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Subset-Specific Reductions in Lung Lymphocyte Accumulation Following Intratracheal Antigen Challenge in Endothelial Selectin-Deficient Mice\textsuperscript{1,2}

Jeffrey L. Curtis,\textsuperscript{3,4,\*8} Joanne Sonstein,* Ronald A. Craig, † Jill C. Todt,* Randall N. Knibbs, † Timothy Polak,* Daniel C. Bullard,\textsuperscript{\*10} and Lloyd M. Stoolman\textsuperscript{3,18}

We previously demonstrated induction and expression of CD62E and CD62P in the lungs of mice primed and then challenged with intratracheal (i.t.) SRBC, The current study examined accumulation of endogenous lymphocytes in the lungs of endothelial E- and P-selectin-deficient (E\textsuperscript{−}P\textsuperscript{−}) mice after i.t. SRBC challenge. Compared with syngeneic wild-type (wt) mice, E\textsuperscript{−}P\textsuperscript{−} mice showed an 85–95% decrease in CD8\textsuperscript{T} T cells and B cells in the lungs at both early and late time points. In contrast, CD4\textsuperscript{T} cell accumulation was reduced by ~60% early, but equivalent to wt levels later. Surprisingly, many γδ T cells were found in lungs and blood of E\textsuperscript{−}P\textsuperscript{−} mice but were undetectable in the lungs and blood of wt mice. Absolute numbers of peripheral blood CD4, CD8, and B lymphocytes in E\textsuperscript{−}P\textsuperscript{−} mice equaled or exceeded the levels in wt mice, particularly after challenge. Trafficking studies using αβ T lymphoblasts confirmed that the recruitment of circulating cells after challenge was markedly reduced in E\textsuperscript{−}P\textsuperscript{−} mice. Furthermore, Ag priming occurred normally in both the selectin-deficient and wt mice, because primed lymphocytes from both groups transferred Ag sensitivity into naive wt mice. Lung production of mRNA for six CC and two CXC chemokines after challenge was equivalent by RT-PCR analysis in wt and E\textsuperscript{−}P\textsuperscript{−} mice. Therefore, reduced lung accumulation of αβ T cells and B cells in E\textsuperscript{−}P\textsuperscript{−} mice did not result from reduced delivery of circulating lymphocytes to the lungs, unsuccessful Ag priming, or defective pulmonary chemokine production. Selectin-dependent lymphocyte recruitment into the lungs following i.t.-SRBC challenge is subset specific and time dependent. \textit{The Journal of Immunology}, 2002, 169: 2570–2579.

Recruitment of lymphocytes into lung parenchyma is centrally involved both in host defense against a wide variety of pathogens and in the development of immunologic lung diseases. Lymphocyte recruitment is a multistep process involving adhesive interactions between lymphocytes and a variety of cell adhesion molecules (CAMs)\textsuperscript{5} on vascular endothelial cells (1). Direct in vitro and intravital study of leukocyte-endothelial adhesive interactions established the sequential roles of individual adhesion receptors to recruitment under high shear flow conditions and identified the physiologically relevant adhesion receptors expressed during inflammatory responses in selected sites. However, many microvascular beds and disease processes are not amenable to in vitro modeling or intravital study. Consequently, the contributions of adhesion receptors must be evaluated in vivo using experimental models of inflammation and immunity.

Such in vivo analysis is particularly important for the lungs, which possess a unique dual vascular supply that impacts directly on leukocyte recruitment. The pulmonary circulation carries the entire output of the right ventricle to the extensive capillary network investing the alveoli. The bronchial circulation branches off the aorta and supplies oxygenated blood directly to the peribronchial and submucosal tissues (2). Depending on the inciting agent and mode of entry, the pulmonary inflammatory response may be alveolar, bronchial and peribronchial, or a combination of both. Recruitment into alveoli generally occurs across capillaries where changes in leukocyte distensibility can arrest cells directly (3–6), although the independence of this process from adhesion molecules has been contested recently (7, 8). In contrast, recruitment into the bronchial wall and peribronchial tissues most likely involves larger vessels, especially intact postcapillary venules (6, 9–12), and hence may use the tethering receptors that mediate immune reactions at other sites with a similar microvascular organization.

