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T Regulatory Cells Control Antigen-Induced Recruitment of Mast Cell Progenitors to the Lungs of C57BL/6 Mice

Tatiana G. Jones,* † Fred D. Finkelman,‡§ K. Frank Austen,* † and Michael F. Gurish* †

In C57BL/6 mice, the recruitment of mast cell progenitors (MCps) to the lung is a feature of Ag-induced pulmonary inflammation that requires sensitization and challenge and is totally inhibited by the administration of anti-CD4 at the time of challenge. When mAb to TGFβ1 or to IL-10R was administered at the time of challenge, the recruitment of MCP/10⁶ mononuclear cells (MNCs) to the lung was inhibited by 56.3 and 69.6%, respectively, whereas mAb to IL-4, IFN-γ, IL-6, IL-17A, and IL-17F had no effect. In sensitized and challenged C57BL/6 mice lacking TGFβRII on CD4⁺ cells, the recruitment of MCP/10⁶ MNCs was reduced by 67.8%. The requirement for TGFβ1 and IL-10 suggested a role for CD4⁺CD25⁺ T regulatory cells. Mice treated with anti-CD25 at the time of Ag-challenge showed a reduction in the recruitment of MCP/10⁶ MNCs by 77.2% without any reduction in MNC influx. These results reveal an unexpected role for T regulatory cells in promoting the recruitment of MCps to the lungs of C57BL/6 mice with Ag-induced pulmonary inflammation. The Journal of Immunology, 2010, 185: 000–000.

Bronchial asthma is characterized by inflammation, airway hyper-responsiveness, and airways remodeling. In addition to cells central to the adaptive immune response, numerous innate cell types, including mast cells (MCs), have been implicated in the disease and in its animal models. The numbers of intraepithelial-type MCs in the mucosa and of connective-tissue-type MCs in the smooth muscle of patients with bronchial asthma are increased, and allergen inhalation challenge causes MC degranulation with mediator release (1–4). Furthermore, MC stabilizing agents have prophylactic benefit, and a mAb to IgE that inhibits its binding to the FceRI receptor is an effective targeted therapy for bronchial asthma (5, 6). In addition, patients with bronchial asthma show a 4-fold increase in circulating progenitors for the MC lineage as assessed by colony-forming assays of peripheral blood cells (7). In mouse models of allergic airways inflammation, there is early recruitment of immature MC progenitors (MCps) to the lung, MC hyperplasia with continued exposure, and MC dependence for many of the remodeling changes that occur with chronic provocation (8–10).

Under normal (basal) conditions, the mouse small intestine contains a large pool of committed MCps with a concentration per 10⁶ mononuclear cells (MNCs) that exceeds that of any other tissue, including lung (11, 12). These MCps are T cell-independent and capable of rapidly providing mature mucosal MCs in a T cell-dependent fashion during helminthic infection (13, 14). In β7 integrin-deficient C57BL/6 mice, the small intestine pool of MCps is absent along with mature mucosal and connective tissue type MCs (13). The maintenance of this MCP population in wild-type (WT) mice requires expression of α4β7 integrin on the blood-borne MCps, which mediates their interaction with the endothelial ligands mucosal addressin cellular adhesion molecule-1 and VCAM-1 in the small intestine. The blockade of each component by mAb prevents the ongoing resupply of MCps from the bone marrow to the small intestine via the circulation (12, 15). This innate pathway for the intestinal MCP pool appears to be strain independent, given that it is critical for migration in the small intestine in both BALB/c and C57BL/6 mice. The basal population of MCs in lung is also innate, being intact in T cell-deficient nude mice, in lymphocyte-deficient Rag-2–deficient mice, and in Rag-2⁻/⁻ double-deficient mice on either background (12, 16, 17).

Although the T cell-independent basal concentration and total number of MCps per lung are minimal, sensitization and challenge with aerosolized OVA elicits a rapid increase in the number of pulmonary MCps in both BALB/c and C57BL/6 mice (8). This recruitment is dependent on both α4 integrins, α4β1 and α4β7, and their only vascular counter-ligand in lung, VCAM-1 (8). Furthermore, the upregulation of VCAM-1 on the lung endothelium of challenged mice depends on the chemokine receptor CXCR2, which is also expressed by pulmonary vascular endothelium (18, 19). The decreased influx of MCps to the lung in CXCR2-deficient BALB/c mice was associated with a decrease in the small number of intraepithelial MCs seen in the trachea of WT mice a week later (19). These sequential findings indicated a role for the adaptive immune response in the recruitment of MCps to the lung.

