may be advantageous, as a critical mechanism that limits immune responses in the lung and in mucosal tissues which are continuously exposed to abundant quantities of antigen.

Methods

Study population. 15 healthy adult volunteers (7 male, 8 female) were selected, all without a history of smoking, asthma, or other respiratory disorders. The median age of study population was 26 yr (range, 22–

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1. *Abbreviations used in this paper:* APC, antigen-presenting cell; BAL, bronchoalveolar lavage; TT, tetanus toxoid.

38). Experimental protocols were approved by the Stanford University Administrative Panel on Human Subjects in Medical Research, and all subjects gave written informed consent.

Antigens, mAbs. TT was obtained from Connaught Laboratories (Swiftwater, PA) and was used in culture at 10 μg/ml. The anti-CD3 hybridoma OKT3 was obtained from American Type Culture Collection (Rockville, MD), and mAb purified from culture supernatants was used in culture at 50 ng/ml. Anti-CD28 ascites fluid (obtained from Dr. Jeff Ledbetter, Bristol Myers/Squibb, Seattle, WA) was used in culture at ~ 1 μg/ml. Ascites fluid of the anti-IL-10 hybridoma, JES3.19F, obtained from ATCC with permission from Dr. John Abrams (DNAX Institute, Palo Alto, CA), was used in culture at 5 μg/ml. Control rat IgG1 (Binding Site, Birmingham, United Kingdom) was used at 5 μg/ml. FACS® analysis was performed using the 133 mAb (an IgM anti-B7-1 mAb) (7) as ascites fluid at a 1:500 dilution. FACS® staining with anti-B7-2 (PharMingen, San Diego, CA), L243 (anti-class II MHC), control IgM mAb (HB130, anti-murine CD8), and control IgG mAb (HB129, anti-murine CD8) was performed at 10 μg/ml, using a fluorescinated anti-mouse Ig second antibody (Jackson ImmunoResearch Labs, Inc., West Grove, PA).

Cytokines. GM-CSF (Genzyme Corp., Cambridge, MA) was used in culture at 20 ng/ml. IFN-γ (Genentech Inc., South San Francisco, CA) was used at 500–1,000 U/ml. IL-12 was generously provided by Stanley Wolf (Genetics Institute, Boston, MA) and used at 50 U/ml.

Bronchoalveolar lavage (BAL). Subjects were admitted to the General Clinical Research Center at Stanford University Hospital and underwent flexible fiberoptic bronchoscopy and BAL under controlled conditions based on the recommendation of the NHLBI Workshop on the Investigative Use of Fiberoptic Bronchoscopy and Bronchoalveolar Lavage in Asthmatics (14). A flexible bronchoscope was passed into the bronchus of a segment of the right middle lobe, and 250 ml of warmed saline was instilled in aliquots into the lung and recovered by gentle aspiration through the bronchoscope. The BAL fluid was chilled on ice until processing, at which time the BAL fluid was pooled and centrifuged at 200 *g* (1,000 rpm) for 10 min. The cell pellet was washed once with PBS and resuspended in complete medium. The cells were then layered over Percoll (45%) (Pharmacia, Alameda, CA) to remove contaminating lymphocytes and red cells, and centrifuged for 45 min at 800 *g* at 4°C. The cells at the interface were collected, washed twice in PBS, and resuspended to a concentration of 1×10^6 cells/ml in complete media. This method resulted in a recovery of 80–85% of cells in the BAL fluid, and the resulting cells were > 95% large granular macrophages.

Mononuclear cell isolation. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by flotation over Ficoll-Hypaque (Sigma Immunochemicals, St. Louis, MO). Cells were washed three times in PBS and then resuspended in Dulbecco's modified Eagle's medium (Hyclone Labs, Logan, UT), supplemented with 2 mM L-glutamine, essential and nonessential amino acids, essential vitamins, 1 mM sodium pyruvate, gentamycin (5 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% AB^+ human serum (Sigma Immunochemicals).

