

L-Selectin is required for the development of airway hyperresponsiveness but not airway inflammation in a murine model of asthma

Lynne C. Fiscus, BA,^a Jenny Van Herpen, BSc (Hon),^b Douglas A. Steeber, PhD,^c
Thomas F. Tedder, PhD,^c and Mimi L. K. Tang, MBBS, PhD^b *Chapel Hill and
Durham, NC, and Parkville, Australia*

Background: Airway inflammation and airway hyperresponsiveness (AHR) are fundamental features of asthma. Migration of inflammatory cells from the circulation into the lungs is dependent on adhesion molecule interactions. The cell surface adhesion molecule L-selectin has been demonstrated to mediate leukocyte rolling on inflamed and noninflamed pulmonary endothelium. However, its role in the development of airway inflammation and AHR in asthma has not been examined. **Objective:** We sought to characterize the role of L-selectin in the recruitment of inflammatory cells to the airway-lung and the development of AHR in a murine model of asthma. **Methods:** An ovalbumin (OVA)-induced allergic airway disease model of asthma was applied to L-selectin-deficient (LKO) mice and C57BL/6 wild-type (WT) control mice. The development of airway inflammation was assessed by examining leukocyte influx into bronchoalveolar lavage (BAL) fluid and the lung. Total and differential BAL leukocyte counts were determined, and the immunophenotype of BAL lymphocytes was assessed by means of flow cytometry. The development of AHR was assessed by means of whole-body plethysmography. **Results:** Airway-lung inflammation was equivalent in LKO and WT mice sensitized-challenged with OVA, as measured by total and differential BAL cell counts and histologic analysis of lung tissue. Numbers of eosinophils, neutrophils, lymphocytes, and monocytes in BAL fluid were equivalent in LKO and WT mice. However, phenotypic analysis of BAL lymphocytes demonstrated significantly reduced CD3⁺ populations and increased B220⁺ populations in LKO compared with WT mice ($P < .05$). Remarkably, despite a fulminant inflammatory response in the airway-lung in LKO mice sensitized-challenged with OVA, AHR was completely abrogated.

Conclusion: L-Selectin plays a crucial role in the development of AHR but not allergic inflammation in an animal model of asthma. L-Selectin represents a potential target for novel asthma therapies specifically aimed at controlling AHR. (*J Allergy Clin Immunol* 2001;107:1019-24.)

Key words: Adhesion molecule, asthma, airway hyperresponsiveness, inflammation

Asthma affects 14% of 13- to 14-year-old children and 12% of 6- to 7-year-old children worldwide, making it one of the most common global illnesses.^{1,2} Airway inflammation is the fundamental abnormality in asthma.³⁻¹⁴ This inflammation is characterized by the influx of lymphocytes, monocytes, and eosinophils into the airway and lung.¹³⁻¹⁵ T_H2 cells are the key orchestrators of this inflammation, initiating and propagating inflammation through the release of their cytokines, IL-4 and IL-5. IL-5, in turn, recruits and activates eosinophils, the effector cells in asthma.¹⁶⁻²³

Migration of leukocytes from the circulation into tissues is dependent on adhesion molecule interactions. The adhesion molecules mediating leukocyte influx to the airway-lung during an allergic inflammatory response remain poorly defined. Video microscopic analysis with blocking antibodies implicate important roles for L-selectin and intercellular adhesion molecule 1 in leukocyte-endothelial interactions within the pulmonary microcirculation.²⁴⁻²⁶ L-selectin has been shown to mediate arteriolar and venular rolling on inflamed and noninflamed pulmonary endothelium,²⁴⁻²⁶ whereas intercellular adhesion molecule 1 has been shown to mediate venular rolling on inflamed pulmonary endothelium.²⁴ Furthermore, we have recently demonstrated a role for L-selectin in lymphocyte migration to the lung during an allergic inflammatory response.²⁷

These findings suggest a key role for L-selectin in leukocyte migration to the lung and hence the development of airway inflammation in asthma. We have characterized the role of L-selectin in the development of airway inflammation by using an animal model of asthma and L-selectin-deficient (LKO) mice. Influx of leukocytes into bronchoalveolar lavage (BAL) fluid and lung tissue and the development of airway hyperresponsiveness (AHR) were examined in LKO and wild-type (WT) mice sensitized-challenged with ovalbumin.

