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Absence of α4 but not β2 integrins restrains development of chronic allergic asthma using mouse genetic models

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Abstract

Objective—Chronic asthma is characterized by ongoing recruitment of inflammatory cells and airway hyperresponsiveness leading to structural airway remodeling. Although α4β1 and β2 integrins regulate leukocyte migration in inflammatory diseases and play decisive roles in acute asthma, their role has not been explored under the chronic asthma setting. To extend our earlier studies with $\alpha4^{\Delta/\Delta}$ and $\beta2^{-/-}$ mice, which showed that both a4 and b2 integrins have nonredundant regulatory roles in acute ovalbumin (OVA)-induced asthma, we explored to what extent these molecular pathways control development of structural airway remodeling in chronic asthma.

Materials and Methods—Control, $\alpha4^{\Delta/\Delta}$, and $\beta2^{-/-}$mouse groups, sensitized by intraperitoneal OVA as allergen, received intratracheal OVA periodically over days 8 to 55 to induce a chronic asthma phenotype. Post-OVA assessment of inflammation and pulmonary function (airway hyperresponsiveness), together with airway modeling measured by goblet cell metaplasia, collagen content of lung, and transforming growth factor β1 expression in lung homogenates, were evaluated.

Results—In contrast to control and $\beta2^{-/-}$ mice, $\alpha4^{\Delta/\Delta}$ mice failed to develop and maintain the composite chronic asthma phenotype evaluated as mentioned and subepithelial collagen content was comparable to baseline. These data indicate that β2 integrins, although required for inflammatory migration in acute asthma, are dispensable for structural remodeling in chronic asthma.

Conclusion—α4 integrins appear to have a regulatory role in directing transforming growth factor β-induced collagen deposition and structural alterations in lung architecture likely through interactions of Th2 cells, eosinophils, or mast cells with endothelium, resident airway cells, and/or extracellular matrix.

In acute asthma, dysregulated immunity triggers a Th2 response by antigen-presenting cells and Th2-derived cytokines, especially interleukin-4 (IL-4) and IL-13, promoting B-cell differentiation into immunoglobulin (Ig) E-sequestering plasma cells. Cross-linking of IgE

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receptors on mast cells releases histamines, prostaglandins, thromboxane, and leukotrienes, leading to bronchoconstriction, vasodilation, and mucus secretion [1]. Thus, a cascade of interactions between cells and soluble molecules in the airways results in bronchial mucosal inflammation and airway hyperresponsiveness (AHR) [2]. In chronic allergic asthma, there is continuous recruitment of Th2 as well as inflammatory cells in the lung and airways. These cells and their secreted products elicit structural changes in resident airway cells, including epithelial desquamation, goblet cell metaplasia, mucus hypersecretion, and thickening of submucosa, manifested as bronchoconstriction and AHR [3,4]. Prominent in the remodeling process is the thickening of the airway wall with development of subepithelial fibrosis from deposition of extracellular matrix proteins, such as collagen, laminin, fibronectin, and tenascin in the lamina reticularis beneath the basement membrane [5–11]. Despite the fact that the histologic features of airway remodeling in chronic asthma have been well-characterized, the immunologic and inflammatory mechanisms that maintain or enhance remodeling are incompletely understood.

Although mouse models of asthma do not completely reproduce all the hallmarks of human disease, and several pathophysiologic responses in mice have been of limited value in humans, these models have provided important insights into the pathophysiology of asthma and have been used for testing new treatments of allergic asthma [12]. Using mouse models, it was found that leukocyte migration into lung is an important early event in the pathogenesis of asthma, and it is mediated by a series of adhesive interactions between leukocytes and airway cells for which integrins (β2 and β1) have been found to be critical participants [13–18]. While CD18 (β2 integrin) null mice have been used to investigate the role of CD18 in allergic asthma [19], studies on α4 integrins have been previously limited to those using monoclonal antibodies or other inhibitors of α4 integrin [13–15,17,18,20,21].

Our recent studies with conditionally ablated α4 knockout mice tested in parallel with $\beta 2^{-/-}$ mice showed that, while β2 integrins control inflammatory migration in the airways, α4 integrins subvert the onset of acute asthma by curtailing the initial sensitization process, as well as by preventing cross-talk between inflammatory leukocytes and their interaction with the endothelium and lung stroma [22]. Because chronic rather than acute asthma appears to be more relevant to human disease [23], it was important to explore the involvement of these two types of integrins in the chronic setting of allergen challenge. Thus, using a repeated-challenge protocol in a more chronic setting, we assessed, in these genetic mouse models, changes associated with structural remodeling of the airways to gain further insight into the contribution of α4 and β2 integrins to the airway remodeling in chronic allergic asthma.

Our data uncover novel information about the differential contribution of β2 vs α4 integrins in the composite phenotype of chronic asthma development and contribute to the understanding of mechanisms by which different cell subsets and molecular pathways participate in the pathophysiology and histopathology of chronic asthma.

Materials and methods

Animals

α4 integrin$^{f/f}$ mice were produced as described previously [24]. These mice were bred with Mx*cre*$^{+}$ mice and the resulting Mx*cre*-+$_{\alpha 4}{}^{flox/flox}$ mice were conditionally ablated as neonates by intraperitoneal injections of poly(I)poly(C) (Sigma Aldrich Co., St Louis, MO, USA) for interferon induction. *cre*$^{-}$α4$^{f/f}$ mice were used as controls and the α4-ablated mice are referred to as $\alpha 4^{\Delta/\Delta}$and only mice with >95% α4 ablation in hematopoietic cells were used for studies. CD18 knockout mice were provided by Dr. Arthur Beaudet, Baylor College of Medicine (Houston, TX, USA). All animal protocols were approved by the University of Washington Institutional Animal Care and Use Committee. Mice were bred and maintained

under specific pathogen-free conditions in University of Washington facilities and were provided with irradiated food and autoclaved water ad libitum.

Induction of chronic allergic asthma

Mice were sensitized and later challenged with ovalbumin (OVA; Pierce Biotechnology, Inc., Rockford, IL, USA) as described previously [25]. Briefly, mice were immunized with 100 μg OVA complexed with aluminium sulfate in a 0.2-mL volume, administered by intraperitoneal injection on day 0. On days 8 (250 μg OVA) and on days 15, 18, and 21 (125 μg OVA), mice anesthetized briefly with inhalation of isoflurane in a standard anesthesia chamber were given OVA by intratracheal administration. Intratracheal challenges were done as described previously [26]. Mice were anesthetized and placed in a supine position on a board. The animal's tongue was extended with lined forceps and 50 μL OVA (in the required concentration) was placed at the back of its tongue. We have previously shown that this protocol results in increased AHR, inflammation of the airways, and Th2 cytokine production [25–27]. Prolonged inflammation was induced by subsequent exposure of mice to 125 μg OVA intratracheally three times a week until groups of mice were sacrificed on day 55 (chronic phase) after the last intratracheal challenge on day 54 (Fig. 1A). The control group [$\alpha 4^{+/+}cre^{-}$ mice also injected with poly(I) poly(C)] received normal saline with aluminium sulfate by intraperitoneal route on day 0 and 0.05 mL 0.9% saline by intratracheal route on days 8, 15, 18, 21 and three times a week until they were sacrificed on day 55.

Bronchoalveolar lavage fluid

Mice underwent exsanguination by intraorbital arterial bleeding and then lavaged (0.4 mL three times) from both lungs. Total bronchoalveolar lavage fluid (BALf) cells were counted from a 50-μL aliquot and the remaining fluid was centrifuged at 200g for 10 minutes at 4°C and the supernatants frozen for assay of BALf cytokines later. Cell pellets were resuspended in fetal bovine serum and smears were made on glass slides. The cells, after air-drying, were stained with Wright-Giemsa (Biochemical Sciences, Inc., Swedesboro, NJ, USA) and differential counts enumerated using a light microscope at 40× magnification. Cell number refers to that obtained from lavage of both lungs/mouse.

