Selective inhibition of T cell proliferation but not expression of effector function by human alveolar macrophages

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Abstract

Background – Alveolar macrophages are thought to play an important part in regulating lung immune responses. While it is clear that human alveolar macrophages suppress T cell proliferation *in vitro*, the mechanisms by which this is achieved are not clear, nor is it known whether alveolar macrophages also inhibit other aspects of T cell function.

Methods – Peripheral blood mononuclear cells were stimulated with phytohaemagglutinin or house dust mite allergen, and cultured with variable numbers of autologous alveolar macrophages obtained by bronchoalveolar lavage from 20 normal subjects.

Results – Alveolar macrophages induced a reversible inhibition of T cell proliferation in response to both mitogen and allergen stimulation, with the latter being considerably more susceptible to inhibition. This was achieved via heterogeneous mechanisms, involving both soluble factors derived from alveolar macrophages and cell-cell contact. Despite inhibiting proliferation, alveolar macrophages had little or no effect on T cell calcium flux, the characteristic changes in CD3, CD2, CD28 and interleukin-2 (IL-2) receptor expression which accompany normal T cell activation, and IL-2 and interferon gamma secretion. In contrast, alveolar macrophages inhibited the tyrosine phosphorylation of proteins which may be involved in IL-2 receptor-associated signal transduction.

Conclusions – The immunoregulatory properties of alveolar macrophages are relatively selective, allowing T cell activation and cytokine secretion while inhibiting T cell proliferation within the lung.

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sponses dependent upon memory T cells of the Th2 phenotype.⁸

While significant progress has been made recently in elaborating the molecular mechanism(s) by which alveolar macrophages regulate T cell function in experimental animals, in particular the importance of the inducible form of nitric oxide (NO) synthase,¹⁰ the corresponding mechanisms in humans remain unclear. Whereas some recent reports suggest that alveolar macrophage-mediated T cell suppression in humans is dependent upon cell-cell contact and is mediated by a component of the alveolar macrophage cell membrane,^{6,11} earlier investigators have proposed that, analogous to the situation observed in experimental animals,¹ human alveolar macrophages inhibit T cell proliferation via secreted factors.³

A second unresolved issue concerns the extent to which human alveolar macrophages are able to suppress other aspects of T cell activation, aside from proliferation. While it has been reported that alveolar macrophages also inhibit T cell activation and cytokine secretion,^{6,11} this is at odds with current knowledge of lung T cells *in vivo*. In contrast to the peripheral blood lymphocyte pool, the normal lung T cell population is dominated by post-activated memory T cells^{12,13} in which cytokine mRNA transcripts are detectable in the absence of exogenous stimulation.^{14,15}

To elucidate further the mechanisms by which human alveolar macrophages suppress T cell proliferation, we have compared mitogen and allergen stimulated T cells and have shown that alveolar macrophages have the capacity to suppress their proliferation by more than one mechanism. Moreover, examination of these "suppressed" T cells indicated that the regulatory properties of alveolar macrophages are selective – while T cell proliferation is inhibited, many early events in the T cell activation process, as well as key effector functions such as cytokine production, proceed relatively unhindered.

Methods

The study population comprised 20 healthy, non-smoking individuals with a mean (SD) age of 37 (11) years. None had experienced symptoms of a respiratory tract infection within the last month, and in all instances spirometric values were normal. House dust mite specific IgE was detectable by RAST in seven subjects. The study was approved by the institutional ethics committee and written informed consent was obtained from each subject.

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Alveolar macrophages are considered to play an important part in regulating T cell responses to inhaled antigens within the lung.¹ This conclusion is based not only on the capacity of alveolar macrophages to suppress T cell proliferation *in vitro*,²⁻⁷ but also on the finding that selective *in vivo* depletion of alveolar macrophages from animals enhances their ability to generate T cell dependent immune responses to inhaled antigen,^{8,9} particularly secondary re-

BRONCHOSCOPY AND CELL PREPARATION

Following premedication with atropine, subjects were sedated with intravenous midazolam and fentanyl. The upper airway and trachea were anaesthetised with topical lignocaine and bronchoalveolar lavage (BAL) was performed as previously described.¹⁶ Recovery of lavage fluid averaged 75%. The BAL specimen was centrifuged at 400g and the cell pellet was washed. Alveolar macrophages were purified to more than 93% on a discontinuous Percoll gradient employing methods detailed elsewhere.¹⁷ In some experiments alveolar macrophages were fixed by incubation in 1% paraformaldehyde for 20 minutes, washed three times, and allowed to incubate for one hour prior to further use. Heparinised blood (30 ml) was collected from each subject and peripheral blood mononuclear cells (PBMC) were isolated on a density gradient using Lymphoprep (Nycomed, Oslo, Norway). Cell viability was assessed using trypan blue and exceeded 90% in all experiments (with the exception of fixed alveolar macrophages where the viability was <15%).

