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The $\alpha 4\beta 7$ -integrin is dynamically expressed on murine eosinophils and involved in eosinophil trafficking to the intestine

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Summary

Background Of the numerous adhesion molecules expressed by eosinophils, the $\alpha 4$ -integrin has been identified as critically involved in eosinophil trafficking in the lung. Most studies have focused on the role of the $\alpha 4\beta 1$ -adhesion complex, but eosinophils also express the $\alpha 4\beta 7$ -integrin complex.

Objective To investigate the role of $\alpha 4\beta 7$, by assessing its membrane expression on eosinophils from different compartments using allergen-challenged mice and IL-4/IL-5 bi-transgenic mice. In addition, we aim to determine the impact of $\beta 7$ -integrin deficiency on eosinophil recruitment to the lungs and intestine in specific experimental allergic models.

Results Evaluation of $\alpha 4\beta 7$ expression on bronchoalveolar lavage fluid (BALF) and lung tissue eosinophils revealed a down-regulation of this integrin as eosinophils migrate through the lungs. Indeed eosinophils isolated from the BALF and lung of allergic mice had low expression of the $\alpha 4\beta 7$ -complex. While expression of the $\alpha 4$ -chain remained unchanged, a significant decrease in $\beta 7$ -surface expression was observed. Intestinal eosinophils, isolated from Peyer's patches, also displayed a down-regulation of the $\alpha 4\beta 7$ -integrin, albeit only modest. In contrast, circulating eosinophils, isolated from the blood and spleen, expressed high levels of the $\alpha 4\beta 7$ -integrin. However, eosinophil trafficking into the lungs of $\beta 7$ -integrin-deficient mice was not significantly impaired in response to respiratory allergen challenges. In contrast, $\beta 7$ -deficient mice had impaired eosinophil recruitment to the intestine.

Conclusion Taken together, these results identify differential expression of the $\alpha 4\beta 7$ -integrin on eosinophils and its critical role in regulating eosinophil responses in the intestine.

Keywords adhesion molecules, allergy, $\beta 7$, eosinophils, integrins, intestine, lung

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Introduction

There are several diseases associated with substantial eosinophil accumulation in various tissues including asthma, Churg-Strauss syndrome, eosinophil-associated gastrointestinal disorders, parasitic infections, and hyper-eosinophilic syndromes [1–5]. Accordingly, understanding the mechanisms involved in regulating eosinophil accumulation is of substantial medical importance, especially because eosinophils have been implicated as essential effector cells in several of these disorders [6–8].

Eosinophil recruitment into mucosal tissues is finely regulated by several processes including the co-ordinate interaction between eosinophil adhesion molecules (e.g.,

selectins and integrins) and their counter ligands (e.g., cell membrane molecules and extracellular matrix (ECM) components) [9–11]. Integrins are heterodimers of α - and β -subunits that non-covalently associate to form receptors for ECM proteins and cell surface ligands. Eosinophils express the integrin $\alpha 4\beta 1$ (very late antigen-4 (VLA-4); CD49d/CD29), which binds VCAM-1 and fibronectin. The $\alpha 4$ -integrin subunit also associates with the $\beta 7$ -subunit, and this complex is capable of binding to VCAM-1 and fibronectin, as well as their main ligand, mucosal addressin cell adhesion molecule (MAdCAM)-1, which is normally expressed by the vascular endothelium of the gastrointestinal tract. Eosinophils also express members of the $\beta 2$ - (e.g., CD18 family of molecules) integrin family, including lymphocyte function antigen (LFA-1) and

Mac-1 that both interact with endothelial cells via intercellular adhesion molecule (ICAM)-1.

Extensive studies have demonstrated that eosinophil recruitment into the lung and skin is generally β 1- and β 2-integrin dependent [8, 12–14], but the role of β 7-integrins in eosinophil recruitment has been less extensively evaluated [15–17]. The α 4 β 7 molecule, which is co-expressed by lymphocytes and eosinophils may be the most important integrin for eosinophil recruitment into the gastrointestinal tract. Notably, the α 4 β 7 receptor MAdCAM-1 is expressed on high endothelial venules in the intestinal lamina propria, lymph nodes and Peyer's patches [15, 18, 19]. Importantly, extensive studies focused on inhibiting the β 7/MAdCAM-1 interaction with neutralizing antibodies or with β 7-deficient mice have supported a central role for this pathway in regulating lymphocyte and mast cell progenitor cell homing into the intestine [20, 21]. The findings that eotaxin-1-induced eosinophil recruitment to the lung and eotaxin-1-mediated chemoattraction *in vitro* are dependent upon VLA-4 (α 4 β 1) [22] suggest that the other α 4-associated integrins on eosinophils (e.g., α 4 β 7) may also be involved in eotaxin-mediated events. Recent studies have shown that the intestinal eosinophilia induced by the eotaxin-1 transgene is dependent upon the β 7-integrin [23]. Consistent with this, there is delayed intestinal hypersensitivity (including lymphocyte and eosinophil infiltrations) in response to intestinal infection with *Trichinella spiralis* in β 7-deficient animals [20]. Additionally, the ability of fibronectin to prolong human eosinophil survival is dependent upon binding of fibronectin peptides to the α 4 β 7-integrin [24]. Determining the role of the α 4- and β 7-integrins on eosinophils has important therapeutic implication since there are several clinical agents that block these molecules [25]; in particular, two recent studies have shown a beneficial effect of anti- α 4 and anti- α 4 β 7 in patients with inflammatory bowel disease [26, 27].

In this study, we aimed to characterize the expression and function of the α 4 β 7-integrin on murine eosinophils. We demonstrate that eosinophils isolated from hematopoietic and lymphoid tissues express high levels of the α 4 β 7-integrin. In contrast, lung eosinophils down-regulate α 4 β 7-integrin resulting in luminal eosinophils that are nearly completely devoid of this integrin. Utilizing mice genetically deficient in β 7-integrin, we demonstrate a significant role for β 7 in eosinophil trafficking to the intestine but not the lungs.

