

# L-selectin and intercellular adhesion molecule 1 mediate lymphocyte migration to the inflamed airway/lung during an allergic inflammatory response in an animal model of asthma

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T lymphocytes play a critical role in the development of allergic inflammation in asthma. Early in the allergic response, T lymphocytes migrate from the circulation into the lung to initiate and propagate airway inflammation. The adhesion molecules that mediate lymphocyte entry into inflamed lung have not been defined. This study directly examined the roles of L-selectin and intercellular adhesion molecule 1 (ICAM-1) in lymphocyte migration to the lung during an allergic inflammatory response in an animal model of asthma. Short-term (1 hour) in vivo migration assays and various combinations of adhesion molecule-deficient and wild-type mice were used. Migration of in vivo activated lymphocytes into inflamed lung was significantly greater than entry of resting lymphocytes into noninflamed lung ( $24.5\% \pm 2.7\%$  vs  $9.5\% \pm 1.3\%$ ,  $P = .001$ ). Migration of activated lymphocytes into inflamed lung was inhibited by 30% in the absence of L-selectin ( $17.3\% \pm 1.3\%$ ,  $P = .04$ ), 47% in the absence of cell surface ICAM-1 ( $13.0\% \pm 2.5\%$ ,  $P = .01$ ), and 47% in the absence of endothelial ICAM-1 ( $13.0\% \pm 2.5\%$ ,  $P = .01$ ). Loss of ICAM-1 on both lymphocytes and lung endothelium inhibited lymphocyte migration by 60% ( $9.8\% \pm 1.8\%$ ,  $P = .002$ ). These findings demonstrate clear roles for both L-selectin and ICAM-1 in lymphocyte migration to the lung during an allergic inflammatory response, with ICAM-1 playing a greater role. (J Allergy Clin Immunol 2001;107:734-8)

**Key words:** Allergic airway disease/asthma, adhesion molecules, L-selectin, intercellular adhesion molecule 1, inflammation, migration, lymphocyte

Inflammation of the airway/lung is the fundamental abnormality in asthma. T lymphocytes initiate and prop-

## Abbreviations used

|                   |                                   |
|-------------------|-----------------------------------|
| AU:               | Arbitrary units                   |
| <sup>51</sup> Cr: | Chromium 51                       |
| ICAM-1:           | Intercellular adhesion molecule 1 |
| LFA-1:            | Leukocyte function antigen 1      |
| OVA:              | Ovalbumin                         |
| WT:               | Wild type                         |

agate this inflammation.<sup>1</sup> Early in the allergic response T lymphocytes are recruited from the circulation to the airway/lung.<sup>2,3</sup> Once present within the airway/lung, these cells orchestrate inflammation through the secretion of cytokines.<sup>4,5</sup>

Migration of leukocytes from the systemic circulation into tissue is mediated by interactions between cell surface and endothelial adhesion molecules.<sup>6</sup> The initial interaction results in leukocyte attachment and rolling along the endothelium. Activation signals then lead to "firm adhesion." Transmigration through endothelium into inflamed tissue follows.<sup>6</sup> Studies of leukocyte-endothelial interactions within the pulmonary microcirculation have reported leukocyte rolling on venular endothelium as seen in the systemic circulation.<sup>7-9</sup> In addition, leukocyte rolling on arteriolar endothelium and leukocyte sequestration within capillaries have been demonstrated in both normal and inflamed lung.<sup>7-9</sup> Leukocyte rolling on pulmonary arterioles is mediated by L-selectin, but not P-selectin or intercellular adhesion molecule 1 (ICAM-1), in both noninflamed lung<sup>7,9</sup> and inflamed lung.<sup>8</sup> Leukocyte rolling on pulmonary venules in inflamed lung is mediated by L-selectin and ICAM-1, but not P-selectin.<sup>8</sup> Interestingly, leukocyte rolling on venules in normal noninflamed lung is not dependent on L-selectin, ICAM-1, or P-selectin.<sup>9</sup> These studies implicate a role for L-selectin and ICAM-1 in lymphocyte recruitment to inflamed lung; however, this has not been directly demonstrated. Identification of specific adhesion molecules that regulate lymphocyte migration to the lung during an allergic inflammatory response may provide novel targets for more effective asthma therapies that selectively inhibit the development of airway inflammation. In this study 1-hour in vivo migration assays and

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various combinations of adhesion molecule-deficient mice were used to directly examine the roles of L-selectin and ICAM-1 in lymphocyte migration to the inflamed airway/lung during an allergic inflammatory response in an animal model of asthma.