To test this hypothesis, we chose an established model of CD4-dependent alveolar and peribronchial lymphocyte recruitment in mice sensitized to SRBC and then challenged with intratracheal (i.t.) SRBC (13–18). Initial studies from our group and others showed that CD62E, CD62P, and VCAM are expressed on the lung microvasculature in sensitized mice after challenge with i.t. SRBC (19, 20). All three receptors are expressed throughout the...
Materials and Methods

Monoclonal Abs

The following epitopes were assessed by three-color flow cytometry analyses using directly conjugated mAbs (BD PharMingen, San Diego, CA) (clone isotopes and fluorochromes are indicated in parentheses): CD4 (RM4-5; rat IgG2a; CyChrome), CD8 (53-6.72; rat IgG2a; FITC), CD19 (RM4-4; rat IgG2a; CyChrome), CD3 (50.7; hamster IgG2a; FITC), CD14 (Gp40; rat IgG1; FITC), and Mac-3 (M38/4; rat IgG1; FITC). The fluorochromes were chosen based on the fluorescence intensities for each epitope and the level of background observed with isotype-matched control reagents.

Mice

Mice containing null mutations for both E- and P-selectin (E−/− mice) were generated by gene targeting in 129/Sv embryonic stem cells, as previously described (22). E−/− mice used in this study had been backcrossed to C57BL6 mice for five generations. Pathogen-free female C57BL6 mice for use as controls were purchased at 7–8 wk from Charles River Laboratory (Wilmington, MA). Although early reports of E−/− mice (also known as Ly-6G) are expressed by murine neutrophils and eosinophils; consequently, the positive cells in the lymphocyte gate using this flow cytometer are most likely degranulated granulocytes. These cells accounted for a variable but significant percentage of the events in the gated region (up to 25%), particularly in the lung specimens. Therefore, the lymphocyte subset percentages used in the following calculations were corrected for the number of Gr-1+ cells contaminating the analysis gate. This type of adjustment was not performed in previous studies using this model system (13, 14, 16, 18, 19, 24, 25), because the light scatter characteristics of the flow cytometers used previously did not result in such contamination of the lymphocyte gates (24). The means of the replicate determinations for each subset were then used to calculate the absolute number of cells as detailed below. The absolute lymphocyte count in each specimen was calculated from the white blood cell count and morphologic differential count. The white blood cell counts were performed with a hemocytometer as previously described (26). Morphologic differential counts of the granulocytes, monocytes/macrophages, and lymphocytes in each specimen were performed on stained filter preparations as previously described (26). The number of lymphocytes in each subset was then calculated from the absolute lymphocyte counts (white blood cell count × percent of lymphocytes from the morphologic differentials), and the lymphocyte subset percentages as measured by flow cytometry. The results for each animal in a cohort were then used for the statistical analyses reported in the figures and tables.

Recruitment assay

Lymph node T cells from normal wild-type (wt) mice were activated using immobilized anti-CD3 mAb, and their numbers were expanded in IL-2 to produce T lymphoblasts, as previously described (16). After 5 days of expansion, T lymphoblasts were labeled with 5-chloromethylfluorescein diacetate (CMFDA) and transferred by tail vein injection into primed recipients, consisting of either wt or E−/− mice, which had been i.t. Ag challenged 4 or 7 days earlier.

Adoptive transfer assay

To confirm the capacity of T cells of E−/− mice to be Ag primed in vivo, in one experiment splenocytes from E−/− donor mice or wt donor mice that had been primed with 1 × 106 SRBC 6 days previously by the i.p. route were transferred to unprimed wt recipient (2 × 107 spleenocytes per recipient mouse in 0.2 ml PBS by the i.v. route). Subsequent Ag-driven accumulation of lung lymphocytes was assayed in BAL 4 days after i.t. SRBC challenge.