Preparation of monocyte containing population. APCs were prepared from peripheral blood by incubating fresh PBMC ($5–10 \times 10^6$ cells/ml) in 60-mm plastic tissue culture dishes in complete medium for 2 h at 37°C in 10% CO_2. Nonadherent cells were removed by gently washing three times with warm RPMI 1630 (GIBCO BRL, Grand Island, NY) containing 5% FCS and used as a source of T cells. The remaining adherent cells (peripheral blood monocytes with small numbers of dendritic cells) were then harvested by washing with iced PBS three times and resuspended in complete medium at a concentration of 1×10^6 cells/ml.

Preparation of CD8-depleted T cells. Nonadherent cells were collected and resuspended in RPMI 1630 containing 10% human AB^+ serum, then incubated on nylon wool columns for 1 h at 37°C. The T cells were collected by eluting the column with 2 vol of RPMI 1630 plus 10% serum. T cells were further purified by the addition of OKT8 mAb (anti-CD8) and L243 mAb (anti-class II MHC), incubation for 45 min on ice, followed by the addition of rabbit complement (Pel-Freez Biologicals, Rogers, AR) at a dilution (1:3) previously found to be optimal. After an additional 30-min incubation at 37°C, cells were washed in RPMI 1630 plus 5% FCS and used in complete medium at a concentration of 2×10^6 cells/ml.

Cell culture/proliferation assay. Alveolar macrophages (5×10^4/well) and/or peripheral blood monocytes (5×10^4/well) were cultured with the purified $CD8^-$ T cells (1×10^5/well) in triplicate in 96-well flat-bottom culture plates with TT (10 μg/ml) and/or cytokines. After 5 d, cultures were pulsed with [^{3}H]thymidine (1 μCi/well) and harvested 18 h later. Incorporation of [^{3}H]thymidine was determined by standard liquid scintillation techniques.

Table I. Alveolar Macrophages Are Ineffective at Presenting Antigen to Autologous $CD4^+$ T Cells

Subject	APC type		
	None	Alveolar macrophage	Peripheral blood monocyte
	cpm	*cpm*	*cpm*
1	312	2,788	10,678
2	455	1,426	8,286
3	225	10,728	38,635
4	627	23,062	70,658
6	3,517	6,738	42,741
7	586	26,793	116,027
8	293	1,233	41,564
9	137	688	8,848
10	1,423	7,265	46,836
11	943	8,364	48,366
12	725	11,245	41,616
Mean	840±290	9,121±2,622	43,114±9,332

Purified $CD4^+$ T cells were cultured with TT (10 μg/ml) and the indicated APC type. The proliferative response was determined by the incorporation of [^{3}H]thymidine over the last 18 h of a 6-d culture. The means are expressed as cpm±SE. The mean of the response induced by alveolar macrophages was significantly different from the mean of the response induced by peripheral blood monocytes, with $P < 0.0008$, using a standard two-tailed paired *t* test.

Results

Antigen presentation by alveolar macrophages is defective. We assessed the ability of alveolar macrophages to present antigen to autologous purified peripheral blood $CD4^+$ T cells. Alveolar macrophages were isolated by bronchoalveolar lavage from the lungs of TT-immune individuals (subjects were boosted with TT within the past 5 yr), and were cultured with purified autologous $CD4^+$ T cells and TT for 6 d. The proliferative response of the T cells was quantitated by examining the incorporation of [^{3}H]thymidine over the last 18 h of culture. Table I shows that the magnitude of the proliferation induced in autologous T cells by alveolar macrophages was only ~ 20% of that induced by an equal number of peripheral blood adherent cells. This indicated that alveolar macrophages were much less effective at inducing antigen-specific proliferation in autologous $CD4^+$ T cells than circulating PBMC from which alveolar macrophages derive (1). The decreased effectiveness of the macrophages at inducing T cell proliferation could not be improved by changing