From ^aUniversity of North Carolina at Chapel Hill School of Medicine, Chapel Hill; ^bthe Department of Immunology, Royal Children's Hospital, Parkville; and ^cthe Department of Immunology, Duke University Medical Center, Durham.

Supported by a grant from Murdoch Children's Research Institute (Tang) and by funds from training grant number 5 T35 DK0386-20 sponsored by the National Institute of Digestive and Diseases of the Kidney-USA (L. C. Fiscus).

Received for publication August 24, 2000; revised January 25, 2001; accepted for publication January 30, 2001.

Reprint requests: Mimi L. K. Tang, MBBS, PhD, Department of Immunology, Royal Children's Hospital, Flemington Rd, Parkville, Victoria 3052, Australia.

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0091-6749/2001 \$35.00 + 0 1/83/114703

doi:10.1067/mai.2001.114703

Abbreviations used

AHR:	Airway hyperresponsiveness
AU:	Arbitrary unit
BAL:	Bronchoalveolar lavage
LKO:	L-selectin deficient
OVA:	Ovalbumin
Penh:	Enhanced pause
WT:	Wild type

METHODS**Mice**

LKO mice were generated as previously described²⁸ and backcrossed onto the C57BL/6 background for over 10 generations. Age-matched WT C57BL/6 mice were obtained from Walter and Elisa Hall Institute (Melbourne, Australia). All mice were maintained in a standard Animal Care Facility (Department of Agriculture, University of Melbourne, Australia), and all procedures were approved by the Royal Children's Hospital Animal Experimentation Ethics Committee.

Ovalbumin-induced allergic airway disease model

An ovalbumin (OVA)-induced allergic airway disease model of asthma was used.²⁹ Briefly, mice were sensitized by means of intraperitoneal injection on day 0 with 10 µg of Grade V OVA (Sigma Chemical, St Louis, Mo) and 1 mg of aluminum potassium sulfate (Sigma Chemical) in 500 µL of saline solution. Mice were then challenged on days 14 to 20 by daily exposure (30 minutes) to an aerosol of 1% (wt/vol) OVA in saline solution. Vehicle control mice were sensitized with a suspension of aluminum potassium sulfate (1 mg) in saline solution (500 µL) and challenged with nebulized saline solution daily from days 14 to 20. Aerosol challenge was carried out on groups of up to 20 mice in a closed-system chamber (24 × 37.5 × 15 cm) attached to an ultrasonic nebulizer (NE-U07; Omron Corporation, Tokyo, Japan) with an output of 1 mL/min and 1- to 5-µm particle size.

OVA-specific IgE ELISA

Serum was obtained by means of orbital bleeding of anesthetized mice on day 21 of the sensitization-challenge protocol, 24 hours after the final OVA or saline challenge. Serum was stored in 100-µL aliquots at -20°C until processed for measurement of OVA-specific IgE.

OVA-specific IgE levels were quantitated by using ELISA, as previously described.²⁹ Briefly, flat-bottomed, 96-well ELISA plates (Immuno Maxisorp; Nalge Nunc International, Roskilde, Denmark) were coated overnight at 4°C with 100 µg/mL OVA in coating buffer (NaHCO₃, 1.94 g/L; Na₂CO₃, 3.52 g/L; and dH₂O, 1 L [pH 9.6]). After 3 washes with 0.5% Tween-20/PBS, plates were blocked with 200 µL/well of 1% BSA/PBS for 1.5 hours at 37°C. After 6 washes with 0.5% Tween-20/PBS, serum samples (1:10, 1:50, and 1:100 dilutions in 1% BSA/PBS) were incubated for 1.5 hours at 37°C. Pooled sera from OVA sensitized-challenged mice served as a positive control, and pooled normal mouse sera served as a negative control. After 6 washes with 0.5% Tween-20/PBS, 100 µL/well of sheep anti-mouse IgE (1:8000 in 1% BSA/PBS; Calbiochem-Novabiochem Corp, La Jolla, Calif) was added for 1.5 hours at 37°C. After 6 washes with 0.5% Tween-20/PBS, 100 µL/well of horseradish peroxidase-conjugated rabbit anti-sheep IgG (1:2000 in 1% BSA/PBS, Calbiochem-Novabiochem Corp) was added for 1.5 hours at 37°C. After a further 6 washes with 0.5% Tween-20/PBS, 100 µL/well of TMB substrate was added to each well. The color reaction was stopped 20 to 30 minutes later by addition of 100 µL/well 2 mol/L H₂SO₄. ODs were read at 450 nm, with a reference wavelength of 620 nm. Levels of OVA-specific IgE in serum samples

were expressed in arbitrary units (AUs), where 1 AU equals the OD of the 1:50 dilution of the positive control sera. Serum OVA-specific IgE levels were then interpolated from the linear part of the OD versus AU standard curve of the positive control sera.

