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Regulation of Pulmonary T Cell Responses to Inhaled Antigen: Role in Th1- and Th2-Mediated Inflammation

Shiour-Ching Lee, Zeina H. Jaffar, Kong-Sang Wan, Stephen T. Holgate, and Kevan Roberts

DO11.10 transgenic mice, expressing an OVA-specific TCR, were used to study pulmonary T cell responses to inhaled Ags. Before OVA inhalation, the activation of lung parenchymal T cells elicited both strong proliferative responses and IL-2 production. However, following Ag inhalation the proliferative responses of the lung T cells, when restimulated in vitro with OVA\textsubscript{323-339} peptide or immobilized anti-CD3, were severely attenuated and associated with a decrease in the level of production of IL-2 but not IFN-\(\gamma\). Such immune regulation was tissue-specific, because T cell responses in the lymph nodes and spleens were normal. This dramatic aerosol-induced attenuation of parenchymal T cell proliferation was also observed in BALB/c mice immunized with OVA and in BALB/c mice following adoptive transfer of DO11.10 T cells bearing either a Th1 or Th2 phenotype. In mice that had received Th2 cells, the reduced proliferative responses were associated with a decrease in IL-2 expression but augmented IL-4 and IL-5 production. Invariably, the inhibition of proliferation was a consequence of the action of F4/80\textsuperscript{+} interstitial macrophages and did not involve alveolar macrophages or their products. These observations demonstrate that clonal expansion of T cells in the lung compartment is prevented following the onset of either Th1- or Th2-mediated inflammation. This form of immune regulation, which appears as a selective defect in IL-2-driven proliferation, may serve to prevent the development of chronic pulmonary lymphoproliferative responses. The Journal of Immunology, 1999, 162: 6867–6879.

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Materials and Methods

Media

Culture media RPMI was used throughout this study and comprised of RPMI 1640 (Life Technologies, Paisley, U.K.) supplemented with penicillin (50 U/ml), streptomycin (50 \(\mu\)g/ml), 5 mM HEPES, 2 mM glutamine, 2 ME (5 \(\mu\)M), and 5% FCS.
Animals
The DO11.10 animals used in this report were provided by Dr. Ethan Shevach (National Institutes of Health, Bethesda, MD). Animals were bred under aseptic conditions in a barrier facility. Mice were originally developed by Dr. D. Y. Loh (Howard Hughes Medical Institute, St. Louis, MO) and bred to normal BALB/c mice in the University of Texas Medical Branch facility (Texas). The expression of TCR transgenes was screened using the anti-clonotypic Ab KJ1-26. BALB/c mice were obtained from Harlan (Loughborough, U.K.), and in some instances they were immunized three times with OVA (100 µg/mouse) using an alum adjuvant as described previously (6).

Animal sensitization and adoptive transfer of DO11.10 T cells
Mice were intranasally challenged by exposure to aerosolized solutions of either PBS or OVA (Grade V; Sigma, Poole, U.K.) for 20 min a day over 8 consecutive days using a Wright’s nebulizer. Mice were sacrificed on day 9, and the lungs, peripheral lymph nodes (PLN), and spleen were harvested for analysis. In some experiments, DO11.10 T cells were adaptively transferred into BALB/c mice before exposure to OVA aerosols. To drive T cell differentiation into a Th1 or Th2 effector phenotype, PLN cells were incubated (5 × 10^7/ml) in the presence of 1 µg/ml OVA123-239 peptide and either 400 ng/ml mouse IFN-γ (R&D Systems, Abingdon, U.K.) or 0.4 µg/ml anti-IL-4 Ab (11B11; American Type Culture Collection, Manassas, VA) or 2 ng/ml murine IL-4 (R&D Systems) plus 0.5 µg/ml of the anti-IFN-γ Ab (R4-6A2; American Type Culture Collection; used as a hybridoma supernatant at 10% final concentration), respectively. After 4 days of culture, cells were then injected i.v. into BALB/c mice (10^7/mouse), and the mice were exposed to aerosolized OVA.

Isolation of lung inflammatory cells
Lung tissue was cut into small fragments and then washed extensively in culture medium RPMI. The tissue was then incubated for 1 h in culture medium RPMI containing 0.1% collagenase (Type IV; Sigma), 0.01% hyaluronidase (Sigma), and 0.002% DNase (Sigma). After digestion, cells were washed in media, and viable mononuclear cells were isolated over a Percoll density gradient (67.6%, 800 × g; 30 min). Bronchoalveolar lavage (BAL) was performed by cannulating the trachea and washing the lungs with 0.5 ml of PBS. In some experiments, lung mononuclear cells (LMCs) were fractionated using magnetic bead separation. This was achieved by pretreating LMC preparations with F4/80 and M1/70 (anti-CD11b) hybridoma supernatants (both from American Type Culture Collection). Cells were washed and mixed with sheep anti-rat IgG Dynabeads (Dynal, Oslo, Norway) before removal by magnetic separation.

