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Activation states of blood eosinophils in asthma

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Abstract

Asthma is characterized by airway inflammation rich in eosinophils. Airway eosinophilia is associated with exacerbations and has been suggested to play a role in airway remodeling. Recruitment of eosinophils from the circulation requires that blood eosinophils become activated, leading to their arrest on the endothelium and extravasation. Circulating eosinophils can be envisioned as potentially being in different activation states, including non-activated, pre-activated or "primed", or fully activated. In addition, the circulation can potentially be deficient of pre-activated or activated eosinophils, because such cells have marginated on activated endothelium or extravasated into the tissue. A number of eosinophil-surface proteins, including CD69, L-selectin, intercellular adhesion molecule-1 (ICAM-1, CD54), CD44, P-selectin glycoprotein ligand-1 (PSGL-1, CD162), cytokine receptors, Fc receptors, integrins including α_M integrin (CD11b), and activated conformations of Fc receptors and integrins have been proposed to report cell activation. Variation in eosinophil activation states may be associated with asthma activity. Eosinophil-surface proteins proposed to be activation markers, with a particular focus on integrins, and evidence for associations between activation states of blood eosinophils and features of asthma are reviewed here. Partial activation of β_1 and β_2 integrins on blood eosinophils, reported by monoclonal antibodies (mAb) N29 and KIM-127, is associated with impaired pulmonary function and airway eosinophilia, respectively, in non-severe asthma. The association with lung function does not occur in severe asthma, presumably due to greater eosinophil extravasation, specifically of activated or pre-activated cells, in severe disease.

Introduction

Asthma is frequently characterized by airway inflammation rich in eosinophils [1–32]. Airway eosinophilia is associated with exacerbations [1, 8, 9, 14, 33–37] and likely plays a role in airway remodeling [1, 8, 36–39]. Recruitment of eosinophils from the circulation requires that blood eosinophils become activated, leading to their arrest on activated endothelium and extravasation [40–44]. This review will discuss cell-surface proteins proposed to report or potentially reporting eosinophil activation. It will particularly focus on the integrin family of cell adhesion receptors [45–50], the activation states or conformations of integrins [47, 48, 50–52], and evidence for associations between activation states of integrins on blood eosinophils and features of asthma, such as pulmonary function, and airway inflammation and eosinophilia. Arrest of eosinophils in vessels and their

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extravasation into the airway wall and through the bronchial tissue and epithelium to the airway lumen are mediated by integrins [12, 41–43, 53, 54]. Thus, there is a biological rationale for integrin conformation states as markers of eosinophil activation in asthma and as potential correlates with disease activity.

Eosinophil-surface proteins proposed to report cell activation

General remarks

The eosinophil surface phenotype, consisting of numerous cell-surface proteins, including adhesion molecules and cytokine, chemoattractant, complement, Fc, and innate immune receptors, has been reviewed extensively [5, 12, 13, 55–57]. Induction or upregulation, in some cases, downregulation, of a number of eosinophil-surface proteins, e.g., CD69 and α_M integrin (CD11b), as well as activated conformations of Fc receptors (FcγRII = CD32) and integrins (β_1 and β_2) potentially report cell activation or have been proposed to be biomarkers in asthma (Table 1) [4, 40, 58–62]. Such suggestions have often been based on the response of blood eosinophils to various cytokines or other factors *in vitro*. Table 1 lists such suggested cell-surface proteins and alterations in the cell-surface protein expression, usually detected by flow cytometry. Unless indicated otherwise, alterations in Table 1 are on blood eosinophils.

“Upregulation” and “downregulation”, etc., in Table 1 and throughout the text of this review refer to increased cell-surface protein expression, regardless of the mechanism in the individual case, which may be mobilization from intracellular stores or the result of increased transcription or translation. Further, upregulation may mean that an increase in average level on all eosinophils has been reported and/or an increase in the percentage of eosinophils positive for a particular protein. Although the percentage positive cells and expression level often appear to correlate, percentage positivity plateaus and is no longer informative beyond a certain level; when positivity reaches close to or 100%, expression level continues to increase and thus has a greater dynamic range [63]. In addition, expression level has been reported in various ways, including by arithmetic or geometric mean or median fluorescent intensity or channel fluorescence (CF). Whenever possible, the dynamic range in percentage positive cells or expression level among subjects, upon stimulation *in vitro*, or, e.g., between airway and blood eosinophis, is given in the text. However, considering the various different ways that data have been reported, the dynamic ranges of different proteins or in different publications are not always directly comparable. Further, Table 1 does not distinguish between different incubation times for the *in vitro* experiments, which range from minutes to days. In some cases, *in vitro* studies of blood eosinophils have been complemented by comparisons of blood eosinophils among subjects with allergic or non-allergic asthma, allergy without asthma, or normal healthy control subjects, or observations of blood eosinophils after whole or segmental lung antigen challenge, BAL eosinophils, or sputum eosinophils.