Lung parenchyma cell recovery

Lung mincing and digestion were performed after lavage as described previously [17] with 100mg/mL collagenase for 1 hour at 37°C, and filtered through a no. 60 sieve (Sigma Aldrich Co.). All numbers mentioned in this article refer to cells obtained from one lung/mouse.

Lung histology

Lungs from other animals of the same group were fixed in 4% paraformaldehyde overnight at 4°C. Tissues were embedded in paraffin and cut into 5-μm sections. A minimum of 15 fields were examined by light microscopy. The intensity of cellular infiltration around pulmonary blood vessels was assessed by hematoxylin and eosin staining. Airway mucus was identified by staining with Alcian blue and periodic acid Schiff staining as described previously [22]. Subepithelial pulmonary fibrosis was detected by Masson's trichrome and Martius scarlet blue stains as described in [28].

Lung immunohistochemical staining

Lungs were processed for immunohistochemical staining following standard procedure [26], then stained with either anti-vascular cell adhesion molecule-1 (VCAM-1; MK/2), anti-β1 (9EG7), or anti-transforming growth factor-β1 (TGF-β1). Briefly, tissues werefixed with 4%

paraformaldehyde in 100 mM phosphate-buffered saline (PBS; pH 7.4) for 6 to 12 hours at 4°C, washed with PBS for 10 minutes three times, and then soaked in 10% sucrose in PBS for 2 to 3 hours, 15% sucrose in PBS for 2 to 3 hours, 20% for 3 to 12 hours at 4°C, and then embedded in OCT compound (Tissue-Tek 4583; Sakura Finetechnical Co., Ltd, Tokyo, Japan) and frozen in acetone-cooled dry ice. Frozen blocks were cut on a freezing, sliding microtome at 4 μm (LEICA CM1850 Cryostat) and air-dried for 30 minutes at room temperature (RT). After washing in PBS three times for 10 minutes at RT, 0.3% hydrogen peroxide was applied to each section for 30 minutes at RT to block endogenous peroxidase activity. Each slide was incubated with blocking solution to block nonspecific reactions, and appropriately diluted primary antibody was applied to each slide and incubated overnight at 4°C. After washing with PBS, slides were incubated with appropriately diluted specific biotin-conjugated secondary antibody solution for 1 hour at RT. After washing with PBS, slides were incubated in AB reagent for 1 hour at RT (ABComplex/ HRP; DAKO, Carpinteria, CA, USA), washed with PBS, and stained with 0.05% 3,3'-diaminobenzidine tetrahydrochloride; Sigma Aldrich Co.) in 0.05 M Tris buffer (pH 7.6) containing 0.01% H_2O_2 for 5 to 40 minutes at RT. Slides were counterstained with Mayer's hematoxylin, dehydrated, and mounted.

Fluorescein-activated cell sorter (FACS) analysis

Cells from hemolyzed peripheral blood, bone marrow (BM), bronchoalveolar lavage, lung parenchyma, spleen, mesenteric lymph nodes, cervical lymph nodes, axillary lymph nodes, and inguinal lymph nodes were analyzed on a FACSCalibur (BD Immunocy-tometry Systems, San Jose, CA, USA) using the CELLQuest program. Staining was performed by using antibodies conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), peridinin chlorophyl protein (PerCP-Cy5.5), and Cy-chrome (PE-Cy5 and PE-Cy7). The following antibodies (BD Biosciences-Pharmingen, San Diego, CA, USA) were used for cell surface staining: APC-conjugated CD45 (30F-11), FITC-conjugated CD3(145-2C11), PE-Cy5 conjugated CD4 (RM4-5), PE-conjugated CD45RC (DNL-1.9), APC-conjugated CD8(53-6.7), PE-Cy5 conjugated B220 (RA3-6B2), FITC-conjugated IgM, PE-conjugated CD19 (ID3), PE-conjugated CD21(7G6), FITC-conjugated CD23 (B3B4), APC-conjugated GR-1 (RB6-8C5), and PE-conjugated Mac1(M1/70). PE-Cy5 conjugated F4/80 (Cl:A3-1[F4/80]) was obtained from Serotec Ltd. (Raleigh, NC, USA). PE-conjugated anti-α4 integrin (PS2) and anti-VCAM-1 (M/K-2) was from Southern Biotechnology (Birmingham, AL, USA). Irrelevant isotype-matched antibodies were used as controls. Among hematopoietic cells, $CD45^+$/ $CD3^+$ were T cells, $CD3^+/CD4^+$ were helper T cells, and $CD3^+/CD8^+$ were cytotoxic T cells. B cells were $B220^+$. $Gr\text{-}1^+/F4/80^-$ cells were granular cells (e.g., neutrophils, eosinophils) and $Gr\text{-}1^-/F4/80^{hi}$ cells were tissue macrophages.

Cytokines

Cytokines (IL-2, IL-4, IL-5, tumor necrosis factor-α [TNF-α], and interferon-γ [IFN-γ]) in BALf and serum were assayed by FACS with Mouse Th1/Th2 Cytokine Cytometric Bead Assay (BD Biosciences) following manufacturer's protocol. Manufacturer's sensitivity for IL-2, IL-4, and IL-5 is 5 pg/mL, for IFN-γ is 2.5 pg/mL, and for TNF-α is 6.3 pg/mL. IL-13 and eotaxin were measured by enzyme-linked immunosorbent assay (ELISA) using Quantikine M kits (R&D Systems, Minneapolis, MN, USA) and the limit of detection is 1.5pg/mL for IL-13 and 3 pg/mL for eotaxin.

OVA -specific IgE and IgG_1 in plasma

Anti-mouse IgE (R35-72) and IgG_1 (A85-1) from BD Biosciences were used for measuring OVA-specific IgE and IgG_1, respectively, by standard ELISA procedures as described previously [29]. The lower and upper limits of detection for IgE and IgG_1 are 3 ng/mL to 10

ug/mL (minimal detectable dose determined by adding 2 standard deviations of the mean OD 405 nm value for 20 replicates of the zero standard and calculating the corresponding concentration.)

Pulmonary fibrosis

Martius scarlet blue and Masson's trichrome stains in paraffin lung sections were used to visualize lung fibrosis [28].

Soluble VCAM-1 and soluble collagen in lung homogenate

Soluble VCAM-1 was determined as described previously [22]. Total amount of soluble collagen in the lung was measured using a Sircol collagen assay kit from Biocolor (Newtownsbury, Northern Ireland, UK), according to the method described [26]. In all experiments, a collagen standard was used to calibrate the assay.

Lung function testing

In vivo AHR to methacholine was measured 24 hours after the last OVA challenge in conscious, free-moving, spontaneously breathing mice using whole-body plethysmography (model PLY 3211; Buxco Electronics, Sharon, CT, USA) as described previously [25]. Mice were challenged with aerosolized saline or increasing doses of methacholine (5, 20, and 40 mg/mL) generated by an ultrasonic nebulizer (DeVilbiss Health Care, Somerset, PA, USA) for 2 minutes. The degree of bronchoconstriction was expressed as enhanced pause, a calculated dimensionless value, which correlates with the measurement of airway resistance, impedence, and intrapleural pressure in the same mouse. P_{enh} readings were taken and averaged for 4 minutes after each nebulization challenge. P_{enh} was calculated as follows:

$$P_{enh} = [(Te/Tr - 1) \times (PEF/PIF)]$$

where T_e is expiration time, T_r is relaxation time, PEF is peak expiratory flow, and PIF is peak inspiratory flow × 0.67 coefficient. The time for the box pressure to change from a maximum to a user-defined percentage of the maximum represents relaxation time. T_r measurement begins at the maximum box pressure and ends at 40%.