Monoclonal Abs
The mAbs used in this study were F4/80, M1/70 (American Type Culture Collection), MOMA-1, MOMA-2 (Serotec, Oxford, U.K.), DX5 (anti-NK cell, Pharmingen), anti-class II (M5/114, rat IgG2b; American Type Culture Collection), anti-FcγRI (2.4G2, IgG2b; American Type Culture Collection), anti-CD3ε (2C11, hamster IgG; American Type Culture Collection), anti-CD8 (53-67.7, rat IgG2a; Pharmingen), anti-CD4 (1.19, rat IgG2b; American Type Culture Collection), and anti-TCR-α (R4-16A2, rat IgG1, American Type Culture Collection), anti-TCR-β (H57-597, rat IgG2b, American Type Culture Collection), anti-CD44 (HM-1, rat IgG2b, American Type Culture Collection), anti-CD45 (30-F11, rat IgG2b; American Type Culture Collection), anti-CD45R (30-H12, rat IgG2b; American Type Culture Collection), anti-CD45R0 (30-H12, rat IgG2b; American Type Culture Collection), and anti-CD103 (M290, a gift from Dr P. J. Kilshaw; Babraham, Cambridge, U.K.). These Abs were employed to deplete or to stain cells by flow cytometric analysis using a FACScan (Becton Dickinson). To analyze T cell function, cells were plated onto microtiter plates (2 × 10^5 per well) and in some experiments stimulated with either whole OVA protein (1 mg/ml), OVA peptide (10 µg/ml of the anti-TCR-α Ab, R4-16A2; American Type Culture Collection; used as a hybridoma supernatant at 10% final concentration), respectively. After 4 days of culture, cells were then injected i.v. into BALB/c mice (10^7/mouse), and the mice were exposed to aerosolized OVA.

LUNG T CELL RESPONSES TO AEROANTIGENS IN MICE

RT-PCR analysis of lymphokine expression
Lung tissue was homogenized and RNA extracted using TRIzol (Life Technologies). Then, 1 µg total RNA was reverse transcribed by AMV reverse transcriptase (RT Systems; Promega, Southampton, U.K.) at 42°C for 1 h using poly d(T)15 as a primer. The cDNA was then amplified by PCR in the presence of PCR buffer, MgCl2 (1 mM), dNTPs (0.2 mM; Pharmacia), 1 U Tag DNA polymerase (Promega), and cytokine-specific primer pair. The PCR was conducted for 35–40 cycles under the following conditions: denaturation at 95°C for 0.5 s, annealing at 54°C for 30 s, and extension at 72°C for 0.5 s. Final extension was at 72°C for 10 min. PCR-amplified products (10 µl) were electrophoresed through 2% agarose gel containing ethidium bromide and visualized by UV illumination. The expression of β-actin (housekeeping gene), IL-2, IL-4, IL-5, IFN-γ, and TNF-α was determined in this way. Primers used were β-actin (5′-TGG AAT CTT GTC GCA TCC AT3 and 5′-TAA ACC GCA GCT CAG TAA CA), IL-2 (5′-ACT TCA AGC TCC ACT TCA AGC and 3′-GCT TGG AGA AAG TAT CCA), IL-4 (5′-GAA TGG ACC AGC CAT ATC and 3′-CCT AGT ACT ACG AGT CCA), IL-5 (5′-CGC TGG ACA CGC TCT GTT GA and 3′-CAG GAT TTG GAA TAG CAT TT), IFN-γ (5′-AAC GCT ACA CAG TGC ATC TTG G and 3′-GAC TTC AAA GAG TGT CAG G), and TNF-α (5′-GCC CTT GCT GTT CTC TGT and 3′-GGC AAC TAT CTC GTC AGT).

Eosinophil peroxidase (EPO) activity
The EPO activity in BAL cells was determined with a colorimetric assay. Five times 10^6 cells/well were added to 96-well U-bottom microtitre plates. The plates were centrifuged at 200 × g at 4°C for 10 min. The supernatants were aspirated, and 100 µl of substrate solution was added consisting of 0.1 mM orthophenylene diamine dihydrochloride in 50 mM Tris-HCl containing 0.1% Triton X-100 and 1 mM hydrogen peroxide. The plates were incubated at room temperature for 30 min, then 50 µl of 0.4 M sulfuric acid was added to stop the reaction. Absorbance was determined with an ELISA reader at 495 nm.

Cell cycle analysis
Cells were washed with PBS for 3 times, resuspended in 1 ml of hypotonic fluorochrome solution (50 µg/ml propidium iodide (PI) in 0.1% sodium citrate plus 0.1% Triton X-100), and then incubated for 1 h at room temperature. Cell cycle was measured using a FACScan flow cytometer (Becton Dickinson).

Analysis of cells isolated from lung tissue
Cyto centrifuge preparations of LMs were stained using Diff-Quick (Baxter, Abingdon, U.K.) and analyzed by microscopy. Alternatively, cells were stained by indirect immunofluorescence, and flow cytometric analysis was performed using a FACScan (Becton Dickinson). To analyze T cell function, cells were plated onto microtiter plates (2 × 10^5 per well) and in some experiments stimulated with either whole OVA protein (1 mg/ml), OVA peptide (1 µg/ml), or anti-CD3 Ab (10 µg/ml). After 3 days, 50 µl of supernatant was removed to analyze for IL-2 or IFN-γ as described below. [3H]Thymidine was added to the wells, and the plates were harvested after 16 h to monitor for proliferation by β counting.