CULTURE MEDIUM

Except where indicated, experiments were performed in specific serum-free medium (AIM-V, Gibco, New York, USA). We elected to do this in order to enhance the detection of antigen-specific T cell proliferation so as to examine the influence of alveolar macrophages on both mitogen-stimulated and antigen-stimulated PBMC. In preliminary experiments it was observed that the ability of alveolar macrophages to inhibit mitogen-stimulated T cell proliferation was similar in AIM-V and RPMI supplemented with 10% fetal calf serum (data not shown). However, the latter medium proved unsuitable for examining antigen-specific T cell proliferation because of high background responses, whereas antigen-specific responses were readily detectable using serum-free medium.¹⁸

T CELL PROLIFERATION ASSAYS

All proliferation assays were performed in 96-well round bottomed plates in 200 µl of AIM-V medium supplemented with 2-mercaptoethanol (5×10^{-5} M; Sigma). Previous investigators who have studied the antigen presenting capabilities of alveolar macrophages have appropriately used purified T cells in their culture systems.^{4,19} However, because the current study focused on the ability of alveolar macrophages to actively suppress T cell proliferation, it was elected to use PBMC without depletion of accessory cells or antigen presenting cells. PBMC (2×10^5 per well) were cultured alone or with variable numbers of alveolar macrophages in order to achieve ratios of alveolar macrophages to PBMCs from 1:32 to 1:1 depending on available cell numbers in each experiment. All assays were performed in triplicate or quintuplicate with medium control. Phytohaemagglutinin (PHA) was obtained from Murex (Dartford, UK) and used at a final

concentration of 1.0 µg/ml. House dust mite (HDM) extract (the kind gift of Dr G Stewart, Perth, UK) was prepared using *Dermatophagoides pteronyssinus* obtained from CSL Ltd (Melbourne, Australia) and was used at a final concentration of 30 µg/ml. Cultures were incubated at 37°C in 5% carbon dioxide for three (PHA) or seven days (HDM). Preliminary experiments had shown that maximal proliferation was present at these times and with these concentrations of PHA or HDM. DNA synthesis was measured by adding 0.5 µCi tritiated thymidine (Amersham, Australia) during the final 16–18 hours of culture. Cells were harvested onto glass fibre filter mats and thymidine uptake was assayed using a Packard Matrix 9600 beta counter. Data were expressed as net counts per minute (Δ cpm) with the background (unstimulated) counts subtracted.

To determine whether alveolar macrophage-mediated inhibition of T cell proliferation is a reversible phenomenon, stimulated PBMCs were removed from alveolar macrophages following overnight co-culture. Cells were gently resuspended, leaving the majority of adherent alveolar macrophages behind, and PBMCs were transferred to new wells with fresh medium. Parallel control cultures contained PBMCs which had been stimulated in the absence of alveolar macrophages and were also transferred to new wells with fresh medium. Thymidine uptake was determined two days (mitogen experiments) or six days (antigen experiments) later.

In order to assess whether the inhibitory activity of alveolar macrophages might be mediated by soluble products, supernatants were generated from alveolar macrophages and stimulated PBMC cultures, with or without alveolar macrophages. These supernatants were harvested at 24–48 h, centrifuged, and stored frozen at -20°C until required. Graded volumes of these supernatants were subsequently added to PBMCs cultured with PHA or HDM.

INFLUENCE OF ALVEOLAR MACROPHAGES ON T CELL ACTIVATION EVENTS

Calcium flux

The determination of intracellular free calcium using the dye Indo-1 (Molecular Probes, Junction City, Oregon, USA) has been described elsewhere.²⁰ Briefly, T cell populations were suspended in RPMI 1640 supplemented with 2% fetal calf serum and 10 mM hepes, pH 7.4, at a concentration of 5×10^6 /ml. Cells were incubated with Indo-1 at a final concentration of 18 µM at 37°C for 35 minutes in the dark. The loaded cells were subsequently maintained for at least two hours prior to analysis in the dark at room temperature (RT). Immediately preceding flow cytometric analysis of calcium flux, the Indo-1 labelled cells were diluted to 1×10^6 /ml in medium containing 1 mM CaCl_2 and equilibrated at 37°C.

For each experiment, measurement of unstimulated baseline levels of intracellular calcium bound Indo-1 was initially established. This was followed by the addition of the ac-

tivating agent and measurement of the increase in the intracellular ionised calcium concentration as determined by the increase in the ratio of blue/violet fluorescence of Indo-1. The results are expressed in two forms: the calcium flux ratio and the percentage of responding cells. Calcium flux, being the ratio of Ca-bound (violet) to unbound (blue) Indo-1, was measured over a five minute interval in response to the stimulant added after one minute. Responding cells were defined as those in which the calcium flux ratio was greater than the mean + 95% confidence interval of resting cells.