Bronchoalveolar lavage

BAL was performed on anesthetized mice on day 21, 24 hours after the last OVA or saline solution challenge. Three 1-mL lavage specimens with cold HBSS were pooled, and the cells were washed and resuspended in 1 mL of 5% FCS in HBSS (FCS/HBSS). Total viable cell counts were determined manually by using trypan blue exclusion. Differential counts of eosinophils, neutrophils, lymphocytes, and monocytes were determined on cytospin smears of BAL samples (4 × 10⁵ cells) stained with DiffQuik (Life Technologies, Auckland, New Zealand) after counting 200 cells. The remaining BAL cells from each experimental group were pooled for flow cytometric analysis.

Flow cytometric analysis

Expression of CD3, CD4, CD8, CD25, and B220 cell surface markers on BAL lymphocytes was examined by using flow cytometry. BAL leukocytes were pooled from up to 4 mice in each experiment. Pooled BAL leukocytes (2.5 × 10⁵) were stained with various directly conjugated antibodies for 30 minutes at 4°C. Appropriate rat IgG isotype controls (PharMingen, San Diego, Calif) were run for each antibody. Samples were analyzed by using a FACStar flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and Cyclops software (Cytomation Inc, Fort Collins, Colo).

Measurement of airway reactivity

Methacholine-induced airway reactivity was assessed on day 21, 24 hours after the final OVA or saline solution challenge in unrestrained mice by means of barometric whole-body plethysmography (Buxco Electronics Inc, Troy, NY), as described by Hamelmann et al.³⁰ A total of 12 to 13 WT mice sensitized-challenged with OVA, LKO mice sensitized-challenged with OVA, and WT mice sensitized-challenged with saline solution and 6 LKO mice sensitized-challenged with saline solution were analyzed in 4 independent experiments. In each independent experiment, 3 or 4 mice were used.

Airway resistance is expressed as an enhanced pause (Penh), a dimensionless value representing a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals and of the timing of inspiration.³⁰ In brief, the biosystem was first calibrated with a rapid injection of 1 mL of air into the mouse chambers. Mice were then placed in individual chambers and allowed to settle for approximately 10 to 15 minutes. Baseline Penh readings were taken and averaged for 5 minutes. Increasing concentrations of aerosolized methacholine (1.5, 3, 6, 12, 25, 50, and 100 mg/mL in saline solution) were nebulized into the chambers for 2 minutes. Penh readings were taken for 6 minutes after each dose challenge and averaged.

Lung histology

Mice were killed within 4 hours of measurement of airway reactivity, and lungs were harvested for histologic analysis. The lungs were perfused with 10% buffered formalin, fixed overnight in 10% formalin at room temperature, and embedded in paraffin. Representative sections of lung (3 × 5-µm sections every 100 µm) were stained with hematoxylin and eosin and examined with light microscopy for evidence of peribronchial inflammation.

Statistical analysis

Data are expressed as mean values ± SEM, except in the case of IgE levels. The 2-tailed Student *t* test was used to compare differences between the sample means, except where otherwise stated. IgE levels were expressed as geometric mean and 95% CI, and the Mann-

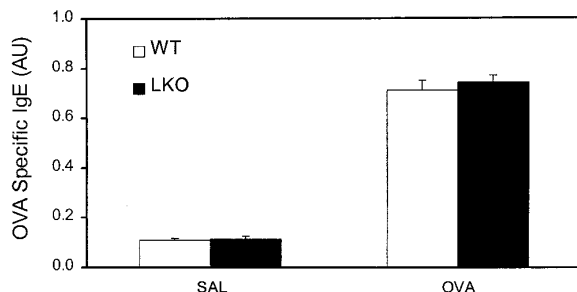


FIG 1. Serum levels of OVA-specific IgE. WT and LKO mice were sensitized-challenged with OVA or saline solution (SAL) control. WT and LKO mice sensitized-challenged with OVA ($n = 17$) demonstrated large increases in OVA-specific IgE levels compared with control mice sensitized-challenged with saline solution ($n = 12$). Values are means $\pm$ SEM.