The proliferation of nonadherent LMCs from aerosol-challenged mice cultured alone or with adherent cells but physically separated from them by a 0.4-µm millipore filter. In some experiments to determine whether physical contact between adherent and nonadherent cells was required for T cell responses, transwells (Costar, Cambridge, MA) were used in which we compared the proliferation of nonadherent LMCs from aerosol-challenged mice cultured alone or with adherent cells but physically separated from them by a 0.4-µm millipore filter.

Cytokine assay
Supernatants were assayed for IL-2 using a CTLL proliferation assay. Briefly, 5 × 10^3 CTLL cells were added to wells of a 96-well microtitre plate. Culture supernatants from the wells were titered and the proliferation of the cells was determined in triplicate by the addition of 1 µCi of [3H]thymidine (Amersham, Little Chalfont, U.K.) to each well. After 18 h, cells were harvested using a Dynatech harvester and tritium incorporation measured by β counting. IFN-γ was measured by ELISA using clone R4.6A2 (Pharmingen) as capture Ab and clone XMG1.2 (Pharmingen) as the detector Ab. IL-4 was measured by a commercially available ELISA kit (Biosource, Lifescreen, Watford, U.K.) according to the manufacturer’s instructions. IL-5 was measured by ELISA using clone TRF-5 as capture Ab and TRF-4 (Pharmingen) as detection Ab.

* Abbreviations used in this paper: PLN, peripheral lymph node; LMCs, lung mononuclear cells; BAL, bronchoalveolar lavage; EPO, eosinophil peroxidase; PI, propidium iodide.
Results

We have analyzed the response of parenchymal T cells in the lung tissue of DO11.10 mice after repeated OVA aerosol challenge. The principle advantages of using TCR transgenic mice were that the T cell response to Ag is largely amplified and that immunization before aerosol challenge was not required to elicit a response. This approach provided the means to evaluate the functional properties of Ag-specific T cells resident in the lung tissue and to determine whether their behavior was influenced by Ag inhalation.

Cytokines mRNA expressed in lung tissue following aerosol exposure

To monitor the T cell response taking place in the lung following Ag inhalation, cytokine mRNA expression in the lung tissue was determined after exposure of the mice to aerosolized OVA. DO11.10 mice were intranasally challenged with OVA for 2, 4, and 8 consecutive days. After challenge, the lung tissue was removed, homogenized, and cytokine mRNA transcripts screened by RT-PCR. We observed mRNA for IL-4, IFN-γ, and TNF-α in the lung tissue of animals that had been intranasally challenged for 2–8 days (Fig. 1A). In contrast, no cytokine expression was observed in the lung tissue from naive mice not exposed to aerosols of OVA. A similar cytokine profile was also observed when T cells isolated from the PLN or LMCs of naive DO11.10 animals were stimulated for 48 h with either whole OVA protein or immobilized anti-CD3. Experiments were repeated twice with similar results.

Pulmonary inflammation in response to intranasal challenge with OVA

The inflammatory response taking place in the lung tissue following Ag inhalation was evaluated by monitoring the magnitude and nature of any cellular infiltrate. This was determined by analyzing the cells present in the BAL and in enzymatically dispersed lung tissue. Aerosol exposure resulted in the development of a mild pulmonary eosinophilia that was clearly evident after 2–4 days of challenge by an increase in EPO activity and the number of eosinophils in the BAL (Table I). The development of a pulmonary eosinophilia was dependent on the concentration of OVA used in the aerosol (Table I), with 0.5% OVA inducing the largest increase in eosinophils in both the tissue and the BAL. Although eosinophils were recruited to the lungs, the total cell yields from the lungs of OVA-challenged animals did not increase significantly when compared with naive or PBS-challenged animals. The number of OVA-specific T cells present in the BAL, lung, spleen, and PLN of challenged mice was determined by flow cytometry using the
KJ1-26 mAb. Interestingly, the frequency and total numbers of OVA-specific T cells in enzymatically dispersed lungs of challenged animals increased only marginally following inhalation of 0.5% OVA (Table II). The number of T cells present in the lungs was not significantly affected by varying the concentration of OVA used in the aerosol (Table II).

The effect of intranasal challenge on lung parenchymal T cell responses

During the course of this study, we were particularly interested in monitoring the effect of OVA aerosol inhalation on the properties of T cells present in the lung tissue. Preparation of LMCs by dispersion of the lung tissue in collagenase provided an opportunity to probe events that were idiosyncratic to the lung environment. Stimulation of LMCs isolated from naive DO11.10 mice with OVA protein and OVA323–339 peptide resulted in strong proliferative responses (Fig. 2). LMCs isolated from mice challenged with the Ag for up to 8 days showed a reduced proliferative response on restimulation in vitro with either whole OVA protein or OVA323–339 peptide (Fig. 2A). The reduction was progressive over consecutive challenges, eventually reaching a maximal attenuation after 8 days of aerosol challenge (Fig. 2A). The proliferative response to immobilized anti-CD3 was also diminished, implying that the reduction of responses was not simply a consequence of a failure to present Ag (Fig. 2A). This effect was not observed in the PLN or spleen cells, where the T cell proliferative response to whole OVA and OVA peptide stimulation remained high (Fig. 2A). Moreover, the number of T cells present in the posterior mediastinal, hilar, and tracheal lymph nodes increased 10- to 20-fold following aerosol challenge, and restimulation with the Ag in vitro resulted in normal proliferative responses (data not shown). The attenuated responses in the lung tissue were dependent on the concentration of OVA used in the aerosol with concentrations of >0.5% resulting in a reduction of 96% (Fig. 2B). The addition of exogenous IL-2 did not restore LMC responses (2741 cpm vs 3070 cpm in response to OVA323–339 peptide in the absence or presence of 2 U/ml IL-2, respectively) implying that the failure to proliferate was not simply due to an inability to produce IL-2. Presentation of OVA323–339 peptide in the LMC cultures was likely to be by B cells and interstitial macrophages because both cell types were present in LMCs from aerosol-challenged animals. The dendritic cell marker NLDC-145 was detected on <1% of LMCs (data not shown).