Immunostaining and flow cytometry

Mitogen-stimulated lymphocytes were studied at baseline and at 24 hours for expression of T cell antigens and markers of activation. Flow cytometric analysis employed an EPICS Elite cytometer equipped with standard analytical software. Because of the importance of accurately distinguishing alveolar macrophages from T cells, the latter were identified on the basis of their forward and side scatter characteristics, together with staining for CD5. (CD5 was included as a relatively stable pan T cell marker, rather than CD3 which was markedly downregulated in a significant proportion of T cells following activation. Preliminary experiments indicated that B cells comprised <5% of the CD5+ population in PBMCs.)

The following monoclonal antibodies were used for immunostaining: anti-CD3 (UCHT1, Dako, Denmark), anti-CD2 (Leu-5b, Becton Dickinson), anti-CD28 (Leu 28, Becton Dickinson), anti-IL-2 receptor alpha chain (IL-2R α ; CD25, Tac), and phycoerythrin (PE) conjugated anti-CD5 (Leu-1, Becton Dickinson). Biotinylated sheep anti-mouse and streptavidin fluorescein isothiocyanate (FITC) were from Amersham, Sydney.

Lymphocytes were retrieved from culture wells by gentle resuspension in order to minimise contamination with adherent alveolar macrophages. Cells were washed and then double stained using optimal concentrations of monoclonal antibodies to CD3, CD2, CD28 or IL-2R α , followed by biotinylated sheep anti-mouse and streptavidin FITC, and finally CD5-PE. An irrelevant rat antibody and an isotype matched PE-conjugated mouse antibody were employed as negative controls.

Measurement of cytokines

Culture supernatants were harvested at 24 hours for determination of interleukin-2 (IL-2) and at 48 hours for interferon gamma (IFN γ) as cytokine levels were maximal at these time points. IL-2 bioactivity was measured using a standard bioassay. Serial dilutions of supernatants were added to CTLL-2 cells (10^4 per well) and compared with a standard curve generated using recombinant IL-2 (Cetus, Emeryville, California, USA). The results were analysed using the probit method. IFN γ was assayed by ELISA as previously described.²¹ This assay was accurate over the range 5–2000 U/ml.

Western blotting for detection of protein phosphorylation

PBMCs were cultured in the presence or absence of alveolar macrophages and stimulated with mitogen as previously described. To remove IL-2 already produced during culture, PBMCs were removed from wells at 24 hours and washed three times in RPMI 1640 supplemented with 5% fetal calf serum. Equivalent cell numbers were then replated in fresh serum-free medium and allowed to equilibrate at 37°C for at least three hours. IL-2 (100 U/ml) was added to the T cell cultures for 15 minutes and stimulation terminated by the addition of ice cold lysis buffer containing 1 mM sodium orthovanadate, 2 mM EDTA, 10 mM iodoacetamide, 25 μ g/ml *p*-nitrophenylguanidinebenzoate, 10 μ g/ml leupeptin, 10 μ g/ml aprotinin, and 0.5% Triton X-100. After addition of the lysis buffer (100 μ l/ 10^7 cells) cells were vortexed and left on ice for 15 minutes. The lysates were clarified by centrifugation at 14 000g at 4°C for 15 minutes. An appropriate amount of $3.3 \times$ concentrated sample buffer was added to the supernatant and the samples were then boiled for five minutes and frozen at -20°C until required.

Samples were electrophoresed on a 10% SDS-polyacrylamide gel and then transferred to a 0.22 μ m nitrocellulose membrane. After blocking at 4°C overnight the membranes were probed for phosphotyrosine activity. Briefly, after washing in Tris-buffered saline (20 mM Tris, 150 mM NaCl) with 0.05% Tween and 0.1 mM Na₃VO₄ (TBS-T_{0.05}, 0.1 mM Na₃VO₄), the membranes were incubated with 1 μ g/ml anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology Inc, Lake Placid, New York, USA) for two hours at room temperature. Membranes were washed a further three times and then incubated with biotinylated sheep-anti-mouse for one hour at room temperature, washed, and finally incubated with streptavidin-horseradish peroxidase for one hour at room temperature. After final extensive washing the membranes were developed by the enhanced chemiluminescence method (ECL, Amersham, Australia) according to the manufacturer's directions.

BLOCKING REAGENTS

The following reagents were employed in experiments involving modulation of the ability of alveolar macrophages to suppress T cell proliferation: recombinant IL-1 (Immunex, Seattle, Washington, USA), anti-TGF β blocking antibody (R&D Systems, USA), indomethacin (Sigma, St Louis, Missouri, USA), and N^G-monomethylarginine acetate (MMA) from Calbiochem (La Jolla, California, USA).

STATISTICAL ANALYSIS

Except where indicated, data are expressed as mean (SD). Statistical comparisons of paired or unpaired data were performed by *t* test. Two-sided *p* values are given and *p* values of <0.05 were regarded as significant.