## METHODS

### Reagents

The following reagents were used: ovalbumin (OVA) (grade V; Sigma-Aldrich Pty Ltd, Perth, Australia); monoclonal rat-antimouse IgE (clone R35-72), mouse IgE antitrinitrophenol (clone IgE-3), biotin-conjugated monoclonal rat-antimouse IgE (clone R35-92), and avidin-peroxidase conjugate (PharMingen, San Diego, Calif); and sheep antimouse IgE ( $\epsilon$ -chain specific) and peroxidase-conjugated rabbit antisheep IgG (Calbiochem-Novabiochem Pty Ltd, Sydney, Australia).

### Animals

Mice deficient in L-selectin were generated as described.<sup>10</sup> Mice deficient in ICAM-1 were purchased from Jackson Laboratory (Bar Harbour, Me).<sup>11</sup> L-selectin-deficient and ICAM-1-deficient mice were backcrossed at least 8 to 10 generations onto C57BL/6 background (H-2<sup>b</sup>). C57BL/6 mice were purchased from the Walter and Elisa Hall Institute (Melbourne, Australia). Mice were aged 6 to 10 weeks for all experiments. Mice were maintained in a standard animal care facility. All procedures were approved by the Royal Children's Hospital Animal Experimentation Ethics Committee (Melbourne, Australia).

### OVA-induced allergic airway disease model

Mice were sensitized on day 0 (intraperitoneal injection of 10  $\mu$ g OVA/1 mg aluminum potassium sulfate [alum]) and challenged daily (30 minutes) from days 14 to 20 (aerosolized 1% OVA/saline solution, ultrasonic nebulizer NE-U07; Omron Corp, Sydney, Australia). Control mice were sensitized with alum alone and challenged with saline solution. Challenges were performed on groups of up to 12 mice in a closed-system chamber (24  $\times$  37.5  $\times$  15 cm).

### In vivo migration assays

Various combinations of adhesion molecule-deficient or wild-type (WT) mice sensitized and challenged (sensitized/challenged) with OVA or saline solution were used as donor and recipient mice. Serum IgE was determined in all mice to confirm sensitization. Splenocytes were isolated from donor mice 24 hours after the final aerosol exposure by density gradient centrifugation, labeled with chromium 51 (<sup>51</sup>Cr) (200  $\mu$ Ci), and injected (10  $\times$  10<sup>6</sup> cells/0.3 mL) into tail veins of recipient mice 24 hours after the last OVA aerosol exposure. Recipient mice were euthanized 1 hour after injection, and various tissues were collected for measurement of radioactivity (1 minute): peripheral lymph nodes (axillary, lateral axillary, and superficial inguinal), mesenteric lymph nodes, Peyer's patches, liver, spleen, lung, thymus, heart, blood, and bronchoalveolar lavage fluid. Migration of lymphocytes into tissues was expressed as a percentage of total radioactivity recovered. The mean percentage of radioactivity recovered for each group ranged from 66.2% to 89.7%. No significant differences in recovery were observed for different strains of mice or different experimental groups. No differences were observed in the distribution of lymphocyte subsets (CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, B220<sup>+</sup>, F480<sup>+</sup>, CD25<sup>+</sup>) within injected cell preparations from adhesion molecule-deficient and WT mice.