Whitney *U* test statistic was used to compare differences between sample populations. In all experiments the numbers of animals used was 12 unless otherwise stated. Differences in lymphocyte subsets were analyzed by using a 1-way ANOVA and Bonferroni posttest.

RESULTS

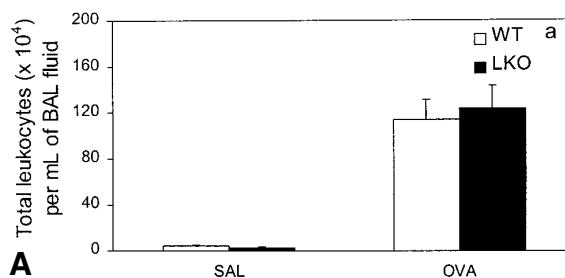
Serum levels of OVA-specific IgE in LKO mice and WT mice

To confirm that LKO mice were adequately sensitized to OVA, serum levels of total and OVA-specific IgE were examined in LKO and WT mice sensitized-challenged LKO with OVA and control mice sensitized-challenged with saline solution. Serum was obtained on day 21, 24 hours after the final OVA or saline solution challenge. Serum levels of OVA-specific IgE were significantly higher in both experimental groups sensitized-challenged with OVA compared with control animals sensitized-challenged with saline solution (Fig 1). Levels of OVA-specific IgE in LKO mice sensitized-challenged with OVA were equivalent to those found in WT control animals sensitized-challenged with OVA.

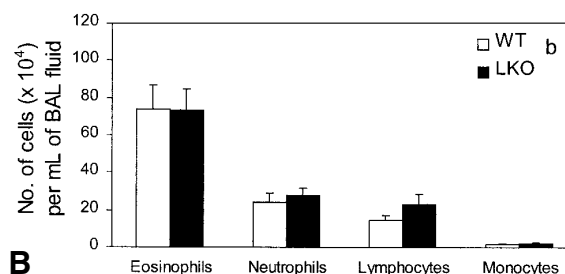
These findings demonstrate that LKO mice mount normal IgE responses equivalent to those of WT mice after sensitization-challenge with OVA.

BAL analysis in LKO and WT mice

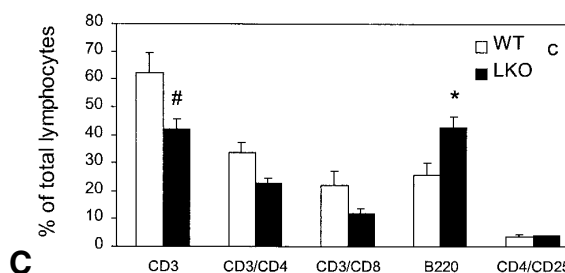
Total BAL leukocyte and differential counts. To assess the influx of leukocytes into the airway lumen in LKO and WT mice sensitized-challenged with OVA, BAL was performed on day 21, 24 hours after the last OVA challenge. Three 1-mL washes with HBSS were performed, and the specimens pooled for each mouse. Total leukocyte numbers in BAL fluid from LKO mice sensitized-challenged with saline solution were equivalent to counts in WT mice sensitized-challenged with saline solution (2.6×10^4 vs 4.2×10^4). Surprisingly, total BAL leukocyte counts in LKO mice sensitized-challenged with OVA were equivalent to BAL leukocyte counts in WT mice sensitized-challenged with OVA (123.2×10^4 vs 113.9×10^4 ; Fig 2, A), with no significant differences in the relative distribution of eosinophils, monocytes, neutrophils, or lymphocytes between the 2 groups (Fig 2, B).



