

$\alpha 4$ and $\beta 2$ integrins have nonredundant roles for asthma development, but for optimal allergen sensitization only $\alpha 4$ is critical

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Objective. Recruitment of effector cell subsets to inflammatory lung, together with airway resident cells responsive to secreted products, play pivotal roles in developing and maintaining asthma. Differential use of adhesion molecules dictates the recruitment patterns of specific cell subsets, yet a clear understanding of the distinctive adhesive molecular pathways guiding them to lung is lacking. To provide further insight into the role of $\alpha 4\beta 1$ /VCAM-1 pathway and to compare this to the role of $\beta 2$ integrin in the development of acute asthma phenotype, we used genetically deficient mice, in contrast to previous studies with anti-functional antibodies yielding ambiguous results.

Methods. Allergen-dependent airway inflammation and hyperresponsiveness was induced in conditional $\alpha 4^{\Delta/\Delta}$, VCAM-1^{-/-}, and $\beta 2^{-/-}$ mice. Cytology, immunocytochemistry, cytokine and immunoglobulin measurements, and cell type accumulation in lung, BAL fluid, plasma, and hemopoietic tissues were carried out.

Results. Asthma phenotype was totally abrogated in $\alpha 4$ - or $\beta 2$ -deficient mice. Adoptive transfer of sensitized $\alpha 4^{\Delta/\Delta}$ CD4⁺ cells into challenged normal mice failed to induce asthma, whereas $\alpha 4^{+/+}$ CD4⁺ cells were able to induce asthma in challenged $\alpha 4^{\Delta/\Delta}$ mice. Parallel studies with $\beta 2^{-/-}$ or VCAM-1^{-/-} mice uncovered novel mechanistic insights in primary sensitization and into redundant or unique functional roles of these adhesion pathways in allergic asthma.

Conclusions. The lack of $\alpha 4$ integrin not only impedes the migration of all white cell subsets to lung and airways, but also prevents upregulation of vascular cell adhesion molecule-1 (VCAM-1) in inflamed lung vasculature and, unlike $\beta 2$, attenuates optimal sensitization and ovalbumin-specific IgE production in vivo. As VCAM-1 deficiency did not protect mice from asthma, interactions of $\alpha 4\beta 1^{+}$ or $\alpha 4\beta 7^{+}$ cells with other ligands are suggested. © 2007 International Society for Experimental Hematology. Published by Elsevier Inc.

Asthma is a chronic allergic airway disease characterized by persistent inflammation and airway hyper-responsiveness (AHR). T cells, especially Th2 cells secreting IL-4, IL-5, and IL-13, are pivotal in orchestrating the disease process, and adoptive transfer of Ag-primed T cells in naïve animals can induce eosinophilia, AHR, and late airway responses [1]. Apart from Th2, other effector cells for AHR and asthma are the eosinophils and mast cells. Under the influence of chemoattractants and IL-5, eosinophils

proliferate, migrate into the lung, and are activated to secrete histamine, leukotrienes, and other mediators [2]. Mature mast cells are sources of histamine, proteases, heparin, and lipid mediators, released upon allergen-induced IgE cross-linking as the initial step in the inflammation process.

Human studies of allergic asthma and animal models of asthma continue to provide conflicting data on the contribution of airway resident cells vs specific cell subsets recruited from circulation in the development of asthma phenotype [1,3,4]. The preferential recruitment of Th2 cells and eosinophils [4] in the lung is mediated by a cascade of adhesive interactions initiated by activated endothelial cells attracted by locally elaborated chemokines/cytokines.

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However, the distinct molecular pathways that dominate these processes are continuously under revision.

A key question is whether hematopoietic cells from circulation (i.e., eosinophils, neutrophils) or resident cells (i.e., tissue macrophages) play a pivotal role in orchestrating the acute allergic asthma phenotype and the role that adhesion molecules play in the interface of such interaction. $\alpha 4$ (very late activation antigen-4 [VLA-4] or $\alpha 4\beta 1$) [5] and $\beta 2$ integrins (lymphocyte function-associated antigen [LFA-1] or $\alpha L\beta 2$; Mac-1 or $\alpha M\beta 2$) [6] mediate hematopoietic cell migration in various inflammatory diseases [7,8]. VLA-4 interacts with extracellular matrix components (fibronectin, osteopontin), and with vascular cell adhesion molecule-1 (VCAM-1) mediating adhesion to activated endothelium. It also interacts with junctional adhesion molecule-2 (JAM-2), with mucosal addressin-associated cell adhesion molecule-1 (MAdCAM-1), and with itself, potentially facilitating interactions among leukocytes in inflammation [9].

Studies on expression of adhesion molecules in migrated cells have not provided definitive answers. Other than the CD18^{-/-} [10] mouse, in vivo and in vitro studies thus far have employed only anti-functional antibodies to several adhesion molecules (i.e., VLA-4, LFA-1, etc.). These studies yielded conflicting data depending on the type and dose of anti- $\alpha 4$ antibody used, its route of administration, or the animal model used [11–18] and examined effects after primary sensitization.

To gain further insight into the contribution of $\alpha 4$ integrin to asthma phenotype and further refine previous results from studies with anti- $\alpha 4$ antibodies, we used conditionally ablated $\alpha 4$ integrin or VCAM-1 mice subjected to ovalbumin (OVA)-induced and Th2 cytokine-driven lung allergic inflammation. Our data examining sensitization issues in the absence of $\alpha 4$ integrin yield novel insights into the involvement of $\alpha 4$ in this process and a comparison of the data to $\beta 2$ knockout (ko) or VCAM-1-deficient mice provides a substantial refinement on their redundant and nonredundant contributions in asthma development.

Methods

Mice

Animal procedures were approved by the Institutional Animal Care Committee of the University of Washington. Mice (C57Bl/6.B129 background) used were: Mxcre⁻ $\alpha 4^{fl/fl}$, Mxcre⁺ $\alpha 4^{\Delta/\Delta}$ ($\alpha 4^{fllox/fllox}$ bred to Mxcre⁺ [19]). Ablation of $\alpha 4$ integrin in Mxcre⁺ $\alpha 4^{fl/fl}$ mice was induced by 3 i.p. (intraperitoneal) injections of poly(I)-poly(C) (Sigma, St. Louis, MO, USA) during the first week of life. Neonatally ablated $\alpha 4^{\Delta/\Delta}$ Mxcre mice were studied as adults. $\beta 2$ ko (CD18^{-/-}) mice were previously described [10]. VCAM-1^{fl/fl} mice were bred to Tie2cre⁺ transgenics to generate VCAM-1-deficient mice as described [20]. In addition, in selected experiments, $\alpha 4^{fl/fl}$ mice also bred to Tie2cre transgenics to generate cre recombination of the $\alpha 4$ gene under Tie2 promoter as described [21] were used.

Induction of acute asthma phenotype

Mice were immunized with 100 μ g OVA (Pierce Chemical Co., Rockford, IL, USA) and complexed with aluminum sulfate (alum, Sigma, [22]) intraperitoneally injected on day 0. On day 8 (using 250 μ g of OVA) and on days 15, 18, and 21 (using 125 μ g of OVA), mice were anesthetized and given OVA by intratracheal (i.t.) administration [23]. Control groups received normal saline (complexed with alum) using the same schedule.

Pulmonary function testing

Twenty-four hours after the last intratracheal allergen (OVA) challenge (day 22), invasive pulmonary mechanics were measured in mice in response to methacholine as previously described [22].

Noninvasive plethysmography

Allergen-induced hyperreactivity in OVA-treated mice was measured on day 22 by pulmonary-function responses to increasing doses of methacholine by noninvasive plethysmography [23] in conscious, free-moving, spontaneously breathing mice. The degree of bronchoconstriction was expressed as enhanced pause (Penh), a calculated dimensionless value that correlates with measurement of airway resistance, impedance, and intra-pleural pressure.

Preparation of cell suspensions

Bone marrow cells were flushed from femurs [24]. Mice were bled retro-orbitally and red cells hemolyzed as described [24]. Bronchoalveolar lavage fluid (BALf) was collected as described [23]. Lung parenchymal cells (LP) were collected from lavaged lungs that were minced and digested as described in [13], then vortexed and filtered through sterile 40- μ m Nitex filter (Sefar America, Depew, NY, USA) before use.