The reduction of proliferation of LMCs from aerosol-challenged animals was progressive upon stimulation with OVA323–339 peptide in culture. This was clearly demonstrated by the observation that the proliferation of LMCs from naive and OVA aerosol-challenged mice, after 24 h stimulation with OVA323–339 Peptide, was equivalent. However, after 48 h and 72 h of stimulation, the degree of proliferation of LMCs from aerosol-challenged mice was markedly lower than the response of LMCs from naive mice (Fig. 2C). These data suggest that the attenuation of the proliferative responses may be a downstream consequence of activation of lung T cells (i.e., parenchymal T cells).

We next evaluated whether the attenuation of lung parenchymal T cell responses was associated with either a Th1 or Th2 response. To resolve this issue, DO11.10 T cells were driven into a Th1 or Th2 phenotype and not host lung parenchymal T cells. LMCs from control mice that had inhalation, it was found that the proliferation and production of IL-2 by LMCs in response to anti-CD3 were severely attenuated in BALB/c mice that had received Ag-specific DO11.10 T cells of either Th1 or Th2 phenotype (Fig. 3A). These data suggest that the attenuation of the proliferative responses may be a downstream consequence of activation of lung T cells (i.e., parenchymal T cells).

We next evaluated whether the attenuation of lung parenchymal T cell responses was associated with either a Th1 or Th2 response. To resolve this issue, DO11.10 T cells were driven into a Th1 or Th2 phenotype in vitro by culture with OVA323–339 peptide and exogenous IFN-γ or IL-4, respectively (Fig. 3A). T cells were injected i.v. into BALB/c recipient mice that were then exposed to aerosolized OVA. After six exposures, the lung tissue was removed and LMCs were prepared. LMCs were restimulated with anti-CD3, and the proliferation and production of IL-2 was then determined. Flow cytometric analysis of LMCs revealed that the proportion of CD4+ T cells that stained with the anti-clonotypic KJ1-26 Ab increased after OVA inhalation from 36.6% to 54.1% for Th1 cells and 26.1% to 44.0% for Th2 cells. Following OVA inhalation, it was found that the proliferation and production of IL-2 by LMCs in response to anti-CD3 were severely attenuated in BALB/c mice that had received DO11.10 T cells of either Th1 or Th2 phenotype (Fig. 3B). These data demonstrate that, OVA inhalation results in the growth arrest of Ag-specific DO11.10 and host lung parenchymal T cells. LMCs from control mice that had received DO11.10 T cells of either Th1 or Th2 phenotype and not inhaled OVA responded normally (Fig. 3B). In mice that received DO11.10 Th2 cells, an increase in the peptide-induced production of IL-4 by LMCs in vitro was observed following OVA inhalation, demonstrating that the Th2 phenotype was maintained in vivo (Fig. 3C). Neutralization of IL-4 or IL-10 had no effect on the proliferative responses of lung parenchymal T cells (data not shown). Collectively, these results show that lung parenchymal T cell responses are dramatically attenuated following OVA inhalation in BALB/c mice that received Ag-specific DO11.10 T cells of either Th1 or Th2 phenotype.

Table I. Intranasal exposure to OVA protein results in the recruitment of eosinophils to the lung tissue and BAL fluid

<table>
<thead>
<tr>
<th>Days of Intranasal Challenge</th>
<th>Eos in lung tissue (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>3.7</td>
</tr>
<tr>
<td>2</td>
<td>5.8</td>
</tr>
<tr>
<td>4</td>
<td>5.7</td>
</tr>
<tr>
<td>8</td>
<td>6.5</td>
</tr>
</tbody>
</table>

* Mice were intranasally challenged 8 times with different concentrations of OVA solution and with different number of aerosol exposures containing a 0.5% OVA solution. After challenge, mice were sacrificed and the number of eosinophils (Eos) in BAL and enzymically dispersed lung tissue were quantified by EPO assay and Diff-Quick staining.