Isolation of RNA

Lungs were homogenized in 2 ml of TRIzol reagent (Life Technologies, Gaithersburg, MD) and RNA was isolated as described in the TRIzol protocol. RNA was quantitated spectrophotometrically. To remove genomic DNA, all RNA samples were treated with DNase (DNA-free method; Ambion, Austin, TX). The integrity of individual RNA samples...
was confirmed by electrophoresing aliquots on a 2% agarose gel containing 0.5 μg/ml ethidium bromide and observing 28S and 18S rRNA bands. RNA samples were stored at −70°C.

RT-PCR detection of cytokine mRNA

Isolated RNA was reverse-transcribed to DNA and amplified by PCR as previously described in detail (17). The primer sequences used are defined in Table I. The amplification scheme was an initial 5 min at 95°C, repeated cycles of 15 s at 95°C, 20 s at 58°C, 30 s at 72°C, and a final extension period of 6 min at 72°C. Positive and negative controls were included in each assay, and the authenticity of reaction products was verified by Southern analysis using a chemiluminescent detection system (ECL; Amersham Life Science, Little Chalfont, U.K.). The amplified DNA was analyzed by electrophoresis on 1.5% agarose containing 0.5 μg/ml ethidium bromide. To quantitate cDNA bands, the ethidium bromide-stained agarose gels were photographed using Polaroid 667 film (Polaroid, Cambridge, MA), scanned using a Scan Jet I1cx (Hewlett Packard, Palo Alto, CA), and analyzed on a Macintosh PowerPC G3 computer (Apple Computer, Cupertino, CA) using the public domain National Institutes of Health Image software (version 1.6; available at http://rsb.info.nih.gov/nih-image/). Results are expressed as a ratio of OD signal for a given cytokine to that for cyclophilin in the same sample.

Statistical analysis

Data were expressed as mean ± SEM. Statistical calculations were performed using Statview and SuperANOVA programs (Abacus Concepts, Berkeley, CA) on a Macintosh PowerPC G3 computer. An unpaired Student t test (for two samples) or ANOVA (for multiple comparisons) was used to evaluate continuous ratio scale data with post hoc analysis by the Tukey-Kramer test (27). Percentage data were arcsine transformed before analysis to convert them from a binomial to a normal distribution using tables in the textbook of Zar (27). Results of RT-PCR experiments were analyzed by unpaired nonparametric Mann-Whitney test. Significant differences were defined as p < 0.05.

Results

Lung lymphocyte accumulation is altered after i.t. challenge of E−P− mice

Lung lymphocyte numbers did not differ significantly between wt control mice and E−P− mice before i.t. challenge, in either BAL (n = 6 mice per group; p = 0.08, unpaired t test) or minced lung preparations (p = 0.2) (Fig. 1). Within groups of either wt mice or E−P− mice, there was no statistical difference in total lymphocyte numbers between untreated mice and mice receiving i.p. priming only, and these two groups were pooled to derive day 0 numbers.

After i.t. SRBC challenge, lymphocyte accumulation in the airway and lung parenchyma was significantly lower in primed E−P− mice than in primed wt control mice (Fig. 1). Total lung lymphocyte numbers differed significantly between the two groups of mice at day 4 after i.t. challenge both in the BAL (63% reduction relative to wt mice) and in the minced lung preparation (44% reduction). Lung lymphocyte numbers were also lower in the minced lung preparation at day 7 (34% reduction), although this difference was not statistically significant (p = 0.11). By contrast, total numbers of BAL lymphocytes were essentially identical in the two groups of mice at day 7 after challenge (p = 0.8). Accumulation of mononuclear phagocytes did not differ significantly between the

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Table I. Primer sequences used for PCR amplification