FACS analysis

Nucleated cells from peripheral blood (PB), bone marrow (BM), BAL fluid, and LP were analyzed on a FACSCalibur (BD Immunocytometry Systems, San Jose, CA, USA) using CELLQuest software [19]. BD Biosciences (San Diego, CA, USA) antibodies used for cell-surface staining included: CD45 (30F-11), CD3 (145-2C11), CD4 (RM4-5), CD45RC (DNL-1.9), CD8 (53-6.7), B220 (RA3-6B2), IgM, CD19 (ID3), CD21 (7G6), CD23 (B3B4), GR-1 (RB6-8C5), and Mac1 (M1/70). F4/80 (Cl:A3-1(F4/80)) was obtained from Serotec Ltd. (Raleigh, NC, USA) and anti- $\alpha 4$ integrin (PS2) and anti-VCAM-1 (M/K-2) were from Southern Biotechnology (Birmingham, AL, USA). Irrelevant isotype-matched antibodies (BD Biosciences) were used as controls.

Smear evaluation

Proportions of eosinophils and mast cells were assessed in Wright-Giemsa-stained smears.

Histology and immunocytochemistry

Hematoxylin and eosin (H&E), Masson's Trichrome stains, Alcian Blue, and Periodic Acid Schiff staining were as described [25]. Lung tissue sections were stained using anti-VCAM-1 (M/K-2) antibody [20].

Goblet cell metaplasia

Using a microscope, percentages of metaplastic goblet cells (detected by Alcian Blue staining) in lung sections were calculated with counts from 10 different fields per mouse as described [22].

ELISPOT

IL-4⁺ and IFN- γ ⁺ cells in single cell suspensions from lung parenchyma and BALf were detected employing standard ELISPOT assays [10] using detection and capture monoclonal antibodies and AEC substrate reagent from BD Biosciences. Dots were counted manually using 40 $\times$ magnification.

Measurement of cytokines

Th1/Th2 cytokines in BALf and plasma were assayed with mouse cytometric bead array (CBA, BD Biosciences). IL-13 and eotaxin were measured by Quantikine M kits from R&D Systems (Minneapolis, MN, USA).

OVA-specific IgE and IgG1 in plasma

Anti-mouse IgE (R35-72) and IgG1 (A85-1) antibodies (BD Biosciences) were used to measure OVA-specific IgE and IgG1 in plasma using sandwich ELISA [26,27].

Adoptive transfer

At day 8 after i.p. sensitization, 5×10^6 CD4⁺ splenocytes from both $\alpha 4^{+/+}$ controls ($\alpha 4^{+/+}$ cre⁻) or $\alpha 4^{\Delta/\Delta}$ mice were purified by

magnetic-activated cell sorting (MACS, Miltenyi Biotec, Auburn, CA, USA), then injected into the tail veins of naïve controls or $\alpha 4^{\Delta/\Delta}$ recipients. The mice were subsequently challenged with 3 i.t. instillations of OVA over the next 72 hours, and sacrificed 24 hours after the last instillation [28]. One group of $\alpha 4^{\Delta/\Delta}$ mice also received unpurified $\alpha 4^{+/+}$ splenocytes from sensitized mice.

CFU-C assay

To quantitate committed hematopoietic progenitors of all lineages, CFU-C assays were performed as described [19].

Proliferation assay

MACS-separated CD4⁺ and CD8⁺ T cells from spleens were stimulated in vitro with various concentrations of stimuli (CD3/CD28, phorbol myristic acetate (PMA)/ionomycin, irradiated antigen-presenting cells (APCs), and lipopolysaccharide (LPS) to assay proliferative responses. After 72 hours, proliferation was measured by either CellTiter96 assay from Promega (Madison, WI, USA) measuring OD at 570 nm, or by [³H]-thymidine incorporation following standard protocols.

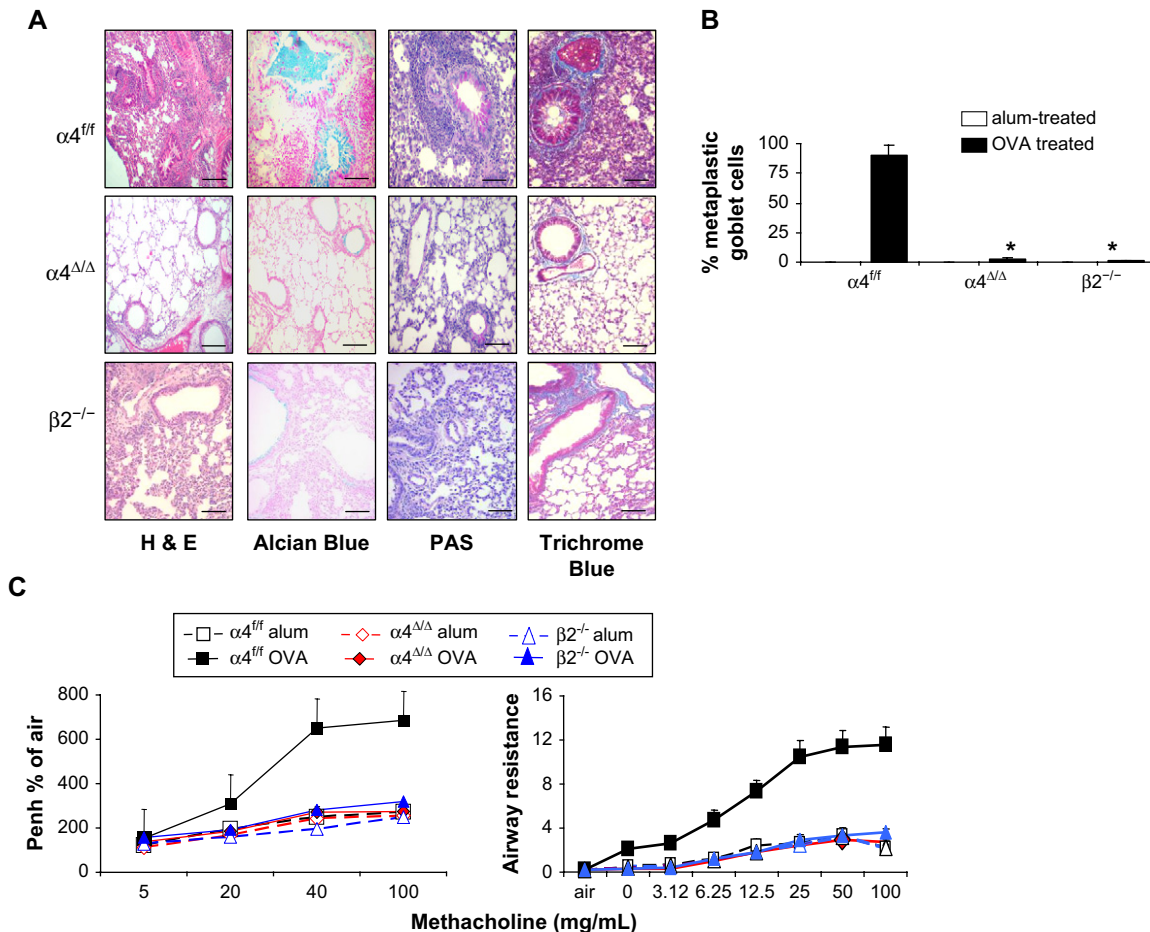


Figure 1. Deletion of $\alpha 4$ and $\beta 2$ integrins abrogates allergic lung disease in mice. Asthma phenotype was not induced in knockout mice as shown by the following: (A) Paraffin sections of lung tissue (H&E, Alcian Blue, PAS, and Masson's Trichrome). (B) % mean $\pm$ SEM of goblet cells [27] with metaplasia (Alcian Blue), * $p < 0.05$ comparing lung tissue from both $\alpha 4^{\Delta/\Delta}$ and $\beta 2^{-/-}$ mice to OVA-treated control lung. (C) In vivo airway responses to methacholine measured by whole-body plethysmography. Left panel: OVA-treated integrin-deficient mice do not show increased AHR to increasing doses of methacholine as control cre- $\alpha 4^{+/+}$ littermates mice do ($p < 0.001$ compared to Penh values in alum-treated control and treated and alum-treated $\alpha 4^{\Delta/\Delta}$ and $\beta 2^{-/-}$ mice). Right panel: Invasive plethysmography of acute asthma in the same groups of mice as in C, except that $\alpha 4^{\Delta/\Delta}$ were from Tie2cre⁺ $\alpha 4^{+/+}$ double-transgenic mice [21].