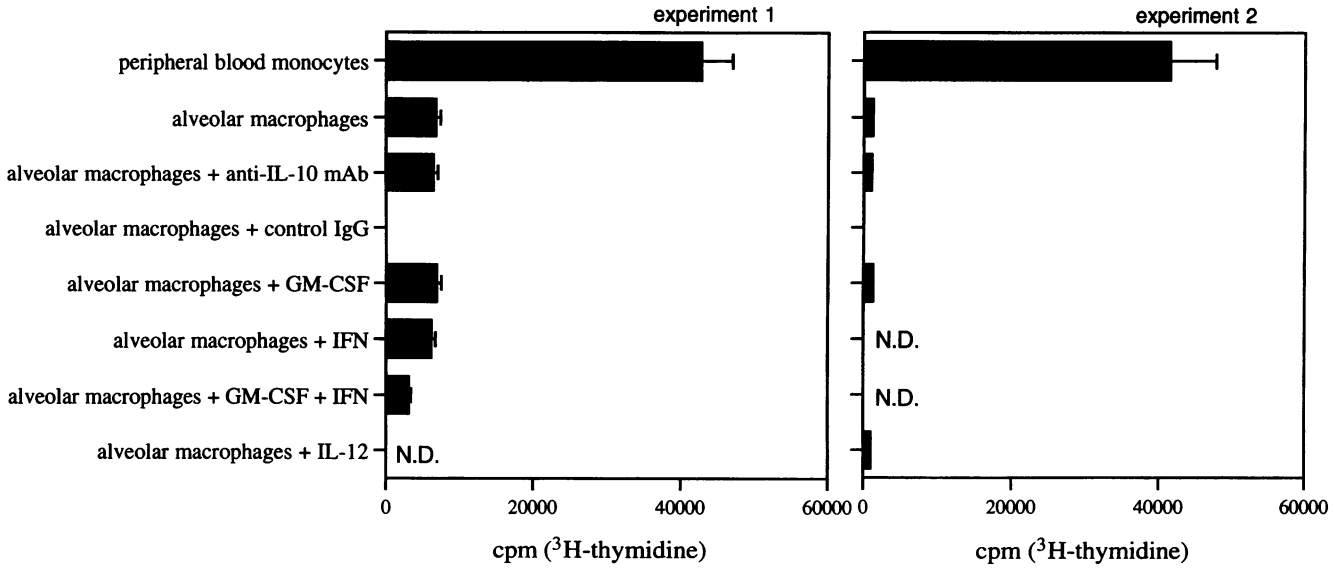

Figure 1. GM-CSF, IFN-γ, IL-12, or anti–IL-10 mAb does not restore the capacity of alveolar macrophages to present antigen to $CD4^+$ T cells. Purified $CD4^+$ T cells (1×10^5/well) were cultured with peripheral blood monocytes (5×10^4/well) or alveolar macrophages (5×10^4/well) in triplicate with TT (10 μg/ml). GM-CSF (20 ng/ml), IFN-γ (1,000 U/ml), IL-12 (50 U/ml), or anti–IL-10 mAb (5 μg/ml) was added to the cultures. Cultures were pulsed with [^{3}H]thymidine on day 5 and harvested on day 6 of culture. Results are representative of four experiments. The T cells plus TT control was 3,517 cpm for experiment 1 and 293 cpm for experiment 2. *N.D.*, not done.

the number of macrophages per culture (1×10^4, 2×10^4, 5×10^4, or 1×10^5 APC/culture), and cultures with higher antigen concentrations had a slightly reduced proliferative response. When alveolar macrophages were mixed with peripheral blood monocytes, alveolar macrophages appeared to inhibit the T cell proliferative response induced with peripheral blood monocytes, but the inhibition was slight and not reproducible.

Anti–IL-10 and IL-12 have no effect on antigen presentation by alveolar macrophages. Since macrophages have been shown to produce IL-10 and since this cytokine has been shown to be inhibitory for T cell proliferative responses (15, 16), we asked if the addition of anti–IL-10 mAb to the cultures might enhance the proliferative response induced by alveolar macrophages. Fig. 1 shows that the addition of anti–IL-10 mAb had no effect on the T cell proliferative response induced by alveolar macrophages. Furthermore, addition of other cytokines such as IFN-γ or GM-CSF or of IL-12, which in other systems have been shown to have beneficial effects on T cell proliferation (17–19), also had no significant effect on the capacity of alveolar macrophages to induce T cell proliferation. Addition of IL-4 to the cultures or pretreatment of alveolar macrophages for 24 h with GM-CSF before culture also had no effect on T cell proliferation induced by alveolar macrophages (data not shown). These results suggest that inadequate amounts of GM-CSF or IL-12 were not directly involved in limiting the ability of alveolar macrophages to activate T cells.