In an initial focus on the BALB/c strain, we recognized that MC recruitment to lung is not dependent on the Th2-linked cytokines involved in maturation of these cells in the small intestine. Rather, we demonstrated that although MCP influx is completely prevented by mAb blockade of CD4⁺ cells at the time of challenge, the prominent components are type 2 or diverse NKT cells and IL-9 (17). In contrast, we now find that these components are not involved in the recruitment of MCps to lung in the C57BL/6 strain. Rather, the Ag-induced recruitment of MCps to the lung of sensitized C57BL/6 mice is driven by CD25⁺ T regulatory (Treg) cells and their associated cytokines, TGFβ1 and IL-10.

Materials and Methods

Animals

Male C57BL/6 6- to 10 wk-old mice were obtained from Taconic Farms (Germantown, NY). Mice deficient in IL-4 (C57BL/6/J-l4⁻/⁻), IFN-γ
Three enzymatic digestions were carried out for 1640 plus 1.25 mg/ml collagenase type 4 (Worthington, Lakewood, NJ). previously described (8, 12). Briefly, the lungs were finely chopped with heat-inactivated FCS (Sigma-Aldrich), and were processed essentially as described. The monoclonal anti–IL-3 (clone MP2-8F8), anti–IL-4 (clone MP5-20F3), anti–IFN-γ (clone XMG1.2), anti–IL-9 (clone D9302C12), anti–IL-10 (clone JES5-2A5), anti–IL-17A (clone TC11-18H10.1), anti–IL-21R clone 4A9), anti–CD4 (clone GK1.5), and the isotype-matched control IgG were obtained from BioLegend (San Diego, CA). The anti–IL-17F (clone 316016), anti–TFGβR1 (purified from chicken egg yolk IgG), and the isotype-matched control Ig were obtained from R&D Systems (Minneapolis, MN). The anti-CD25 mAb (clone PC61.5) and anti–IL-10R mAb (clone 1B1.3) or isotype-matched control mAb were generated inhouse (21, 22). All mAb were sodium azide free.

Ab blocking and cell depletion

The monoclonal anti–IL-3 (clone MP2-8F8), anti–IL-4 (clone 11B11), anti–IL-6 (clone MP5-20F3), anti–IFN-γ (clone XMG1.2), anti–IL-9 (clone D9302C12), anti–IL-10 (clone JES5-2A5), anti–IL-17A (clone TC11-18H10.1), anti–IL-21R clone 4A9), anti–CD4 (clone GK1.5), and the isotype-matched control IgG were obtained from BioLegend (San Diego, CA). The anti–IL-17F (clone 316016), anti–TFGβR1 (purified from chicken egg yolk IgG), and the isotype-matched control Ig were obtained from R&D Systems (Minneapolis, MN). The anti-CD25 mAb (clone PC61.5) and anti–IL-10R mAb (clone 1B1.3) or isotype-matched control mAb were generated inhouse (21, 22). All mAb were sodium azide free. For blocking, mice were injected i.p. with 100 μg mAb 5 min before each aerosol challenge, unless otherwise indicated. To block IL-10R, the mAb was injected i.p. at a dose of 350 μg per injection. To deplete CD45+ cells, 1.0 mg anti-CD25 mAb or isotype-matched control Ig was injected i.p. prior to each aerosolized OVA challenge. For depletion of CD4+ cells, 500 μg mAb was injected i.p. on days 15 and 18 of the above protocol.

Flow cytometry

For flow cytometry, we used FITC-conjugated anti–CD4 (RM4-5, BD Biosciences, San Jose, CA), FITC-conjugated anti–CD8a (53-6.7, BD Biosciences), allophycocyanin-conjugated anti–CD25 (PC61.5, eBioscience, San Diego, CA), PE-conjugated anti–Foxp3 (FJK-16s, eBioscience), FITC-conjugated anti–TGFβRII (FAB532P, R&D Systems), PE-conjugated anti–FceRIα (MAR-1, BioLegend), and PE-conjugated anti–TGFRβII (FAB532P, R&D Systems). Cellular staining was performed as previously described (19).