Th differentiation, intracellular staining, and ELISA assay for IL-17A and IFN-γ

Cell suspensions from spleen were enriched for $CD4^+$ T cells using anti-CD19 and anti-CD8 antibodies and BioMag goat anti-rat IgG Fc beads (Qiagen Inc, Valencia, CA, USA). Naïve $CD4^+$ $CD62L^+$ cells were sorted by flow cytometry and $\alpha4^+$ cells were excluded from $\alpha4^{\Delta/\Delta}$ cells (4.1%). Five-hundred thousand naı̈ve $CD4^+$ $CD62L^+$ T cells were cultured under Th0 conditions: anti-CD3 [1 mg/mL] and anti-CD28 [2 mg/mL]; or Th17 polarization conditions: anti-CD3 [1 mg/mL], anti-CD28 [2 mg/mL], IL-6 [10 ng/ mL], (Peprotech, Rocky Hill, NJ, USA), TGF-β [5 ng/mL], (Peprotech), anti-IL-4 [5 mg/mL] (clone 11B11) and anti-IFN-γ [5 mg/ mL] (clone XMG1.2); or inducible regulatory T-cell conditions: anti-CD3 [1 mg/mL], anti-CD28 [2 mg/mL], TGF-β [5 ng/mL], anti-IL-4 [5 mg/mL], anti-IFN-γ [5 mg/mL], and IL-2 [100 U/ mL] (eBioscience, Inc., San Diego, CA, USA). After 4 days, cells were restimulated with phorbol myristate acetate (PMA) (50 ng/ mL; Sigma Aldrich) and ionomycin (1 mg/mL; Sigma Aldrich) and monensin (2 μM; eBioscience) for 5 hours. Cells were stained with CD4-FITC and/or CD103-PE. Intracellular staining was performed using Perm and Fix solutions from eBioscience, anti-IL-17A-PE (clone eBio17B7), anti-IFN-γ-APC (clone XMG1.2), and anti-Foxp3-FITC. These antibodies were obtained from eBioscience. For ELISA analysis, supernatants were harvested after 4 days. IL-17A and IFN-γ levels were analyzed using monoclonal antibodies and recombinant cytokine standards from eBioscience. Detection limits were IL-17A (125 pg/mL) and IFN-γ (100 pg/ mL).

Statistics

Mean ± standard error of the mean was calculated using Student's *t*-test in Excel Software (Microsoft, Redmond, WA, USA). A p value < 0.01 was considered statistically significant.

Results

$\alpha4^{\Delta/\Delta}$ mice fail to develop AHR to chronic airway challenge by allergen

To determine pulmonary function in the OVA-treated mice, we measured AHR to increasing doses of methacholine by noninvasive whole-body plethysmography (P_{enh}) (Fig. 1B). While both post-OVA control and $\beta2^{-/-}$ mice show significantly increased P_{enh} value over saline-treated controls at 40 and 100 mg/mL methacholine, OVA-treated $\alpha4^{\Delta/\Delta}$ mice have a P_{enh} value similar to baseline values from untreated or saline-treated mice. Using the same genetic models ($\alpha4^{\Delta/\Delta}$, $\beta2^{-/-}$), we have previously shown [22] that measurements of airway resistance by invasive plethysmography were in full agreement with responses by noninvasive whole-body plethysmography, as measured here.

Migration of leukocytes from circulation to lung and to airways

Total number of lymphoid and myeloid cells present in BM, peripheral blood circulation, spleen, or in BALf and lung parenchyma were assessed through measurements of total nucleated cell counts (Fig. 1C and Fig. 1D) and their differentials were determined by FACS based on specific antigen expression and by cell morphology from smears (Fig. 2 and Suppl. Table 1). Both total cell numbers and differentials varied among the animal groups and tissues studied. It is worth noting that differences among the groups also exist at baseline (i.e., increase in leukocytes in peripheral blood and spleen), as previous studies with these mice indicate [19,24]. In BM and peripheral blood, the total number of different leukocyte subsets was higher post-OVA in $\beta2^{-/-}$ mice compared to controls. $\alpha4^{\Delta/\Delta}$ mice also had higher levels than controls in BM, with one exception, i.e., eosinophils. In lung parenchyma, $\beta2^{-/-}$ mice again had the highest numbers of leukocytes, reflecting both recruitment and accumulation in lung. In BALf, all leukocyte subsets were low in $\alpha4^{\Delta/\Delta}$ mice, whereas only lymphocytes and eosinophils were reduced in $\beta2^{-/-}$ mice (Fig. 2) compared to controls. It is of interest that, as in acute asthma, eosinophils were not increased in BALf of both $\beta2^{-/-}$ and $\alpha4^{\Delta/\Delta}$ mice, suggesting that for the interstitial airway migration of these cells, β2 and α4 are both important and nonredundant. However, chronic accumulation of eosinophils in lung parenchyma is α4- and β2-integrin–independent. Nevertheless, despite minimal eosinophil presence in BALf and increased presence of eosinophils in lungs of both genetic models, AHR was induced in $\beta2^{-/-}$ mice only, but not in $\alpha4^{\Delta/\Delta}$ mice.

Inflammation and fibrosis in lungs in response to chronic OVA challenge

To assess the degree of inflammation and structural fibrotic remodeling in response to chronic allergen challenge, paraffin-embedded lung sections from untreated and OVA-treated mice were stained with hematoxylin and eosin to detect inflammatory cell migration into lung, Alcian blue to detect goblet cell metaplasia and mucus secretion in the luminal spaces of the lung and Masson's trichrome and Martius scarlet blue to assess changes primarily in lung collagen content (Fig. 3). While OVA-treated control and $\beta2^{-/-}$ lungs show considerable inflammatory changes and increased deposition of collagen, indicating subepithelial fibrosis compared to aluminium sulfate-treated mice, in OVA-treated $\alpha4^{\Delta/\Delta}$ mice no significant changes from non-OVA-treated control mice were noted. Mononuclear cell accumulation in lungs of $\alpha4^{\Delta/\Delta}$ mice was frequently in a nodular pattern within the parenchyma (Fig. 3, right panel), unlike the other models.

Th2/Th1 cytokines in BALf and plasma and IgE and IgG1 levels in plasma

Levels of Th1 and Th2 cytokines and eotaxin were measured in plasma and BALf as described in Materials and Methods. All Th2 cytokines (i.e., IL-4, IL-5, IL-13), eotaxin, and TNF-α levels were low in BALf (Fig. 4A, left panels) of $\alpha4^{\Delta/\Delta}$ mice compared to both control and β2 mice post-OVA, whereas in plasma (Fig. 4A, right panels), IL-13 levels were similar to other groups. $\beta2^{-/-}$ mice developed high levels of cytokines in circulation, like control mice, but in BALf (Fig. 4), only TNF and eotaxin were significantly lower than controls (see Suppl. Table 2), commensurate with the lower eosinophil levels (Fig. 2). Furthermore, OVA-sensitization responses were largely attenuated in $\alpha4^{\Delta/\Delta}$ mice in contrast to the other two groups, as indicated by levels of OVA-specific IgE in plasma (Fig. 4C), corroborating previous findings in acute asthma [22].

Soluble VCAM-1 in BALf and plasma and VCAM-1 expression in lung

Because increases in cytokine levels (e.g., IL-4, IL-13, TNF-a) upregulate expression of VCAM-1 and increased VCAM-1 is a hallmark of allergic asthma phenotype, we next investigated whether the presence or absence of asthma phenotype affected the levels of soluble VCAM-1 in plasma and BALf or its expression in the lung in the two genetic models compared to controls. Soluble VCAM-1 levels post-OVA in both plasma and BALf were increased in control and $\beta2^{-/-}$ mice, but in $\alpha4^{\Delta/\Delta}$ mice were quite low by comparison (Fig. 5A and Fig. 5B). Histochemical evidence of VCAM-1 protein in lung was also increased in response to OVA in both control and $\beta2^{-/-}$ lung (Fig. 6C, left panel), similar to changes seen in acute asthma [22]. However, in $\alpha4^{\Delta/\Delta}$ lung post-OVA expression of VCAM-1 (Fig. 6C, right panel) was almost unchanged from baseline and lower than what was observed in the other two groups post-OVA. Therefore, although reduced levels of relevant cytokines could be responsible for decreased upregulation of VCAM-1 in $\alpha4^{\Delta/\Delta}$ mice, other reasons, e.g., unresponsive endothelium in $\alpha4^{\Delta/\Delta}$ mice, cannot be excluded.