Figure 1 Inhibition by alveolar macrophages of proliferation of T cells stimulated with optimal concentrations of either antigen (house dust mite, HDM) or mitogen (phytohaemagglutinin, PHA). Thymidine uptake is expressed as a percentage of that in control cultures which contained only stimulated PBMCs. Solid lines are means and dashed lines 95% CI. Inhibition of HDM responses was similar in mite allergic and non-allergic subjects (data not shown). Mean (SD) Δ cpm in control cultures were 26 142 (12 374) and 64 963 (19 397) for HDM and PHA, respectively. Mean background thymidine uptake in unstimulated wells for all experiments described in this study were as follows. For three day cultures backgrounds were invariably <1000 cpm while in seven day cultures backgrounds were 3649 (3384) cpm.

Results

INHIBITION OF T CELL PROLIFERATION BY ALVEOLAR MACROPHAGES

When we compared the capacity of alveolar macrophages to inhibit T cell responses to antigen or mitogen (fig 1), alveolar macrophages inhibited proliferation induced by both stimuli in a dose dependent manner. However, proliferation due to HDM was considerably more susceptible to the inhibitory influence of alveolar macrophages than proliferation induced by PHA ($p < 0.0001$ at ratios of 1:16, 1:8, and 1:4).

There is evidence that alveolar macrophage-mediated inhibition of T cell proliferation is a reversible phenomenon in that lymphocytes removed from alveolar macrophages within the first 24 hours will respond to re-stimulation with mitogen.⁷ In order to determine whether such re-stimulation is necessary for proliferation, parallel replicate cultures were established in which mitogen-stimulated or antigen-stimulated PBMCs were incubated overnight in the presence or absence of alveolar macrophages and then transferred to new wells with fresh medium. (It was estimated that approximately 10% of the original concentration of mitogen or antigen remained in the medium in these new wells. Particularly in the case of antigen, this is well below the normal threshold for optimal stimulation). As depicted in fig 2, these "rescued" cells exhibited levels of proliferation which were at least half those of the controls.

Figure 2 Reversibility of alveolar macrophage-mediated inhibition of T cell proliferation. PBMCs were stimulated with antigen or mitogen and cultured in the presence or absence of alveolar macrophages (AM). After 24 hours PBMCs were gently resuspended ("rescued") and transferred to new wells with fresh medium. Fresh antigen or PHA was not added. Results shown are mean (SD) thymidine uptake of a representative of three experiments. * $p < 0.01$ compared with PBMC which remained in the presence of AM throughout the culture period.

USE OF HETEROGENOUS MECHANISMS TO INHIBIT T CELL PROLIFERATION

It is clear that the capacity of alveolar macrophages to inhibit T cell proliferation in vitro is an active process which is not due to overcrowding of culture wells by the large alveolar macrophages.¹⁶ Nonetheless, the mechanism by which this phenomenon is mediated in humans remains unclear. Rich *et al* have proposed that suppression of T cell proliferation by human alveolar macrophages is mediated primarily by contact dependent mechanisms involving a lipid-rich component of the cell membrane of the alveolar macrophages and that suppression is not dependent on the viability of alveolar macrophages.⁶ However, on the basis of our observations that alveolar macrophages inhibited antigen-specific proliferation at extremely low alveolar macrophage:PBMC ratios (fig 1), it seemed likely that alveolar macrophages would also secrete factors which suppress T cell proliferation. When we compared live alveolar macrophages with alveolar macrophages which had been fixed in paraformaldehyde it was evident that fixation reduced the ability of alveolar macrophages to inhibit T cell proliferation (fig 3). Although fixed alveolar macrophages retained some capacity to inhibit mitogen responses at high alveolar macrophage:PBMC ratios, they had no effect on antigen responses which suggests that the ability of alveolar macrophages to inhibit these latter responses is largely dependent on the viability of alveolar macrophages.

We then addressed the related issue of whether suppressive activity is present in supernatant derived from alveolar macrophages. Supernatant was added to PBMCs at the initiation of cultures and the results expressed as the equivalent alveolar macrophage:PBMC ratio. If 50% supernatant derived from wells containing 1.0×10^5 alveolar macrophages ($\pm 2 \times 10^5$ PBMCs; i.e. alveolar macrophage:PBMC ratio

Figure 3 Fixation of alveolar macrophages (AM) reduces their ability to inhibit T cell proliferation. * $p < 0.05$, proliferation significantly greater than in cultures with live alveolar macrophages. Fixed alveolar macrophages inhibited proliferation of PHA-stimulated PBMCs (A) below control at ratios of 1:4 and 1:2 ($p < 0.02$) but did not inhibit antigen-stimulated PBMCs (B) at any of the ratios tested.