MNC preparation and MCP assessment

Mice were euthanized, the lungs were perfused with 10 ml HBSS administered via the right ventricle, and both lungs and spleen were harvested. Lung and spleen tissues were placed separately in 20 ml RPMI 1640 containing 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin, 2 mM l-glutamine, 0.1 mM nonessential amino acids, and 10% heat-inactivated FCS (Sigma-Aldrich), and were processed essentially as previously described (8, 12). Briefly, the lungs were finely chopped with scalpels and transferred to 50-ml plastic tubes with 20 ml complete RPMI 1640 plus 1.25 mg/ml collagenase type 4 (Worthington, Lakewood, NJ). Three enzymatic digestions were carried out for 1640 plus 1.25 mg/ml collagenase type 4 (Worthington, Lakewood, NJ). The undigested tissue clumps were collected after each digestion and washed in complete RPMI 1640. The number of viable cells was determined by trypsin blue dye exclusion on a hemocytometer. The cells were serially 2-fold diluted in complete RPMI 1640, and 100 μl of each dilution was added to each well of standard 96-well flat-bottomed microtiter plates (Corning, Corning, NY). Twenty-four wells were plated for each cell concentration. Lung MNCs were plated at concentrations beginning at 20,000 cells/well. Then, each well received 100 μl gamma-irradiated (30 Gy) splenic feeder cells plus cytokines (mouse rIL-3 at 20 ng/ml and mouse recombinant stem cell factor at 20 ng/ml). The cultures were placed in humidified 37°C incubators with 5% CO2 for 10–12 d, and wells containing MNC colonies were counted with an inverted microscope. The MNC colonies were easily distinguished as large colonies of nonadherent, small- to medium-sized round cells (11, 23). The MNC concentration is calculated as the number of MNCs isolated from the tissue. The number of lung MNCs per mouse is derived by multiplying the concentration of MNCs by the number of MNCs obtained from the lung.

Flow cytometry showed that the administration of the CD4 blocking mAb just before and during the challenges reduced the number of CD4+ T cells in the lung MNC population from 11.3% in mice treated with isotype-matched control Ig to undetectable levels. Whereas the MNCs of sensitized C57BL/6 mice and their C57BL/6 controls, as well as TGFRβRII (B6.Cg-TgCd4-TGFBRII(1685/j) mice and their C57BL/6 controls were obtained from The Jackson Laboratory (Bar Harbor, ME). Jo18-deficient mice were kindly provided by Dr. M. Tani-guchi (20) (RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan) and bred in-house.

**OVA sensitization and OVA aerosol challenge protocol**

Individual groups of two to three mice received i.p injections of 10 μg OVA (Sigma-Aldrich, St. Louis, MO) adsorbed to 1 mg alum (Pierce, Rockford, IL) in 200 μl sterile HBSS on days 0 and 7. Mice were challenged with 2% aerosolized OVA in HBSS for 30 min per day using a PARI nebulizer (Midlothian, VA). For most experiments, mice were challenged on days 17–19 and were euthanized on day 20 for analysis. Histologic analysis of the lungs at this time point shows no increase in mature MCs and thus the assay of lineage progenitors by limiting dilution and clonal expansion is directed to tissue MCps (8, 19). Assessment for mature MCs in this model requires an interval of ~7 d after challenge (19). In addition, the limited 3 d of challenge allows for both enhancement of the MCp response and inhibition depending on the respective mutation (17).

**Results**

**CD4+ T cells are required for Ag-induced pulmonary MCP recruitment in sensitized C57BL/6 mice**

The recruitment of MCps to the lung with aerosolized Ag challenge of C57BL/6 mice required prior sensitization with the Ag, thereby implying a role for T cells. The most direct approach to identifying whether CD4+ T cells were required at the time of challenge in sensitized mice was by mAb blockade of CD4+ cells as compared with an isotype control Ig. Flow cytometry showed that the administration of the CD4 blocking mAb just before and during the challenges reduced the number of CD4+ T cells in the lung MNC population from 11.3% in mice treated with isotype-matched control Ig to undetectable levels. Whereas the MNCs of sensitized C57BL/6 mice and their C57BL/6 controls, as well as TGFRβRII (B6.Cg-TgCd4-TGFBRII(1685/j) mice and their C57BL/6 controls were obtained from The Jackson Laboratory (Bar Harbor, ME). Jo18-deficient mice were kindly provided by Dr. M. Tani-guchi (20) (RIKEN Research Center for Allergy and Immunology, Kanagawa, Japan) and bred in-house.

### Statistical analysis

All experiments were repeated at least three times unless otherwise indicated. Data are expressed as the mean + SE when derived from three or more values. Significance was determined with a two-tailed Student t test where three or more values were available for analysis. The p < 0.05 was considered significant.

**FIGURE 1.** Recruitment of MCps to lung is prevented by mAb blockade of CD4+ cells. Sensitized C57BL/6 mice were given isotype-matched control IgG or anti-CD4 before and during the time of challenge and either were not challenged or were challenged with aerosolized OVA as indicated. The values are means ± SE of the concentration of MNCs derived from three or more values. Significance was determined with a two-tailed Student t test where three or more values were available for analysis. The p < 0.05 was considered significant.
the total number of lung MCps per mouse in mice treated with the control Ig increased by 5.5- and 10.8-fold with challenge, respectively, there was no increase in MCP concentration or in the total number of lung MCps in mice treated with the anti-CD4 (Fig. 1). These findings indicated a CD4⁺ cell requirement beyond the sensitization step but did not reveal selectivity for MCps because the recruitment of MNCs was also significantly prevented in the anti-CD4-treated mice.