Table II. Numbers of KJ1-26+ T cells in the lung tissue of DO11.10 mice exposed to OVA aerosols

<table>
<thead>
<tr>
<th>Concentration of OVA Used in the Aerosol (%)</th>
<th>Total cell nos. (10^6)</th>
<th>KJ1-26+ T cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>16.9</td>
<td>20.8</td>
</tr>
<tr>
<td>PBS</td>
<td>18.5</td>
<td>21.4</td>
</tr>
<tr>
<td>0.125</td>
<td>24.0</td>
<td>19.5</td>
</tr>
<tr>
<td>0.5</td>
<td>22.0</td>
<td>23.2</td>
</tr>
<tr>
<td>2.0</td>
<td>29.0</td>
<td>18.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Days of Intranasal Challenge</th>
<th>Total cell nos. (10^6)</th>
<th>KJ1-26+ T cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>19.3</td>
<td>17.2</td>
</tr>
<tr>
<td>2</td>
<td>15.9</td>
<td>21.9</td>
</tr>
<tr>
<td>4</td>
<td>19.9</td>
<td>26.5</td>
</tr>
<tr>
<td>8</td>
<td>11.9</td>
<td>20.9</td>
</tr>
</tbody>
</table>
Invariably, the failure of parenchymal T cells to proliferate was associated with a reduction in the level of IL-2 produced in response to Ag stimulation. When stimulated in vitro with either OVA\textsubscript{323–339} peptide or immobilized anti-CD3, LMCs isolated from aerosol-challenged DO11.10 mice showed a marked reduction in the level of IL-2 produced (Table III). PLN or spleen cells from aerosol-challenged DO11.10 animals produced high levels of IL-2 (data not shown). Although the production of IL-2 by LMCs from challenged mice was severely reduced, the expression of IFN-\(\gamma\) was high on stimulation in vitro with 1000 and 100 ng/ml of OVA peptide. A total of 10 ng/ml of OVA\textsubscript{323–339} peptide elicited the production of IFN-\(\gamma\), which was not observed by LMCs from naive animals under the same conditions (Table III).

The number of F4/80\textsuperscript{+} interstitial macrophages in the lungs of DO11.10 mice is increased following OVA inhalation

Coincident with the reduction in proliferative responses to OVA\textsubscript{323–339} peptide and anti-CD3 was a 2- to 3-fold increase in the number of F4/80\textsuperscript{+} cells present in the lung tissue digest of DO11.10 mice after OVA inhalation (Fig. 4A). The F4/80 Ab is specific for a highly glycosylated protein expressed by splenic and tissue macrophages, Langerhans cells, and dendritic cells. Isolation of plastic adherent LMCs revealed that they were predominantly F4/80\textsuperscript{+} cells (94%), which coexpressed CD80, CD86, CD54, high levels of class II (Ia) (Fig. 4B), and were morphologically indistinguishable from macrophages. The remaining cells comprised mainly of stromal cells. Following aerosol challenge, the level of expression of Ia, ICAM-1, and CD86 by F4/80\textsuperscript{+} cells was increased.

Adherent cells inhibit T cell proliferative responses by a mechanism requiring cell-cell contact

Because the loss of T cell proliferative responses to Ag was coincident with an increase in the number of interstitial F4/80\textsuperscript{+} macrophages, we evaluated whether adherent cells mediated the attenuation of T cell responses. Depleting adherent cells from LMC

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**FIGURE 2.** Proliferative responses of lung mononuclear T cells following aerosol exposure. DO11.10 mice were challenged with OVA aerosols either (A) daily for 2, 4, and 8 consecutive days using a 0.5% solution of OVA or (B) eight times with aerosolized OVA solutions of 0.125%, 0.5%, and 2.0%. After challenge, lung tissue was removed and dispersed in collagenase. Spleen and PLN cells were also harvested for analysis. Cells were cultured in either media, whole OVA protein, OVA\textsubscript{323–339} peptide, or plate-bound anti-CD3. The [\textsuperscript{3}H]Tdr incorporation was determined after 3 days. C, The kinetics of the proliferative responses in LMCs from naive (filled circle) and aerosol-challenged (filled triangle) mice stimulated with OVA\textsubscript{323–339} peptide for 24, 48, and 72 h. Open symbols refer to responses in media. Proliferation was determined by [\textsuperscript{3}H]Tdr incorporation.
preparations of aerosol-challenged DO11.10 animals resulted in
the restoration of the OVA 323–339 peptide and anti-CD3 responses
(Fig. 5A). Removal of adherent cells from LMCs of naive animals
did not affect OVA323–339 peptide or anti-CD3 responses. Cell cy-
cycle analysis by flow cytometry revealed that unfractionated
(whole) LMCs from aerosol-challenged mice remained predomi-
nantly in G0/G1 phase following stimulation with OVA323–339
peptide, and only 10.4% were found to be in G 2 -M phase (Fig. 5
B).

In contrast, removal of adherent cells restored the proliferative
response to OVA323–339 peptide, with 31.8% of cells found to be
in G 2 -M phase. Following stimulation with OVA323–339 peptide,
36.2% and 34.4% of apoptotic cells were observed in whole
and adherent-depleted LMCs, respectively. Alveolar macrophage-me-
diated inhibition of T cell responses has previously been shown to
be nitric oxide-dependent and not requiring physical contact be-
tween the cells (19). To determine whether physical contact be-
tween adherent and nonadherent cells was required for attenuation
of the T cell response, transwells were used in which we compared

Table III. Aerosol inhalation inhibits proliferative responses and IL-2 production but not IFN-γ
production

<table>
<thead>
<tr>
<th>Treatment</th>
<th>OVA323–339 Peptide Concentration (ng/ml)</th>
<th>Anti-CD3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000</td>
<td>100</td>
</tr>
<tr>
<td>Proliferation (cpm)</td>
<td>Naive</td>
<td>40043</td>
</tr>
<tr>
<td></td>
<td>Post aerosol</td>
<td>245</td>
</tr>
<tr>
<td>IL-2 (U/ml)</td>
<td>Naive</td>
<td>238</td>
</tr>
<tr>
<td></td>
<td>Post aerosol</td>
<td>7</td>
</tr>
<tr>
<td>IFN-γ (pg/ml)</td>
<td>Naive</td>
<td>1800</td>
</tr>
<tr>
<td></td>
<td>Post aerosol</td>
<td>2250</td>
</tr>
</tbody>
</table>