TGF-β1 and soluble collagen in BALf

TGF-β1 has been thought to be instrumental in fibrosis development and consequent collagen deposition in lung. Therefore, because subendothelial collagen deposition was present in control and $\beta2^{-/-}$ mice by immunohistochemistry (Fig. 3), we measured TGF-b1 in BALf (Fig. 5C) and soluble collagen levels in lung (Fig. 5D) of these mice. Significant increases in TGF-β1 and soluble collagen from aluminium sulfate-treated mice were only seen in control and $\beta2^{-/-}$ mice post-OVA. In contrast, in $\alpha4^{\Delta/\Delta}$ mice, levels were very low consistent also with immunohistochemical documentation of TGF-β1 in lung sections (Fig. 6A, right panel).

Discussion

Although a body of literature is available describing the development of acute asthma in animal models, data comparing asthma and airway remodeling in both acute and chronic asthma settings by employing the same genetic mouse models are rare. As chronic asthma is considered more relevant to the human disease, molecular pathways involved in its development are important for designing targeted therapies [12,30]. Changes in chronic asthma are perpetuated because of a continuous dialogue between inflammatory cells and resident cells and matrix components in airways. The preferential recruitment of effector cells (eosinophils, Th2 cells) in the lung and airways is mediated by a cascade of adhesive interactions initiated by activated endothelial cells. However, the distinct molecular pathways that dominate these processes are continuously being revised. Studies with antifunctional antibodies yielded conflicting data, depending on the type, dose and route of administration of the antibody, or on the animal model used [13–18,20,21,31,32]. Data with CD18-deficient mice suggested an absolute requirement of CD18 for recruitment of

eosinophils in the airways and for AHR [19]. Our parallel comparison of CD18 with conditional $\alpha4^{\Delta/\Delta}$ mice in acute asthma showed that, in the latter, not only was AHR and migration of cells to airway lumen prevented, as in the $CD18^{-/-}$ mice, but there was attenuation of the sensitization process, minimal recruitment of eosinophils and lymphocytes in the lung parenchyma, and no upregulation in VCAM-1 expression [22]. As reported here, attenuation of all features of acute asthma observed in the absence of α4 integrins were, by and large, maintained during the chronic allergen challenge, although there was a higher leukocyte accumulation in the lungs of chronically challenged $\alpha4^{\Delta/\Delta}$ mice (Fig. 1D and Fig. 2). Surprisingly, however, and in contrast to acute asthma, absence of β2 integrins (CD18) did not prevent chronic asthma development or its accompanied structural changes and AHR, despite the fact that migration of eosinophils to airways (BALf) (Fig. 1D and Fig. 2) was restrained in these mice. Although one cannot theoretically exclude the possibility that eosinophil degranulation differences among the two genetic models influence the cell numbers recorded, there is no experimental evidence supporting this notion.

Thus, the migratory flow of eosinophils from the systemic circulation to lung and then through the lung interstitium to airway lumen may be dictated by differences in adhesive interactions between pulmonary vasculature and that of systemic bronchial circulation. Our data highlight the fact that α4 and β2 integrins play critical but nonredundant roles in facilitating this pathway of inflammatory cell migration from lung interstitium to airway lumen. Cooperativity between α4β1 (ligating domains 1 and 4 of VCAM-1) and αMβ2 (recognizing only domain 4 of VCAM-1) in mediating adhesion and release from luminal ligands [23] may provide a mechanistic basis for such an outcome. However, the fact that the asthma phenotype was not prevented in VCAM-1-deficient mice [22] suggests that VCAM-1 upregulation and its interaction with integrins is not of critical importance in this process and that ligands other than VCAM-1 in airways and lung (i.e., E-selectin or fibronectin) may play pivotal roles in the absence of VCAM-1.

Total cell and total eosinophil accumulation in lung was high in $\beta2^{-/-}$ mice, even higher than in control mice. Such a high accumulation of inflammatory cells in lung, not necessarily in BALf, may be contributing to chronic asthma development in these mice. However, our data with $\alpha4^{\Delta/\Delta}$ mice would argue that mere accumulation of inflammatory cells, including eosinophils, in lung might be necessary but not sufficient for asthma development. It is possible that the state of activation of accumulated cells is more important than their presence. For example, $\alpha4^{\Delta/\Delta}$ mice did not show the elevated levels of TGF-β1, presumably because in the absence of α4 integrins, activation of the latent TGF-β1 in eosinophils (by mast-cell tryptase or αvβ6 on epithelia [4]) was not induced [33]. Furthermore, in vitro studies have previously implicated α5β1 expressed by airway smooth muscle cells in regulating fibronectin deposition after TGF-β1 stimulation [34]. Thus, although the α5β1-dependent pathway is intact in $\alpha4^{\Delta/\Delta}$ mice, the low levels of TGF-β1 are consistent with reduced fibrin deposition seen in $\alpha4^{\Delta/\Delta}$ mice. The role of eosinophils in acute or chronic asthma and their role in maintenance of chronic inflammation and airway remodeling is currently a subject of debate. Nevertheless, a large body of published evidence suggests a compelling role of eosinophils in chronic asthma, especially in humans [4]. Although caveats exist in the contribution of eosinophils in different strains of mice [35], our genetic models were of the same genetic background.

The recently appreciated role of anti-stem cell factor (SCF) antibody or oral imatinib mesylate in the attenuation of airway responses in both acute and chronic asthma is intriguing [36]. Interaction of fibroblasts with eosinophils that leads to increased production of cytokines is mediated through SCF [37]. Because of the known influence of SCF on integrin activation [38] and expression of SCF and c-kit in human asthmatic airways [39], it could be speculated that SCF effects are at least partially integrin-dependent, as both c-kit

and α4 integrin signaling are linked to the same pathways that regulate migration and activation of mast cells [40]. The role of mast cells, a constant component in allergic inflammatory response in lung, has been controversial in airway disease development [41]. However, abundant experimental evidence in murine models have shown that mast cells adhere to mucosal surfaces through α4β1/ α4β7 and VCAM-1 interactions [42] and secrete important mediators like TNF or CCL1, which can activate Th2 cells, or histamine and leukotriene B4 involved in recruitment of effector T cells in lung [41]. Mice with mast cell deficiency display marked reduction in lung mucosal inflammation, similar to mice with CCR8 deficiency or depletion of CD4 T lymphocytes [43], not unlike our findings with $\alpha4^{\Delta/\Delta}$ mice. Also consistent with our findings, pretreatments with anti-very late antigen-4 antibody attenuated early response after OVA challenge through inhibition of mast cell activation [44].

New insights about the mast cell-dependent IL-17 activation and its role in asthma have been recently uncovered. IL-17 is thought to be produced by a distinct T cell lineage (Th17 cells), and its production is negatively regulated by IFN-γ and IL-4 [45,46]. After antibody neutralization of IL-17 [47] or in IL-17 knockout mice [48], the OVA-induced initiation of asthma is prevented. Furthermore, mice not susceptible to asthma development (C3H) do not produce IL-17 [49]. These data suggest that IL-17 signaling is essential during antigen sensitization to establish asthma; however, in mice already sensitized, IL-17 seems to attenuate the allergic response [48]. It is of interest that, rather than Th17 cells, the main producers of IL-17 in asthma are alveolar macrophages [47]. Their activation and upregulation of IL-17A is mediated by products secreted by IgE/ OVA-activated mast cells. As the primary sensitization and OVA-specific IgE production in our $\alpha4^{-/-}$ mice is significantly impaired, one may speculate that a decrease in IL-17A is expected in these mice. Although we have not measured levels of IL-17A in acute or chronic asthma in our mice, the ability of naïve $CD4^{+}/CD62L^{+}/\alpha4^{-}$ cells to produce IL-17 is not compromised (Suppl. Fig. 1B and C) under in vitro conditions. Any influence of IL-17, if any, in our mouse model may be secondary to their impaired in vivo activation by mast cells or other cells and lack of migration of effector cells in BALf. Furthermore, induction of $Foxp3^{+}/CD103^{+}$ regulatory T cells in vitro under the influence of TGF-β is also not impaired in $\alpha4^{-/-}$ T cells (18.3% in $\alpha4^{+/+}$and 17.8% in $\alpha4^{\Delta/\Delta}$).