A



B



C

FIG 2. BAL analysis in LKO and WT mice sensitized-challenged with OVA. LKO and WT mice were sensitized with OVA/aluminum potassium sulfate intraperitoneally on day 0 and then challenged with nebulized OVA from days 14 to 20. On day 21, 24 hours after the last OVA challenge, BAL was performed. **A**, Total leukocyte count per milliliter of BAL fluid recovered from LKO and WT mice sensitized-challenged with saline solution and OVA. Total BAL leukocyte counts in LKO mice sensitized-challenged with both saline solution and OVA were equivalent to those in WT control mice sensitized-challenged in the same way ($n = 12$). **B**, Differential leukocyte counts in BAL fluid from LKO and WT mice sensitized-challenged with OVA. Differential BAL counts were determined in LKO and WT mice on Diffquick-stained cytospin smears of BAL leukocytes. Numbers of eosinophils, neutrophils, lymphocytes, and monocytes from LKO mice were equivalent to the numbers in BAL fluid from WT mice ($n = 12$). **C**, Phenotypic analysis of BAL leukocytes from LKO and WT mice sensitized-challenged with OVA. BAL leukocytes from up to 4 mice were pooled, stained with antibodies against various cell surface markers, and analyzed by means of flow cytometry. Results are from 5 independent experiments. The percentage of CD3⁺ cells was significantly reduced in LKO mice compared with WT control animals. A corresponding increase in the percentage of B220⁺ cells in LKO mice was observed. Values are means $\pm$ SEM (# $P < .01$, * $P < .05$).

Immunophenotype of BAL lymphocytes by means of flow cytometric analysis. To further characterize the BAL leukocyte influx in LKO mice, phenotypic analysis was performed by using flow cytometry after staining with antibodies against various lymphocyte markers (CD3, CD4, CD8, CD25, and B220). BAL samples were pooled from up to 4 mice, and the analysis was repeated in 5 independent experiments (Fig 2, C).

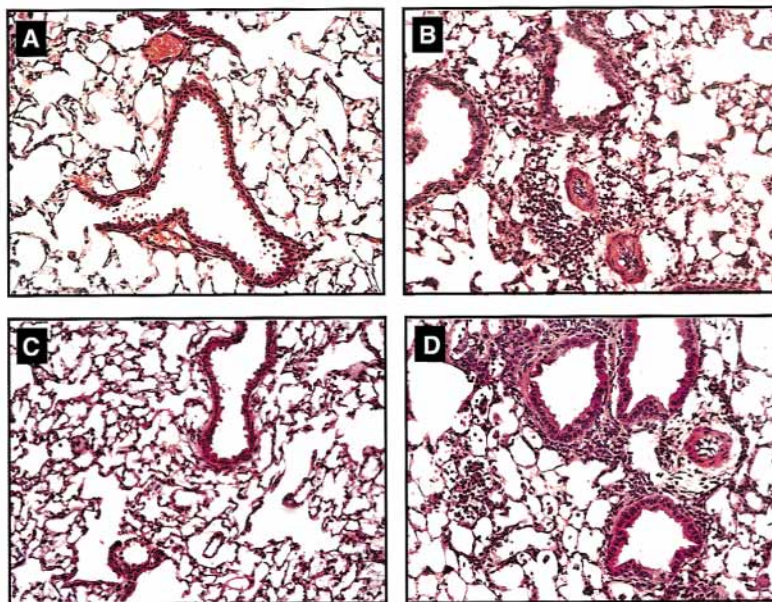


FIG 3. Histologic analysis of lung sections from LKO and WT mice sensitized-challenged with OVA and saline solution. Twenty-four hours after the last OVA or saline solution challenge, lungs were perfused with formalin and fixed overnight. Representative 5- μ m sections of lung tissue were stained with hematoxylin and eosin and examined by means of light microscopy. **A**, A representative lung section ($\times 20$) from a WT mouse sensitized-challenged with saline solution. **B**, A representative lung section ($\times 20$) from a WT mouse sensitized-challenged with OVA. Marked peribronchial, perivascular, and alveolar inflammatory infiltrates with eosinophils and mononuclear cells are evident, as well as marked bronchial wall edema. Representative sections of lung from an LKO mouse sensitized-challenged with saline solution, **C**, and an LKO mouse sensitized-challenged with OVA, **D**, are similar to those from WT mice sensitized-challenged with saline solution and OVA, respectively.

The percentage of CD3⁺ lymphocytes in LKO mice sensitized-challenged with OVA was reduced by 32% compared with that of WT mice (42.2% vs 62.4%, $P < .01$). The reduction in CD3⁺ cells is reflected in reduced percentages of both CD3⁺/CD4⁺ (22.8% vs 33.8%, $P > .05$) and CD3⁺/CD8⁺ cells (11.8% vs 22.0%, $P > .05$). Correspondingly, the percentage of B220⁺ B cells in BAL fluid from LKO mice sensitized-challenged with OVA was increased compared with that of WT mice sensitized-challenged with OVA (42.9% vs 25.8%, $P < .05$). The percentage of activated CD4⁺/CD25⁺ cells in BAL fluid from LKO mice sensitized-challenged with OVA was equivalent to that found in WT mice sensitized-challenged with OVA (3.7% vs 4.0%, $P > .05$).