### Total IgE and OVA-specific IgE ELISA

**Total IgE ELISA.** Flat-bottomed 96-well plates were coated (overnight, 4°C) with monoclonal rat-antimouse IgE (clone R35-72,

2  $\mu$ g/mL in 0.1 mol/L NaCO<sub>3</sub>, pH8.1) and blocked with 1% BSA (30 minutes). Serum samples were incubated (1 hour, room temperature) at various dilutions (1:10, 1:50, 1:100) followed by secondary antibody biotin-conjugated rat-antimouse IgE (clone R35-92, 2  $\mu$ g/mL) (1 hour, room temperature) and avidin-peroxidase conjugate (1:1000) (30 minutes, room temperature). The reaction was developed with 3,3',5,5'-tetramethylbenzidine substrate (100  $\mu$ L, 20 minutes) and stopped with 2 mol/L H<sub>2</sub>SO<sub>4</sub>. Purified mouse IgE (clone IgE-3) was used to generate a standard curve.

**OVA-specific IgE ELISA.**<sup>12</sup> Flat-bottomed 96-well ELISA plates were coated with 100  $\mu$ g/mL OVA (overnight, 4°C, 0.06 mol/L Na<sub>2</sub>HCO<sub>3</sub>/NaHCO<sub>3</sub> buffer, pH 9.6) and blocked with 1% BSA (2 hours, 37°C). Serum samples (neat, 1:5, 1:10, 1:50) were incubated (2 hours, 37°C), followed by sheep-antimouse IgE (1:8000, 2 hours, 37°C) and peroxidase-conjugated rabbit-antisheep IgG (1:2000, 2 hours, 37°C). The reaction was developed with 3,3',5,5'-tetramethylbenzidine substrate (20 minutes) and stopped with 2 mol/L H<sub>2</sub>SO<sub>4</sub>. Pooled serum from OVA-sensitized/challenged WT mice was used as a positive control. OVA-specific IgE levels were determined by converting optical density values of samples to arbitrary units (1 AU being equal to the optical density of as 1:10 dilution of pooled OVA-sensitized sera).

### Statistical analysis

Data are shown as mean values  $\pm$  SEM unless otherwise stated. A 2-tailed, equal variance Student *t* test was used to determine the significance of differences between population means.

## RESULTS

### Short-term (1 hour) migration of in vivo activated or resting lymphocytes in OVA-sensitized/challenged mice and control mice

The migration of activated lymphocytes from OVA-sensitized/challenged mice or resting lymphocytes from control saline-sensitized/challenged mice into inflamed or normal lung was examined in short-term (1 hour) in vivo migration assays. <sup>51</sup>Cr-labeled splenocytes from OVA-sensitized/challenged or control WT mice were injected into individual OVA-sensitized/challenged mice or control saline-sensitized/challenged WT mice. After 1 hour recipient mice were euthanized, and radioactivity was measured in various tissues (Fig 1). Migration of resting lymphocytes into normal noninflamed lung was 9.5%  $\pm$  1.3%. Migration of activated lymphocytes into inflamed lung was increased 2.6-fold (24.5%  $\pm$  2.7%, *P* = .001). This increased migration was dependent on both activation of lymphocytes and the presence of lung inflammation (Fig 1). Migration of resting lymphocytes to inflamed lung was increased approximately 2-fold (17.9%  $\pm$  0.8%, *P* = .001) but remained lower than that of activated cells entering inflamed lung (*P* = .03) (Fig 1). This suggests that increased migration to inflamed lung is mediated by both constitutively expressed cell surface adhesion receptors and adhesion receptors that are induced and/or upregulated on lymphocyte activation. However, because migration of activated lymphocytes to noninflamed lung was similar to that of resting lymphocytes migrating to noninflamed lung (13.4%  $\pm$  1.6% vs 9.5%  $\pm$  1.3%, *P* = .1), the increased migration of activated lymphocytes to inflamed lung appears to be