To seek a role for polarized T cells in MCp recruitment with aerosolized Ag challenge, we used mainly immunologic approaches to eliminate issues of sensitization. We found no inhibition of MCp recruitment to lung with mAb blockade of IL-4, IFN-γ, IL-17A, or IL-17F at the time of challenge, suggesting that the critical CD4⁺ cell was not a classic Th1, Th2, or Th17 phenotype (Table I). We observed no inhibition with mAb blockade of IL-3 or IL-9, which are known to mediate survival and/or proliferation of immature MCs (24, 25). We also saw no effect on MCp recruitment in mice lacking all NKT cells (CD1d-deficient mice) or the invariant NKT cell subset (Jα18-deficient mice). The lack of a requirement for IL-9 and CD1d-restricted NKT cells distinguished the response in the C57BL/6 strain from the response of the BALB/c strain, which also was abolished by mAb depletion of CD4⁺ cells (17).

With our current protocol, the concentration of MCp/10⁶ MNCs and the total number of lung MCps per mouse were consistently greater in BALB/c mice than in C57BL/6 mice, although the basal levels were also higher in BALB/c mice, as noted previously (8). To evaluate the current level of recruitment in these two strains, we calculated the average response obtained in 10 separate experiments performed in these two strains over the past year. In sensitized BALB/c mice, the mean concentration of lung MCp/10⁶ MNCs was 75 ± 7 and the total number of lung MCps per mouse was 121 ± 7 (mean ± SE, n = 10). After three aerosolized Ag challenges, these values increased to 420 ± 46 MCp/10⁶ MNCs and 1217 ± 183 total lung MCps per mouse. By comparison, in sensitized C57BL/6 mice there were 43 ± 5 lung MCp/10⁶ MNCs and 59 ± 12 total lung MCps per mouse, and these values

![FIGURE 2. Recruitment of MCps to lung is inhibited by mAb blockade of TGFβ or absence of TGFβRII on T cells. A. Sensitized C57BL/6 mice were given isotype-matched control IgG or anti-TGFβ1 mAb at the time of challenge and either were not challenged or were challenged with aerosolized OVA as indicated. The values are means ± SE of the concentration of MCp/10⁶ MNCs (top panel), the number of lung MCps per mouse (middle panel), and the number of lung MNCs per mouse (bottom panel) from seven separate experiments with 14, 14, and 18 mice per treatment group, respectively. **p < 0.05; ***p < 0.001. B, C57BL/6 WT and TGFβRII−CD4⁺ dominant negative transgenic mice were sensitized, and half of each group was challenged with aerosolized OVA as indicated. The values are means ± SE of the concentration of MCp/10⁶ MNCs (top panel), the number of lung MCps/mouse (middle panel), and the number of lung MNCs/mouse (bottom panel) from three separate experiments with six, eight, and eight mice per group, respectively. **p < 0.01.](http://www.jimmunol.org/)

### Table I. Evaluation of cytokine requirements for MCp recruitment to the lung in sensitized and challenged C57BL/6 mice

<table>
<thead>
<tr>
<th>Factor or Cell Tested</th>
<th>Knock-Out Strain or mAb</th>
<th>Mean % of Control Lung MCp/10⁶ MNC</th>
<th>Mean % of Control Lung MCp/Mouse</th>
<th>No. of Experiments (No. of Mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ (Th1)</td>
<td>αIFN-γ Ab</td>
<td>196</td>
<td>569</td>
<td>1 (2)</td>
</tr>
<tr>
<td>IL-4 (Th2)</td>
<td>αIL-4 Ab</td>
<td>193</td>
<td>448</td>
<td>1 (2)</td>
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<tr>
<td>IL-17 (Th17)</td>
<td>αIL-17A Ab</td>
<td>135</td>
<td>202</td>
<td>1 (2)</td>
</tr>
<tr>
<td>IL-3</td>
<td>αIL-3 Ab</td>
<td>118</td>
<td>357</td>
<td>1 (1)</td>
</tr>
<tr>
<td>IL-9</td>
<td>αIL-9 Ab</td>
<td>75</td>
<td>102</td>
<td>2 (4)</td>
</tr>
<tr>
<td>NKT</td>
<td>CD1d⁻/⁻ (N13)</td>
<td>128</td>
<td>148</td>
<td>2 (4)</td>
</tr>
<tr>
<td></td>
<td>Jα18⁻⁻⁻⁻ (N12)</td>
<td>114</td>
<td>181</td>
<td>2 (4)</td>
</tr>
</tbody>
</table>

*All mice listed were on a C57BL/6 background and were sensitized and challenged. The increment in concentration (MCp/10⁶ MNCs) over baseline was >5-fold in the different experiments. For mAb inhibition, mice were injected with 100 μg of the indicated mAb before each challenge.