Figure 4 Ability of culture supernatants to inhibit T cell proliferation. Supernatants were derived from alveolar macrophages (AM, ○) or co-cultures of AM + PBMC (▲) as described in the Methods section and added to (A) PHA-stimulated and (B) antigen-stimulated PBMCs at time 0. Data are from a representative experiment ($n = 3$). Supernatants derived from stimulated PBMCs had no significant effect on the uptake of ^3H -thymidine (data not shown). * $p < 0.05$ compared with control cultures which received medium instead.

of 1:2) was added subsequently to 2×10^5 PBMCs, this was expressed as an “equivalent alveolar macrophage:PBMC ratio” of 1:4. If 25% was added, this was expressed as an “equivalent alveolar macrophage:PBMC ratio” of 1:8. Similarly, supernatant from wells containing 0.5×10^5 alveolar macrophages $\pm 2 \times 10^5$ PBMC (i.e. alveolar macrophage:PBMC ratio of 1:4) was diluted to 50%, 25%, and 12.5%, yielding “equivalent alveolar macrophage:PBMC ratios” of 1:8, 1:16, and 1:32. In mitogen stimulated cultures, supernatants derived from alveolar macrophage + PBMC co-cultures had only a slight inhibitory effect on PHA responses (fig 4A), suggesting that secreted factors play a minor role in this situation. In contrast, HDM-specific T cell proliferation was readily inhibited by added supernatants derived from alveolar macrophages cultured alone or alveolar macrophage:PBMC co-cultures (fig 4B). Indeed, it appeared that factors secreted spontaneously by alveolar macrophages can account for most of their in vitro suppressive activity in this latter culture system.

Alveolar macrophages from various species have been shown to secrete a variety of mediators which are capable of inhibiting T cell proliferation, including IL-1 receptor antagonist protein,²² nitric oxide (NO),¹⁰ transforming growth factor β (TGF β),²³ and prostaglandins.³ In order to determine the possible contribution of each of these in the present model, co-cultures of alveolar macrophages + PBMCs were established in the presence of saturating concentrations of either recombinant IL-1 (100 U/ml), the NO synthase inhibitor MMA (0.5 mM), anti-TGF β blocking antibody (10–20 $\mu\text{g/ml}$), or indomethacin (1.0 $\mu\text{g/ml}$). However, neither of these alone nor the combination of anti-TGF β antibody plus indomethacin had any significant influence on the degree of inhibition of mitogen or antigen-driven T cell proliferation ($n = 3$ for each experiment; data not shown).

CHARACTERISTICS OF “SUPPRESSED” T CELLS

We then examined the characteristics of T cells which were “suppressed” by alveolar macro-

Figure 5 Influence of alveolar macrophages (AM) on calcium flux by mitogen-stimulated PBMC. Data are from a representative experiment and are expressed as either (A) calcium flux ratio or (B) percentage of responding cells. Ionomycin, used as a positive control, produced a flux ratio of 5.7 and a response in 100% of cells.

phages to determine whether alveolar macrophages suppress all aspects of the T cell activation process. To obtain sufficient stimulated cells for these analyses it was necessary to study mitogen (rather than antigen) stimulated PBMCs as the concentration of antigen-specific T cells is impractically low in the peripheral blood (precursor frequency <0.1%). We focused on the initial 24 hours following stimulation because it appears that this is the critical period for alveolar macrophage-induced inhibition of T cell proliferation.^{7,24} In the following series of experiments PHA-stimulated PBMCs were compared with PBMCs co-cultured with sufficient alveolar macrophages to reduce thymidine uptake to less than 30% of control in parallel culture wells.

Calcium flux

Engagement of the T cell receptor/CD3 complex leads to the generation of "second messengers" including a rapid increase in intracellular calcium. Both IL-2 production and T cell proliferation are thought to be dependent on this calcium flux.²⁵ In order to examine the influence of alveolar macrophages on calcium flux, PBMCs were incubated for one hour or for 24 hours in the presence of alveolar macrophages at an alveolar macrophage: PBMC ratio of 1:1. However, alveolar macrophages did not significantly alter calcium flux in response to PHA, whether the data were expressed as calcium flux ratio or as the percentage of responding cells (fig 5). This occurred even though subsequent T cell proliferation was markedly inhibited.

Activation associated antigens

During normal T cell activation characteristic changes occur in the expression of a number of T cell antigens. In particular, the intensity of CD3 expression diminishes while CD2 and CD28 are upregulated.²⁶⁻²⁸ (CD2 is an activation and adhesion molecule, while CD28 is a receptor through which co-stimulatory signals are received from antigen-presenting cells.

Lymphokine gene expression appears to be dependent on a CD28-mediated signal.) Additionally, IL-2R becomes apparent on the surface of the majority of T cells. Because the foregoing changes are necessary components of normal T cell activation, it is possible that alveolar macrophages could inhibit proliferation by interfering at one or more of these sites.

However, the experiments performed here suggested that alveolar macrophages had little influence on the above parameters of T cell activation. As expected, PHA-stimulated T cells which were cultured alone exhibited downregulation of CD3 and upregulation of CD2, CD28 and IL-2R α (fig 6A and B). These same changes were also present on T cells which were co-cultured with sufficient alveolar macrophages to inhibit their subsequent proliferation markedly. Alveolar macrophages did not significantly alter the normal pattern of activation-associated changes in CD2, CD28 and IL-2R expression, and had a minor (though statistically significant) effect on the activation-associated reduction in CD3 expression. Similar results were seen regardless of whether the data were expressed as the mean fluorescence intensity (fig 6) or as the percentage of T cells which stained positively for these four antigens (latter not shown).