Among cytokine levels in BALf, there is a striking reduction of TNF-α in $\alpha4^{\Delta/\Delta}$ mice compared to other groups. Considerable in vitro and in vivo evidence suggests that TNF-α plays a key role in development of AHR, however, the molecular mechanisms linking TNF-α to AHR are not precisely defined [50]. Nevertheless, although we believe that the low levels found in $\alpha4^{\Delta/\Delta}$ are likely contributing to lack of AHR in these mice, the reasons for the presence of low TNF-α are unclear and require further studies. IL-13 and its downstream target signal transducer and activator of transcription 6 exert a pivotal role in chronic asthma by affecting the function of resident airway cells (e.g., epithelia, fibroblasts, smooth muscle cells, mast cells) and, together with IL-4, by facilitating selective recruitment of inflammatory cells [51,52]. Upregulation of VCAM-1 in airway vessels [14,18] and increase of several chemokines (eotaxin or monocyte chemotactic protein-1, etc.) work in concert to establish chemotactic gradients between different compartments in the lung. In addition, direct participation of IL-13 in fibrosis has been advocated because of IL-13-mediated induction of arginase 1 or TGF-β and platelet-derived growth factor by endothelial cells and monocyte/macrophages and the effects of these mediators on fibroblast proliferation [53,54]. Nevertheless, despite the critical role of IL-13 in chronic asthma [55], $IL\text{-}13^{-/-}$ mice still had AHR, albeit with less fibrosis, less inflammatory changes, and intraepithelial eosinophil accumulation, thus dissociating the presence of these changes from AHR [56]. Of interest, IL-13, along with other cytokines, was decreased in BALf of $\alpha4^{\Delta/\Delta}$ mice, consistent with a decrease in total cells in BALf in these animals, but levels of IL-13 were not different in

plasma compared to controls, highlighting the importance of local changes in cytokine/chemokine milieu for asthma development.

A phenotypic response similar to the one seen in our $\alpha4^{\Delta/\Delta}$ animals in acute and chronic asthma was reported for sphingosine kinase inhibition [57] with suppression of eosinophil migration to airways, Th2 cytokine, and chemokine secretions and decreased AHR. Sphingosine 1-phosphate [57], like α4 integrin, is important in mast cell, neutrophil, and eosinophil chemotaxis, and its inhibition may have an effect on cellular migration machinery involving adhesion molecules.

It is of great interest that, despite the effectiveness of several inhibitors of integrins (i.e., antibodies, small molecules, peptides) in animal models of asthma (i.e., mouse, rat, sheep, guinea pig), human trials have been largely disappointing [23]. It is possible that movement of leukocytes or eosinophils in humans involves other molecules in addition to integrins, or that it involves a combination of integrins (i.e., both β1 and β2 integrins and possibly even some selectins) for dampening the inflammatory response. Furthermore, it should be pointed out that integrin inhibitors largely prevented disease in animals and may not be able to greatly influence already established disease in humans, although they should curtail either the frequency or severity of episodes. Along these lines, our data with the two genetic models ($\beta2^{-/-}$,$\alpha4^{\Delta/\Delta}$) may provide important preclinical relevance. It could be argued that a major impact of α4 integrin is to dampen the initial sensitization process. As a result, the effectiveness in established processes in humans may be less than that seen in animal models. Further, it is possible that even in the absence of additional inflammatory cell recruitment, the remodeling process persists through sustained activation of airway structural cells [58]. In addition, results we have reported with combined β2 and α4 deficiencies in leukocyte migration to inflammatory sites [59] are instructive in terms of the effectiveness of using combinations of integrin inhibition.

Overall, in chronic asthma there is parallel induction of a complex array of genes in a variety of cells recruited to the airways and in airway resident cells. The contribution of individual players (cells or cytokines) seems to be variable and under independent regulation [60]. We believe that our present studies addressing some of these issues by exploiting genetic murine models expand on the knowledge of the molecular understanding of the pathophysiology of chronic asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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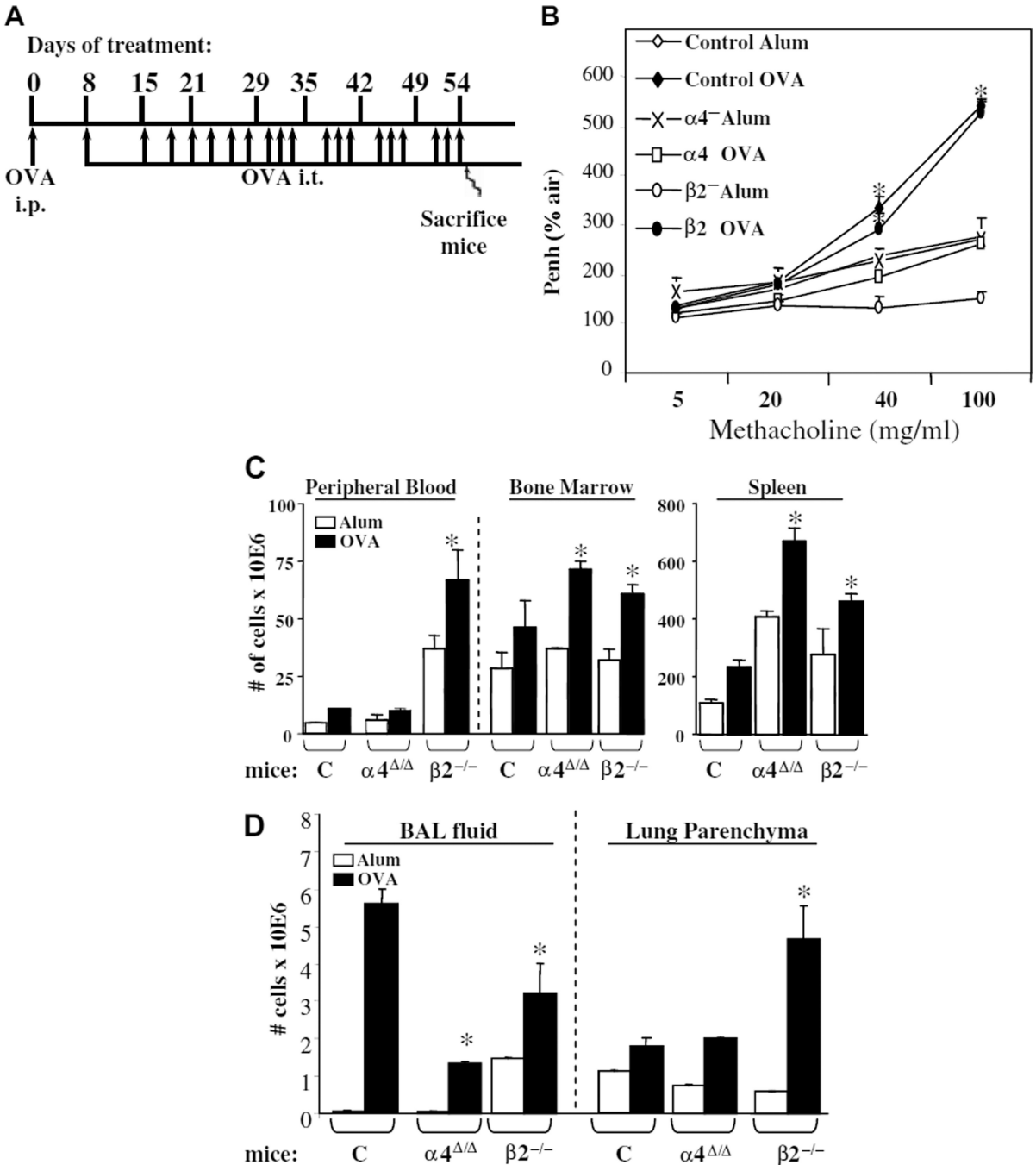

Figure 1.