Antigen presentation by alveolar macrophages is greatly enhanced by the addition of anti-CD28 mAb. Inasmuch as cross-linking of the CD28 antigen on T cells has been shown to provide a potent costimulatory signal, we hypothesized that alveolar macrophages might be deficient in their capacity to cross-link CD28 on T cells. Anti-CD28 mAb was therefore added to cultures of alveolar macrophages and purified autologous T cells to provide help in cross-linking CD28 and stimulating T cell proliferation. Fig. 2 shows that alveolar macrophages were significantly less effective than peripheral blood monocytes/adherent cells in presenting TT to autologous T cells, but that in the presence of anti-CD28, alveolar macrophages became significantly more competent at inducing TT-specific proliferation. Addition of anti-CD28 mAb alone in the absence of antigen did not induce T cell proliferation. Similar results were obtained using soluble anti-CD3 mAb instead of TT to cross-link the T cell receptor/CD3 complex, in that alveolar macrophages, which can display anti-CD3 mAb via cell surface Fcγ receptors, were ineffective at inducing anti-CD3 mAb mediated T cell proliferation. However, when anti-CD28 mAb was added to the cultures with soluble anti-CD3 mAb, the alveolar macrophages induced T cell proliferation that was equal in magnitude to that observed in response to anti-CD3 mAb with peripheral blood monocytes/adherent cells as APC (Fig. 2). These results indicate that alveolar macrophages are ineffective at activating T cells because they cannot provide the CD28 costimulatory signal to T cells.

Alveolar macrophages are defective in their expression of the costimulator molecules B7-1 and B7-2. To explain the lack of CD28 costimulatory ability of alveolar macrophages, we directly examined the expression by alveolar macrophages of the cell surface molecules B7-2 and B7-1, which bind to CD28 on T cells. Fig. 3 shows that alveolar macrophages activated by overnight incubation in IFN-γ did not express B7-2, although B7-2 was expressed at low levels on resting alveolar macrophages from 50% of subjects (absent on macrophages from the other 50% of subjects), when stained immediately after isolation. In contrast, peripheral blood monocytes activated with IFN-γ expressed substantially increased quantities of B7-2, above the significant levels already expressed on resting monocytes. Expression of B7-1 on alveolar macrophages was also anomalous compared with that on peripheral blood monocytes, in that resting alveolar macrophages expressed little or no B7-1 (very low levels of B7-1 were present on alveolar macro-