Methods

Animals

C57BL/6 mice were obtained from the National Cancer Institute (Frederick, MD, USA) while β 7-deficient mice

(in the C57BL/6 background) were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice over expressing murine IL-4 under the regulation of the Clara Cell 10 promoter (a kind gift of Dr Jeffrey Whitsett, Cincinnati Children's Hospital, OH, USA) [28], were crossed with IL-5 (CD2-promoter) transgenic mice [29] in order to obtain mice that were bitransgenic for IL-4 and IL-5 in a pure BALB/c background. Animals involved in these studies were housed under specific pathogen-free conditions and treated according to institutional guidelines. Water was available continuously through automatic ports and a commercial mouse diet was provided *ad libitum*.

Experimental protocols

In the first experimental asthma model, two intraperitoneal (ip) sensitizations 2 weeks apart (50 μ g of ovalbumin (OVA) per 1 mg of alum) were followed by two or three OVA (50 μ g) or saline intranasal challenges 3 days apart, as previously described [30]. In the second model, sensitized mice were challenged following intranasal delivery of 100 μ g of *Aspergillus fumigatus* extracts (Bayer Corp., Spokane, WA, USA) three times per week as described elsewhere [31]. Mice were killed 24 h following a given allergen challenge. Finally, in the eosinophilic gastrointestinal allergy model, a single ip sensitization was followed by two oral allergen challenges as previously described [8, 32]. Briefly, on days 12 and 15, mice were lightly anesthetized by Isoflurane inhalation and orally administered 20 mg of encapsulated OVA enteric-coated beads or placebo enteric-coated beads, followed by oral administration of 300 μ L of acidified double distilled water (pH = 2). Seventy-two hours after the last antigen challenge, mice were killed and parameters analyzed.

Mucosal eosinophil quantification

Lung tissue was collected 18 h after the last intranasal challenge while jejunum tissue was collected 10 cm distal to the stomach, 72 h after the last oral challenge. Tissue samples were then fixed in 10% formalin and processed using standard histological techniques. Briefly, 5 μ m sections were quenched with H₂O₂, and then blocked with 3% normal goat serum, and stained with a rabbit anti-murine eosinophil MBP antiserum (a kind gift of J. and N. Lee, Mayo Clinic, Scottsdale, AZ, USA) over night at 4 °C. The slides were washed and incubated with biotinylated goat anti-rabbit antibody and avidin-peroxidase complex (Vectastain ABC Peroxidase Elite Kit; Vector Laboratories, Burlingame, CA, USA). The slides were then developed by nickel diaminobenzidine to form black precipitates (DAB Kit; Vector Laboratories), and counterstained with nuclear fast red. At least three random fields were analyzed per tissue section as previously described [8, 32]. Quantification of stained cells per square millimeter of tissue was

performed by morphometric analysis using Image-Pro Plus (Media Cybernetics, Silver Spring, MA, USA).

Bronchoalveolar lavage

Allergen challenged mice and IL-4 and/or IL-5 transgenic mice were killed and lungs were lavaged two times with 1 mL of sterile lavage buffer (phosphate-buffered saline (PBS), 1% FCS and 0.2 mM EDTA). The bronchoalveolar lavage fluid (BALF) was centrifuged at 300 g for 5 min and re-suspended in 200 μ L of lavage buffer. Viable cells were quantitated following trypan blue staining. Differential cell counts were performed on cytopsin preparations stained with Diff-Quick (Dade Behring, Newark, DE, USA).

Cell isolation

BALF, lung, blood, spleen, and Peyer's patches were collected in cold PBS. Spleen and Peyer's patches were directly dissociated into single-cell suspensions, filtered through a nylon strainer (70 μ m), centrifuged at 300 g for 5 min, and resuspended in PBS. Red blood cells present in the different cell suspensions were lysed (RBC Lysing Buffer; Sigma-Aldrich, St Louis, MO, USA). Remaining cells were centrifuged at 300 g for 5 min before being re-suspended in FACS buffer and counted with a hemacytometer.

Flow cytometric analysis

Cells (2×10^5) were washed with FACS-buffer (2% BSA, 0.1% NaN₃ in PBS) and then incubated for 45 min at 4 °C with a combination of 150 ng (1.5 μ g/mL) FITC-conjugated anti-murine CCR3 antibody (FAB729; rat IgG2a; R&D Systems, Minneapolis, MN, USA) and one of the following antibodies: 300 ng (3 μ g/mL) anti-murine $\alpha 4$, $\beta 1$, $\beta 2$, $\beta 7$, $\alpha 4\beta 7$, c-kit, CD-3 (10 μ g/mL) (BD Biosciences Pharmingen, San Diego, CA, USA) or the isotype-matched control antibody. After two washes, cells were incubated with the viability dye 7ADD (Molecular Probes, Eugene, OR, USA) just before being subjected to flow-cytometry on a FACScalibur Flow Cytometer and analyzed using the CELLQuest software (BD Biosciences, San Jose, CA, USA).

Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical significance comparing different sets of mice was determined by Student's unpaired *t*-test.

Results

Development of a dual IL-4/IL-5 transgenic system for analysis of pulmonary eosinophils

We were interested in developing an experimental system that would allow us to reproducibly and conveniently examine lung eosinophils without the need for a prolonged allergen-induced experimental asthma protocol. Accordingly, we generated a dual transgenic mouse line by mating CD2-driven IL-5 transgenic mice with Clara Cell Protein 10 (CC10)-driven IL-4 transgenic mice [28]. Bi-transgenic mice had synergistic increases in BALF eosinophils compared with IL-4 and IL-5 single transgenic mice, and reached levels similar to allergen-challenged mice (Fig. 1a).

Following either repeated intranasal *A. fumigatus* antigen challenges [31] or a classic OVA challenge model [30], BALF eosinophils were identified by FACS and compared with BALF eosinophils from IL-4/IL-5 transgenic mice (Fig. 1b). When compared with eosinophils from allergen challenged mice, BALF eosinophils from IL-4/IL-5 transgenic mice were found to be similar as assessed by their characteristic light scatter appearance as well as their high expression of the eotaxin receptor CCR3 and the surface marker Gr-1 (Ly6G-C).