*LMCs from naive and aerosol-challenged mice were stimulated in culture with either different concentrations of OVA323–339
peptide or immobilized anti-CD3. After 48 h, supernatants were collected, and IL-2 and IFN-γ levels were determined. Proliferation
was measured by [3H]Tdr incorporation after 36 h.
the proliferation of nonadherent LMCs from aerosol-challenged mice cultured alone or with adherent cells but physically separated from them by a 0.4-μm millipore filter (Fig. 6). The proliferation of the nonadherent LMCs was unaffected by adherent cells when they were physically separated from them by the 0.4-μm filter, implying that cell-cell contact was required for attenuation of parenchymal T cell responses. However, when adherent cells were added to nonadherent LMCs, the proliferative response was severely attenuated (Fig. 6).

**F4/80**

interstitial macrophages inhibit proliferation and IL-2 production but not IL-4 or IL-5

Because the adherent cells were heterogeneous, it was important to determine the specific contribution of interstitial macrophages to the attenuation of the responses. Using the macrophage-specific mAbs F4/80, M1/70, MOMA-1, or MOMA-2 with anti-rat Ig Dynabeads, it was possible to specifically deplete interstitial macrophages. Removal of tissue macrophages using the F4/80 Ab resulted in a 77% restoration of the T cell response when compared with preparations depleted of adherent cells (Fig. 7). The removal of CD11b+ cells (using M1/70) completely restored responses. In contrast, the depletion of NK cells using the DX5 Ab or subpopulations of macrophages using the MOMA-1 and MOMA-2 Abs had no effect. These data demonstrate that mature interstitial macrophages are principally responsible for the immune modulation observed. Interstitial macrophages expressed CD80, CD86, and class II, and thus it is likely that they are effective at Ag-presentation. However, adherent LMCs isolated from OVA-challenged animals were poor at driving proliferative responses by both lung parenchymal and lymph node T cells (data not shown).

We next evaluated whether F4/80+ interstitial macrophages were responsible for regulation of proliferative responses in BALB/c mice that have received transgenic T cells. DO11.10 T cells bearing a Th2 phenotype were adoptively transferred into

![Figure 4](http://www.jimmunol.org/) A. Aerosol-induced changes in the cellular infiltrate into the lungs of DO11.10 mice. A. Mice were challenged with an aerosolized 0.5% OVA solution for 8 consecutive days. LMCs from naive and challenged mice were analyzed by flow cytometry using the F4/80 mAb. B. Flow cytometric analysis of adherent cells purified from LMCs of naive and challenged animals is shown.
BALB/c mice. The lung parenchymal T cell responses of control BALB/c mice (which had either received DO11.10 Th2 cells and not inhaled OVA or had not received Th2 cells but inhaled OVA) remained unchanged (Fig. 8A). In contrast to the control mice, OVA inhalation by animals that had received DO11.10 Th2 cells resulted in a dramatic reduction in lung parenchymal T cell proliferative responses and in IL-2 production (Fig. 8A and B). OVA inhalation by mice that had received the Th2 cells resulted in a 5-fold and 22-fold increase in the level of IL-4 and IL-5 production, respectively (Fig. 8B). These mice also developed a pronounced airway eosinophilia (data not shown). Importantly, growth arrest and attenuation of IL-2 production was reversed by the removal of F4/80+ interstitial macrophages. However, their depletion had no effect on the production of IL-4 or IL-5 (Fig. 8B).

To examine whether the tissue-specific attenuation occurred in nontransgenic mice, we analyzed the responses of BALB/c mice that had been immunized with OVA before OVA inhalation. Following repeated immunization of BALB/c mice, in vitro stimulation of LMCs with OVA protein demonstrated Ag-specific proliferative responses (Table IV). Inhalation of OVA aerosol resulted in the reduction of proliferative responses of LMCs to OVA protein, but responsiveness was restored by the removal of adherent cells (Table IV). In contrast, the responses of spleen cells to OVA protein was not influenced by Ag inhalation. LMCs from primed animals produced high levels of IL-2; however, following aerosol challenge the production of this cytokine was diminished. Interestingly, stimulation of LMCs from challenged mice with the Ag induced the production of IL-5. These data suggest that similar mechanisms of regulation of parenchymal T cell responses are evident in nontransgenic mice.

The observation that proliferative responses are reduced only after 24 h Ag stimulation in vitro may reflect a requirement for cellular activation before attenuation of the response. Neutralizing Abs to TNF-α, TGF-β, GM-CSF, IFN-γ, IL-4, IL-10, or IL-12 did not restore responses (data not shown), and coculture experiments failed to implicate the involvement of soluble factors. CTLA-4 has
been shown to down-regulate T cell responses; however, parenchymal T cells did not express CTLA-4 before or following activation. Similarly, blocking Abs to CD40, CD40 ligand, CD80, CD86, CD28, Fas ligand, VCAM-1, CD31, and CD44 failed to down-regulate T cell responses of aerosol-challenged DO11.10 mice, most of which express endogenous TCR α-chains (21). In the present study, the proportion of T cells in the lungs of animals that stained with the anti-clonotypic Ab KJ1-26 was found to be 9.8% ± 9.8%.