The values represent the influx of MCps as measured by lung MCp/10⁶ MNCs or by total lung MCps per mouse as a percent of the values obtained from control mice treated in parallel. The controls were WT mice for deficient strains and WT mice treated with isotype-matched IgG for WT mice receiving blocking mAb.

The values are the mean of single mice in separate experiments or separate mice in one experiment. Number of experiments and in parentheses the number of mice evaluated.
increased after Ag challenge to 212 ± 30 MCp/10^6 MNCs and 489 ± 73 total lung MCps per mouse (mean ± SE, n = 10). Thus, in the BALB/c strain, there was a 5.6-fold increase in the concentration of the MCps in the MNCs isolated from the lung after challenge and a 10-fold increase in the number of lung MCps per mouse, which is similar to the 4.9-fold increase in the concentration of MCps in the MNC population and the 8.3-fold increase in total number of lung MCps per mouse observed in the C57BL/6 mice. The increased concentration of MCp/10^6 MNCs indicates a selective recruitment process for these cells at this early time point in both strains.

Ag-induced recruitment of MCps to lung requires TGFβ1

The dependence on CD4^+ cells at the time of challenge and lack of attenuation by the signature cytokines of Th1, Th2, and Th17 or by elimination of NKT cells suggested that another CD4^+ T cell was the critical component in this response. Because we had not assessed for Treg cells, we began with a mAb blockade to TGFβ1. Blockade of TGFβ1 with mAb at the time of Ag challenge in sensitized WT mice significantly reduced the concentration of lung MCp/10^6 MNCs and the total number of lung MCps per mouse by 56.3 and 45.7%, respectively, as compared to mice treated with isotype-matched control Ig (Fig. 2A). The reduction of MCps within the MNC population was selective as the increase in harvested lung MNCs over unchallenged controls was similar in mice treated with isotype-matched control Ig or with mAb to TGFβ1.

To seek support for this finding, we evaluated TGFβRII-CD4^+ dominant negative transgenic mice that lack expression of TGFβRII on all CD4^+ and CD8^+ T cells and thus lack Treg cells (26). The basal concentration of lung MCp/10^6 MNCs and the total lung MCps per mouse were slightly lower in the TGFβRII-deficient mice and the number of lung MNCs higher as compared with WT controls, but none of these changes reached a level of significance. The increase in MCp/10^6 MNCs was reduced by 67.8% and for total lung MCps per mouse by 56.1% in TGFβRII-deficient mice as compared with the response in sensitized and challenged WT mice treated in parallel (Fig. 2B). The increase in recruited MNCs with Ag challenge was not significantly different in the WT and TGFβRII-deficient mice. Taken together, the findings for mAb blockade of TGFβ1 in WT mice and for TGRβRII-deficient mice favor a role for Treg cells in Mcp recruitment in the sensitized and challenged C57BL/6 strain.

Ag-induced recruitment of MCps to lung does not require IL-6

TGFβ is known to drive Th17 development in the presence of IL-6. Although we found no inhibition of MCp recruitment in mice treated with anti–IL-17A or anti–IL-17F, we further evaluated this possibility by assessing recruitment in C57BL/6 mice given anti–IL-6 at the time of challenge and in IL-6–deficient mice. The increases in pulmonary MCp concentration, in total number of lung MCps per mouse, and in pulmonary MNCs in WT mice treated with anti–IL-6 at the time of challenge were similar to those in WT mice given isotype-matched control Ig and treated in parallel (Fig. 3A). Similarly, there was no inhibition of the recruitment of MCps after challenge of sensitized IL-6–deficient mice as compared with WT C57BL/6 mice treated in parallel (Fig. 3B). Thus, these findings supported a role for TGFβ-dependent Treg cells rather than Th17 cells in driving the pulmonary recruitment of MCps with Ag challenge of sensitized C57BL/6 mice.

**FIGURE 3.** Recruitment of MCps to lung is not dependent on IL-6. *A*, Sensitized C57BL/6 mice were given isotype-matched IgG control or anti–IL-6 mAb at the time of challenge and either were not challenged or were challenged with aerosolized OVA as indicated. The values are means ± SE of the concentration of MCps/10^6 MNC (top panel), the number of lung MCps/mouse (middle panel), and the number of lung MNCs/mouse (bottom panel) from two separate experiments with four to five mice per treatment group. *B*, C57BL/6 WT and IL-6−/− mice were sensitized, and half of each group of mice was challenged with aerosolized OVA as indicated. The values are the mean ± SE of the concentration of MCp/10^6 MNCs (top panel), the number of lung MCps per mouse (middle panel), and the number of lung MNCs per mouse (bottom panel) from four separate experiments with 8, 6, 9, and 10 mice per group, respectively.
CD25+ T cells regulate Ag-induced MCp recruitment to the lung of C57BL/6 mice

To directly address a possible key role for Treg cells in MCp recruitment, we administered anti-CD25 mAb or an isotype-matched control Ig at the time of each aerosolized Ag challenge. This mAb depleted or attenuated Treg cell function in the lung when treatment was conducted at the time of sensitization and again before challenge in another Ag-induced pulmonary inflammation model (22), and we observed that it increased IgE responses in BALB/c mice treated before both sensitization and challenge using our procedure (17).