Statistics

Data were analyzed using two-tailed Student's *t*-test and *p* values less than 0.05 were considered statistically significant.

Results

$\alpha 4^{\Delta/\Delta}$ mice fail to develop the composite asthma phenotype in response to acute allergen challenge

Following initial sensitization and subsequent OVA challenges, control ($\alpha 4^{+/+}$ Mxcre⁻) mice develop the characteristic hallmarks of acute asthma, including eosinophil and lymphocyte sequestration in lung and BALf, mucus hypersecretion, goblet cell metaplasia, and AHR to methacholine measured by either invasive or noninvasive plethysmography. None of the above changes were noted in our $\alpha 4^{\Delta/\Delta}$ mice (Figs. 1 and 2).

Cells migrating to lung interstitium and airway lumen were quantified and their phenotypes analyzed by FACS (Fig. 2A) and cytospin smears. In OVA-treated controls, cells recovered from BALf were 10-fold over alum-treated, but in $\alpha 4^{\Delta/\Delta}$ mice were unchanged from alum-treated mice (Fig. 2A). Likewise, fewer cells were recovered from lung parenchyma in OVA-treated $\alpha 4^{\Delta/\Delta}$ mice compared to similarly treated controls (Fig. 2A). If cells migrating to lung or BALf are expressed as a fraction of total circulating leukocytes (recruitment index), they represent 3% in lung and about 1% in BALf (Table 1) in OVA-treated $\alpha 4^{\Delta/\Delta}$ mice, but 29% and 33% respectively in OVA-treated controls. Eosinophils, whose role in allergic asthma remains controversial [29,30], were 18% and 41% of total white blood cells (WBCs) recovered from lung and BALf respectively in controls, but only 2% and 1% in $\alpha 4^{\Delta/\Delta}$ mice (Fig. 2A). Further, IL-4⁺ cells were low in LP and BALf, and IFN- γ ⁺ cells were low in BALf in $\alpha 4^{\Delta/\Delta}$ mice (Fig. 2B).

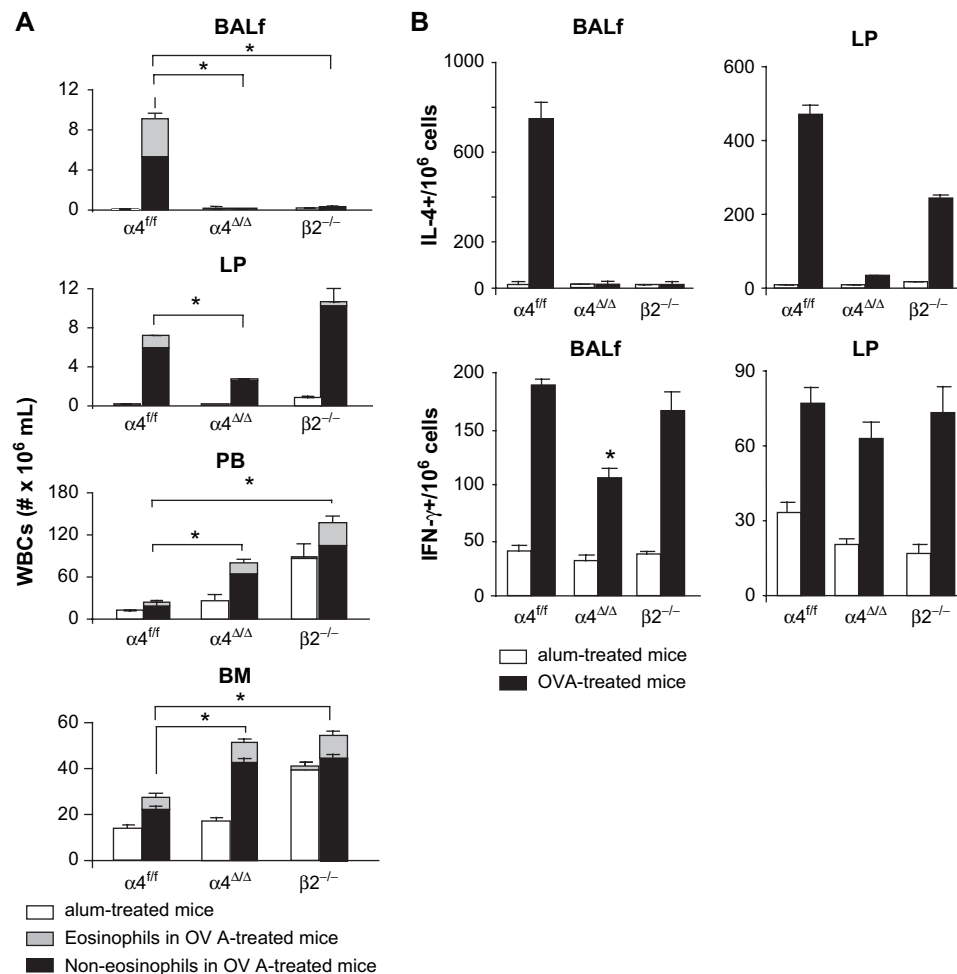


Figure 2. Local and systemic cellular and humoral responses to OVA challenge in control and OVA-treated mice. (A) Numbers of total WBCs in alum-treated mice (white bars) and OVA-treated mice (black/gray bars) are shown. Height of gray bars denotes number of eosinophils and black bars represent noneosinophils in BALf from both lungs, LP from both lungs, peripheral blood and bone marrow from both femurs, **p* < 0.05 post-OVA in integrin-deficient mice compared to post-OVA control. Cell numbers are represented as mean $\pm$ SEM, 12 mice were tested per group. (B) Top panel: Numbers of IL-4⁺ cells in 10⁶ cells from BALf and LP as detected by ELISPOT from alum-treated mice (white bars) and OVA-treated mice (black bars). Lower panel: Numbers of IFN- γ ⁺ cells in 10⁶ cells from BALf and LP in the same mice, *n* = 12/group, **p* = 0.05 post-OVA in integrin-deficient mice compared to post-OVA control.

Table 1. Cellular responses of leukocyte subsets to OVA treatment in all groups of mice studied

BLOOD								
	Total	Lymphocytes		Monocytes	Neutrophils	Eosinophils	Basophils	
	WBCs	CD3 ⁺	B220 ⁺					
WT	25.92 ± 6.41	7.69 ± 2.45	6.25 ± 1.07	1.7 ± 2.48	8.38 ± 2.05	0.79 ± 1.56	1.11 ± 0.03	
$\alpha 4^{\Delta/\Delta}$	79.45* ± 22.24	26.3* ± 1.18	19.6* ± 3.75	5.72 ± 1.18	21.42* ± 4.85	6.37* ± 2.05	0.04* ± 0.001	
$\beta 2^{-/-}$	147.28* ± 60.16	43.3* ± 8.22	14.6* ± 1.53	8.41* ± 0.13	57.87* ± 3.97	22.76* ± 2.73	0.34* ± 0.01	
VCAM-1 ^{-/-}	18.79 ± 3.58	5.108 ± 1.97	4.83 ± 1.35	1.72 ± 0.89	5.66 ± 1.43	0.65 ± 0.07	0.875 ± 0.01	
LUNG								
	Total	Lymphocytes		Monocytes	Neutrophils	Eosinophils	Basophils	
	WBCs	CD3 ⁺	B220 ⁺				Mast	
WT	7.18 ± 0.12	2.84 ± 0.05	0.06 ± 0.01	1.02 ± 0.03	1.36 ± 0.01	1.3 ± 0.002	0.028 ± 0.001	1.8 ± 0.07
$\alpha 4^{\Delta/\Delta}$	2.64* ± 0.04	0.42* ± 0.07	0.0076* ± 0.0001	0.592* ± 0.04	1.238 ± 0.02	0.06* ± 0.001	0	0.36* ± 0.002
$\beta 2^{-/-}$	10.7 ± 0.97	0.008* ± 0.02	0.2 ± 0.001	1.66 ± 0.05	3.96* ± 0.06	0.58* ± 0.01	0	3.4* ± 0.02
VCAM-1 ^{-/-}	6.62 ± 1.01	2.11 ± 0.65	0.05 ± 0.002	0.82 ± 0.15	1.23 ± 0.018	0.92 ± 0.06	0.06 ± 0.002	1.405 ± 0.001
BALf (2 lungs)								
WT	8.5 ± 0.42	1.48 ± 0.05	1.39 ± 0.17	1.14 ± 0.07	0.78 ± 0.04	3.83 ± 0.08	0.04 ± 0.001	0.501 ± 0.001
$\alpha 4^{\Delta/\Delta}$	0.82* ± 0.17	0.09* ± 0.002	0.04* ± 0.016	0.06* ± 0.001	0.46* ± 0.07	0.019* ± 0.001	0.002* ± 0.0001	0.05* ± 0.001
$\beta 2^{-/-}$	0.72* ± 0.03	0.08* ± 0.002	0.048* ± 0.001	0.141* ± 0.04	0.066* ± 0.001	0	0	0.649 ± 0.05
VCAM-1 ^{-/-}	8.59 ± 1.67	1.59 ± 0.54	1.46 ± 0.03	0.97 ± 0.21	0.63 ± 0.004	2.54 ± 0.001	0.02 ± 0.001	0.58 ± 0.02