(**A**) Diagram illustrating the experimental design for chronic asthma development. i.p. = intraperitoneal injection, i.t. = intratracheal instillation. (**B**) Lung function testing using whole-body plethysmography. Measurement of airway hyperresponsiveness at 24 hours after the last ovalbumin (OVA) challenge on day 54. The degree of bronchoconstriction to increasing doses of aerosolized methacholine was expressed as P_{enh} (percentage of air as control). *$p < 0.01$ compared to baseline values in saline-treated control mice, n = 8 mice per group. Control mice, α4-deficient mice, and β2-deficient mice were treated with aluminium sulfate (alum) and OVA. Measurements of airway resistance by invasive

plethysmography [22] were found to be in full agreement with P_{enh} data. (**C**) Numbers of lymphoid and myeloid cells in blood (n/mL), bone marrow (n/femur), and spleen, and (**D**) total numbers of cells in bronchoalveolar lavage (BAL) fluid and lung parenchyma from the two genetic models and controls treated with alum or OVA. Data are from two independent experiments expressed as average ± standard error of the mean. n = 8 mice per group.

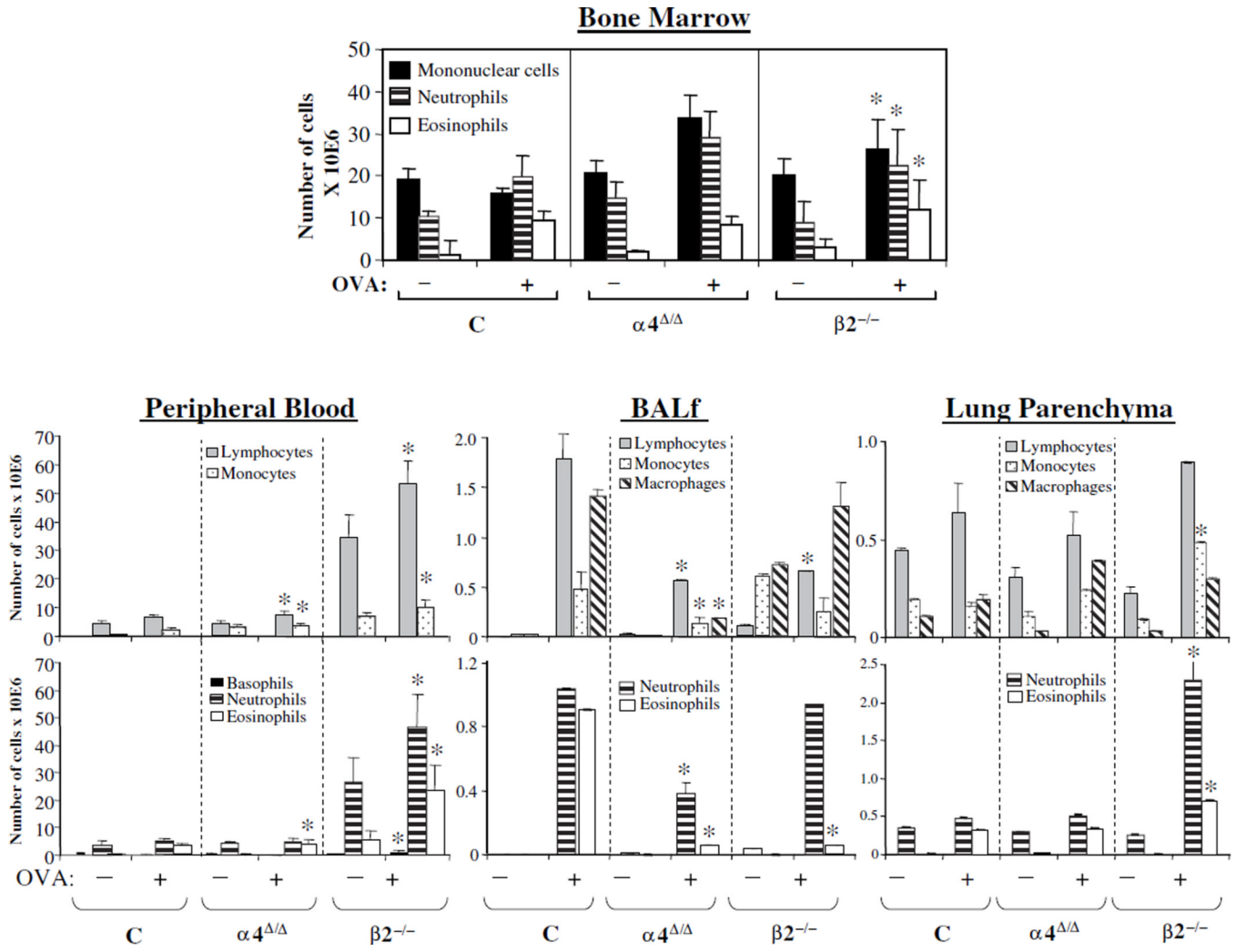

Figure 2.

Total numbers of all leukocytes in bone marrow, peripheral blood, bronchoalveolar lavage fluid (BALf), and lung parenchyma in control, α4-deficient (α4Δ/Δ), and $\beta 2^{-/-}$ mice pre- and post-chronic ovalbumin (OVA) treatment. Total numbers (× 10E6) of cells were counted in a Beckman Coulter Counter and differentials (neutrophils, eosinophils, monocytes, etc.) were assessed by fluorescein-activated cell sorting (specific antigen expression) and morphology in smears. *$p < 0.01$ from control values post-OVA, n = 8 mice per group, C = Control mice.