Lung histology

The development of inflammation in the lungs of LKO mice was assessed by means of histologic examination of hematoxylin and eosin-stained sections of lung tissue. Lungs were isolated on day 21 from LKO and WT mice sensitized-challenged with OVA and saline solution. Representative 5- μ m paraffin sections of lung tissue (3 $\times$ sections every 100 μ m) were examined.

Marked bronchial wall edema and inflammatory influx into the peribronchial, perivascular, and alveolar tissue were observed in lung tissue sections from WT mice sensitized-challenged with OVA (Fig 3, *B*). Lungs from LKO mice sensitized-challenged with OVA demonstrated a similar

degree of inflammation to that found in WT mice sensitized-challenged with OVA, with edema and peribronchial, perivascular, and alveolar inflammatory infiltrates noted (Fig 3, *D*). Evaluation of peribronchial infiltrates at higher magnification revealed similar numbers of eosinophils and mononuclear cells in lungs from LKO and WT mice sensitized-challenged with OVA. No inflammation was observed in control lungs from LKO and WT mice sensitized-challenged with saline solution (Fig 3, *A* and *C*).

Methacholine-induced AHR

AHR was examined in LKO and WT mice sensitized-challenged with OVA and saline solution on day 21, 24 hours after the final OVA or saline solution challenge. Penh was measured in response to increasing concentrations of inhaled methacholine. Despite demonstrating marked leukocyte inflammation in the airway-lung equivalent with that found in WT mice, LKO mice sensitized-challenged with OVA demonstrated significantly reduced methacholine-induced AHR, with a shift in the dose-response curve to the right, compared with that of WT control mice sensitized-challenged with OVA (Fig 4). Furthermore, the airway reactivity responses in LKO mice sensitized-challenged with OVA were returned to levels below those observed in WT and LKO mice sensitized-challenged with saline solution, with maximal Penh readings in LKO mice sensitized-challenged with OVA being lower than those in the WT and LKO control mice sensitized-challenged with saline solution.

DISCUSSION

These studies demonstrate that leukocyte inflammation of the airway-lung, in the murine model of asthma described, persists in the absence of the cell-surface adhesion molecule L-selectin. Surprisingly, however, despite fulminant inflammation, AHR was dramatically reduced in LKO mice sensitized and challenged with OVA.

The persistence of inflammation in the absence of L-selectin is perhaps surprising because L-selectin is a major adhesion molecule mediating leukocyte rolling in the pulmonary microcirculation.²⁴⁻²⁶ In other *in vivo* models of inflammation where a role for L-selectin in leukocyte rolling has been demonstrated, the absence of L-selectin has resulted in reduced inflammation.³¹⁻³⁵ Our studies, however, indicate that L-selectin is not required for the migration of eosinophils, monocytes, neutrophils, and most populations of lymphocytes into the airway-lung during an allergic inflammatory response (Fig 2, B, and Fig 3). They further suggest that alternative adhesion molecules may mediate leukocyte rolling to inflamed lung in the absence of L-selectin, that leukocyte rolling is not an essential requirement for leukocyte migration across pulmonary endothelium into tissue, or both. Although no major reduction in overall leukocyte influx into the airway-lung was observed, the absence of L-selectin was associated with a selective reduction in CD3⁺ lymphocytes within the BAL fluid (Fig 2, C). This suggests that L-selectin plays a key role in the selective migration of CD3⁺ T-lymphocyte populations to inflamed airway-lung during an allergic inflammatory response.

We have previously demonstrated a greater dependence of T lymphocytes compared with B lymphocytes on L-selectin for migration to peripheral lymphoid tissues.³⁵ T lymphocytes selectively migrate to peripheral lymphoid tissues, and this selective migration is regulated by an increased expression of cell surface L-selectin on T lymphocytes compared with that found on B lymphocytes. Consequently, the absence of L-selectin inhibited T-lymphocyte migration to peripheral lymphoid tissues to a greater extent than B-lymphocyte migration.³⁵ The selective inhibition of T-lymphocyte migration to inflamed lung compared with that of B lymphocytes may reflect a similar phenomenon whereby T lymphocytes have a greater dependence on L-selectin for migration into the airway-lung compared with B lymphocytes.