Bronchoalveolar lavage fluid eosinophils differ from spleen eosinophils

In order to characterize the role and regulation of pulmonary eosinophils, we isolated eosinophils from the BALF of allergen challenged mice or IL-4/IL-5 transgenic mice and assessed surface expression of common eosinophil surface molecules by FACS. When compared with eosinophils from the spleen, BALF eosinophils were found to have similar light scatter appearance and Gr-1 expression (Figs 1b and c). However, a decrease in CCR3 expression was observed on BALF eosinophils as assessed by mean fluorescence intensity most likely due to internalization following binding with its ligands [33]. Indeed high levels of eotaxin-1 and -2 have been reported in the lungs of IL-4 transgenic mice [34] as well as allergen challenged mice [35, 36].

Surface expression of several eosinophil adhesion molecules was assessed on BALF and spleen eosinophils following allergen challenge. Expression levels of $\beta 1$ and $\beta 2$ were similar on BALF and spleen eosinophils, with respectively $94.1 \pm 0.9\%$ vs. $94.2 \pm 1.2\%$ for $\beta 1$ and 100% vs. 99.9% for $\beta 2$ (mean $\pm$ SEM). Only $\beta 7$ -expression appeared down-regulated on BALF eosinophils when compared with spleen eosinophils (Fig. 2). We therefore concentrated our analysis on surface expression of the $\beta 7$ -integrin family.

The $\alpha 4\beta 7$ -integrin is down-regulated in the allergic lung

In order to further examine the down-regulation of the $\beta 7$ -integrin on BALF eosinophils, we assessed the expression

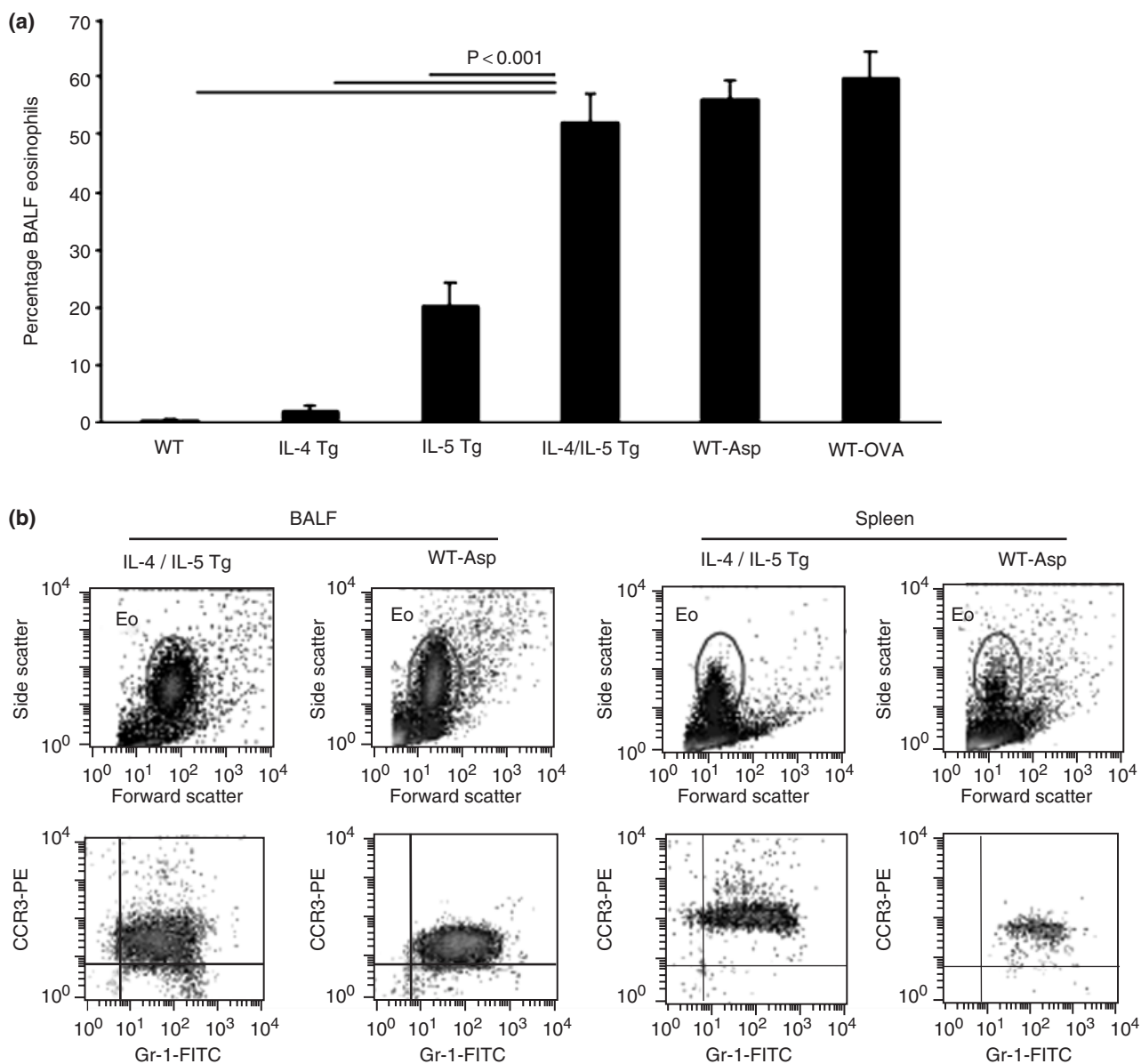


Fig. 1. Characterization of airway and spleen eosinophils. (a) The percentage of bronchoalveolar lavage fluid (BALF) eosinophils in wild-type (WT), IL-4 and IL-5 single transgenic (Tg) mice, IL-4/IL-5 bi-transgenic mice, as well as mice repeatedly challenged with *Aspergillus fumigatus* and ovalbumin (OVA)/alum-sensitized mice intranasally challenged twice with OVA. The IL-5 transgene is under the CD2 promoter and the IL-4 transgene under the CC10 promoter. Results are expressed as mean $\pm$ SEM of 7–14 mice per group. (b) Using FACS analysis, eosinophils were characterized by side and forward scatter as well as by surface marker expression. Almost all cells within the eosinophil gate (Eo) were positive for the eotaxin receptor CCR3 and for Gr-1 (Ly6G-C).