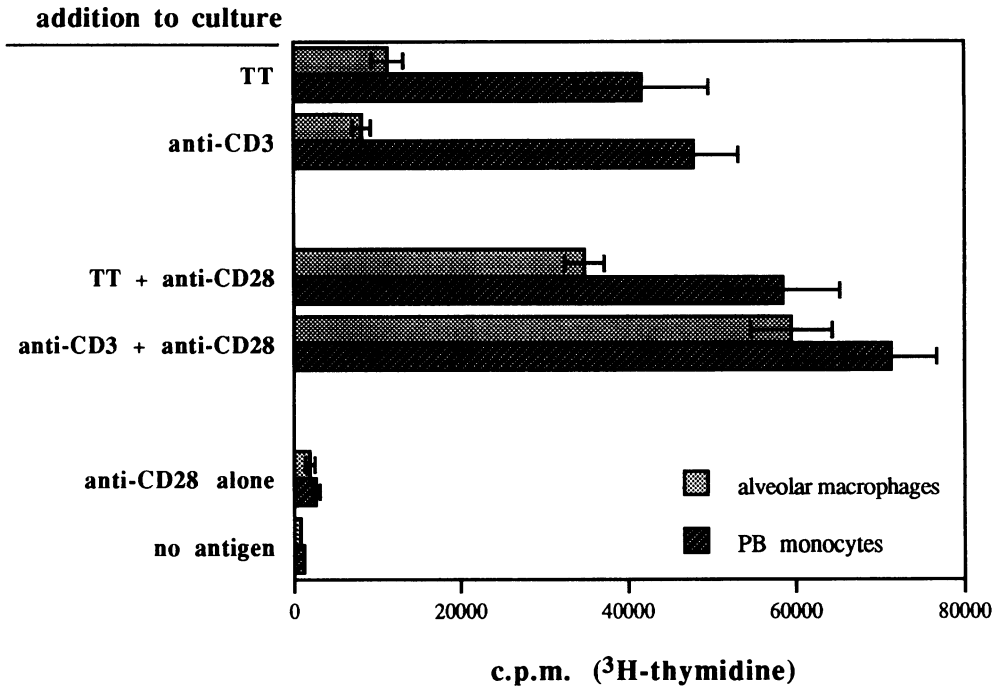

Figure 2. Anti-CD28 mAb restores the ability of alveolar macrophages to present antigen. $CD4^+$ T cells were cultured as described in Fig. 1, with TT (10 μg/ml) or with anti-CD3 mAb (50 ng/ml). Anti-CD28 mAb (1 μg/ml) was added to the indicated cultures. Results are representative of two experiments.

phages from 50% of subjects), but any such expression was abolished after incubation of the macrophages with IFN-γ (Fig. 3) or by culture with T cells and antigen (data not shown). In contrast, little or no B7-1 was expressed by resting peripheral blood monocytes (very low levels of B7-1 were present on monocytes from 50% of subjects), but expression was significantly enhanced after incubation of the monocytes with IFN-γ (Fig. 3) (8) and by culture with T cells and antigen (data not shown).

Because IL-10 inhibits expression of B7-1 and B7-2 in monocytes (20, 21), and because alveolar macrophages may produce significant quantities of IL-10, we cultured alveolar macrophages in the presence of anti-IL-10 mAb and IFN-γ. After overnight culture, expression of B7-1 and B7-2 on alveolar macrophages was still absent (data not shown), consistent with our observations that anti-IL-10 mAb had minimal effect on the capacity of the macrophages to stimulate T cell proliferation (Fig. 1), and indicating that B7-1 and B7-2 expression was not directly inhibited by elevated production of IL-10 in our cultures.

Discussion

The mechanisms that limit the capacity of alveolar macrophages to activate T cells are poorly understood, but could be due to the failure of alveolar macrophages to provide sufficient activation signals to T cells. Activation of $CD4^+$ T cells, evidenced by proliferation and cytokine production, requires at least two signaling events: one triggered by the ligation and cross-linking of the T cell receptor complex by antigen peptide/class II MHC on the APC; the other signaling event generated by the ligation and cross-linking of T cell costimulatory molecules, e.g., CD28, by counterreceptors (e.g., B7-1 and B7-2) on APC such as macrophages, B cells, and dendritic cells (7, 8, 11, 22). The ligation of the CD28 antigen on T cells provides an extremely important signal that upregulates the synthesis of IL-2 and IFN-γ in T cells (23–25) and prevents the induction of T cell anergy or tolerance (12, 13, 26–28).

In our studies, we examined the failure of alveolar macrophages to activate T cells and determined the role of B7-1 and B7-2 on alveolar macrophages in this failure. We found that the inability of alveolar macrophages to activate T cells was not due to a failure to process antigen, since alveolar macrophages could induce a TT-specific proliferative response when a costimulatory signal was provided by anti-CD28 mAb. Furthermore, the inability of alveolar macrophages to activate T cells was not due to a failure to process antigen, because alveolar macrophages could not activate T cells via anti-CD3 mAb, which directly ligates the T cell receptor/CD3 complex and is independent of antigen processing. Inasmuch as the failure of alveolar macrophages to activate T cells was reversed by the addition of anti-CD28 mAb, and since activated alveolar macrophages failed to express B7-1 and B7-2 antigens, we suggest that the sole reason for the failure of alveolar macrophages to activate T cells is their inability to provide costimulation via the CD28 pathway.