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<tr>
<th>Mouse</th>
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<th>Synonym</th>
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<td>MCP-1</td>
<td>CCL2</td>
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<td></td>
<td></td>
<td>Antisense</td>
<td>CAGGATTGGAGCAGTAG</td>
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</tr>
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<td>Sense</td>
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<td></td>
<td>Antisense</td>
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<td></td>
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<td>Sense</td>
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<td>25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antisense</td>
<td>TGGTGTCCTTGGCCTGACTTG</td>
<td></td>
</tr>
</tbody>
</table>

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FIGURE 1. Lung lymphocyte numbers are reduced in E−P− mice after Ag challenge. Lung lymphocytes were collected by BAL (A) or from enzymatically digested minced preparation of the perfused and lavaged lungs (B) of SRBC-primed mice 4 or 7 days after i.t. SRBC challenge. ■, wt C57BL/6 mice; □, E−P− mice. Absolute lymphocyte numbers were determined as the product of total BAL cell count (by hemocytometer) and lymphocyte percentage on differential cell count of H&E-stained preparations. Note differences in scales between A and B. Data represent mean ± SEM of 6 mice per group (days 4 and 7) or 11–12 mice per group (day 0) assayed individually in at least 2 experiments. **, p < 0.01, unpaired Student t test.
two groups of mice in either BAL or minced lung preparation at any time point (data not shown).

Detailed analysis of lung lymphocyte subset distribution revealed the full extent of the abnormal lymphocyte accumulation in the selectin-deficient mice. Total leukocyte counts, leukocyte differentials (200-cell count of stained filter preparation), and lymphocyte subset differentials (flow cytometry) were performed on leukocytes recovered from the BAL, interstitial mince, and peripheral blood. The absolute counts for CD4, CD8, CD19, and γδ T cell subsets were then calculated as described in Materials and Methods. CD4+ T cells, CD8+ T cells, and B cells were reduced in both the BAL and the lung interstitium of E- selectin− mice throughout the period of maximal lymphocyte recruitment (Fig. 2). The magnitude of the decrease was subset dependent: accumulation of the CD4+ subset was reduced by 2- to 3-fold, whereas the CD8+ subset and B cells fell by 8-fold to 10-fold at the 4-day time point. The CD8+ subset and B cells remained low in both compartments at the 7-day time point. In contrast, the CD4+ subset remained low in the interstitial compartment but returned to wt levels in the BAL.

An unexpected finding was a massive increase in the recovery of γδ T cells from both lung compartments in the E- selectin− mice after i.t.-SRBC challenge (Fig. 2). Indeed, γδ T cells accounted for ~40% of all lymphocytes recovered from the BAL of E- selectin− mice at both day 4 and day 7. This subset constituted a much smaller percentage of the lymphocytes recovered from the interstitium of the E- selectin− mice, but the level remained markedly higher than that obtained from wt animals. To further investigate the significance of pulmonary γδ T cell accumulation after challenge, we compared numbers of lymphocytes in BAL and lung mince in wt mice and E- selectin− mice, both untreated and after i.p. priming only. There were no differences between the two groups of mice from either treatment state or in either lung compartment in numbers of CD4, CD8, or CD19 cells (data not shown). In comparing the untreated with the primed state within a given group of mice, slight (≤2-fold) differences, which were for the most part not statistically significant, were noted for each of these three lymphocyte subsets in both lung compartment. By contrast, γδ T cells were significantly increased in numbers in both lung compartments of untreated E- selectin− mice relative to wt mice (Table II). Similar disparity between the two groups of mice was seen after i.p. priming in BAL, but no significant difference was found between the two groups in numbers of γδ T cells recovered from lung minces after i.p. priming (Table II). Thus, E- selectin− mice had baseline increases in numbers of lung γδ T cells that further increased markedly after i.t. challenge (in E- selectin− mice, BAL γδ T cells, 1.55 ± 0.15 × 10^6 on day 4 and 2.63 ± 0.32 × 10^6 on day 7; in lung mince γδ T cells, 4.13 ± 0.04 × 10^5 on day 4 and 2.86 ± 0.05 × 10^5 on day 7).