Many of the studies are on purified eosinophils, whereas some are on whole blood or BAL cells. Use of unfractionated cells has several advantages, including the requirement for only a small volume of blood making repeated sampling in the same subject possible, and the fact that purified eosinophils are not a completely accurate reflection of eosinophils *in vivo*. For

instance, the signal for mAb N29, reporting the intermediate-activity conformation of β_1 integrins [41], increases upon purification of blood eosinophils [64]; thus, β_1 becomes more activated during the purification process. Similarly, CD35 and CD81 expression on purified eosinophils is higher and less variable among subjects than that on eosinophils in whole blood [65].

As will become evident below, high expression levels or activation states of cell-surface proteins achieved on BAL eosinophils are often not observed on circulating eosinophils. In addition, concentrations of cytokines or other factors required to produce a fully activated state *in vitro*, e.g., with highly activated $\alpha_M\beta_2$ integrin or high degree of adhesion, are often relatively high, ≥ 10 ng/ml interleukin (IL)-5, granulocyte macrophage-colony stimulating factor (GM-CSF), or IL-3 [66–68]. In asthmatic lung, extrapolated from levels found in BAL [63, 69], IL-5 family cytokines range from 0.1 to 100 ng/ml [63, 70–74], whereas levels in peripheral blood in asthma are lower, e.g., 1–10 pg/ml [75–80]. Also for other stimuli, such as eotaxins, IL-4, IL-13, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α, the same discrepancy between lower concentrations in blood [77–85] and higher concentrations in inflamed airway [63, 73, 86–90] exists, supporting a scenario in asthma where eosinophils likely are exposed to high mediator concentrations only after entering vasculature of the lung or airway tissue.

Finally, it is worth mentioning in this context, although it is not the focus of this review, that in addition to expression of potential activation markers, pre-activation or “priming” of blood eosinophils as a result of systemic inflammation have been evaluated using functional assays [40, 91, 92]. Such experiments have shown that blood eosinophils from subjects with allergy or asthma, particularly after antigen challenge, have a greater degree of adhesion or transendothelial migration or greater responsiveness to chemoattractants for chemotaxis or activation of the respiratory burst, whereas blood eosinophils from normal donors can be “primed” for greater responses by IL-5 family cytokines *in vitro* [40, 93–99]. A disadvantage with the functional assays is the need for isolated eosinophils; cell purification requires a larger blood volume and may in itself promote more activation, as discussed above. It is possible that the “priming” seen with the functional assays and believed to be a result of exposure to IL-5 or similar cytokines *in vivo* [40, 91, 92] is associated with changes in activation markers, such as seen in the IL-5-dependent (decreased by anti-IL-5 *in vivo*) presence of the intermediate-activity state of β_2 integrins or the upregulation of α_L, α_L, and β_2 after segmental antigen challenge (see below and Table 1) [100].

CD69

CD69, an early activation antigen of T cells, was suggested in the beginning of the 1990s as a marker of eosinophil activation [4, 59, 60, 101, 102]. It is absent or expressed at a low level on unstimulated blood eosinophils, with from 0% to about 30% positive cells [65, 101–109]. The expression level, although low, varies up to 50-fold among subjects (by median CF, defined as described [66]) [110]. It is induced *in vitro* by IL-5 or the other IL-5 family cytokines IL-3, GM-CSF, or by cytokines of other classes (Table 1) [65, 101–110]. In different studies percentage positive cells were increased to 50–90% and level about 3–50-fold by IL-5 family cytokines (by fluorescence intensity or CF) [65, 101, 104–110]. Further,

CD69 is induced transiently on blood eosinophils after whole-lung antigen challenge (from 1–4% to 10–20% positive cells) [111] and on BAL eosinophils (up to four-fold level compared to blood eosinophils) [101, 103, 105]. In one study of approximately 350 mAbs, only those against CD69 reacted with cytokine-stimulated blood eosinophils and BAL eosinophils but not with unstimulated blood eosinophils [103], supporting the suggestion of CD69 as an eosinophil activation marker [59, 60]. However, a comparison by Johnsson and others among patients with asthma or airway allergy, other eosinophilic diseases, and normal healthy control subjects of expression levels of multiple surface proteins on eosinophils in blood did not find any differences in CD69 expression among the groups [77].