Values represent total numbers (in millions) of different cells and their subsets that migrated from blood (~2 mL) to lung interstitium (both lungs) and to BALf (both lungs) of WT (wild-type), $\alpha 4^{\Delta/\Delta}$, $\beta 2^{-/-}$, and VCAM-1^{-/-} mice. Recruitment of total WBCs (white blood cells) is less in $\alpha 4^{\Delta/\Delta}$ lung as well as in BALf compared to WT and VCAM-1 mice. In $\beta 2^{-/-}$ lungs, except for T cells and eosinophils, all other cells were increased in number, * $p < 0.01$ compared with post-OVA control. Recruited T cells post-OVA (both CD4⁺ and CD8⁺) were CD45RC-negative (memory) in control lung and BALf, but the few T cells in $\alpha 4^{\Delta/\Delta}$ and $\beta 2^{-/-}$ mice were mostly CD45RC-positive (naïve). Note the differences between $\alpha 4$ - and $\beta 2$ -deficient mice in total LP (lung parenchyma) cell content. Mφ denotes macrophage, n = 12/genotype in $\alpha 4^{\Delta/\Delta}$, $\beta 2^{-/-}$, and WT and 7/group in VCAM-1^{-/-}.

To explore whether hematopoietic progenitor cells (HPCs), which are found to be increased post-OVA in circulation [31], migrate to lung or BALf along with mature cells, we quantitatively assessed them by clonogenic CFU-C assays. We found increased progenitor cells in both lung and BALf in control, but not in $\alpha 4^{\Delta/\Delta}$ mice (Fig. 3), despite the higher circulating HPC levels in the latter. Thus, the migratory behavior of progenitor cells to lung followed a pattern similar to that of mature cells in all mice.

OVA-specific sensitization responses in $\alpha 4^{\Delta/\Delta}$ mice

To examine whether $\alpha 4^{\Delta/\Delta}$ mice responded optimally to OVA sensitization, we evaluated the elaboration of OVA-specific IgE in these mice. OVA-specific IgG1 was similar in all three genotypes, but OVA-specific IgE was significantly lower only in $\alpha 4^{\Delta/\Delta}$ mice (Fig. 4A). Since the class

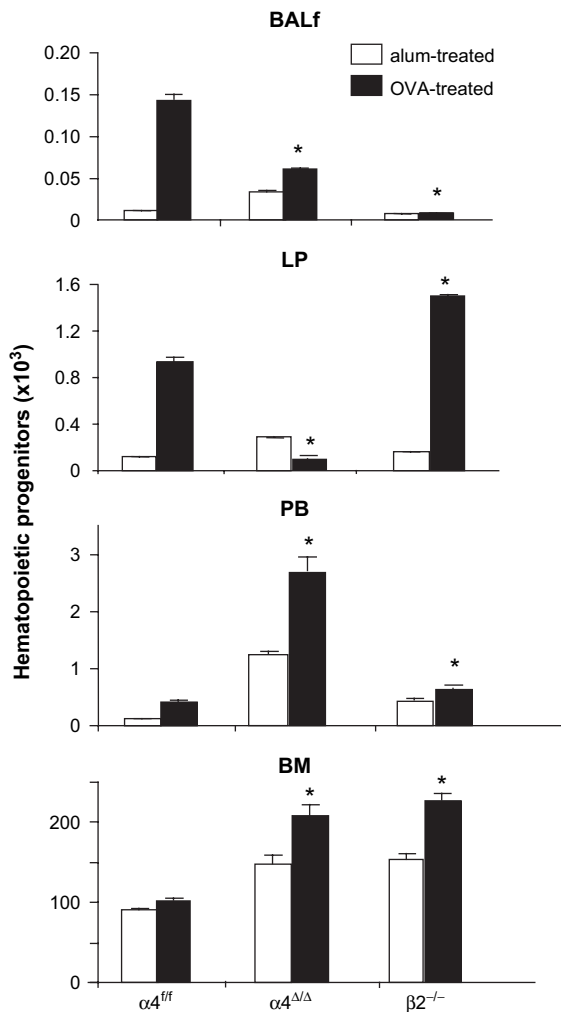


Figure 3. CFU-Cs from alum-treated and OVA-treated mice. Total number of CFU-C per organ was calculated using the number of colonies/number of cells plated, normalized to the total number of cells counted in single cell suspensions obtained from 2 femurs (BM), 2 mL of blood (PB), lung parenchyma (LP), and BALf from both lungs from alum-treated and OVA-treated mice, * $p < 0.05$ compared to OVA-treated control.

switch from IgG1 to IgE is largely Th2 cytokine-dependent [32], we measured the systemic elaboration of Th1- (IL-2, IFN- γ , TNF- α) and Th2- (IL-4, IL-5, IL-13) dependent cytokines and eotaxin in plasma and in BALf in all OVA-treated mice (Fig. 4B). After OVA challenge in control mice, a marked increase was seen in Th2 cytokine levels (i.e., IL-5, IL-13, and eotaxin) in plasma and BALf, but very low levels in $\alpha 4^{\Delta/\Delta}$ mice, consistent with absence of effector cell recruitment in BALf.

$\alpha 4^{\Delta/\Delta}$ T cells fail to show proliferative response to OVA but not to non-Th2 stimuli

To ascertain the intrinsic functional status of $\alpha 4$ -deficient T lymphocytes, proliferative responses to specific (OVA) (Fig. 4C) and to nonspecific stimuli (anti-CD3/CD28, PMA/ionomycin) (Fig. 5) were assayed. Although $\alpha 4^{\Delta/\Delta}$ splenocytes from OVA-sensitized mice proliferate in response to nonspecific stimuli, they failed to proliferate in response to increasing concentrations of OVA, in contrast to control cells, which efficiently proliferate in response to both nonspecific and Th2 stimuli. When Th2 cytokines were measured in the culture supernatant of splenocytes 4 days post incubation with OVA, levels of all Th2 cytokines remained at baseline levels when $\alpha 4^{\Delta/\Delta}$ cells were used (Fig. 4D), in contrast to controls.

Adoptive transfer of $CD4^{+}$ splenocytes from sensitized $\alpha 4^{\Delta/\Delta}$ mice fails to restore asthma in naïve control mice upon their subsequent challenge

To test whether the failure of $\alpha 4^{\Delta/\Delta}$ mice to develop asthma is due to an intrinsic inability of $\alpha 4^{\Delta/\Delta}$ splenocytes to respond to OVA and recruit all other cell subsets to lung and BALf, we adoptively transferred (Fig. 6) sensitized $\alpha 4^{\Delta/\Delta}$ donor cells (either unpurified $\alpha 4^{+}$ splenocytes or $CD4^{+}$ -purified $\alpha 4^{+}$ splenocytes) from $\alpha 4^{+/+}$ Mxcre⁻ ($\alpha 4^{+/+}$) mice to naïve $\alpha 4^{+/+}$ or $\alpha 4^{\Delta/\Delta}$ mice by i.v. injection. While sensitized $\alpha 4^{+/+}$ splenocytes restored asthma phenotype after challenge in both naïve $\alpha 4^{+/+}$ and $\alpha 4^{\Delta/\Delta}$ recipients, sensitized $CD4^{+}\alpha 4^{\Delta/\Delta}$ splenocytes transferred to both naïve $\alpha 4^{+/+}$ and $\alpha 4^{\Delta/\Delta}$ recipients failed to cause asthma upon further OVA challenge (Fig. 6A, lower line graphs). Analysis of BALf after the adoptive transfer of $\alpha 4^{+}$ cells and OVA challenge revealed an increase in Th2 cytokines (Fig. 6B) and an accumulation in BALf of a mononuclear cell population ($4.45 \pm 0.12 \times 10^6$), predominantly of lymphocytes and a few other cells (macrophages and neutrophils), but no eosinophils.