**Endothelial selectin deficiency does not impair lung granulocyte accumulation.**

Before i.t. SRBC challenge, granulocyte numbers in E- selectin− mice were elevated 3-fold (BAL) and >7-fold (in lung mince) relative to wt control mice (Fig. 3). As with lymphocyte numbers, within groups of either wt mice or E- selectin− mice, there was no statistical difference in granulocyte numbers between untreated mice and mice receiving i.p. priming only, and these two groups were pooled to derive day 0 numbers.

After i.t. SRBC challenge, granulocyte numbers in the BAL was similar in E- selectin− mice and wt mice (p = 0.23) (Fig. 3A). However, the number of granulocytes in the minced lung preparations of the E- selectin− mice was markedly greater than in wt mice at both the 4-day (p < 0.001) and 7-day time points (p < 0.001) (Fig. 3B). As in most previous studies in this model system, we did not separate neutrophils and eosinophils in this enumeration, given that distinguishing the two types of granulocytes in the mouse is problematic. Caution should be exerted in interpreting these data. Although...
the pulmonary vasculature of both groups of mice was perfused in an identical manner, E−P− mice have previously been reported to have a marked peripheral blood leukocytosis (28). Thus, we cannot exclude the possibility that granulocyte numbers recovered by mincing lung tissue of E−P− mice were spuriously increased by incomplete removal of cells trapped in pulmonary capillaries. Supporting this possibility, histologic analysis confirmed identical overall anatomic distribution and magnitude of interstitial inflammation in the two groups of mice (data not shown), with prominent perivascular and peribronchial accumulation of mononuclear cells and granulocytes (9). Nevertheless, these data clearly indicate that lung granulocyte accumulation after Ag challenge is not impaired by absence of endothelial selectins.

Peripheral blood analysis shows a leukocytosis in E−P− mice

To assess the delivery of circulating lymphocytes to the lung, PBL counts in wt mice and E−P− mice were compared. In the absence of i.p. priming and of i.t. challenge, the numbers of CD4+ T cells and CD19+ cells were significantly higher in E−P− mice than in wt mice (Fig. 4A), and this difference increased following SRBC sensitization and i.t. challenge. At the 7-day time point, the absolute counts for these subsets were 2- to 4-fold higher in E−P− mice than in wt mice (Fig. 4B). γδ T cells were below the level of detection in both untreated wt mice and SRBC-challenged wt mice. In contrast, γδ T cells constituted ~7% and ~5% of the circulating pool in untreated and i.t.-SRBC challenged E−P− mice, respectively. Thus, the decreased accumulation of CD4, CD8, and CD19 subsets in the E−P− lungs did not result from reduced numbers of circulating cells. In contrast, the increased accumulation of γδ T cells reflected, in part, a systemic expansion of this subset in the E−P− mice.

Recruitment of wt T lymphoblasts into the lungs of E−P− mice is reduced

Steady state lymphocyte counts in the lungs during a pulmonary immune response reflect the dynamic balance between lymphocyte

Table II. γδ T cell numbers are increased in lungs of E−P− mice before IT challenge

<table>
<thead>
<tr>
<th></th>
<th>BAL (cells/mouse)</th>
<th>Lung Mince (cells/mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
<td>i.p. only</td>
</tr>
<tr>
<td>E−P− mice wt</td>
<td>11,347 ± 509b</td>
<td>32,450 ± 5,344b</td>
</tr>
<tr>
<td>wt mice</td>
<td>3,663 ± 178</td>
<td>5,662 ± 384</td>
</tr>
</tbody>
</table>

a Absolute numbers of γδ T cells in BAL and lung mince were calculated as the product of the total leukocyte count, the differential lymphocyte counts (adjusted as described in Materials and Methods), and the percentage of γδ TCR+ cells by flow cytometry. Mean ± SEM, n = 3 mice per group assayed individually.

b Significantly different, p < 0.05, unpaired t test, compared with wt mice of same treatment.