Cytokine production

Alveolar macrophages had no effect on the levels of bioactive IL-2 secreted into culture supernatants by activated T cells (fig 7). In contrast, a modest reduction in the concentration of IFN γ was found when PBMCs were stimulated in the presence of alveolar macrophages. After subtracting the amount of IFN γ present in wells containing alveolar macrophages alone, levels of this cytokine averaged 41% of that in controls, but the production levels can nevertheless still be classified as relatively high.

Phosphorylation of intracellular proteins

Because saturating amounts of IL-2 do not overcome the ability of human alveolar macrophages to inhibit T cell proliferation,^{7,29} it is possible that alveolar macrophages interfere with the normal functioning of the IL-2 receptor. Consequent upon the binding of IL-2 to its receptor, a number of intracellular proteins undergo phosphorylation by tyrosine kinases.³⁰ Preliminary data concerning the influence of alveolar macrophages on this process are displayed in fig 8. Under conditions of normal T cell activation (as shown in lanes 3 and 4), several proteins have undergone tyrosine phosphorylation. Attempts were made to demonstrate that these proteins were phosphorylated directly as a result of IL-2 by removing IL-2 from the cultures for a period of time. Cells were washed, rested in fresh medium for three hours, and then IL-2 was added for 15 minutes. However, even when IL-2 was not added, a high degree of phosphorylation was present in the samples (lane

Figure 6 Influence of alveolar macrophages on expression of activation-associated antigens on mitogen-stimulated CD5+ T cells. (A) CD3, CD2 and CD28 expression. Data are expressed as mean fluorescence intensity (MFI) normalised to that on resting T cells, arbitrarily set at 1.0. * $p < 0.05$ versus resting T cells. Data are shown as mean (SD); $n \geq 4$ experiments. (B) Effect of alveolar macrophages (AM) on upregulation of IL-2R α (CD25) expression on mitogen-stimulated CD5+ T cells. A histogram from a representative experiment is shown ($n = 5$). Fluorescence intensity is on the horizontal axis and relative cell number on the vertical axis. The vertical line indicates the upper limit of background staining using an irrelevant primary (rat) antibody.

Figure 7 Influence of alveolar macrophages (AM) on cytokine production by PHA-stimulated PBMCs. * $p < 0.05$ compared with PBMC. Data are expressed as mean (SD); $n = 8$.

4), either as a result of ongoing mitogen stimulation or in response to the continuous production of IL-2 by activated T cells. It was thus difficult to ascertain with any certainty which proteins were phosphorylated specifically as a result of IL-2 stimulation. Nonetheless, two major bands were readily apparent in stimulated PBMCs (lanes 3 and 4; arrowed) but were undetectable in PBMCs co-cultured with alveolar macrophages (lane 2). In addition, a faint band of approximately 165 kDa was consistently present in stimulated PBMCs but absent in co-cultures of alveolar macrophages with PBMCs. A diffuse band of phosphorylated protein was evident in lysates from cultures containing alveolar macrophages (lanes 1 and 2). As this band was more prominent in lane 2, it is possible that activated PBMCs are acting upon alveolar macrophages. However, in the context of the experiments described in figs 5–8 which focused on the influence of alveolar macrophages on stim-

ulated lymphocytes, the bands highlighted by the arrow appear to be the most important.

Discussion

The salient findings to emerge from this study may be summarised as follows: (1) human alveolar macrophages mediate reversible inhibition of T cell proliferation in response to both mitogen and antigen stimulation, and the latter is much more susceptible to inhibition; (2) the mechanisms by which suppression of antigen-induced T cell proliferation is achieved appear to involve the secretion of as yet undefined effector molecules by viable alveolar macrophages, whereas fixed alveolar macrophages only appear capable of blocking mitogen-induced proliferation at very high ratios of alveolar macrophages to T cells; (3) rather than a non-specific suppression of all aspects of T cell function, alveolar macrophages selectively inhibit proliferation, while allowing

Figure 8 Effect of alveolar macrophages (AM) on tyrosine phosphorylation of intracellular proteins. Lane 1: AM + PHA + IL-2; lane 2: AM + PBMC + PHA + IL-2; lane 3: PBMC + PHA + IL-2; lane 4: PBMC + PHA. Two major bands (arrow) were apparent in lanes 3 and 4 but were absent in lanes 1 and 2. A representative experiment is shown ($n=3$).

many other facets of the initial T cell activation cascade to proceed unhindered.