Lack of increased VCAM-1 expression in response to OVA in $\alpha 4^{\Delta/\Delta}$ lung

Upregulated VCAM-1 expression in lung vasculature in response to OVA is another hallmark of allergic asthma [12,25], believed to be implicated in the enhancement of inflammatory cell interactions with the endothelium and the preferential recruitment of memory cells or mast cells [33]. Additionally, soluble VCAM-1 (sVCAM-1) may be

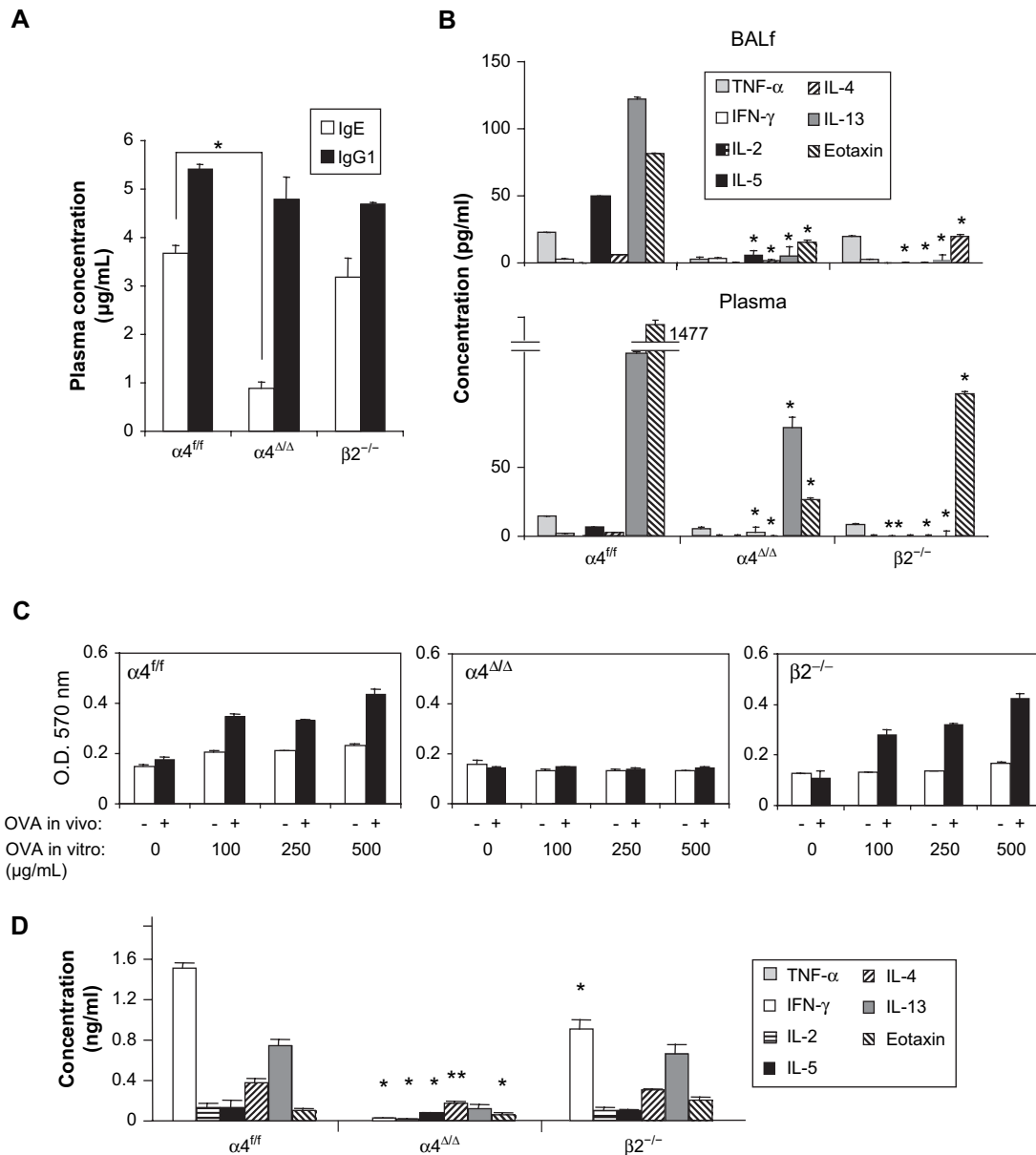


Figure 4. OVA sensitization responses in control ($\alpha 4^{f/f}$) vs. $\alpha 4^{\Delta/\Delta}$ and $\beta 2^{-/-}$ mice. **(A)** Neonatally ablated $\alpha 4^{\Delta/\Delta}$ mice show significantly decreased OVA-specific IgE levels ($*p < 0.05$ relative to control) in plasma, but IgG1 levels were similar in all genotypes, $n = 12$. Levels were undetectable in plasma from alum-treated mice in all groups and are not shown. **(B)** Th1 and Th2 cytokines and eotaxin levels in BALf and plasma of OVA-treated mice measured by ELISA and cytometric bead array. Th2 cytokines were lower for $\alpha 4^{\Delta/\Delta}$ and $\beta 2^{-/-}$ mice compared to controls, $*p < 0.01$ compared with post-OVA control, $n = 12$ /genotype. All cytokines in alum-treated mice were below the level of detection. **(C)** Splenocytes from mice of three genotypes with either in vivo saline sensitization or OVA sensitization/challenge were allowed to proliferate in vitro with increasing concentrations of OVA over the course of 4 days. Proliferation was assessed by incorporation of MTT tetrazolium (O.D. 570 nm). Only $\alpha 4^{\Delta/\Delta}$ cells failed to show appreciable proliferative changes, $*p < 0.001$ relative to control cells, $n = 6$ /genotype. **(D)** Cytokines in supernatants from the in vitro OVA-stimulated splenocyte cultures (500 $\mu\text{g/mL}$ OVA) in Figure 3C. Only $\alpha 4^{\Delta/\Delta}$ cells failed to generate increased levels of cytokines, $*p < 0.001$ relative to control, $n = 6$ mice/genotype.

important in promoting the allergic late-phase eosinophilia in a positive feedback loop of interaction between $\alpha 4$ integrin and its ligand VCAM-1 [34]. We documented that VCAM-1 expression in $\alpha 4^{\Delta/\Delta}$ lung, as well as sVCAM-1 in BALf and plasma of $\alpha 4^{\Delta/\Delta}$ mice after OVA challenge, were significantly reduced, compared to that found in control samples (Fig. 7).