Following repeated OVA aerosol challenge of DO11.10 mice, the number of lung parenchymal T cells, labeled with the anti-clonotypic Ab KJ1-26, did not change. More protracted Ag challenge did not elicit severe inflammatory responses as defined by evidence of T cell activation (such as expression of IL-2R or CD69). It is difficult to comment on the degree of T cell recruitment to the tissue because the dynamics of migration to, and from, the lungs is unknown. However, it appeared that severe pulmonary T cell responses were prevented as a consequence of the modulation of T cell function following Ag inhalation. The most clear demonstration of this phenomena was that parenchymal T cells isolated from lungs of mice exposed to OVA aerosols, when restimulated in vitro with OVA323–339 peptide or immobilized anti-CD3, displayed severely attenuated proliferative responses. This effect was dependent on the dose of aerosol administered and could not be explained by changes in the number of OVA-specific T cells in the tissue. Such immune regulation was tissue-specific because the majority of the T cells are of a memory phenotype (3). Memory T cells have been described previously in the DO11.10 mouse, most of which express endogenous TCR α-chains (21). In the present study, the proportion of T cells in the lungs of animals that stained with the anti-clonotypic Ab KJ1-26 was found to be 81.7% ± 9.8%.

Discussion

We have used a TCR transgenic mouse to study the development of an inflammatory response in the lung tissue to inhaled aeroantigens. Exposure of DO11.10 mice to OVA aerosols induced the expression of mRNA for the cytokines IL-4, IFN-γ, and TNF-α in the lung tissue following 2–8 days of challenge. Coincident with the induction of cytokine expression was the generation of a mild pulmonary eosinophilia. In this context, TNF-α, IL-4, and IL-5 have been implicated in the recruitment of eosinophils in other mouse models (20).

During the course of this study we were particularly interested in monitoring the effect of OVA aerosol inhalation on the properties of T cells present in the lung tissue. Conceivably, responses occurring in the lung mucosa display characteristics which are idiosyncratic to the lung environment. T cell responses in the pulmonary tissue are likely to be secondary rather than primary, because the majority of the T cells are of a memory phenotype (3). Memory T cells have been described previously in the DO11.10 mouse, most of which express endogenous TCR α-chains (21). In the present study, the proportion of T cells in the lungs of animals that stained with the anti-clonotypic Ab KJ1-26 was found to be 81.7% ± 9.8%.

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The failure to proliferate was not only in response to OVA 323–339 peptide but also following stimulation with a polyclonal T cell activator such as immobilized anti-CD3. A similar phenomena was also evident in OVA-primed BALB/c mice, demonstrating that the down-regulation of pulmonary T cell proliferative responses following OVA inhalation is not restricted to the DO11.10 mice used in this study.

On monitoring the kinetics of proliferation, stimulation of parenchymal T cells in vitro with OVA 323–339 peptide revealed that the loss of response was progressive, with maximal inhibition occurring after 24 h of stimulation. This possibly reflects a requirement for activation of the T cells before the onset of inhibition. The attenuated proliferative responses of LMCs from OVA-challenged DO11.10 mice, and from BALB/c mice that had received DO11.10 T cells, was associated with a failure of the parenchymal T cells to produce IL-2 in response to Ag stimulation in vitro. However, the production of IFN-γ by LMCs from aerosol-challenged DO11.10 mice was not diminished on restimulation with OVA 323–339 peptide, demonstrating that not all effector functions of the parenchymal T cells were lost. Similarly, in BALB/c mice that had received DO11.10 Th2 cells and inhaled OVA, stimulation of lung parenchymal T cells with either OVA peptide or anti-CD3 cross-linking induced growth arrest and reduced IL-2 production. Interestingly, this onset of growth arrest did not prevent the production of IL-4 or IL-5. Moreover, these responses were typically associated with the development of a pronounced pulmonary eosinophilia. To our knowledge, this is the first demonstration of this phenomena. Collectively, these observations imply that the onset of growth arrest

FIGURE 8. Inhalation of OVA by BALB/c mice that have received Th2 DO11.10 T cells results in the attenuation of proliferative responses and IL-2 production but continued production of IL-4 and IL-5. DO11.10 T cells bearing a Th2 phenotype were generated as detailed previously. Cells (10⁷) were injected i.v. into naive BALB/c mice that were then exposed to OVA aerosols for 6 days. Lung tissue was harvested, and the response of LMCs in response to OVA 323–339 peptide (1 μg/ml) and plate-bound anti-CD3 (10 μg/ml) was evaluated after 3 days. A. Proliferative responses. Controls comprised of mice that had received DO11.10 T cells but had not inhaled OVA or had inhaled OVA but had not been given cells. LMCs prepared from mice that had received DO11.10 T cells and exposed to OVA aerosols were depleted of interstitial macrophages using the F4/80 Ab and anti-rat Ig-coated Dynabeads. B. IL-2, IL-4, and IL-5 production in mice that had received DO11.10 Th2 cells and inhaled OVA. Upon stimulation with OVA 323–339 peptide, the level of IL-4 and IL-5 produced by LMCs from mice that had simply received Th2 DO11.10 T cells but not not exposed to OVA aerosols was 2.1 and 7.4 pg/ml, respectively.
Consequently, the attenuation of the proliferative responses was achieved only if live cells were used. The role of the adherent cells in limiting clonal expansion of T cells in the lung parenchyma was further demonstrated by the observation that adherent-depleted LMCs from aerosol-challenged mice were stimulated with OVA 323–339 peptide (1 μg/ml). The effect of indomethacin (10 μg/ml) and monomethyl arginine (250 μM) on the proliferation after 3 days of stimulation was determined by [3H]TdR incorporation. Comparisons were made with LMCs following the removal of adherent cells by incubation on plastic for 1 h. Numbers in parentheses denote standard deviations of triplicate wells.