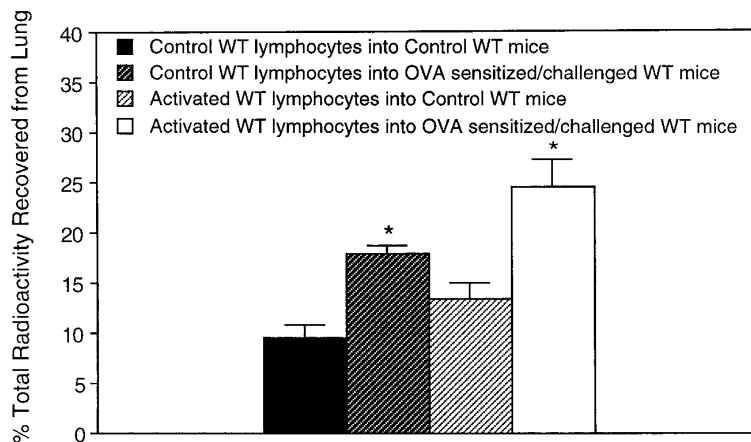


**FIG 1.** Migration of in vivo activated and control WT lymphocytes into tissues of OVA-sensitized/challenged and control WT mice. In total,  $10 \times 10^6$  splenocytes from OVA-sensitized/challenged control WT mice were labeled with  $^{51}\text{Cr}$  and injected into the tail veins of OVA-sensitized/challenged control WT mice ( $n = 5$ ). After 1 hour, tissues were harvested and radioactivity was measured. Migration of injected cells into tissues was expressed as the percentage of total radioactivity measured. In vivo activated and control lymphocytes demonstrated increased migration to inflamed lung. Migration of activated lymphocytes to noninflamed lung was not significantly different from migration of control lymphocytes. \* $P < .001$ .

largely dependent on induction of adhesion molecules on lung endothelium. No significant differences were observed in the migration of activated or resting lymphocytes to other tissues in either OVA-sensitized/challenged or control mice (data not shown).

### Role of L-selectin and ICAM-1 in migration of activated lymphocytes to inflamed lung

To examine the role of L-selectin in the migration of activated lymphocytes to inflamed lung activated splenocytes from WT or L-selectin-deficient mice sensitized/challenged with OVA were injected into tail veins of WT recipient mice sensitized/challenged with OVA. Migration of activated lymphocytes from L-selectin-deficient mice into inflamed lungs from WT mice was reduced by 30% as compared with migration of activated lymphocytes from WT mice ( $17.3\% \pm 1.3\%$  vs  $24.5\% \pm 2.7\%$ ,  $P = .04$ ) (Fig 2). A corresponding increase in migration to spleen was observed ( $50.3 \pm 2.8$  vs  $41.9 \pm 2.8$ ,  $P = .07$ ).

To examine the role of cell surface ICAM-1 in the migration of activated lymphocytes to inflamed lung, we injected activated splenocytes from WT or ICAM-1-deficient mice sensitized/challenged with OVA into the tail veins of WT mice sensitized/challenged with OVA. Loss of lymphocyte ICAM-1 inhibited migration to inflamed lung by 47% as compared with migration of WT lymphocytes ( $12.9\% \pm 2.5\%$ ,  $P = .01$ ) (Fig 2). To examine the role of endothelial ICAM-1 in this process, we injected activated splenocytes from WT mice into ICAM-1-deficient or WT mice that had been sensitized/challenged with OVA. Loss of ICAM-1 expression on lung endothelium resulted in a 47% reduction in lymphocyte migration to inflamed lung as compared with

WT lymphocytes entering inflamed lung in WT mice ( $13.0\% \pm 2.5\%$ ,  $P = .01$ ) (Fig 2). To examine the combined roles of lymphocyte and endothelial ICAM-1, activated lymphocytes from ICAM-1-deficient mice were injected into ICAM-1-deficient mice. Loss of ICAM-1 on both lymphocytes and lung endothelium resulted in a 60% reduction of lymphocyte migration to inflamed lung ( $9.8\% \pm 1.8\%$ ,  $P = .002$ ). A corresponding increase in migration to the spleen was observed ( $59.7 \pm 4.6$  vs  $41.9 \pm 2.8$ ,  $P = .01$ ). No significant differences were observed in the migration of activated adhesion molecule-deficient or WT lymphocytes to other tissues in adhesion molecule-deficient or WT mice (data not shown).

Migration of unactivated lymphocytes to noninflamed lung was not inhibited in the absence of L-selectin or ICAM-1 (Fig 2).