Differences between responses of $\alpha 4^{\Delta/\Delta}$, $\beta 2^{-/-}$, or VCAM-1 $^{\Delta/\Delta}$ mice to acute OVA challenge

In addition to control ($\alpha 4^{f/f}$), $\beta 2^{-/-}$ (CD18 KO) mice [10,35] were used for comparison, to explore whether these two integrins have overlapping or redundant roles in inflammatory recruitment of leukocytes in acute asthma. Like the other groups, $\beta 2^{-/-}$ mice responded systemically by

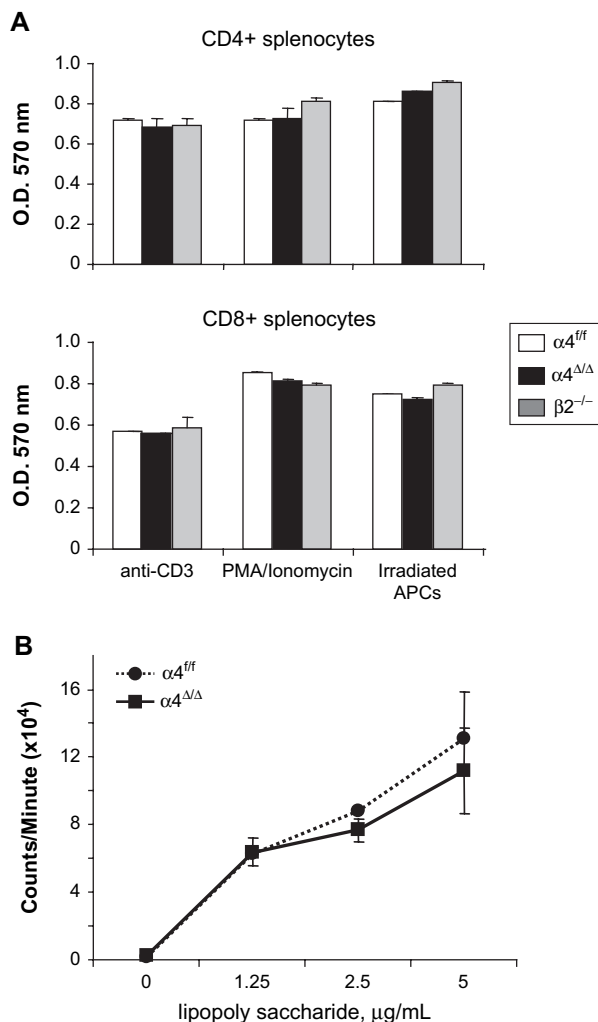


Figure 5. Proliferation responses of splenocytes from control vs knockout mice. CD4^+ and CD8^+ splenocytes were positively enriched using MACS and activated in vitro with different stimuli: 1 $\mu\text{g/mL}$ anti-CD3/CD28, 10 ng/mL PMA/1 μM ionomycin and allogeneic irradiated stimulator splenocytes (APCs). (A) shows similar proliferative responses (measured by Cell-Titer96 Assay and OD measurement at 570 nm) in all three genotypes. (B) CD19^+ cells from control and $\alpha 4^{\Delta/\Delta}$ mice enriched by MACS were assayed for proliferative responses to increasing doses of *E. coli* LPS by [^3H]-thymidine incorporation. Similar proliferative responses are seen. (n = 5 mice/group.)

increasing nucleated cells and progenitors in their BM and PB (Figs. 2A and 3). However, as previously described [10], $\beta 2^{-/-}$ mice failed to develop AHR and to accumulate eosinophils and lymphocytes in BALf (Fig. 2A). In contrast to BALf, we found significant recruitment of both mature myeloid cells (Table 1) and progenitors in the lung of $\beta 2^{-/-}$ mice post-OVA (Figs. 2A and 3). Nevertheless, the proportion of recruited cells in lung was 8% of circulating cells, in contrast to 29% in control mice, suggesting that the recruitment of $\beta 2^{-/-}$ cells in allergic lungs, despite the significant numbers, was not as efficient as that seen with normal cells.

In addition to the levels of cytokines (in response to acute OVA challenge) obtained from ELISA, we also measured Th1 vs Th2 cell numbers in $\alpha 4^{\Delta/\Delta}$ and $\beta 2^{-/-}$ mice to exclude the possibility that low levels of IL-4 in BALf may be due to bound IL-4 in the lung interstitium. IL-4 $^+$ (Th2) and IFN- γ^+ (Th1) cells in BALf and lung quantitated by ELISpot (Fig. 2B) were again decreased in both $\alpha 4^{\Delta/\Delta}$ and $\beta 2^{-/-}$ mice. TNF- α was also reduced in BALf of $\alpha 4^{\Delta/\Delta}$ compared to controls, whereas Th2 cytokines in plasma were increased only in OVA-treated control mice, but not in similarly treated $\alpha 4^{\Delta/\Delta}$ and $\beta 2^{-/-}$ mice (Fig. 4B).

To clarify the role of VCAM-1 upregulation in asthma, we induced and similarly evaluated asthma phenotype in VCAM-1-deficient mice [36]. In contrast to $\alpha 4$ -deficient mice, all the cellular and humoral hallmarks of asthma were present in these mice (Fig. 8).

Discussion

Because prior data from studies using antibodies to $\alpha 4$ integrins provided inconsistent results varying with the type, dose, and route of administration of antibody used [11–18], in the present study we explored the role of $\alpha 4$ integrins in the development of acute allergic asthma using a conditional $\alpha 4$ -integrin knockout mouse. Further, parallel studies in $\beta 2^{-/-}$ and VCAM-1-deficient mice provided novel mechanistic insights about the selective roles of the two classes of integrins in allergen sensitization and inflammatory cell recruitment to allergic lung and the role of VCAM-1 in asthma.

Studies addressing OVA sensitization were of interest in $\alpha 4^{\Delta/\Delta}$ mice. Although an adequate systemic cellular response was seen in BM and PB (Fig. 2A), OVA-specific IgE in plasma (Fig. 4A) was only modestly increased. Normal levels of OVA-specific IgG1 (but low IgE) in $\alpha 4^{\Delta/\Delta}$ mice support differential regulation (i.e., differential sensitivity to IL-4) of IgG1 vs IgE production noted previously [37–39]. It is of interest that although both IgE and IgG1 are IL-4 dependent, IL-4/IL-13 double-deficient mice and IL-4/R $\alpha^{-/-}$ mice produced IgE and IgG1 responses [40], suggesting IL-4/IL-13-independent responses. Therefore, adequate production of IgG1 and decreased IgE are neither incompatible with low levels of IL-4 nor do they predict on their own the asthma outcome. For the systemic BM response, we speculate that increased sensitivity of $\alpha 4^{\Delta/\Delta}$ cells to tested or untested cytokines (e.g., IL-3, IL-9) and/or decreased levels of inhibitory cytokines may have been responsible, whereas the modest IgE levels seen in these mice could reflect an inefficient sensitization process largely derived from regional lymph node cells [32].

In addition to reduced IgE, low levels of Th2 cytokines were seen in plasma samples from OVA-treated $\alpha 4^{\Delta/\Delta}$ (Fig. 4B) mice. As the initiation of asthmatic reaction is Th2 dependent, it would seem that $\alpha 4$ -deficient mice are unable to sensitize a sufficient number of Th2 cells to raise