As shown in fig 1, alveolar macrophages inhibited T cell proliferation in a dose dependent fashion. Because antigen-specific T cell proliferation was inhibited at very low alveolar macrophage:PBMC ratios in vitro, we think it likely that alveolar macrophages are able to inhibit T cell proliferation following antigen exposure in vivo, even in a particular microenvironment within the lung where alveolar macrophages are outnumbered by T cells. The greater susceptibility of antigen compared with mitogen-induced proliferation has been noted previously,^{7,31} and there appear to be at least two possible explanations for this difference. Firstly, the kinetics of these T cell responses differ. Whereas antigen requires time-dependent processing by antigen-presenting cells, the T cell receptor can be immediately cross-linked by unprocessed mitogen. It is noteworthy that the suppressive activity is directed at events occurring within the first 24 hours after T cell stimulation and that, once a critical stage of activation has been reached, alveolar macrophages are no longer able to inhibit proliferation.⁷ Accordingly, alveolar macrophages may have less time to inhibit mitogen (compared with antigen) stimulated T cells before they proceed past "the point of no return". Secondly, it is possible that the susceptibility to suppression differs because alveolar macrophages inhibit antigen-presenting cell function³² and thereby indirectly inhibit T cell proliferation. Compared with mitogen, T cell responses to antigen have much more exacting requirements with respect to antigen processing and presentation, and it is possible that these are particularly vulnerable to the inhibitory influences of alveolar macrophages. Others have shown that the extent of alveolar macrophage-mediated suppression does vary to a minor degree according to the concentration of mitogen employed.⁷ However,

the extent of this variation is relatively small, and certainly much less than the differences between antigen and mitogen cultures as shown in fig 1.

This suppressive phenomenon is essentially reversible (see fig 2) such that T cells are able to proliferate following their removal from alveolar macrophages. In vivo, if activation is initiated within the lung in the presence of alveolar macrophages, "suppressed" T cells might therefore retain the ability to undergo clonal expansion if and when they migrate out of the lung to regional lymph nodes. Such a mechanism might serve a dual purpose – it would permit the development of T cell memory to antigens encountered on the epithelial surfaces of the lung while minimising the physiological sequelae of local T cell proliferation within the lung.

By examining both mitogen and antigen-stimulated T cells it became clear that human alveolar macrophages possess at least two distinct immunoregulatory mechanisms. In experiments with mitogen-stimulated T cells, suppression required high ratios of alveolar macrophages to PBMCs and was partially reproduced by paraformaldehyde fixed alveolar macrophages. These findings are in agreement with the notion that alveolar macrophages can inhibit T cell proliferation via a contact dependent mechanism.^{6,11} However, the existence of another considerably more potent mechanism became evident in experiments employing antigenic stimulation, where suppression was observed at low alveolar macrophage:PBMC ratios, was absolutely dependent upon alveolar macrophage viability, and was reproduced with supernatants derived from alveolar macrophages. These latter findings suggest that alveolar macrophages can also inhibit T cell proliferation by the secretion of "suppressive factors" into the medium. Thus, the mechanisms by which human alveolar macrophages inhibit T cell proliferation appear to be heterogeneous, analogous to the situation in the rabbit.³¹ While alveolar macrophages from a variety of experimental animals have been shown to secrete soluble suppressive factors, this is not spontaneous but requires an IFN γ signal from T lymphoblasts.^{2,10,31} In contrast, it appears that the supernatants derived from human alveolar macrophages cultured alone contain suppressive factors (see fig 4). This suggests that human alveolar macrophages produce such factors constitutively, or that the culture conditions (including adherence to plastic) are sufficient to "activate" human alveolar macrophages³³ and thus induce secretion of suppressive factors.

Nonetheless, these lymphocytostatic mechanisms remain only partially defined. Whereas nitric oxide mediates much of the suppressive activity of rodent alveolar macrophages,^{10,29} this appears less likely for human alveolar macrophages as concentrations of MMA which are known to inhibit nitric oxide synthase did not modulate their suppressive activity. Neither is it likely that the IL-1 receptor antagonist, which has also been shown to contribute to the T cell suppressive activity of supernatants derived

from rodent alveolar macrophages,³⁴ plays a significant role in the human process as the presence of high concentrations of recombinant IL-1 in the human cultures did not modify suppression either. Another report suggests that human alveolar macrophages use a combination of TGF β and prostaglandins to block induction of lymphokine activated killer (LAK) cell activity in response to high concentrations of IL-2.²³ However, our findings on the lack of effects of adding blocking anti-TGF β antibodies suggest that alveolar macrophages employ other (as yet undefined) mechanisms to inhibit T cell proliferation in response to stimulation via the T cell receptor.

When we examined the properties of PBMCs which had been stimulated with mitogen in the presence of alveolar macrophages, it appeared that many of the earliest steps in T cell activation were relatively unaffected. The characteristic changes in expression of CD3, CD2, CD28, and IL-2R α which accompany normal T cell activation were not substantially altered by the presence of alveolar macrophages, despite marked inhibition of their subsequent proliferation. Even though we have not examined these same parameters under conditions of antigen stimulation, studies comparing the effects of alveolar macrophages on mitogen versus antigen stimulation of T cells from hyperimmune rats indicate an identical pattern of surface marker expression in both systems.³⁴ This provides circumstantial evidence that the sequelae for the suppressed T cell are the same, regardless of the mechanisms by which alveolar macrophages suppress T cell proliferation.