The mechanism by which alveolar macrophages, long-lived phagocytic cells that derive from peripheral blood monocytes (1), paradoxically limit their expression of B7-1 and B7-2 is not clear. IL-10 has been shown previously to downregulate B7-1 expression on macrophages (20, 21). Therefore the production of IL-10 in the lung might play a role in downregulating B7 expression on alveolar macrophages, and in downregulating inflammation, as it does in other tissues, particularly in the intestinal tract, where recent studies indicate that ablation of IL-10 expression results in severe inflammatory bowel disease (29). In our experiments the addition of anti-IL-10 mAb to cultures of alveolar macrophages for > 24 h did not enhance the expression of B7-1 and B7-2 and did not enhance antigen presentation by alveolar macrophages. It is possible, however, that anti-IL-10 mAb had no effect in vitro because a potent IL-10 signal occurred in vivo, or that more prolonged in vitro

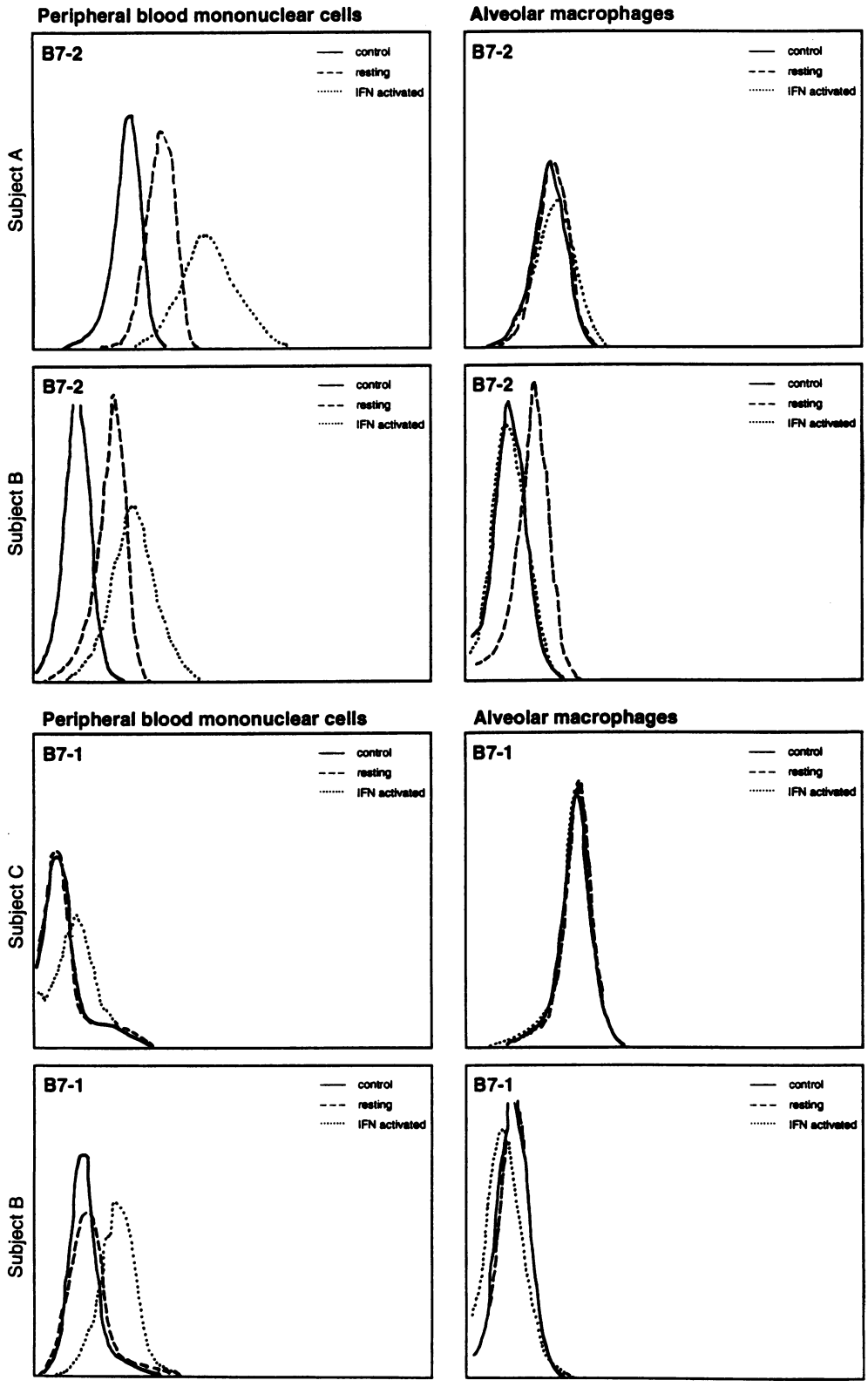

Figure 3. Abnormal expression of B7-1 and B7-2 on alveolar macrophages. FACS® analysis of peripheral blood monocytes (*left panels*) or alveolar macrophages (*right panels*), stained for B7-2 or B7-1. Each horizontal pair of panels represents the results with cells from an individual subject. Results are representative of seven experiments.

culture of alveolar macrophages in the presence of anti–IL-10 mAb is required for the appearance of B7 antigens on alveolar macrophages.

We observed that resting alveolar macrophages from some individuals expressed low levels of B7-1 or B7-2 (Fig. 3). The limited expression of B7-1 or B7-2 by resting alveolar macrophages may permit the induction of limited T cell proliferation (~ 20% of that observed with peripheral blood monocytes, Table I) and may permit alveolar macrophages to induce proliferation in already activated T cells and in T cell clones

(3, 5). However, in our studies, expression of B7-1 or B7-2 by resting macrophages was ablated by activation with IFN-γ, which is in direct contrast with our results with peripheral blood monocytes, which expressed increased quantities of B7-1 and B7-2 when activated (7, 9, 10). This is consistent with our observations that IFN-γ, which greatly increased class II MHC expression on PBMC, did not increase class II MHC expression on alveolar macrophages (data not shown). Thus taken together, these observations indicate that B7-1 and B7-2 expression is regulated differently in alveolar macrophages than in peripheral blood monocytes and suggest that the role of alveolar macrophages in immune responses is distinct from that of other types of APC, i.e., alveolar macrophages may be involved in the induction of peripheral tolerance rather than in the activation of T cells.