Treatment of sensitized C57BL/6 mice with anti-CD25 mAb during the challenge phase resulted in a 77.2% reduction in the increase of MCp/10⁶ MNCs and an 81.3% reduction in the increase of the total lung MCps per mouse but did not alter the increase in the influx of MNCs (Fig. 4). There was a significant ~2-fold increase (p < 0.05, n = 4 mice) in the levels of total serum IgE in the mice treated with anti-CD25 as compared with the control mice, 2.0 ± 0.4 versus 1.1 ± 0.2, respectively (mean ± SEM). Thus, the reduction of MCps within the MNC population was selective. In contrast, this same protocol did not reduce the recruitment of pulmonary MCps in the BALB/c strain (data not shown).

IL-10R blockade and genetic deficiency of IL-10 reduce Ag-induced recruitment of MCps to the lung

IL-10 along with TGFβ is involved in the development and immunosuppressive functions of CD25+ Treg cells (21, 27, 28). Therefore, we assessed the involvement of IL-10 using both immunologic and genetic approaches. The administration of anti–IL-10R mAb at the time of aerosolized Ag challenge of sensitized C57BL/6 mice caused a significant inhibition of the recruitment of MCps with a 69.6% reduction in the increase of MCp/10⁶ lung MNCs, and an 80.1% reduction in the increase of the total lung MCps per mouse without altering the influx of MNCs (Fig. 5A). These findings indicated a selective effect of anti–IL-10R mAb on MCp recruitment to the lung.

When we administered anti–IL-10 mAb to OVA-sensitized C57BL/6 mice at the time of Ag challenge, there was no inhibition in pulmonary MCp recruitment or in MNC influx to the lung (data not shown). The inability of the mAb JES5-2A5 to suppress the IL-10–dependent function of Treg cells has been reported by others (27). In contrast, in sensitized and challenged IL-10–deficient mice there was a significant 62.3% reduction in MCp/10⁶ MNCs, but the reduction in the total number of lung MCps per mouse of 24.6% was not significant. Furthermore, the fall in MCp concentration is not necessarily an inhibition in their recruitment because of a striking and significant increase in the number of recruited lung MNCs in the aerosolized Ag-challenged IL-10−/− mice, as compared with the WT mice treated in parallel. When we administered mAb to TGFβ to IL-10−/− mice, the increased recruitment of MNCs was curtailed. However, the effect of the mAb on the recruitment of MCps in WT mice was too great to see any additive inhibition with the combination (data not shown).

Discussion

We have used a mouse model of Ag-induced pulmonary inflammation to examine the requirements for the recruitment of MC lineage progenitors from the circulation to the lung (8). We have found consistent increments of ~5-fold in concentration of MCp/10⁶ lung MNCs and of ~9-fold in total number of lung MCps per mouse in both BALB/c and C57BL/6 mice with the same protocol of OVA/alum i.p. sensitization and aerosolized OVA challenges, thereby indicating that this process is not strain dependent. Although the accumulation of MCps in the lung also may involve some proliferation, cytoprotection, and/or impaired emigration of these cells, the time course and magnitude of the increase, and its dependence on MCp α4 integrins and VCAM-1 favor recruitment as the principal response (8, 19). The fact that MC lineage progenitors are also increased in the peripheral blood of patients with asthma implies that their participation is common to the inflamed airways response in both humans and mice (7). Although the dependence of MCp recruitment on the integrity of MCp α4 integrins and pulmonary VCAM-1 was anticipated as essential for their transendothelial migration, we were surprised by the strict nature of a CD4+ cell requirement at the time of challenge in previously sensitized mice. In sensitized mice of both strains, the increases in lung MCps and lung MNCs were fully inhibited by blockade with anti-CD4 at the time of aerosolized Ag challenge.

The nature of the CD4+ cell types involved in expansion of the lung MCp concentration (per 10⁶ MNCs) or the number of MCps per mouse was established in BALB/c and now in C57BL/6 mice by blocking the cytokines required for their development and/or function. Our analyses of the CD4+ cell types required for MCp recruitment have emphasized mAb blockade in sensitized WT mice at the time of Ag-inhalation challenge and used genetic approaches for support of any positive findings. In the absence of conditional mutant strains, a genetic approach can be limited by developmental effects, which can either dampen the sensitization step or allow spontaneous autoimmune inflammation (29, 30). In the BALB/c strain, both mAb blockade and genetic approaches excluded a role for classic Th2 and Th1 cells and for the cytokines associated with Th17 or Treg cell development and/or actions (17). Instead, these approaches established that ~70% of the MCps accumulated after challenge were dependent on the function of IL-9 and CD1d-restricted type 2 NKT cells but not Jα18+.