L-selectin

L-selectin (CD62L) is another proposed eosinophil activation marker [40, 59, 60]. L-selectin is constitutively expressed by blood eosinophils, with level varying about six-fold among subjects (by fluorescence intensity) [112]. It is downregulated in response various mediators (Table 1) (to an 0.2–0.6-fold level, by intensity or CF), through a mechanism involving metalloproteinase-mediated shedding [112–116]. It is also downregulated on BAL eosinophils (to an 0.2-fold level of that on blood eosinophils or from about 70% to 20% positive cells) [116–118] and sputum eosinophils (to an 0.1–0.3-fold level) [119]. However, comparing subjects with severe or mild asthma, or normal subjects, no difference in blood eosinophil L-selectin expression was found [112, 119].

ICAM-1

ICAM-1 (CD54) is not expressed or only expressed at a low level on blood eosinophils [60, 118, 120, 121]. ICAM-1 is induced by IL-5 family cytokines and other cytokines, about 3–20-fold (by fluorescence intensity) (Table 1) [114, 121–123]. It is expressed on BAL (1.8-fold of the level on blood eosinophils) [118] and sputum [120] eosinophils. Patients with asthma or airway allergy as a group do not have higher expression than normal subjects [77].

CD44

CD44, a hyaluronan receptor and another suggested activation marker [59, 60, 110], is normally expressed on blood eosinophils [59, 60, 103, 124] (with 40–60% positive cells [106] and level varying about at least four-fold among subjects, by fluorescence intensity [124], more by CF [110]). CD44 is upregulated by IL-5 (1.6-fold and to 60–70% positivity) (Table 1) [103, 106]. Further, it is modestly upregulated on blood eosinophils after segmental lung antigen challenge (less than two-fold by CF) [110] and upregulated on BAL (five-to-six-fold) [110] and sputum (about 1.4-fold by intensity) [124] eosinophils. No differences have been found between CD44 expression on blood eosinophils in patients with asthma as a group and normal subjects [77, 125]. However, interestingly, Sano and colleagues found that the level of blood eosinophil CD44 expression was higher in patients with well-controlled than poorly controlled asthma (about 1.7-fold) and suggested that this implies that the transmigration of activated, CD44-high eosinophils from the circulation is facilitated [124]. Extravasation of eosinophils with the highest levels of CD44 is compatible with a contribution for CD44 to eosinophil recruitment to the airway in a mouse asthma model [126–128]. Further, CD44 becomes redistributed on blood eosinophils within minutes

after addition of IL-5, GM-CSF, IL-3, or eotaxin-1, when the cell undergoes shape change and polarization, concentrating at and covering one pole of the cell, the nucleopod, which constitutes a specialized uropod occupied by the nucleus; such reorganization of CD44 and other receptors may promote eosinophil arrest, extravasation, and migration [68].

PSGL-1

P-selectin glycoprotein ligand-1 (PSGL-1, CD162), the eosinophil counter-receptor for P-selectin [129], is constitutively expressed at a high level on blood eosinophils [64, 100, 130, 131] (varying about two-fold among subjects, by geometric mean CF) [64, 130]. It is downregulated *in vitro* in response to platelet-activating factor (PAF), presumably by shedding [131]. However, whether such shedding occurs *in vivo* is uncertain. Unlike L-selectin, PSGL-1 is not downregulated on BAL eosinophils [100]. It is modestly upregulated on blood eosinophils 48 h after segmental antigen challenge (1.1-fold by CF); this increase is ablated after anti-IL-5 administration, indicating that IL-5 can be responsible for PSGL-1 upregulation *in vivo* [100]. After whole-lung antigen challenge, which is a more major insult and a model of asthma exacerbation [132, 133], blood eosinophil PSGL-1 is first modestly decreased at 8 h (to about 0.8-fold of the baseline level) followed by a recovery and increase at 48 h to about 1.1-fold above baseline, supporting a scenario in which the cells with the highest PSGL-1 expression extravasate [130]. Like CD44, PSGL-1 on blood eosinophils becomes localized at the nucleopod during IL-5-stimulated cell polarization [68].