FIGURE 3. Endothelial selectin deficiency does not impair lung granulocyte accumulation. Granulocytes recovered by BAL (A) or enzymatic digestion (B) of the lungs of mice after i.p. priming only (day 0) or after i.t. SRBC challenge were enumerated as described in the legend to Fig. 1. Note the marked difference in scales between the two panels. ■, wt mice; □, E−P− mice. Data represent mean ± SEM of 6 mice per group (days 4 and 7) or 11–12 mice per group (day 0) assayed individually in at least 2 experiments. *, p < 0.05; **, p < 0.001, unpaired Student t test.

FIGURE 4. Peripheral blood analysis indicates a lymphocytosis in E−P− mice. PBL were analyzed by flow cytometry. ■, CD4 cells; □, CD8 cells; □, CD19 cells; γδ cells. Data represent mean ± SEM of 6 mice per group (day 7) or 11–12 mice per group (untreated) assayed individually in at least 2 separate experiments. **, p < 0.01, unpaired Student t test compared to wt.
accumulation of CD8

Chemokine elaboration is intact in E

Lung lymphocyte recruitment in the i.t.-SRBC model system is Ag

Chemokine elaboration is intact in E

Adoptive transfer shows T cells of E

Lymph node T cells from normal wt mice were activated using immobilized anti-CD3 mAb, and their numbers were expanded in IL-2 to produce T lymphoblasts, as previously described (16). After 5 days of expansion, T lymphoblasts were labeled with CFMFD and transferred by tail vein injection into primed recipients consisting of either wt or E

Discussion

The principal finding of this study is that selectin-mediated lymphocyte accumulation in the lungs following i.t. SRBC-challenge in primed animals is subset dependent. In the E

cells are problematic in the mouse; however, our data indicate that both constitutive and inflammation-induced trafficking of this subset into the lung is not invariably selectin dependent in the mouse. The attenuated lymphocyte accumulation in the E

These findings extend previous work from our group showing that i.t.-SRBC challenge of sensitized mice induced P- and E-selectin expression on the pulmonary vasculature, increased the number of circulating T cells expressing selectin-ligands, and recruited selectin ligand-positive circulating T cells into the lung (16, 19, 21). The current results are in agreement with our previous demonstration that lung lymphocyte recruitment is partially dependent on binding to endothelial cell selectins (16). That conclusion was based on analysis of in vitro activated T lymphoblasts derived from gene-targeted mice lacking fucosyltransferase VII (FucT VII

FIGURE 5. Recruitment of T lymphoblasts into lungs of E

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more directly by analyzing lung accumulation of individual lymphocyte subsets in response to i.t. SRBC in sensitized FucT VII−/− mice themselves. The current results are also consistent with those of Pan et al. (31), who found that ganglioside analogs of the endothelial selectin ligand sialyl-Lewis X inhibited lung inflammation in a murine model of hypersensitivity pneumonitis in response to *Saccharopolyspora rectivirgula*. Selectin-mediated Th1 lymphocyte recruitment has been documented in cutaneous and peritoneal immunologic lesions as well (32–34). However, selectins were not required for lymphocyte recruitment during immunologic reactions in the colon (35), liver (36), and brain (37). Indeed, Bartholdy et al. (38) found that meningeal accumulation of CD8+ T cells and CD8-mediated viral clearance from multiple organs was identical in E−/− and wt mice lethally infected with lymphocyte choriomeningitis virus.

These seemingly disparate results may reflect the partial redundancy of adhesion receptors involved in lymphocyte recruitment and changes in receptor expression/utilization that occur as immunologic reactions progress. On T cells, at least six receptors or receptor families mediate tethering interactions and at least two additional families can support the arrest/transmigration of tethered cells (39). Furthermore, several studies document changes in adhesion receptor expression and utilization during pulmonary immune responses. In SRBC-primed mice, i.t. SRBC challenge was followed by transient expression of E-selectin and prolonged expression of P-selectin and VCAM on the microvasculature of the lung (19). In OVA-sensitized mice, i.t. OVA-induced peribronchial inflammation was P-selectin dependent during the acute phase and CD49d dependent during the late phase response (40–42). In the current study, time-dependent changes were observed for the CD4 subset but not for the CD8 subset. In light of these complexities, it is not surprising that immune reactions use a wide variety of adhesion receptors and that receptor utilization is not uniform across organs, disease processes, or lymphocyte subsets.