of the $\alpha 4\beta 7$ complex on BALF, lung, blood and spleen eosinophils following repeated intranasal *A. fumigatus* antigen challenges [31]. Notably, the $\alpha 4$ -integrin expression on lung and BALF eosinophils was comparable to the spleen, while the $\beta 7$ -integrin was significantly down-regulated on BALF and lung eosinophils (Figs 2a and b; $P < 0.05$). $\beta 7$ expression on blood and spleen derived eosinophils were similar, respectively $97.5 \pm 1.3\%$ and $94.8 \pm 2.1\%$ ($n = 5$). Finally, levels of $\alpha 4$ (data not shown) and $\beta 7$ expression on BALF, lung and spleen

derived eosinophils were similar between IL-4/IL-5 bi-transgenic mice and *Aspergillus*-challenged mice (Fig. 2b).

In order to assess the impact of the observed $\beta 7$ down-regulation on lung and BALF eosinophils on the formation of the $\alpha 4\beta 7$ complex, we took advantage of the unique ability of the DATK32 antibody to bind this complex [37]. Surprisingly, less than 20% of BALF eosinophils had detectable $\alpha 4\beta 7$ expression following allergen challenges (*Aspergillus*: $10.1 \pm 1.7\%$ $n = 4$; OVA: $8.7 \pm 3.5\%$ $n = 4$). In

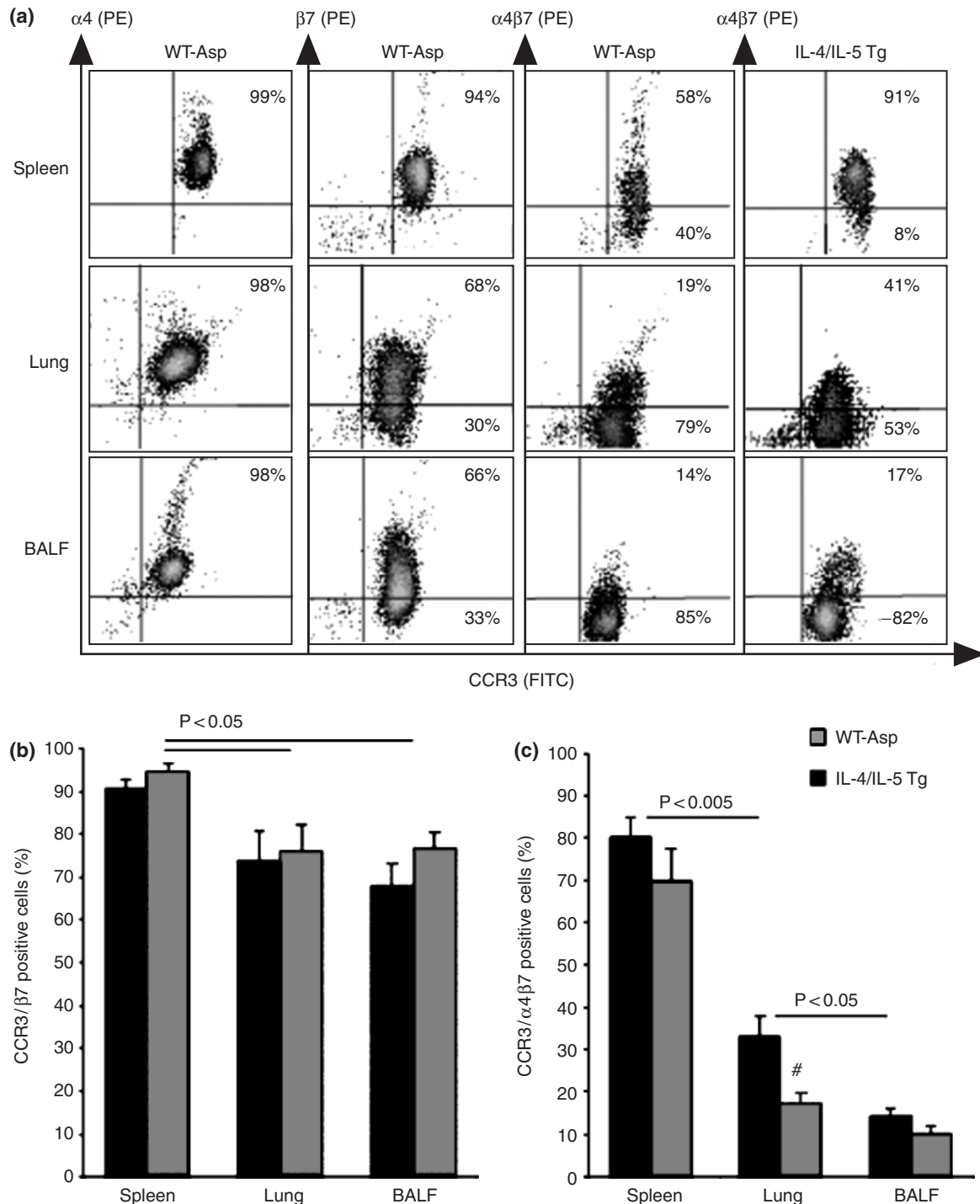


Fig. 2. The $\alpha 4\beta 7$ -integrin is down-regulated in the lung and bronchoalveolar lavage fluid (BALF). BALF cells and cells isolated from the lung tissue were compared with splenocytes of *Aspergillus* challenged mice and IL-4/IL-5 bi-transgenic mice by FACS analysis. (a) Double staining for CCR3 and $\alpha 4$ (9C10), $\beta 7$ (FIB27) or the $\alpha 4\beta 7$ complex (DATK32) was performed on spleen, lung and BALF cells within the eosinophil gate and negative for 7AAD (alive). Representative density plots of three to five independent experiments. The percentage of (b) $\beta 7$ /CCR3 double positive cells and percentage of (c) $\alpha 4\beta 7$ /CCR3 double positive cells within the eosinophil gate of spleen, lung and BALF is expressed as mean $\pm$ SEM ($n = 2-6$ mice/group; $\# P < 0.05$).

contrast, the majority of spleen-derived eosinophils were positive for $\alpha 4\beta 7$ following *Aspergillus* exposure (Fig. 2a).