![FIGURE 4. Recruitment of MCps to lung is prevented by mAb blockade of CD25+ cells. Sensitized C57BL/6 mice were given isotype-matched IgG control or anti-CD25 mAb at the time of challenge and either were not challenged or were challenged with aerosolized OVA as indicated. The values are the means ± SE of the concentration of MCp/10⁶ MNCs (top panel), the number of lung MCps per mouse (middle panel), and the number of lung MNCs per mouse (bottom panel) from three separate experiments with six to eight mice per group. ***p < 0.0001.](http://www.jimmunol.org/brv/article-pdf/168/15/5922/2783304/168-5922.pdf)
Recruitment of MCps is inhibited by mAb blockade of IL-10R or absence of IL-10. A, Sensitized C57BL/6 mice were given 350 μg isotype-matched IgG control or anti-IL-10R mAb at the time of challenge and either were not challenged or were challenged with aerosolized OVA as indicated. The values are the means ± SE of the concentration of MCp/10⁶ MNCs (top panel), the number of lung MCps per mouse (middle panel), and the number of lung MNCs per mouse (bottom panel) from two separate experiments with five to six mice per treatment group. ***p < 0.001. B, C57BL/6 WT and IL-10⁻/⁻ mice were sensitized, and half of each group was challenged with aerosolized OVA as indicated. The values are the means ± SE of the concentration of MCp/10⁶ MNCs (top panel), the number of lung MCps per mouse (middle panel), and the number of lung MNCs per mouse (bottom panel) from three separate experiments with six, two, seven, and six mice per group, respectively. **p < 0.01.

FIGURE 5. Recruitment of MCps is inhibited by mAb blockade of IL-10R or absence of IL-10.

As Treg cells were an obvious remaining candidate for the CD4⁺ T cell involved in the recruitment of MCps in the C57BL/6 strain, we sought independent verification and turned to mAb inhibition with anti-CD25. Indeed, the administration of blocking mAb to CD25 at the time of aerosolized Ag challenge in sensitized WT mice provided almost total inhibition of MCp recruitment to lung whether assessed as the concentration of MCp/10⁶ MNCs or as the total number of lung MCps per mouse. The blocking with mAb to CD25 did not impair the CD4⁺ T cell-dependent inflammatory response as defined by the numbers of MNCs obtained from these lungs. Furthermore, the CD25 blocking at the time of challenge increased the level of total serum IgE indicating an enhancement of Th2 cell function in association with blockade of MCp recruitment. The anti-CD25 treatment of naive mice did not reduce the basal T cell-independent concentration or numbers of MCps in lungs suggesting the absence of a direct effect (T. Jones and M. Gurish, unpublished data). Taken together our findings indicate a preferential action of anti-CD25 on Tregs in this protocol for MCp recruitment, although we cannot exclude that a proximate TGFβ- and/or CD25-dependent signal could favor both Treg development and/or function and MCp recruitment in parallel. These findings further highlighted the differences in the two strains, as neither anti-CD25 nor anti-TGFβ1 blockade had any effect on MCp recruitment in sensitized and challenged BALB/c mice (17) (T. Jones and M. Gurish, unpublished data).

Our evidence for a role for IL-10 in MCp recruitment is indirect and depends wholly on the finding that mAb to IL-10R at the time of Ag challenge selectively suppressed MCp recruitment in sensitized C57BL/6 mice. These findings were not confirmed in IL-10⁻/⁻ mice or with blockade of IL-10 in WT mice. In the IL-
10-deficient mice, the >2-fold expansion of the MNC population as compared with WT mice after sensitization and challenge may account for the fall in MCP concentration without a change in total lung MCP number per mouse. Thus, these data do not provide an independent confirmation of a role for IL-10. We also did not inhibit the MCP influx by treatment of sensitized WT mice with the anti–IL-10 mAb JESS-2A5 at the time of challenge. Others also have reported an inability to demonstrate a role for IL-10 in Treg cell-related functions based on suppression of its presumed action by this mAb (27) and have relied on the activity of the anti–IL-10R to demonstrate an IL-10–dependent effect. Kim et al. (38), in a model of OVA-induced airway inflammation, found that administration of anti–IL-10R mAb increased inflammatory changes in the lung as well as the levels of IL-4 and IL-5 in the lung tissue and of OVA-specific IgE in the serum; and they attributed the proinflammatory actions of anti–IL-10R mAb to its ability to suppress IL-10 function in the lung tissue. Furthermore, Herbert et al. (21), using anti–IL-10R in combination with anti-TGFβ1 blocking Ab in an acute schistosomiasis model, demonstrated a redundancy in protective mechanisms dependent on both IL-10 and TGFβ1. Thus, we also indirectly implicate IL-10 in our model based on inhibition of MCP recruitment with mAb to IL-10R.