Cytokine receptors

Several cytokine receptors, upregulated or downregulated, have been proposed to report eosinophil activation (Table 1) [4, 59, 60, 110]. IL-2 receptor (IL-2R, CD25) expression on blood eosinophils (varying from 3% to about 60% positive cells [134, 135]) is upregulated by GM-CSF [134, 135] but downregulated by IFN-γ [134]. IL-2R is upregulated on BAL eosinophils (about 1.4-fold by median channel fluorescence) [110], but not increased on blood eosinophils after segmental antigen challenge [110]. It was found not be different between asthma and allergy, and normal subjects [77].

IL-5Rα (CD125) on blood eosinophils is downregulated *in vitro* by its own ligand IL-5 as well as by the related cytokines GM-CSF and IL-3 (to about 0.1–0.3-fold level, by intensity or CF, or from about 80–90% to 10% positive cells) [107, 108, 136, 137], through the involvement of metalloproteinase-mediated shedding [107]. Further, it is downregulated on BAL eosinophils (to 0.4-fold of the blood eosinophil level and to 10% positivity) [137]. Blood eosinophil IL-5Rα expression has been reported to be increased after administration of anti-IL-5 mepolizumab [138], supporting the scenario that IL-5 regulates the expression of its own receptor *in vivo*.

In contrast to IL-5Rα, IL-3Rα (CD123), expressed on blood eosinophils at a level varying about four-fold among subjects (by CF) [110], is upregulated by its own ligand and the other IL-5 family cytokines (about three- to ten-fold by fluorescence intensity) [108, 136]. It is modestly upregulated on blood eosinophils after segmental antigen challenge (about 1.2-fold) and more highly upregulated on BAL eosinophils (about 2.3-fold) [110].

IL-13Rα1 (CD213a1) on blood eosinophils is upregulated by various cytokines (up to about 2.5-fold) (Table 1) but downregulated by its own ligand IL-13 and the related cytokine IL-4 (to about a 0.5-fold level) [139].

Finally, expression of the subunits of the IL-25 receptor, IL-17RA and IL-17RB (varying among subjects from about 0% to 100% positive blood eosinophils) was recently found to be elevated in patients with mild allergic asthma compared to atopic non-asthmatic patients and normal subjects (median about 100% and 50% positive for IL-17RA and IL7RB, respectively, in asthma versus about 70% and 20% in the other groups) [140].

To summarize, various cytokine receptors are up- or down-regulated on blood eosinophils in response to their own ligands or other cytokines *in vitro*, have altered expression levels on BAL eosinophils, and may have moderately altered expression on blood eosinophils after antigen challenge or when comparing subjects with asthma to normal subjects.

Fc receptors

Various Fc receptors (i.e., receptors for immunoglobulins), their induction, upregulation, or activation, have been suggested to report eosinophil activation (Table 1) [4, 40, 58, 60, 61]. Expression of FcαRI (CD89), an immunoglobulin (Ig) A receptor, on blood eosinophils is higher in subjects with asthma and/or allergic rhinitis than in normal subjects (about two-fold, by fluorescence intensity) [141]. FcεRII (CD23), an IgE receptor, on blood eosinophils (varying from 0% to about 50% among subjects and three-fold, by intensity) is upregulated by IL-5 family cytokines (1.1–1.3-fold) [65], but was found not to be different between subjects with asthma or airway allergy compared to normal subjects [77]. The IgG receptor FcγRI (CD64) is induced on blood eosinophils by IFN-γ (about eight-fold) [142]. FcγRII (CD32), another IgG receptor, on blood eosinophils is upregulated by IFN-γ or IL-3 (1.7-fold by intensity or from 15–59% to 32–72% positive cells) [142, 143], but is not different in subjects with asthma and/or allergic rhinitis from normals [141].

FcγRIII (CD16), a third IgG receptor, is not expressed or expressed at a low level on unstimulated blood eosinophils [141, 142, 144, 145] but is induced by various chemoattractants and other mediators (from 0% up to about 30% positive cells) (Table 1) [142, 144]. CD16 expression of blood eosinophils is increased after whole-lung antigen challenge [145]. Monteiro and colleagues as well as Davoine and others found it to be higher in allergic asthma and/or allergic rhinitis than in normal subjects (ranging about 0–30% in allergic asthma and 0–10% in normals) [141, 145], whereas Johnsson et al. found no difference between asthma or airway allergy as a group and normals [77].