In order to determine if the down-regulation of the $\alpha 4\beta 7$ -integrin also occurred in the lung tissue itself,

eosinophils were examined from single cell lung tissue suspensions. Following challenge, $\alpha 4\beta 7$ expression on lung derived eosinophils was also dramatically down-regulated (*Aspergillus*: $17.2 \pm 2.6\%$ $n = 3$; OVA: 24.3 ± 1.4

$n = 2$). Of note, when allergen challenged mice were compared with IL-4/IL-5 bi-transgenic mice, $\alpha 4\beta 7$ expression levels were similar on BALF, spleen and blood eosinophils (Fig. 2c and data not shown). In contrast, surface expression of $\alpha 4\beta 7$ on lung eosinophils was significantly lower following *Aspergillus* challenge (Fig. 2c).

Taken together, these results demonstrate that the $\alpha 4\beta 7$ complex is progressively down-regulated when eosinophils are recruited to the lung and airway lumen, and that the mechanism mainly involves down-regulation of the $\beta 7$ -integrin itself.

$\beta 7$ -integrin is not required for eosinophil recruitment to the bronchoalveolar lavage fluid

In order to dissect a role for $\alpha 4\beta 7$ down-regulation, we subjected $\beta 7$ -deficient mice to two distinct experimental asthma models, hypothesizing that they would have a change in eosinophil levels and/or distribution. In an OVA-induced model of experimental asthma, we observed no significant difference in the level of BALF eosinophils after two or three intranasal allergen challenges (Fig. 3a). Additionally, using an independent model of experimental asthma, induced by repeated intranasal *A. fumigatus* antigen exposure [31], BALF eosinophilia in $\beta 7$ -deficient mice was similar to that in wild type mice (Fig. 3b). Additionally, the distribution of eosinophils in the asthmatic lung was comparable between $\beta 7$ -deficient and wild type mice in these two independent experimental asthma models (data not shown). These results suggest that $\alpha 4\beta 7$ is not critical for eosinophil recruitment into the BALF.

$\alpha 4\beta 7$ -integrin on intestinal and pulmonary eosinophils

Following these observations, it became important to determine if $\alpha 4\beta 7$ had a functional role in allergic responses in other tissues. We thus focused our attention on the gastrointestinal tract where the major $\alpha 4\beta 7$ ligand MAdCAM-1 is expressed [19]. As we have previously reported that eosinophils are recruited to Peyer's patches in IL-5 transgenic mice [38], we chose to examine this population of eosinophils. Thus, we compared $\alpha 4\beta 7$, $\beta 7$, and $\alpha 4$ expression on eosinophils from Peyer's patches with eosinophils from the spleen and blood of IL-5 transgenic mice (Fig. 4a). The $\alpha 4\beta 7$ -integrin expression was significantly, albeit modestly, lower on Peyer's patches compared with eosinophils from the spleen and blood (Fig. 4b).

In order to assess if $\alpha 4\beta 7$ expression on circulating eosinophils was affected by intranasal allergen challenge or IL-5 over-expression, we compared $\alpha 4\beta 7$, $\beta 7$, and $\alpha 4$ expression on blood-derived eosinophils from IL-5 transgenic mice, allergen challenged mice and unchallenged wild type mice. Expression levels of $\alpha 4\beta 7$ were similar between wild type and IL-5 transgenic mice (Figs 4a and c). Furthermore, allergen challenges did not significantly