The dramatic reduction in AHR observed in LKO mice sensitized-challenged with OVA (Fig 4) suggests a critical role for L-selectin in the development of AHR in allergic airway disease. Furthermore, the finding that LKO mice also demonstrated a selective reduction in CD3⁺ lymphocytes to the airway-lung suggests that the reduced AHR may occur as a result of reduced influx of a critical population of CD3⁺ lymphocytes that regulate the development of AHR. Indeed, recent studies suggest that the development of AHR in allergic airway disease is regulated by CD3⁺ T lymphocytes. T_H2 lymphocytes and their respective cytokines IL-4 and IL-5 have been implicated in the development of AHR,¹⁶⁻²³ with antibody

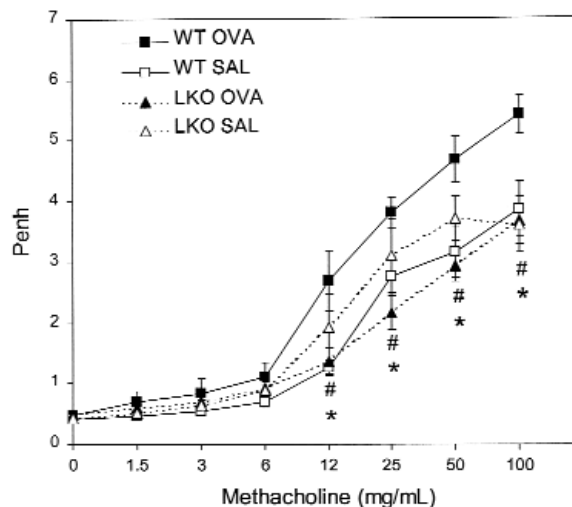


FIG 4. Methacholine-induced airway responses in WT and LKO mice sensitized-challenged with saline solution (SAL) and OVA. On day 21, 24 hours after the last OVA or saline solution challenge, methacholine-induced airway responses were assessed by means of whole-body box plethysmography (Buxco systems). WT mice sensitized-challenged with OVA (WT OVA, $n = 12$) demonstrated significantly increased AHR compared with WT control mice sensitized-challenged with saline solution (WT SAL, $n = 12$). LKO mice sensitized-challenged with OVA (LKO OVA, $n = 13$) demonstrated markedly attenuated AHR, with Penh values similar to those of LKO mice sensitized-challenged with saline solution (LKO SAL, $n = 6$) and WT control mice sensitized-challenged with saline solution. No significant difference was observed between AHR in LKO mice sensitized-challenged with saline solution and WT mice sensitized-challenged with saline solution. Values are means $\pm$ SEM (* $P < .05$ LKO OVA vs WT OVA, # $P < .05$ WT OVA vs WT SAL).

inhibition of IL-5 attenuating AHR in mice.^{21,22} Our finding of reduced CD3⁺ lymphocyte influx into the airway-lung and the associated reduced AHR in LKO mice supports a key role for T lymphocytes in the development of AHR in asthma and further suggests that L-selectin may be an important mediator of the migration of AHR-inducing T lymphocytes to the lung. Other mechanisms by which L-selectin may contribute to the development of AHR include promotion of airway smooth muscle hypertrophy and subepithelial fibrosis,³⁶ although these mechanisms are unlikely because the OVA-induced model used in this study was a short-term model of airway inflammation, and structural changes are generally associated with models of chronic asthma.

In conclusion, we have demonstrated a marked attenuation of AHR associated with a selective reduction of CD3⁺ lymphocyte influx into the airway-lung of LKO mice in an animal model of asthma. Because different strains of mice have been demonstrated to respond differently to immunization protocols,³⁷ these results may not be generalized to other mice strains or immunization protocols. Nevertheless, these findings implicate L-selectin as a potential target for novel therapies that selectively inhibit AHR in asthma. Current treatment regimens focus on controlling airway inflammation; however, reduction of airway

inflammation may not necessarily result in reduced AHR.³⁸ Because AHR is a central feature of clinical asthma, novel therapies that can selectively inhibit AHR may represent a major advance in asthma treatment, particularly for those patients with chronic and severe disease that is refractory to maximal anti-inflammatory therapy.

We thank Toby Merson for technical assistance.

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