Table V. Role of nitric oxide and PG production on lung parenchymal T cell responses

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Media</th>
<th>OVA 323–339 Peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media</td>
<td>4245 (143)</td>
<td>942 (285)</td>
</tr>
<tr>
<td>Monomethyl arginine</td>
<td>3675 (177)</td>
<td>15,756 (1629)</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>5163 (545)</td>
<td>14,375 (1269)</td>
</tr>
<tr>
<td>Monomethyl arginine + indomethacin</td>
<td>4944 (419)</td>
<td>22,697 (2370)</td>
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</table>

Adherent-depleted

| Media | 4578 (368) | 102,384 (1379) |

Unfractionated

<table>
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<tbody>
<tr>
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<td>IL-2 (cpm)</td>
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<tr>
<td>IL-5 (pg/ml)</td>
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<td>Spleen Proliferation (cpm)</td>
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<td>IL-2 (cpm)</td>
<td>14,428</td>
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</table>

Adherent Cell Depleted

<table>
<thead>
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</thead>
<tbody>
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<tr>
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<tr>
<td>IL-5 (pg/ml)</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Spleen Proliferation (cpm)</td>
<td>592</td>
</tr>
<tr>
<td>IL-2 (cpm)</td>
<td>734</td>
</tr>
</tbody>
</table>

* BALB/c mice were immunized three times with OVA using an alum adjuvant. Mice were either exposed to OVA aerosols for 7 consecutive days or left unchallenged. LMCs and spleen cells were prepared from both groups of animals. Unfractionated and adherent-depleted cells were stimulated with OVA protein and supernatants from cultures harvested for determination of IL-2 (using CTL assay) and IL-5 levels (using ELISA). The level of proliferation was determined after 3 days by [3H]TdR incorporation. Results are representative of three separate experiments.
nitric oxide induces growth arrest by reducing tyrosine phosphorylation of Jak3 and STAT5 in T cells, which are events downstream of IL-2 signaling through its receptor (19). The growth arrest that we have observed following OVA inhalation is invariably associated with the loss of IL-2 production but continued production of IL-4, IL-5, and IFN-γ. Consistent with this observation, we found that the inhibition of nitric oxide production did not restore proliferative responses. This leads us to conclude that during the onset of Th1- and Th2-mediated inflammation the expansion of T cells is prevented by a mechanism not involving nitric oxide production.

Separation of the parenchymal T cells from the adherent cells by a 0.4-μm filter prevented the inhibition of parenchymal T cell responses. Moreover, supernatants from cultures of LMCs were ineffective at inhibiting these T cell responses, thus demonstrating that the immune modulation required cell-cell contact. Neutralizing Abs to TNF-α, TGF-β, GM-CSF, IFN-γ, IL-4, IL-10, or IL-12 did not restore responses. CTLA-4 has been shown to down-regulate T cell responses (41); however, parenchymal T cells did not express CTLA-4 before or following activation. Similarly, blocking Abs to CD40, CD40 ligand, CD80, CD86, CD28, Fas ligand, VCAM-1, CD31, and CD44 failed to restore responses. Interestingly, the addition of the ECCD-1 Ab to E-cadherin partially restored proliferative responses and was found to be expressed predominantly on low numbers of epithelial cells in the dispersed LMCs. Given the importance of E-cadherin in the interaction between T cells and the epithelial cells, its involvement is noteworthy.

Our studies have demonstrated that following Ag inhalation the responses of lung parenchymal T cells, as measured by proliferation and IL-2 production, were severely attenuated. The unresponsiveness was tissue-specific and a consequence of active inhibition of T cell function by interstitial macrophages. We speculate that this immunoregulation is representative of the normal mucosal T cell function. Of particular interest is the fact that inhibition of these T cell responses, thus demonstrating that the immune modulation required cell-cell contact. Neutralizing Abs to TNF-α, TGF-β, GM-CSF, IFN-γ, IL-4, IL-10, or IL-12 did not restore responses. CTLA-4 has been shown to regulate T cell responses (41); however, parenchymal T cells did not express CTLA-4 before or following activation. Similarly, blocking Abs to CD40, CD40 ligand, CD80, CD86, CD28, Fas ligand, VCAM-1, CD31, and CD44 failed to restore responses. Interestingly, the addition of the ECCD-1 Ab to E-cadherin partially restored proliferative responses and was found to be expressed predominantly on low numbers of epithelial cells in the dispersed LMCs. Given the importance of E-cadherin in the interaction between T cells and the epithelial cells, its involvement is noteworthy.

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Acknowledgments

References