In the lungs and in the intestinal tract, antigen exposure has been shown previously to cause immunological tolerance (26, 30–32). Although these mucosal surfaces are continuously exposed to abundant quantities of antigen, immune responsiveness must be restricted or downregulated at these sites since inflammatory reactions inevitably produce significant damage to the mucosal surface. The mechanisms responsible for the induction of oral/mucosal tolerance have not been fully elucidated, but are thought to involve the generation of active suppression or the induction of clonal anergy (33–38). Although it is thought that antigen dose can modulate oral tolerance (33), in view of the fact that antigen presentation in vitro in the absence of B7 costimulation results in T cell unresponsiveness or tolerance (12, 13, 26–28), we suggest that the principal mechanism that regulates the induction of peripheral tolerance in vivo (in lung and bronchial-associated lymphoid tissue or in gut-associated lymphoid tissue [30, 39]) is the restriction of B7-1 and B7-2 expression on APC. Moreover, we suggest that alveolar macrophages, which have reduced expression of B7-1 and B7-2, play a major role in downregulating immune responses in the lung and in inducing immunological tolerance to inhaled antigens. In this regard, preliminary studies in our laboratory indicate that alveolar macrophages can indeed induce antigen-specific tolerance in peripheral blood T cells (Hwang, P. T., et al., manuscript in preparation).

Since alveolar macrophages are limited in their capacity to initiate inflammatory responses, how are antigen-specific immune responses elicited in the lung? This is not necessarily a problem, since dendritic cells, which are present in the peribronchial connective tissues (but not on the epithelial surface in the lung nor in bronchial alveolar lavage fluid [40]), as well as neutrophils, can be recruited into the lung quite readily (41). However, the balance between activation of immune responses by dendritic cells and the inhibition of inflammatory responses by alveolar macrophages is precarious: overly zealous inhibition may result in overwhelming infection, whereas insufficient inhibition may result in allergic or inflammatory disease. Nonetheless, alveolar macrophages appear well suited to defend the lung, since they are very efficient at phagocytizing antigen and microorganisms but at the same time are ineffective at activating T cells and initiating inflammatory responses.

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