### L-selectin-deficient and ICAM-1-deficient mice have normal immune response to OVA

To confirm that the reduced migration of lymphocytes to inflamed lung seen in the absence of L-selectin and ICAM-1 related specifically to inhibition of adhesive mechanisms rather than to differences in the afferent arm of the immune response to OVA, serum total IgE and OVA-specific IgE were examined in adhesion molecule-deficient and WT mice. Total serum IgE and OVA-specific IgE levels in L-selectin-deficient and ICAM-1-deficient mice were not significantly different from those found in WT mice (geometric mean  $\pm$  95% CIs: IgE, WT  $449.4 \pm 13.7$  ng/mL, L-selectin deficient  $915.9 \pm 28.1$  ng/mL, ICAM-1 deficient  $394.5 \pm 8.1$  ng/mL; OVA-specific IgE, WT  $11.4 \pm 0.002$  AU, L-selectin deficient  $19.4 \pm 0.1$  AU [ $P = .05$ ], ICAM-1 deficient  $27.4 \pm 0.03$  AU).

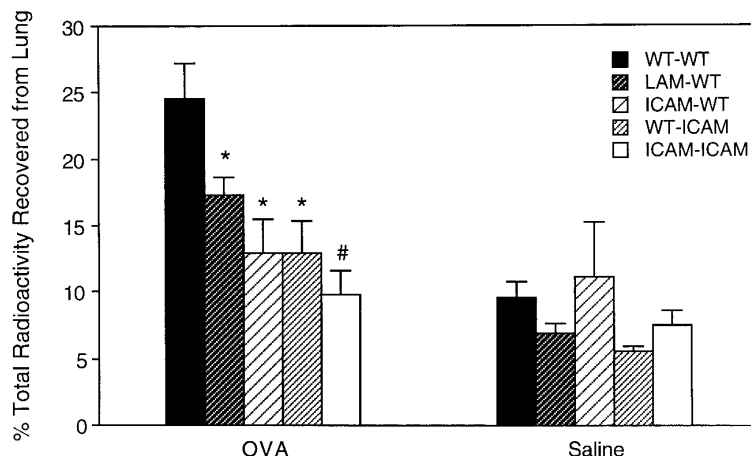


**FIG 2.** Migration of activated or resting lymphocytes into activated or resting lungs of WT and adhesion molecule-deficient mice. In total,  $10 \times 10^6$  in vivo activated (OVA) or resting (Saline) splenocytes from WT, L-selectin-deficient (LAM) or ICAM-1-deficient (ICAM) mice were labeled with  $^{51}\text{Cr}$  and injected into recipient WT or ICAM-1-deficient mice ( $n = 5$ ). After 1 hour, tissues were harvested and radioactivity was measured. Migration of injected cells into tissues is expressed as the percentage of total radioactivity recovered. \* $P < .01$ ; # $P < .001$ .

## DISCUSSION

Recent studies suggest important roles for L-selectin and ICAM-1 in lymphocyte recruitment to several sites of inflammation in vivo.<sup>13-16</sup> However, the adhesion molecules that mediate lymphocyte migration to sites of allergic inflammation in the airway/lung have not been defined. Video microscopic studies in mice implicate roles for L-selectin and ICAM-1 in leukocyte interactions with inflamed pulmonary microvasculature.<sup>8</sup> L-selectin is important for sequestration of neutrophils within pulmonary capillaries and noncapillary microvessels in noninflamed and inflamed lung.<sup>17</sup> However, there have been no studies examining the adhesion molecules that mediate lymphocyte migration to inflamed lung.

Using in vivo homing assays and adhesion molecule-deficient mice, we have directly assessed the roles of L-selectin and ICAM-1 in lymphocyte migration to sites of allergic inflammation in the lung. The presence of inflamed lung resulted in increased influx of both normal and activated lymphocytes to lung tissue, with a greater influx of activated lymphocytes than resting lymphocytes. In contrast, activated and normal lymphocytes demonstrated a similar ability to migrate to normal noninflamed lung (Fig 1). These findings indicate (1) that induction and/or upregulation of adhesion receptors on tissue endothelium is a major factor regulating increased lymphocyte recruitment to the lung during an allergic inflammatory response, and (2) that the ligand(s) for these endothelial adhesion receptors are both constitutively expressed on circulating lymphocytes and induced or upregulated on activation of lymphocytes.