Moreover, our findings suggest that the initial calcium flux which precedes T cell activation is also relatively unaffected by the presence of alveolar macrophages. This conclusion contrasts with that of a previous report,¹¹ but is internally consistent with the observation that alveolar macrophages did not reduce IL-2 levels in our system (see fig 6), given that IL-2 secretion is dependent upon calcium flux.²⁵ Similar results have also been obtained in the rat.³⁴

It has been reported that alveolar macrophages inhibit expression of the activation markers IL-2R and the transferrin receptor, but not HLA-DR, on T cells stimulated with mitogen for 48 hours.⁷ (It is not clear whether a similar effect was seen at 24 hours.) However, in that study only 22–35% of T cells cultured in the absence of alveolar macrophages expressed IL-2R. Because the authors chose to purify lymphocytes over a nylon wool column prior to flow cytometry, it is possible that activated T cells may have been lost in the process, leading to an underestimate of the degree of expression of IL-2R α .

The effect of alveolar macrophages on T cell cytokine production has received relatively little attention compared with their effects on T cell proliferation. Our own finding that levels of bioactive IL-2 were not affected by alveolar macrophages differs from a prior report in which human alveolar macrophages reduced, but did not abolish, IL-2 production.⁶ In contrast to the latter, it appears that alveolar macro-

phages do not inhibit the ability of alloantigen-stimulated T cells to provide help for B cell immunoglobulin production; neither IFN γ nor IL-4 production were suppressed by alveolar macrophages in that study.³⁵ Indeed, our observation that alveolar macrophages partially inhibited production of IFN γ , but not IL-2, implies that alveolar macrophages may exert differential effects on particular T cell cytokines. It is possible that alveolar macrophages may secrete factors which inhibit the CTLL cell line, such that our assay has underestimated IL-2 concentrations. If this were the case, it would strengthen the conclusion that alveolar macrophages have little effect on IL-2 production, despite marked inhibition of DNA synthesis.

In view of the finding that human alveolar macrophages have little effect on the earliest sequelae of T cell stimulation (see figs 5 and 6), and that IL-2 secretion and IL-2R α chain expression are normal, it would appear that alveolar macrophages exert their suppressive influence at a more "distal" site in the activation cascade. Given that alveolar macrophage-mediated suppression is not overcome by the presence of large concentrations of IL-2,^{7,29} it seems likely that alveolar macrophages interfere with the normal functioning of the IL-2R at a point downstream from IL-2 binding; similar suggestions have been made in relation to recent animal studies from our laboratory demonstrating identical levels of IL-2 binding to normal and "alveolar macrophage suppressed" rat T cells.³⁴ It is also noteworthy that Yarbrough *et al* have proposed that lung macrophages inhibit the activation of LAK cells at a point distal to the binding of IL-2 to the β chain of the IL-2R.³⁶

Our preliminary results concerning tyrosine phosphokinase activity in mitogen-stimulated lymphocytes, as shown in fig 8, are consistent with this hypothesis. Alveolar macrophages appear to impede phosphorylation of certain intracellular proteins, though further studies will be required to characterise these proteins and their exact relationship with the IL-2R. Nevertheless, the marked differences observed between PBMCs and co-cultures of alveolar macrophages + PBMCs suggest that further more detailed studies on protein phosphorylation and associated signal transduction via the IL-2R are warranted.

The characteristics of "alveolar macrophage suppressed" T cells described herein resemble in many respects the profile of freshly isolated lung T cells. As noted earlier, this latter T cell population is dominated by post-activated memory cells^{12,13} which contain cytokine mRNA transcripts^{14,15} and which proliferate poorly upon *ex vivo* stimulation.^{13,16,21} As shown in the current study, these are features of peripheral blood T cells which have been stimulated *in vitro* in the presence of alveolar macrophages, consistent with the notion that alveolar macrophages selectively inhibit T cell proliferation but not activation, and implying that alveolar macrophages contribute to the development of these characteristics in the lung. *In vivo* it is likely that other im-

munoregulatory mechanisms synergise with alveolar macrophages, including surfactant³⁷ and type II alveolar epithelial cells.³⁸ Interestingly, while the latter inhibit T cell proliferation, they do so in a manner which permits the expression of activation markers on mitogen stimulated T cells, which is similar to our own findings with respect to alveolar macrophages.

The conclusion that alveolar macrophages inhibit some, but not all, aspects of T cell function has important consequences for our understanding of the possible role of alveolar macrophages in immune mediated lung disease. In asthma, for example, there is some evidence that alveolar macrophages are less able to suppress T cell proliferation than normal alveolar macrophages.³⁹ Equally important will be studies which compare the capacity of alveolar macrophages from normal and diseased human subjects to regulate the production of particular disease-relevant cytokines, in particular those derived from Th2 cells which, in the animal model, appear to be particularly sensitive to the effects of alveolar macrophages.⁸

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