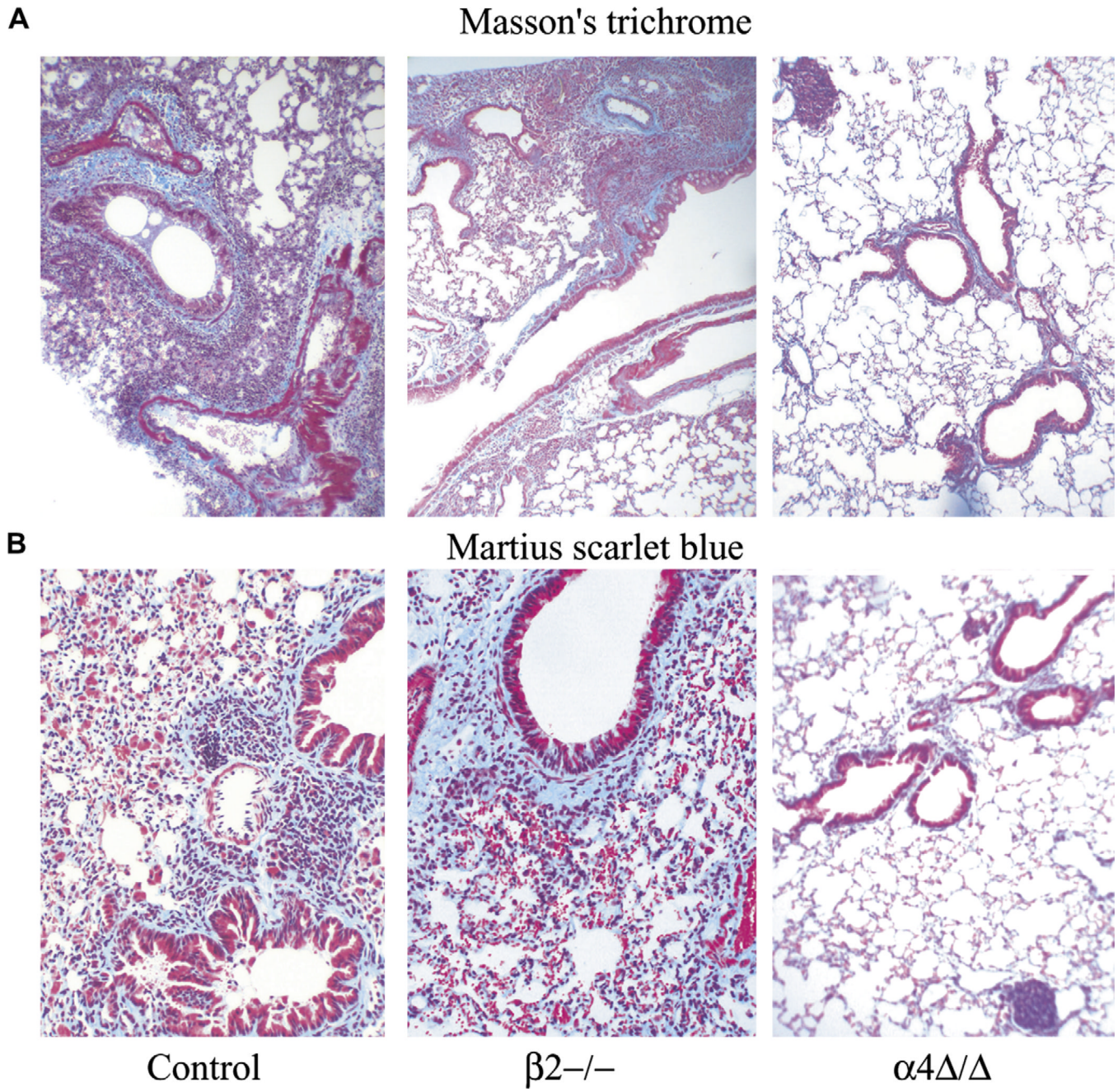

Figure 3.
Paraffin sections of lungs from ovalbumin-treated mice were stained with (**A**) Masson's trichrome stain revealing the collagen depositions around the airways and (**B**) Martius scarlet blue for collagen and fibrin deposition in interstitial spaces of the parenchyma. Adjustments for brightness, contrast, and color balance using Adobe Photoshop were made in order to match in all six photographs.

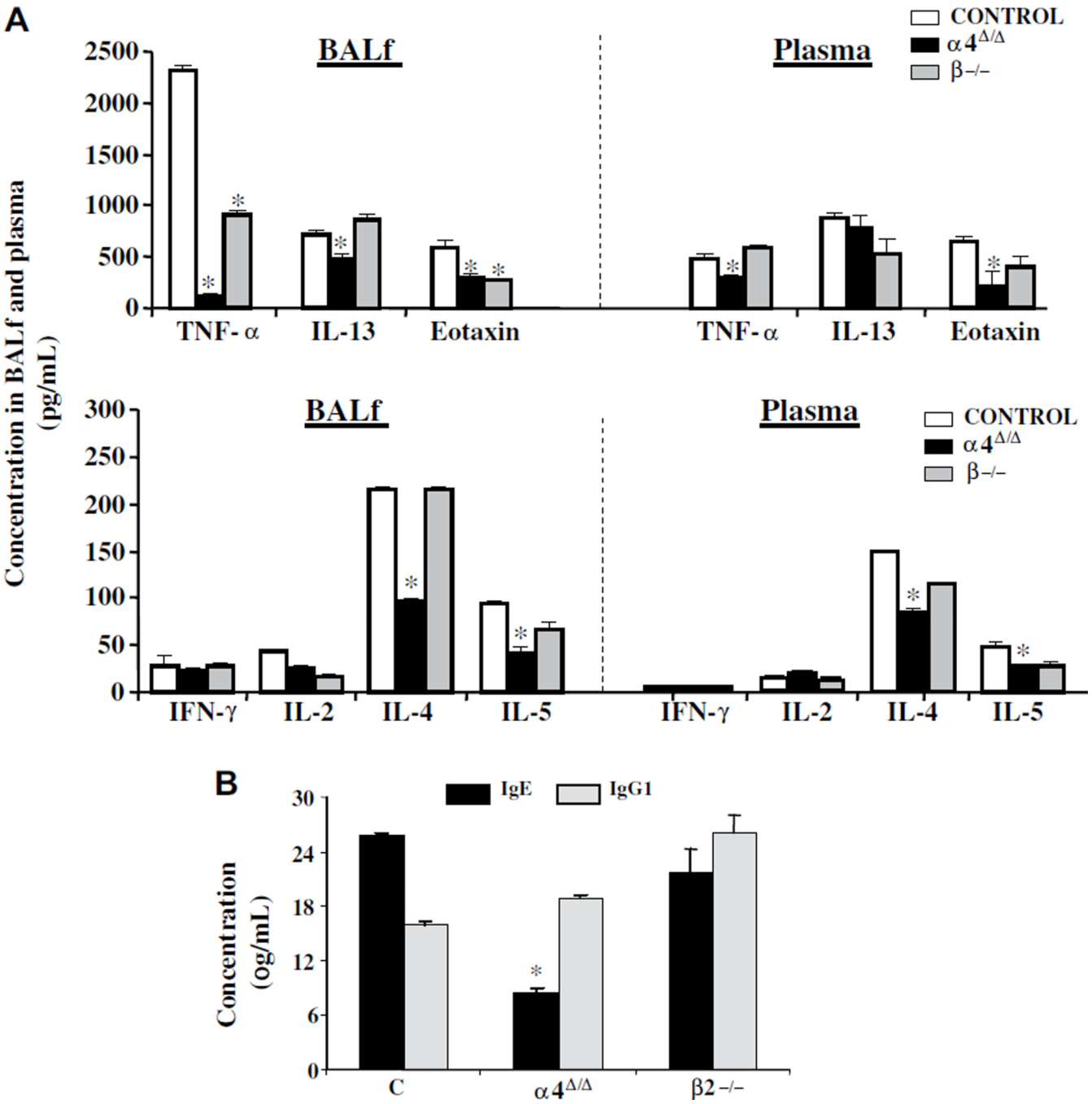

Figure 4.

(**A**) Concentration of cytokines (pg/mL) post-ovalbumin (OVA) (upper two panels) in bronchoalveolar lavage fluid (BALf) and plasma in control (white bars), $\alpha 4^{\Delta/\Delta}$ (black bars), and $\beta 2^{-/-}$ (gray bars) mice (*$p < 0.01$ compared to post-OVA control), and (**B**) concentrations of OVA-specific immunoglobulin (Ig) E and IgG_1 (μg/mL) in plasma of OVA-treated mice in the three genotypes. Data (see Suppl. Table 2) are averaged from two independent experiments, all assays were run in triplicate, n = 8 mice per group, and *$p <$ 0.01 compared to post-OVA control values. IFN-γ = interferon-γ; IL = interleukin; TNF-α = tumor necrosis factor-α.