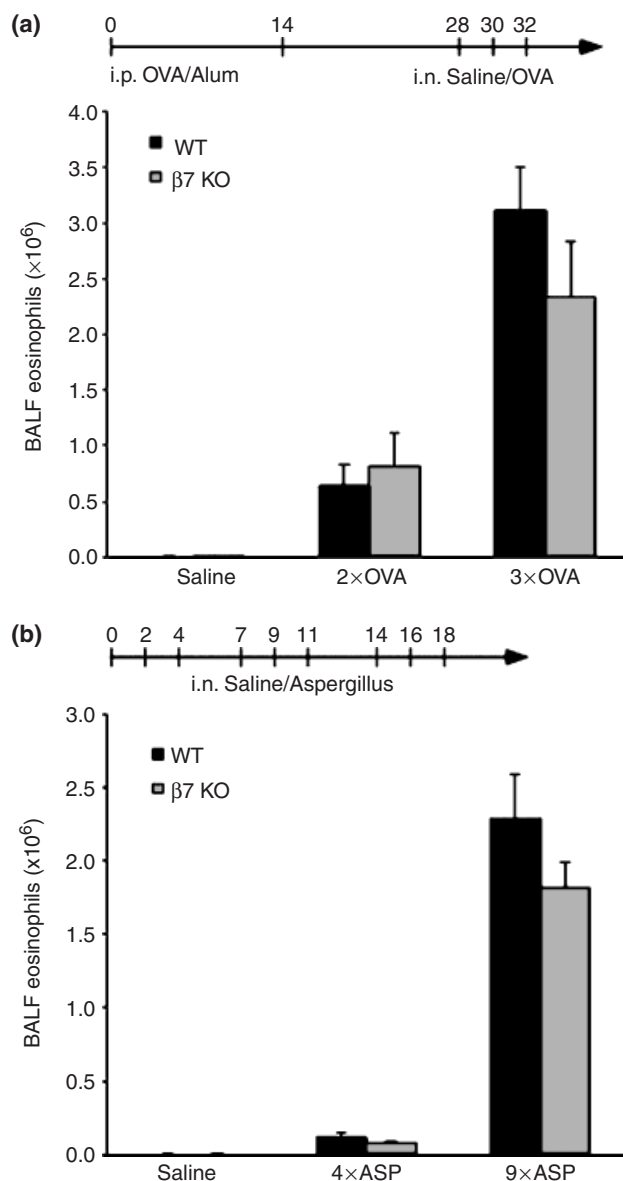


Fig. 3. The $\alpha 4\beta 7$ -integrin is not required for eosinophil recruitment to the bronchoalveolar lavage fluid (BALF). BALF eosinophilia was assessed in $\beta 7$ -deficient mice and their wild type C57BL/6 controls in two separate models of allergic lung inflammation. (a) Mice were sensitized twice ($50 \mu\text{g}$ of ovalbumin (OVA) per 1 mg of alum) followed by two or three OVA ($50 \mu\text{g}$) or saline intranasal challenges 3 days apart. The total number of cells was collected 24 h after the last challenge from $\beta 7$ -deficient and control mice. Data is expressed as mean $\pm$ SEM ($n = 5\text{--}8$ mice/group). (b) Mice were intranasally challenged with saline or *Aspergillus fumigatus* antigen ($100 \mu\text{g}$) three times a week for up to 3 weeks. The total number of eosinophils in the BALF of WT and $\beta 7$ -deficient mice is expressed as mean $\pm$ SEM ($n = 8\text{--}12$ mice/group).

affect $\alpha 4\beta 7$ expression on eosinophils from OVA challenged mice (data not shown) or *A. fumigatus* challenged mice (Fig. 4c).

Taken together, these results show lower expression levels of $\alpha 4\beta 7$ on Peyer's patch eosinophils compared with levels of this integrin on circulating eosinophils.

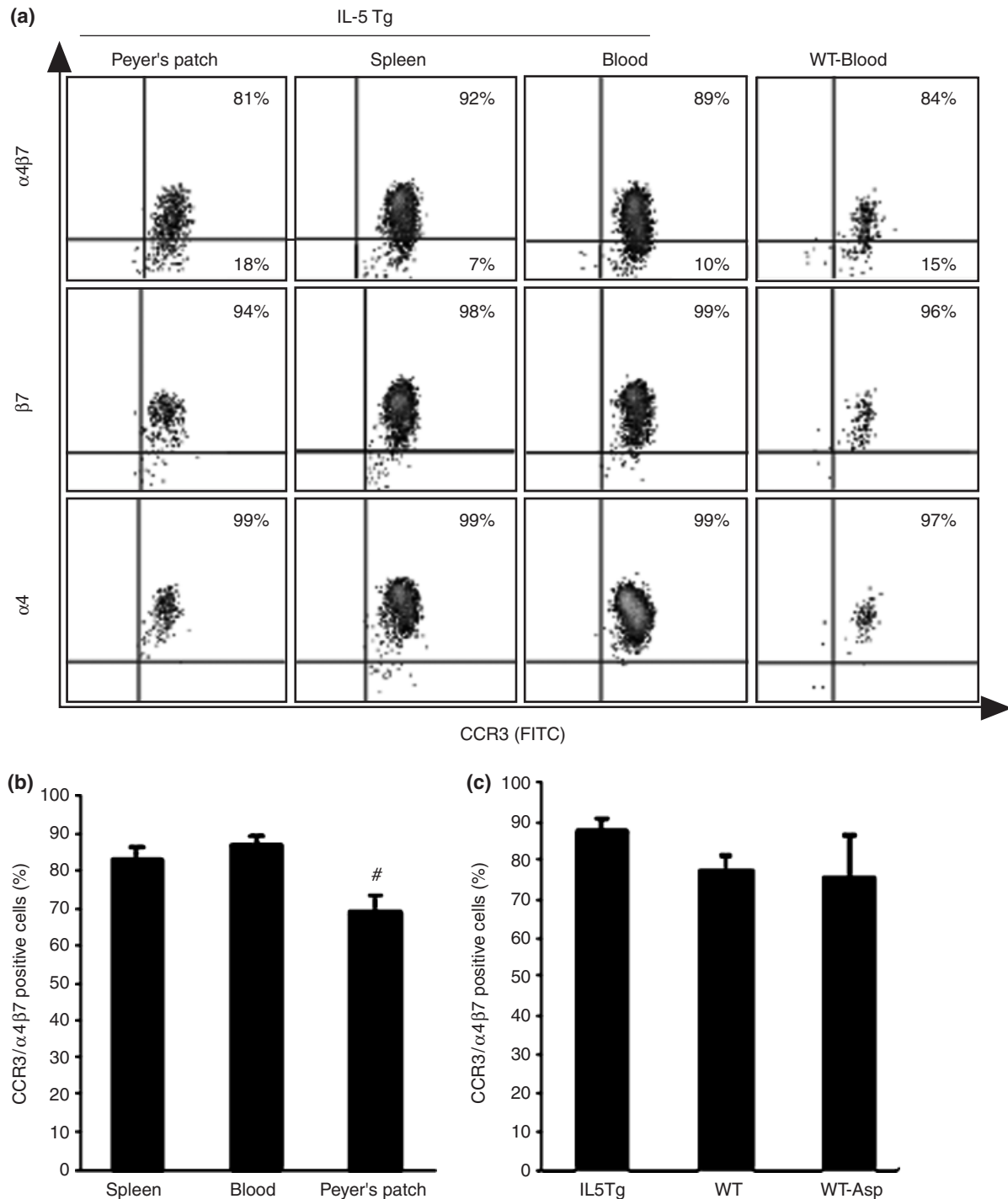


Fig. 4. The $\alpha 4\beta 7$ -integrin is down-regulated on tissue eosinophils. (a) Representative FACS density plots from cells isolated from Peyer's patches, spleen and blood of IL-5 transgenic mice and blood of unchallenged wild type mice. Double staining for CCR-3 and $\alpha 4$ (9C10), $\beta 7$ (FIB27) or the $\alpha 4\beta 7$ complex (DATK32) was performed on cells within the eosinophil gate and negative for 7AAD. (b) The percentage of $\alpha 4\beta 7$ /CCR-3 double positive cells within the eosinophil gate of spleen, blood and Peyer's patches is expressed as mean $\pm$ SEM ($n = 5-7$ mice/group; $\# P < 0.05$). (c) The percentage of $\alpha 4\beta 7$ /CCR-3 double positive blood eosinophils from IL-5 transgenic mice, wild type mice and *Aspergillus fumigatus* challenged mice is expressed as mean $\pm$ SEM of three to four separate experiments.