This experimental model system examines the pulmonary response to the classic particulate Ag, SRBC (21). This system is relevant to human lung disease because many naturally occurring inhaled or aspirated substances are complex particulates, the response to which may be poorly simulated by results from experimental systems using soluble Ags of lower complexity. SRBC do not proliferate or directly cause tissue damage but do present a variety of glycopeptide and glycolipid moieties (notably the Forsmann Ag) in a three-dimensional context that may stimulate recognition by the innate immune system (43). The response to i.t. challenge is dose dependent and Ag specific (24, 44). An i.t. challenge induces intense inflammatory cell accumulation, predominantly in the bronchovascular bundles and around veins (9), that most closely resembles a type IV Gell and Coombs response. Although priming does not involve adjuvants, initial i.t. challenge induces a predominately type 2 cytokine response with prominent lung eosinophilia (17, 26). Repeated i.t. challenge evolves to a distinctly type 1 and waning pulmonary response (17), thus appearing to duplicate the spontaneous tolerance seen in most animal models of repeated i.t. Ag challenge (45–48). 

The SRBC model

FIGURE 6. T cells of E−/− mice can be Ag primed by i.p. injection. A, In this experiment, the transferred cell population was not CFMDF labeled, and the experimental condition assayed was total Ag-driven lung lymphocyte accumulation. KO, Knockout. B, Data are means ± SEM of three to five mice per group analyzed individually. Adpt Tx, adoptive transfer. *, p < 0.01, ANOVA, with post hoc analysis by the Tukey-Kramer test.

FIGURE 7. Whole lung chemokine production by E−/− mice is intact. Total lung RNA was extracted from SRBC-primed wt C57BL/6 mice (■) or E−/− mice (□) at 4 and 7 days after i.t. challenge. RNA from individual mice was reverse-transcribed, and aliquots of cDNAs were amplified by PCR. Data are the ratio of the OD of the individual chemokine to the OD of cyclophilin as means ± SEM of three to five mice. Note the difference in scale between individual chemokines. *, p < 0.05, unpaired nonparametric Mann-Whitney test comparing groups at the same time point.
system has been used by a number of laboratories to analyze the anatomic and molecular mechanisms of lymphocyte recruitment to lung parenchyma (19, 20), the cytokine requirements for airways hyperresponsiveness (49), and the role of neuropeptides in development of lung inflammation (15).

In the SRBC model, lung lymphocyte accumulation is dependent on continuous recruitment from the periphery. This conclusion is based on the high rate of lymphocyte elimination by in situ apoptosis (50), the very low rate of in situ proliferation (26), and direct evidence that circulating T lymphoblasts are trafficking into the lung throughout the immune response (16). Furthermore, previous experiments in this model indicated that systemic depletion of CD4+ T cells before i.t. challenge with SRBC reduced the peri-bronchial accumulation of all leukocytes except CD8+ T cells (13). Conversely, CD8 depletion before i.t. SRBC challenge did not alter the accumulation of either the CD4 subset or B cells (25). Thus, the early recruitment of the CD4 and CD8 subsets in the SRBC model are mutually independent. These findings, coupled with the persistence of multiple T cell-directed chemokines in the lungs of E- P- animals, support the hypothesis that recruitment of CD8 and, to a lesser extent, the CD4 subset of T cells from the altered trafficking of CD4+ T cells. Ligands for both E- and P-selectin are synthesized by human B cells and mediate tethering interactions in vitro (51-53); therefore, selectin-mediated B cell recruitment in vivo is a theoretical possibility. Alternatively, diminished accumulation of a critical T cell subset might delay expression of endothelial adhesion receptors or chemokines essential for subsequent B cell recruitment. For example, Gonzalo et al. (40) proposed that CD4+ T cells recruited early following i.t. OVA administration in sensitized mice induced subsequent VCAM-mediated eosinophil recruitment. B cells express α4-integrins and interact with VCAM in vitro (54, 55). The α4-integrins clearly mediate memory B cell trafficking in vivo, as shown for α4β7: mucosal addressin CAM-dependent localization of IgA-specific B cells in the gastrointestinal tract (56, 57). Thus, the current results are compatible with either a direct or an indirect role for selectins in B cell recruitment.