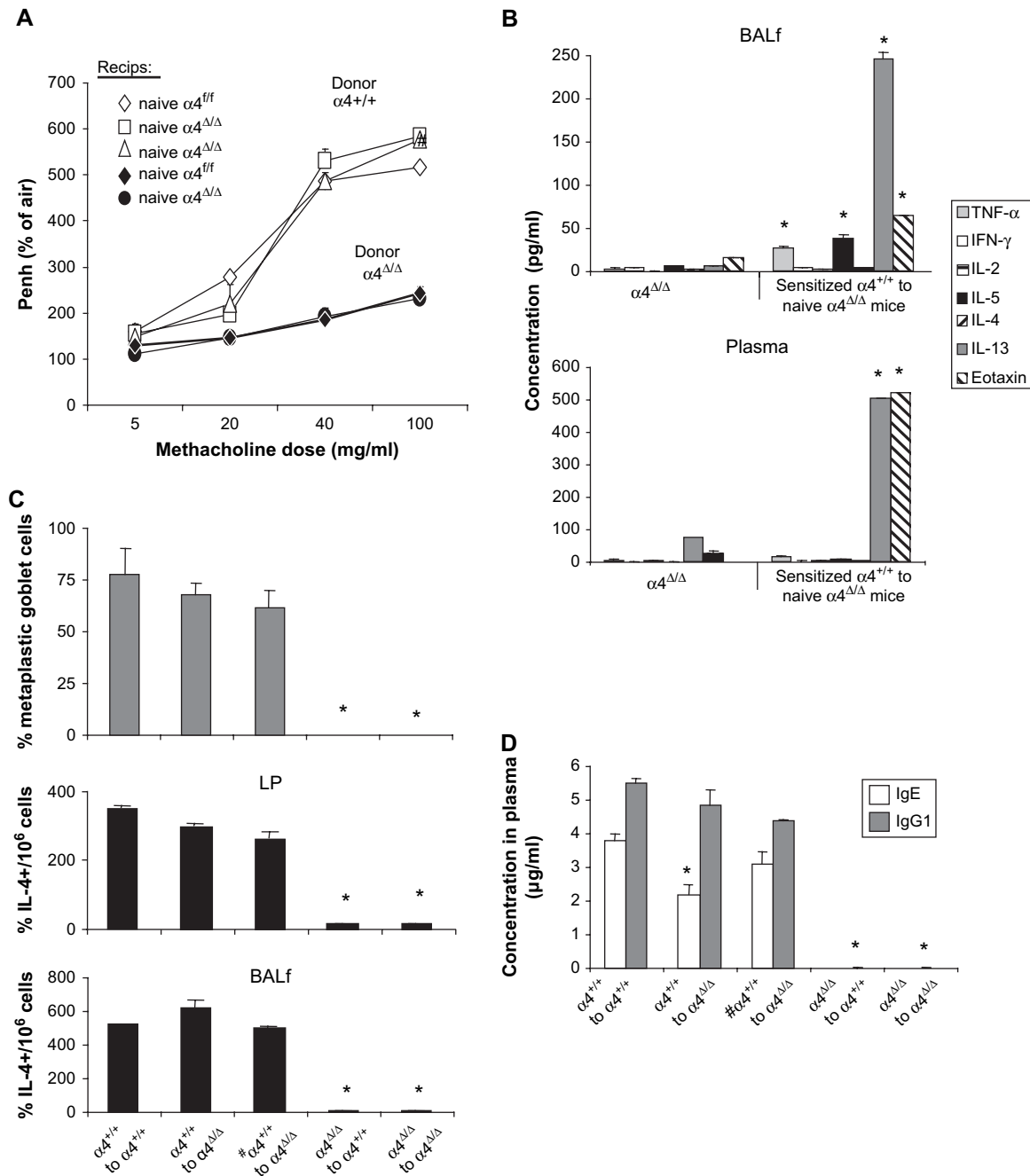


Figure 6. Adoptive transfer of sensitized CD4 $^{+}$ $\alpha 4^{+}$ splenocytes in naive $\alpha 4^{\Delta/\Delta}$ mice restores asthma response. (A) Naive $\alpha 4^{\Delta/\Delta}$ recipients receiving CD4 $^{+}$ splenocytes (□) or unpurified splenocytes (△) from sensitized $\alpha 4^{+/+}$ donors showed increased AHR after challenge. By contrast, when sensitized $\alpha 4^{\Delta/\Delta}$ donor cells were given to naive recipient mice, neither control $\alpha 4^{+/+}$ (◆) nor $\alpha 4^{\Delta/\Delta}$ (●) showed increased AHR after challenge. Naive $\alpha 4^{+/+}$ recipients given sensitized CD4 $^{+}$ $\alpha 4^{+/+}$ splenocytes (◇) were considered positive controls. (B) Th1 and Th2 cytokines and eotaxin levels from plasma or BALf from naive $\alpha 4^{\Delta/\Delta}$ mice after the adoptive transfer of sensitized CD4 $^{+}$ $\alpha 4^{+/+}$ splenocytes are shown compared to nontransplanted OVA-treated $\alpha 4^{\Delta/\Delta}$ mice, * p < 0.001 relative to $\alpha 4^{\Delta/\Delta}$ without adoptive transfer. (C) Upper panel: Development of Alcian Blue–stained mucus-laden metaplastic goblet cells in lung airway lumina of unsensitized mice given cells from sensitized $\alpha 4^{+/+}$ donors (A, upper line graphs). Middle and lower panels: Percentage of IL-4 $^{+}$ cells/10 ^{6} cells (ELISpot assay) show a similar trend in LP and BALf, n = 5/group. * p < 0.05 comparing naive recipients given cells from sensitized Δ/Δ donors to recipients given cells from sensitized $+/+$ donors. (D) OVA-specific IgE and IgG1 in plasma were increased in all recipients of $\alpha 4^{+/+}$ donor cells, but not in recipients receiving $\alpha 4^{\Delta/\Delta}$ cells (#purified CD4 $^{+}$ cells, others are unpurified). For A–D: n = 5 mice/group.

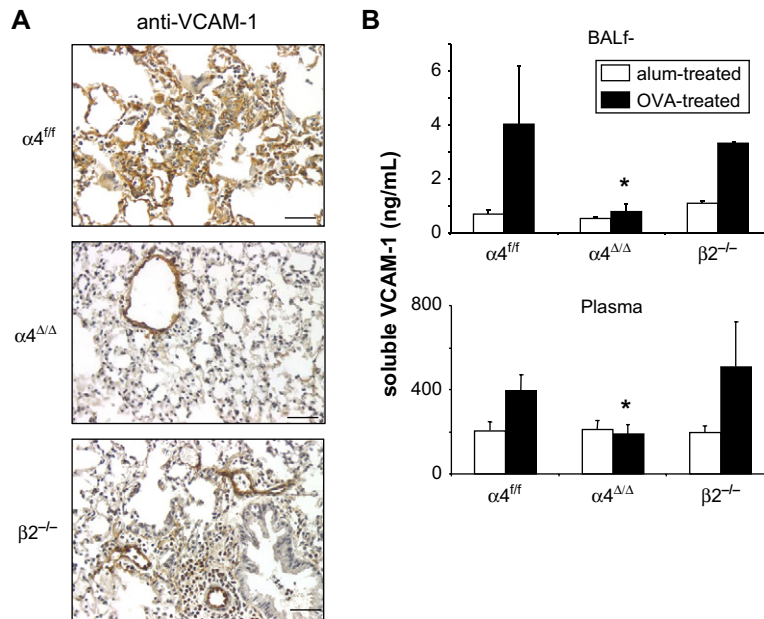


Figure 7. VCAM-1 expression in lung sections and sVCAM-1 levels in BALf and plasma in response to OVA. **(A)** Upregulation of VCAM-1 is seen in control and $\beta 2^{-/-}$ but not in $\alpha 4^{\Delta/\Delta}$ prefixed, frozen lung tissue sections. **(B)** Soluble VCAM-1 levels in BALf and plasma show comparable increases in control and $\beta 2^{-/-}$ mice but remain unchanged in $\alpha 4^{\Delta/\Delta}$ mice. * $p < 0.001$ compared to OVA-treated control, $n = 6$ mice/group.

PB cytokine levels similar to those seen in controls. $CD4^{+}$ and $CD8^{+}$ splenocytes from $\alpha 4^{\Delta/\Delta}$ mice after primary in vivo OVA sensitization were unable to proliferate in response to OVA in vitro (Fig. 4C), although their proliferation to nonspecific stimuli, like anti-CD3/CD28, PMA/ionomycin, or allogeneic splenocytes from BALB/c mice (APCs), remained similar to that of control cells (Fig. 5A). The response of $\alpha 4^{\Delta/\Delta}$ $CD19^{+}$ splenocytes to LPS in vitro was also normal (Fig. 5B). In contrast to $\alpha 4^{\Delta/\Delta}$, $\beta 2^{-/-}$ splenocytes showed similar proliferative responses to both specific (OVA) and nonspecific stimuli (anti-CD3, PMA, etc.) compared to controls [30]. Likewise, Th1 and Th2 cytokines and eotaxin were increased in culture supernatants of both control and $\beta 2^{-/-}$ cells, but not in culture supernatants from $\alpha 4^{\Delta/\Delta}$ splenocytes (Fig. 4D). Nevertheless, in $\beta 2^{-/-}$ mice, circulating Th2 cytokines were surprisingly low for unclear reasons. We speculate that an increased uptake by tissue-wide distribution of inflammatory cells at steady state in these mice may be responsible.