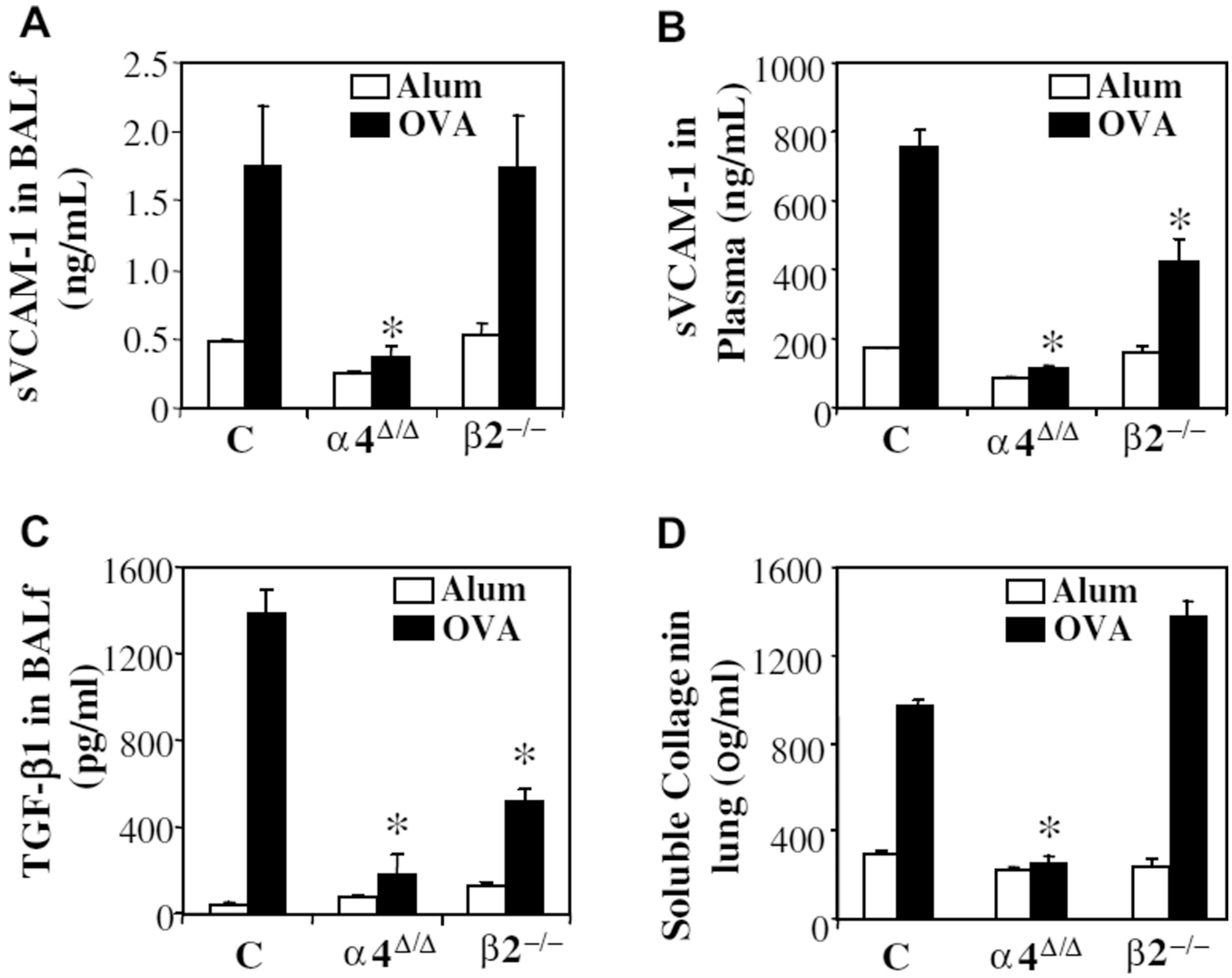

Figure 5.

(**A**) Soluble vascular cell adhesion molecule-1 (sVCAM-1) concentrations (ng/mL) in bronchoalveolar lavage fluid (BALf) and (**B**) in plasma measured by enzyme-linked immunosorbent assay (ELISA), (**C**) transforming growth factor-β1 (TGF-β1) levels (pg/mL) in BALf measured by ELISA, and (**D**) soluble collagen content (μg/mL) of lung measured by Sircol dye kit. Data are averaged from two independent experiments ± standard error of mean. All assays were run in triplicate, n = 8 mice/group, and *$p < 0.01$ compared to post-ovalbumin (OVA) control values.

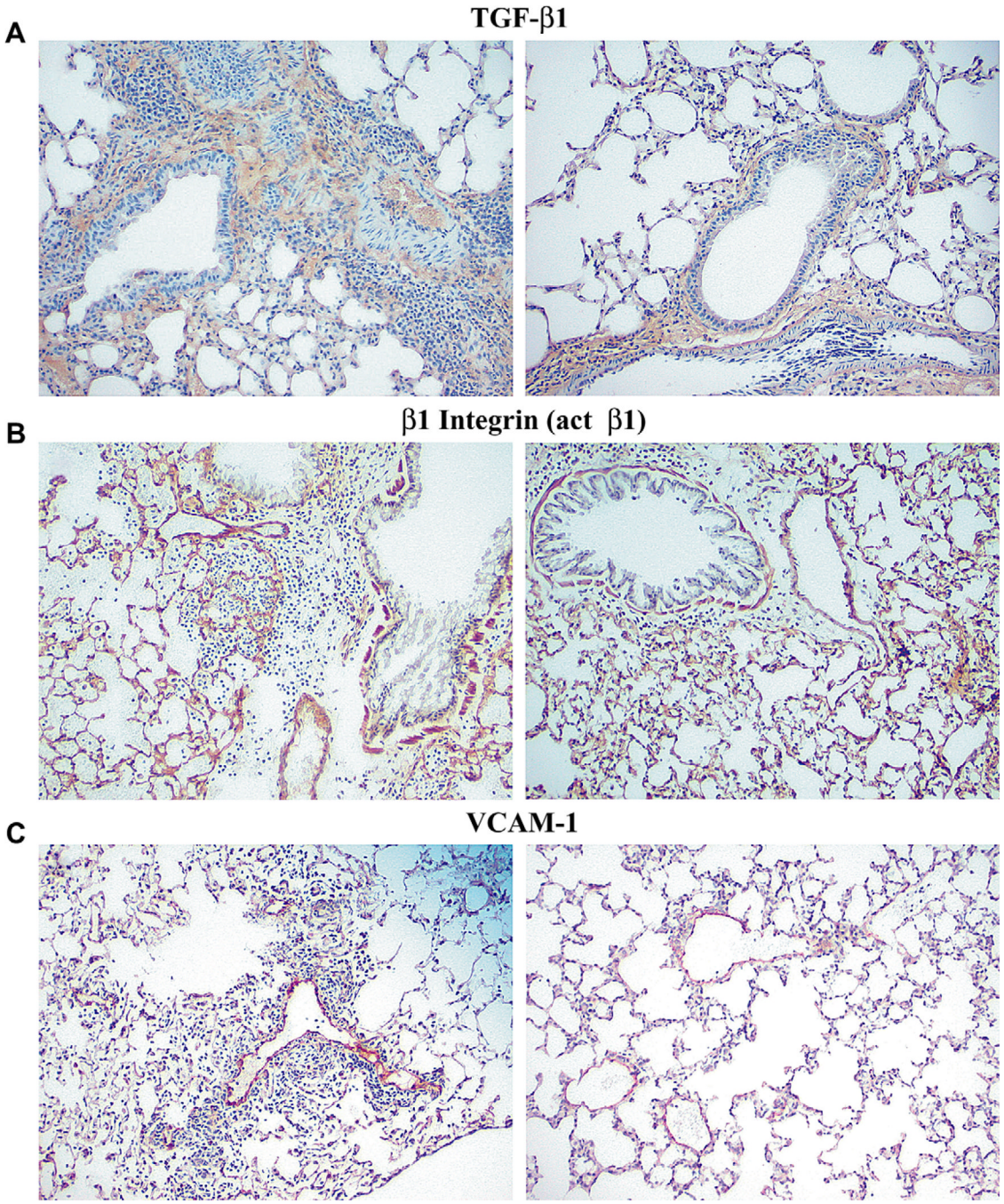

Figure 6.

Lung tissue sections, stained for transforming growth factor-β1 (TGF-β1) (anti-TGF-β1), 9EG7 (activated anti-β1 antibody), and vascular cell adhesion molecule-1 (VCAM-1) (anti-VCAM-1, MK/2) as described in Materials and Methods. (**A**) Images from Control (left) and $\alpha 4^{\Delta/\Delta}$ lung (right) post-ovalbumin (OVA) treatment and challenge stained with anti-TGF-β1. (**B**) Images of $\beta 2^{-/-}$ lung (left) and $\alpha 4^{\Delta/\Delta}$ lung (right) post-OVA stained with 9EG7. (C) Images of $\beta 2^{-/-}$ lung (left) and $\alpha 4^{\Delta/\Delta}$ lung (right) post-OVA stained with anti-VCAM-1. There were no significant differences in labeling with 9EG7, but labeling for TGF-β and VCAM-1 was less intense in $\alpha 4^{\Delta/\Delta}$ mice.