In the aggregate, our data for suppression of MCP recruitment by mAb blockade of CD25 and/or TGFβ1 in sensitized and challenged WT mice strongly support a role for Treg cells in MCP recruitment, whereas the requirement for functional TGFβRII–expressing CD4+ cells provides a mechanism. As TGFβ1 has been shown to be chemotactic for immature bone marrow–derived mouse MCs and mature rat peritoneal MCs in vitro (39), such an additional contribution to Treg-dependent regulation of MCps in vivo is possible. Our data supporting a role for IL-10 in the mechanism of the Treg cell effect are incomplete, but that does not diminish the importance and novelty of the finding that Treg cells are positive regulators of MCp recruitment, a function that would be distinct from their downstream role to suppress the full inflammatory response with remodeling of the lung (40). Our report of a role for Treg cells in orchestrating cellular trafficking is not the first. In a model of mucosal infection by the HSV, Lund et al. (41), found that Treg cells could facilitate early local protective responses by driving the trafficking of several types of immune cells, NKT cells, dendritic cells, and effector T cells, from the draining lymph nodes into the peripheral tissue. The authors further attributed this control by Treg cells to their ability to modulate the chemokine gradients that control movement of effector T cells from the draining lymph nodes. Moreover, Venet et al. (42), using a CD4-dependent model of acute lung injury and IL-10R blockade in C57BL/6 mice, showed that a specific population of CD4+ CD25+ IL-10–dependent Treg cells was responsible for neutrophil recruitment to the lung.

Our studies on the early Ag-induced influx of MCps to the lung in BALB/c and C57BL/6 mice have uncovered both common and unique requirements. Both strains show a similar fold increase in the number of MCps recruited to the lung of sensitized mice after three consecutive days of aerosolized Ag challenge and a dependence on the α4 integrins of the MCps interacting with VCAM-1 on the vascular endothelium (8). We also show a strong requirement for a CD4+ T cell during their accumulation in lung, and this requirement is not due to the activation of the classic Th subsets of Th1, Th2, or Th17 as defined by blockade of their signature cytokines and certain companion cytokines. Instead, we found an unexpected dependence on other CD4+ T cell phenotypes. Importantly, the cytokines that we have identified as critical for MCp recruitment in each strain are not those believed to be involved with maturation of MCps to a mucosal phenotype in gut and thus implicated for lung maturation in the respective strains. Using helmint infection, this group includes for the small intestine, IL-3, and IL-4 in BALB/c mice (43) and IL-4 and IL-9 in C57BL/6 mice and IL-9 in the lung of C57BL/6 mice (44, 45). With an inhibition of the role of IL-9 and NKT for BALB/c mice, and TGFβ1 and Treg for C57BL/6 mice in the recruitment of MCps, we can now evaluate other mutant mouse strains and blocking mAbs to recognize if the same or different cytokines and T cell subsets are involved in the maturation step to mucosal MCs. These studies require a robust appearance of mucosal MCs obtained both by more than three challenges and by a >7 interval for assessment by microscopy of mature MCs as observed by others noting abundant MC in association with airways remodeling (9, 10).

The BALB/c and C57BL/6 strains have significant differences in inflammatory responses as noted by others with different models that did not focus on mast cells. Previous studies of Ag-induced pulmonary hyper-responsiveness to methacholine have identified the eosinophil as a critical component in the C57BL/6 strain but not in the BALB/c strain (46–48). Differences in chromosome 11 regions coding for the T cell Ig and mucin domain-containing molecules, and T cell Ig and mucin domain-containing–1, in particular, have been related to the more prominent Th2 response in the BALB/c strain as compared with the Th1-prone C57BL/6 or the DBA/2 mice (49, 50). The difference in survival of these strains when infected with the intracellular pathogen Listeria monocytogenes highlights the tendency toward Th2 responses in the BALB/c mice versus protective Th1 responses in the C57BL/6 mice (51). Of particular relevance is the finding that infection of the C57BL/6 strain but not the BALB/c strain with Sendai virus elicits a delayed and second inflammatory response that is NKT cell-dependent and due to the appearance of alternatively activated macrophages (52, 53). Our findings now reveal that strain differences between the C57BL/6 and BALB/c strains also translate into entirely different mechanisms for CD4+ T cell–mediated regulation of pulmonary MCP recruitment to the lung in a model of allergic inflammation.

Disclosures
The authors have no financial conflicts of interests.

References
Treg CONTROL OF MCp RECRUITMENT