Migration impairments noted in $\alpha 4^{\Delta/\Delta}$ mice could be attributed to either intrinsic migratory defects of all $\alpha 4^{\Delta/\Delta}$ leukocyte subsets (lymphocytes, monocytes, eosinophils), or migratory deficiency of only one population upstream (i.e., lymphocytes), that normally triggers a chemotactic response for other populations downstream in a sequential fashion. The data from adoptive transfer of both $\alpha 4^{+/+}$ and $\alpha 4^{\Delta/\Delta}$ cells (Fig. 6) provide further insight into the OVA-specific unresponsive state of $CD4^{+}$ $\alpha 4^{\Delta/\Delta}$ cells and collectively indicate: 1) the APCs in $\alpha 4^{\Delta/\Delta}$ mice were able to reactivate transferred $\alpha 4^{+/+}$ $CD4^{+}$ cells for asthma

induction, 2) eosinophil migration to airway lumen even in the presence of chemotactic stimuli is dependent primarily on $\alpha 4$ integrin, as no eosinophils migrated with transferred $\alpha 4^{+/+}$ $CD4^{+}$ cells, 3) eosinophils are not necessary for the development of the acute asthma phenotype consistent with recent data [29,30]), and 4) the lack of asthma phenotype development in $\alpha 4^{\Delta/\Delta}$ mice is not contributed by defective responses from airway resident cells including alveolar macrophages in this model, as previously suggested [15,41]. Thus, the failure of asthma development upon transfer of sensitized $\alpha 4^{\Delta/\Delta}$ cells suggests intrinsic defects in $CD4^{+}$ responses and putative inefficiency in T- and B-cell interactions. Lack of OVA-specific proliferation in vitro by sensitized splenocytes from $\alpha 4^{\Delta/\Delta}$ mice likely reflects inefficient responses from $\alpha 4$ -deficient $CD4^{+}$ T cells. Consistent with these data are previous studies suggesting VLA-4-dependent costimulatory effects on $CD4^{+}$ T-cell activation [42–45]. Recent independent studies also show evidence of an important role of VLA-4 in the generation of the immune response through its involvement in the formation of immune synapse [46], where $\alpha 4\beta 1$ is relocated to the peripheral supramolecular activation complex (pSMAC) during immune synapse formation between APCs and T lymphocytes. An inhibition of asthma similar in many aspects (i.e., cytokine responses, IgE responses) to that seen in our $\alpha 4^{\Delta/\Delta}$ mice was recently described after engagement (by agonistic Ab) of CD137 (4-1BB), an inducible T-cell costimulatory receptor [47]. In this case, a more complete anergy of OVA-specific $CD4^{+}$ T cells was induced than the one seen in $\alpha 4^{\Delta/\Delta}$ mice.

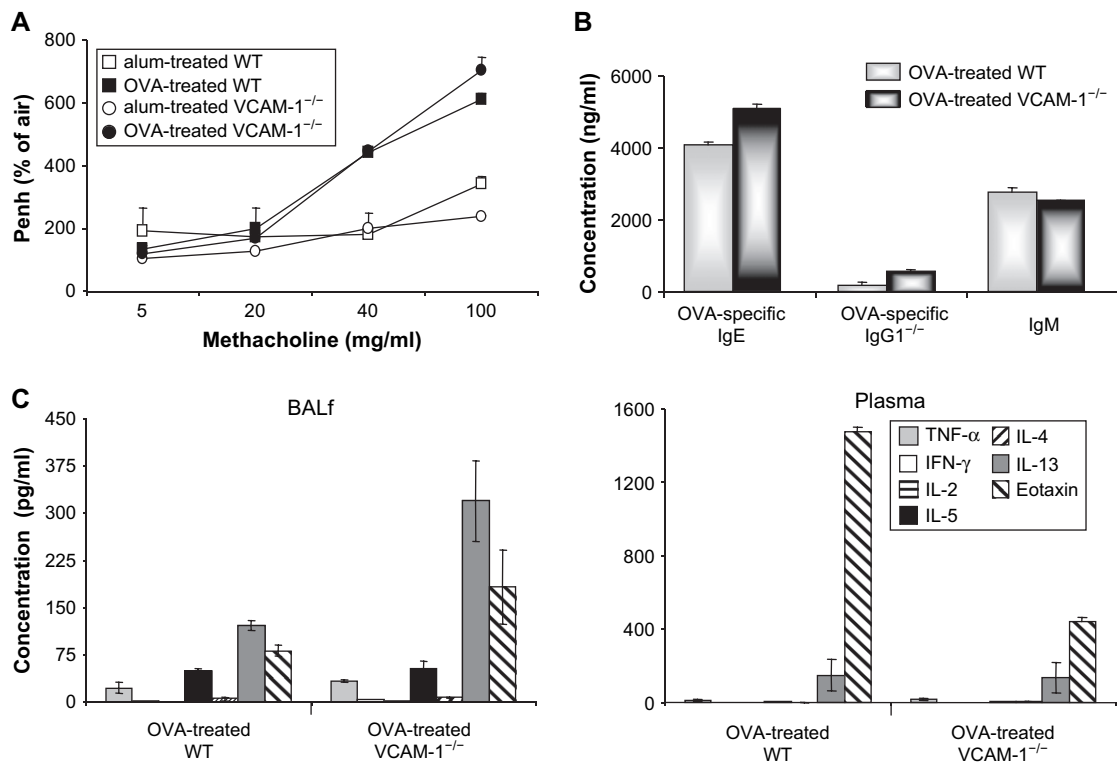


Figure 8. Acute asthma induction in VCAM-1-deficient mice compared to OVA-treated controls. (A) Response to methacholine was determined by non-invasive plethysmography. (B) Plasma levels of IgE, IgG1, and IgM in VCAM-1^{-/-} mice and their controls after OVA sensitization. (C) The concentrations of Th1 and Th2 cytokines in BALf and plasma were determined by ELISA (as in Fig. 4). The number of mice studied was 7/genotype.

Impairment in IL-4 and/or TNF- α secretion or other cytokines by $\alpha 4^{\Delta/\Delta}$ cells in response to OVA may also be responsible for lack of VCAM-1 upregulation in $\alpha 4^{\Delta/\Delta}$ lung post-OVA (Fig. 7), thereby preventing further cell sequestration and cytokine responses [48]. In addition to lung endothelial cells, VCAM-1 is upregulated in bronchial epithelial cells through their interaction with eosinophils in normal mice [49]. Lack of $\alpha 4$ /VCAM-1 interaction, likely due to a negative feedback loop in the $\alpha 4^{\Delta/\Delta}$ mice, was considered significant in the inhibition of development of the Th2 effector arm critical for asthma onset and maintenance, and $\alpha 4$ deficiency may prevent cross-talk between the inflammatory leukocyte and the endothelium important for acute asthma development. Whether additional factors, i.e., an increase in ADAM8, a sheddase for VCAM-1, are responsible for increase in sVCAM-1 in plasma [50] in other mice except $\alpha 4^{\Delta/\Delta}$ is also unclear. Nevertheless, asthma was not inhibited in our VCAM-1-deficient mice, suggesting that VCAM-1 upregulation in normal mice is likely a secondary event with no causative involvement in asthma. Of interest, anti-VCAM-1 treatment did not block IL-4-induced eosinophil accumulation in rat pleural cavities, in contrast to anti-rat $\alpha 4$ mAb. These and our data suggest dissociation between VCAM-1 upregulation and eosinophil accumulation, which is dependent on $\alpha 4$ integrin.

In the present study, both $\alpha 4^{\Delta/\Delta}$ and $\beta 2^{-/-}$ mice failed to develop a typical asthma phenotype in response to acute OVA challenge (Figs. 1 and 2). Upon dissecting the various criteria jointly leading to the composite asthma phenotype, we found that the migratory behavior of different cell subsets in the two types of deficient mice was quite distinct. In fact, the pattern of migratory responses from blood to lung and to airway lumen displayed by $\alpha 4^{\Delta/\Delta}$ mice differed from both control and $\beta 2^{-/-}$ mice. In contrast to control and in contrast to Ab studies [16], all $\alpha 4^{\Delta/\Delta}$ leukocyte subsets (T cells, B cells, monocytes, neutrophils, eosinophils) failed to migrate from circulation to both lung and airway lumen, whereas in $\beta 2^{-/-}$ mice, only T cells and eosinophils failed to migrate to lung and lumen. For other $\beta 2^{-/-}$ cells (B cells, monocytes, neutrophils, and macrophages), $\alpha 4$ integrins mediate all of the CD18-independent migration to lung [16], but there is interstitial migratory failure from lung to airway lumen (Table 1).

In summary, our data demonstrate that $\alpha 4$ integrins are necessary for all cell subset migration to allergic lung, and their absence attenuates the sensitization process and ancillary functions of sensitized cells. Further, our data clarify the subset-specific integrin usage for recruitment of cells from blood to lung interstitium and then to airway lumen. In agreement with prior data [29,51,52], eosinophil migration to BAL fluid is not essential for AHR and is an $\alpha 4$ - but not a VCAM-1-dependent process.

Acknowledgments

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