$\beta 7$ -integrin is important for eosinophil recruitment to the intestinal tract under inflammatory conditions

In order to establish if the $\beta 7$ -integrin was involved in eosinophil recruitment into the gastrointestinal tract, we

subjected $\beta 7$ -deficient mice to the induction of experimental gastrointestinal allergy. Experimental allergic responses were induced by intragastric exposure of OVA/alum sensitized mice to OVA (in the form of enteric coated microbeads) [8, 32]. Intestinal eosinophil levels were

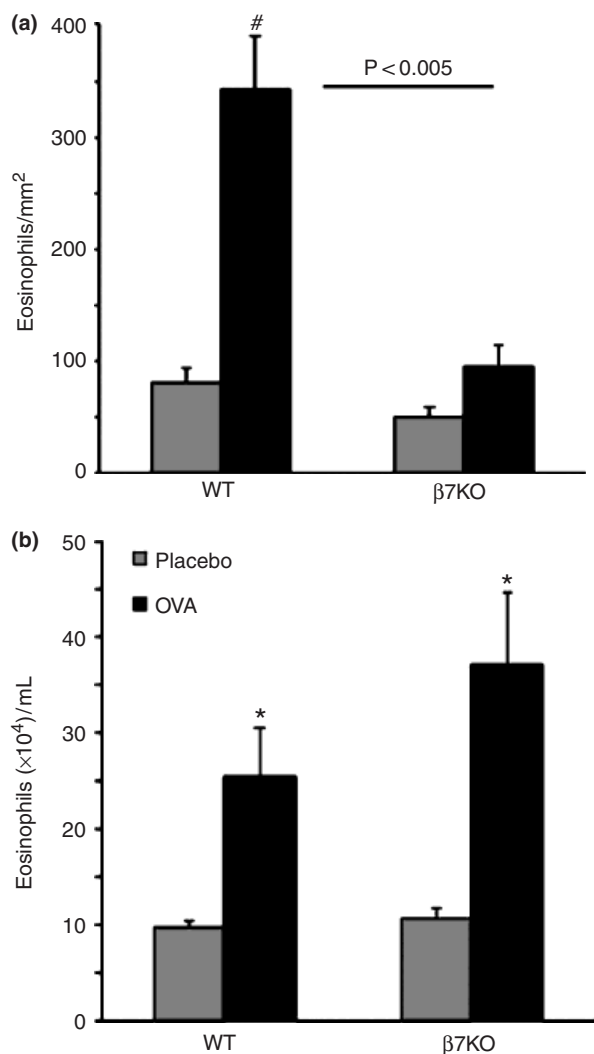


Fig. 5. $\beta 7$ -integrins are important for eosinophil recruitment to the intestinal tract under inflammatory conditions. Ovalbumin (OVA)/alum sensitized mice challenged twice with OVA or placebo enteric coated beads. Three days after the second oral challenge, and blood and intestinal eosinophilia assessed. (a) Eosinophil levels in the jejunum of $\beta 7$ -deficient mice and their wild-type (WT) C57BL/6 controls were assessed by morphometric analysis of MBP-stained cells per mm² of lamina propria and expressed as mean $\pm$ SEM with a total of 4–5 mice/group. (b) Peripheral blood eosinophil levels were assessed by Discombe's staining and expressed as mean $\pm$ SEM with a total of 4–5 mice/group. Statistical significance was assessed by unpaired Student's *t*-test between C57BL/6 mice and $\beta 7$ -deficient mice and between placebo and OVA challenged mice ([#] $P < 0.005$; ^{*} $P < 0.05$).

significantly increased in the jejunum of OVA challenged mice (Fig. 5a; $P < 0.005$). Importantly, $\beta 7$ gene-deficient mice displayed impaired eosinophilia compared with wild type mice ($P < 0.005$). Accordingly, eosinophil levels were also significantly lower in the colon of $\beta 7$ -deficient mice (27.9 ± 4.6 vs. 61.9 ± 8.2 ; $P = 0.005$). However, blood eosinophil levels were not significantly affected (Fig. 5b).

Taken together these results demonstrate an important role for $\alpha 4\beta 7$ in eosinophil recruitment to the gastrointestinal tract during an allergic gastrointestinal response.

Discussion

Our observation that eosinophils lose their surface expression of the $\alpha 4\beta 7$ -integrin during their migration into the airways suggested a role for this integrin in allergic lung inflammation. However, while we demonstrated that the $\alpha 4\beta 7$ -integrin is important for gastrointestinal eosinophil trafficking, the absence of the $\beta 7$ gene did not significantly affect eosinophil recruitment into the lung of allergen-challenged mice. While the main $\alpha 4\beta 7$ receptor, MAdCAM-1, can be expressed in non-gastrointestinal organs like the pancreas or the brain, there is no report of MAdCAM-1 expression in the lung, even under inflammatory conditions [19]. However, $\alpha 4\beta 7$ also binds to VCAM-1 and fibronectin [16] and these molecules have been implicated in supporting eosinophil rolling under conditions of shear flow [39]. Studies using blocking antibodies have implicated $\alpha 4\beta 1$ and not $\alpha 4\beta 7$ in eosinophil lung recruitment [12]. In accordance with these findings, we could not demonstrate a decrease in BALF eosinophilia in the absence of $\beta 7$ following allergen challenge to the lung, indicating that other integrins ($\alpha 4\beta 1$) in combination with CCR3 binding chemokines are likely to be responsible for allergen-induced pulmonary eosinophilia [40, 36].