The existence of large numbers of γδ T cells in the peripheral blood and alveoli of E- P- mice has not been reported previously. The generalized increased in myelopoiesis observed in selectin-deficient mice may reflect either direct involvement of selectins in B cell recruitment or indirect changes in the lung microenvironment resulting from the altered trafficking of CD4+ T cells. Ligands for both E- and P-selectin are synthesized by human B cells and mediate tethering interactions in vitro (51-53); therefore, selectin-mediated B cell recruitment in vivo is a theoretical possibility. Alternatively, diminished accumulation of a critical T cell subset might delay expression of endothelial adhesion receptors or chemokines essential for subsequent B cell recruitment. For example, Gonzalo et al. (40) proposed that CD4+ T cells recruited early following i.t. OVA administration in sensitized mice induced subsequent VCAM-mediated eosinophil recruitment. B cells express α4-integrins and interact with VCAM in vitro (54, 55). The α4-integrins clearly mediate memory B cell trafficking in vivo, as shown for α4β7: mucosal addressin CAM-dependent localization of IgA-specific B cells in the gastrointestinal tract (56, 57). Thus, the current results are compatible with either a direct or an indirect role for selectins in B cell recruitment.

The finding of abundant γδ T cells in the peripheral blood and alveoli of E- P- mice is noteworthy because this cell type may contribute to host responses as both an effector and regulatory cell (61). γδ T cells react directly with unprocessed Ag (62) and mediate effector functions including cytokine production, cytotoxicity, and presentation of Ag to αβ T cells (63-65). Pulmonary γδ T cells comprise a heterogeneous population that are found both in normal mice and in a variety of infectious models (66-68). Unlike the case in the skin, where γδ T cells lack TCR diversity, pulmonary γδ T cells show considerable clonal diversity (66, 69). γδ T cells may also play important immunoregulatory roles in autoimmunity and allergic inflammation (70). In both murine and rat model systems, small numbers of γδ cells from Ag-exposed donors transferred Ag-specific tolerance to naive recipients via immune deviation (71, 72). In addition, γδ T cells augmented IL-4 dependent, type 2-mediated airway inflammation to peptide Ags in some murine models (73). Therefore, the elevated numbers of γδ T cells in E-P- mice and their capacity for recruitment to the lungs in the absence of endothelial selectins must be considered when using these mice for in vivo experimental model systems.

The current study found that granulocyte recovery from the lungs of E-P- mice was either unaltered (BAL) or significantly increased (minced tissues) compared with wt animals. This finding is consistent with previous reports documenting selectin-independent pathways for neutrophil recruitment into the lungs (3-6, 74). However, one cannot completely rule out a role for selectins in neutrophil recruitment. As previously reported (28), neutrophil counts were constitutively elevated in the E-P- mice. After i.t. SRBC challenge, the absolute number of circulating neutrophils reached levels as high as 10- to 50-fold above the measurements in wt control animals (data not shown). Because neutrophil delivery to the lungs is markedly increased in E-P- mice, the relatively low numbers of neutrophils recovered from the BAL in particular may indicate that the extraction efficiency is actually decreased in the absence of endothelial selectins.

In summary, the absence of endothelial selectins significantly reduces lymphocyte accumulation in the lungs following i.t. Ag challenge of sensitized mice. The impact is greatest on CD8+ cells and B cells, less marked on CD4+ cells, and without apparent effect on γδ T cells. Thus, agents designed to block the endothelial selectins may both diminish and skew pulmonary immune responses.

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References