Migration of activated lymphocytes to inflamed lung was shown to be mediated in part by cell surface L-selectin (Fig 2). This is consistent with a role for L-selectin in lymphocyte rolling on inflamed pulmonary venules and arterioles.<sup>8</sup> However, recruitment of lymphocytes to the lung during an allergic inflammatory response appears to be predominantly regulated by increased expression of ICAM-1 and its ligands on activated lymphocytes and inflamed lung endothelium (Fig 2).

Indeed, absence of ICAM-1 on both lymphocytes and lung tissue endothelium inhibited the increased migration of lymphocytes to inflamed lung down to levels of migration seen for resting lymphocytes entering noninflamed lung. This is consistent with the finding that ICAM-1 contributes to both leukocyte rolling and sequestration in inflamed pulmonary microcirculation.<sup>8</sup> Reports of increased ICAM-1 expression on bronchial endothelium in inflamed lungs from patients with asthma during acute attacks of asthma further support this.<sup>18,19</sup> Similarly, we have found increased ICAM-1 expression on vascular endothelium within inflamed lung as compared with noninflamed lung (unpublished data).

Taken together, our findings suggest that during an allergic inflammatory response in the lung, there is increased expression of L-selectin ligands and ICAM-1 on vascular endothelium. These adhesion receptors may then interact with cell surface L-selectin expressed constitutively on circulating lymphocytes and leukocyte function antigen 1 (LFA-1)-1/ICAM-1 expressed constitutively on resting lymphocytes and/or upregulated on activated lymphocytes, respectively. The mechanisms by which cell surface ICAM-1 mediates lymphocyte migration to inflamed lung are unclear because LFA-1 is not expressed at high levels on either inflamed or resting lung tissue.<sup>13</sup> One possibility is that cell surface ICAM-1 mediates cell-cell interactions on inflamed pulmonary endothelium in the initial stages of capture/rolling, thereby allowing endothelial ICAM-1 to mediate sequestration and diapedesis through interaction with cell surface LFA-1. Leukocyte rolling on leukocytes has been documented previously, and this process may allow further activation through lymphocyte-lymphocyte interaction.

The increased migration of activated lymphocytes to inflamed lung was specifically mediated by adhesion-dependent events and did not reflect changes in blood flow or altered immune response to OVA. Lymphocyte migration to inflamed lung was inhibited to varying degrees by the absence of specific adhesion molecules, loss of ICAM-1 inhibited migration to that seen in noninflamed lung, and both L-selectin-deficient and ICAM-1-deficient mice generated normal immune responses to OVA. Although L-selectin is required for lymphocyte homing to peripheral lymph nodes<sup>10</sup> and antigen-presenting cells from ICAM-1-deficient mice are poor stimulator cells *in vitro*,<sup>13</sup> numerous studies have confirmed normal immune responses in these mice. L-selectin-deficient mice have normal antibody and proliferative responses after immunization with keyhole limpet hemocyanin<sup>15</sup> and normal cytotoxic T-lymphocyte responses after allogeneic skin graft.<sup>14</sup> ICAM-1-deficient T cells respond well in mixed lymphocyte reactions, and ICAM-1-deficient mice have normal immune function with normal *in vivo* antibody and cellular (delayed-type hypersensitivity) responses to collagen type II, normal humoral and proliferative responses to keyhole limpet hemocyanin after *in vivo* immunization, and normal cytotoxic T-lymphocyte responses after allogeneic skin graft.<sup>13,15,20</sup>

In summary, the findings in this study directly demonstrate important roles for both L-selectin and ICAM-1 in lymphocyte migration to the lung during an allergic inflammatory response in an animal model of asthma with ICAM-1 playing a dominant role. Because lymphocyte recruitment to the airway/lung is a critical event in the initiation and propagation of allergic airway inflammation in asthma, these molecules may represent potential targets for novel therapies that selectively inhibit allergic inflammation without inducing unwanted systemic effects. Such selective therapy would represent a major advance in current asthma management.

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