The function of $\alpha 4\beta 7$ on murine and human eosinophils has not been extensively studied. $\beta 7$ up-regulation occurs during eosinophil differentiation [17]. Circulating blood eosinophils from normal donors constitutively express $\alpha 4\beta 7$ and higher levels of $\alpha 4\beta 7$ are observed in hypereosinophilic patients [41]. Accordingly, we demonstrate constitutive expression of $\alpha 4\beta 7$ on murine blood and spleen eosinophils. However, circulating eosinophils from IL-5 transgenic mice or allergen challenged mice did not present a significant alteration in their $\alpha 4\beta 7$ surface expression compared with eosinophils from unchallenged wild type mice. This suggests that IL-5 stimulation has no effect on $\alpha 4\beta 7$ expression levels on eosinophils.

Interestingly, while eosinophils migrating through the lung tissue into the BALF eventually lost most of their surface expression of the $\alpha 4\beta 7$ -integrin, the down-regulation of the $\alpha 4\beta 7$ -integrin on lung-derived eosinophils from allergen challenged mice was more dramatic than on eosinophils of IL-4/IL-5 bi-transgenic mice, suggesting that the inflammatory environment of the allergic lung has a crucial role in the observed $\alpha 4\beta 7$ down-regulation on recruited eosinophils.

In order to measure the impact of BALF components on eosinophil $\alpha 4\beta 7$ expression, we injected splenocytes from IL-5 transgenic mice (40–50% eosinophils) intratracheally into wild type mice and retrieved them 4 h later with only

a very modest down-regulation of $\alpha 4\beta 7$ (data not shown). Since the environment of a wild type lung is dramatically different from that of an allergic lung, we also cultured spleen eosinophils from IL-5 transgenic mice for 4 h in the presence of BALF from allergen challenged mice. However, culture in BALF only modestly decreased $\alpha 4\beta 7$ expression on eosinophils when compared with freshly isolated cells (data not shown).

In another approach, we reasoned that the migration through the epithelial layer into the BALF could be responsible for the dissociation of the complex. We further speculated that interactions with epithelial cells possibly through E-cadherin would require the transient formation of $\alpha E\beta 7$ on transmigrating eosinophils. Indeed a small subpopulation of $\alpha E/CCR3$ double positive cells could be observed in the BALF. However this population was only about 10% of all BALF eosinophils (7AAD negative and CCR3 positive cells within the eosinophil gate). Furthermore, transmigration of spleen eosinophils (with high levels of $\alpha 4\beta 7$) through a layer of A549 epithelial cells did not significantly affect $\alpha 4\beta 7$ expression on these eosinophils (data not shown). This suggests that eosinophil interactions with epithelial cells is unlikely to account for the majority of $\alpha 4\beta 7$ down-regulation. In accordance with our observations, an *in vitro* study analyzing eosinophil adherence to human airway epithelial cells demonstrated that while anti- $\alpha 4$ and anti- $\beta 1$ antibodies inhibited eosinophil adhesion, the anti- $\beta 7$ -antibody had no effect [42].

Another explanation for the dramatic down-regulation of the $\alpha 4\beta 7$ -integrin on lung-derived eosinophils might be interaction with its ligands VCAM-1, fibronectin and/or MAdCAM-1. Indeed, several studies have shown that upon interaction with their ligands, CCR3 and the IL-5 receptor are down-regulated on eosinophils [33, 43]. Accordingly, when comparing circulating and lung-derived eosinophils, a decrease in CCR3 expression was observed on BALF eosinophils [44]. A decrease in IL-5 receptor expression on human BALF eosinophils compared with blood eosinophils has also been reported [45]. However, interaction with its main ligand MAdCAM-1, which is expressed on HEV from Peyer's patches [19], only induces a modest down-regulation of $\alpha 4\beta 7$. These studies suggest that while interaction with its ligand may contribute to a transient internalization of this integrin, it does not account for the majority of the observed down-regulation. Perhaps, the dissociation of the $\alpha 4\beta 7$ complex on the surface of lung-derived eosinophils is the result of numerous interactions of these eosinophils with the allergic tissue environment thus leading to an activation of these eosinophils and the loss of the $\alpha 4\beta 7$ integrin. Indeed, when compared with blood eosinophils, airway eosinophils have been reported to be activated [46–49]. In addition to expressing increases of several activation markers including CD69, BALF eosinophils display increased survival [46]. Importantly, engagement of the

$\alpha 4\beta 7$ -integrin by its ligands has been shown to enhance human blood eosinophil survival *in vitro* via autocrine production of GM-CSF [24].

Extending our prior observation that $\beta 7$ was important in regulating intestinal eosinophilia in a murine model over expressing eotaxin-1 specifically in the small intestine [23], we demonstrate a role for $\beta 7$ in recruiting eosinophils to the small and large intestine following oral allergen challenge. Thus, the central role of $\alpha 4\beta 7$ in intestinal eosinophil recruitment is consistent with the expression pattern of its ligand MAdCAM-1 [19]. This suggests that both eotaxin-1 (acting through CCR-3) and $\alpha 4\beta 7$ (binding to MAdCAM-1) are essential signals for eosinophil recruitment to the small intestine. Furthermore, studies using $\beta 7$ -deficient mice have already demonstrated the importance of the $\alpha 4\beta 7$ -integrin in lymphocyte as well as mast cell progenitor cell recruitment to the small intestine [20, 21].

Taken together, we demonstrate that BALF eosinophils differ significantly from blood eosinophils in their expression of the $\alpha 4\beta 7$ -integrin. While the absence of $\beta 7$ had little impact on BALF eosinophilia, the recruitment of eosinophils to the gastrointestinal tract is dependent on the $\beta 7$ -integrin. While an anti- $\alpha 4$ -integrin has been proposed for treating patients with inflammatory bowel disease [26], blocking $\beta 7$ -integrins ($\alpha 4\beta 7$ and $\alpha E\beta 7$) might be more specific for gastrointestinal diseases [27, 50], abating not only eosinophil but also mast cell and lymphocyte recruitment to the inflamed gastrointestinal tract.

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