Interestingly, two activation-sensitive mAbs, A17 and A27, have been developed [146] that recognize an activated form of FcγRII (CD32) [40, 112]. Using these mAbs, the signal of unstimulated blood eosinophils varied at least 20-fold among subjects [112, 146], and CD32 was found to become more activated in response to IL-5, GM-CSF, or fMLF *in vitro* (up to about tenfold, by intensity) [112, 146]. CD32 on eosinophils in blood from patients with mild asthma is more activated than on cells from normal subjects (two-four-fold) [112, 146]. Blood eosinophil CD32 becomes more activated after whole-lung challenge in patients with

a dual-response asthma phenotype (up to 1.8-fold) [112, 146]. Finally, CD32 is more activated on BAL eosinophils than on blood eosinophils (about three-fold) [146].

Taken together, Fc receptors are induced, upregulated, or, at least in the case of CD32, activated by cytokines or chemoattractants and may be upregulated or activated by antigen challenge, in subjects with asthma, or on BAL eosinophils.

Integrins

Integrins are heterodimers of α and β subunits [45, 46]. Eosinophils express seven integrins, $\alpha_4\beta_1$ (CD49d/CD29), $\alpha_6\beta_1$ (CD49f/CD29), $\alpha_L\beta_2$ (CD11a/CD18), $\alpha_M\beta_2$ (CD11b/CD18), $\alpha_X\beta_2$ (CD11c/CD18), $\alpha_D\beta_2$, and $\alpha_4\beta_7$ [5, 12, 13, 41, 42, 55, 128], which potentially interact with ligands including vascular cell adhesion molecule (VCAM)-1, ICAM-1, laminin, fibrinogen/fibrin, vitronectin, and periostin, on other cells or in the extracellular matrix (ECM) [41, 42, 67]. The platelet integrin $\alpha_{IIb}\beta_3$ (CD41/CD61) [147] is not synthesized by eosinophils [110] but can be detected by flow cytometry or immunofluorescence microscopy staining on a variable proportion of eosinophils (in humans and mice), due to association of platelets or platelet fragments with some of the cells [64, 130, 148–152]; activated platelets are known to bind leukocytes via P-selectin [153]. Some workers have detected low levels of additional integrins, including $\alpha_2\beta_1$ (CD49b/CD29), which is also not synthesized by eosinophils [110], on the eosinophil surface [154–156]; this is similarly likely due to platelet "satellitism". It has been reported that (human and mouse) eosinophils with platelet satellitism also can have increased levels of endogenous eosinophil integrins, including α_4, α_M, and β_2 (Table 1) [149–151]. Similarly, eosinophils with high level of surface-associated P-selectin (presumably primarily derived from activated platelets, most of these cells also have platelet satellitism [64]) have increased reactivity with mAb N29, demonstrating that they have a higher number of their β_1 integrins in the intermediate-activity conformation [64] (see Table 1 and more below).

Studies with β_2 integrin-deficient and conditionally α_4 integrin-deficient mice, or with mAbs in wild-type mice, indicate that both α_4 and β_2 integrins mediate eosinophil recruitment to the airway [53, 54, 157, 158]. Such data together with results from *in vitro* adhesion experiments on human cells [41, 42, 66, 67, 159, 160] indicate that $\alpha_4\beta_1$ and $\alpha_M\beta_2$ are the principal integrins mediating eosinophil adhesion, with $\alpha_4\beta_1$ largely responsible for arrest of blood eosinophils on VCAM-1 on activated endothelium in vessels of the asthmatic lung, with a more minor contribution by $\alpha_M\beta$; whereas activated $\alpha_M\beta_2$, by interacting with periostin and possibly other ligands, is involved in subsequent eosinophil movement to and persistence in the ECM of the bronchi in asthma [41] (see Fig. 1 for model).

The expression level of several integrins has been proposed to report eosinophil activation (Table 1) [4, 40, 59, 60]. α_L expression on blood eosinophils (varying about 20-fold, by CF), was found to be higher (about five-fold) in patients with asthma than in normal subjects [161]. It was modestly upregulated on blood eosinophils after segmental antigen challenge (1.2-fold by CF); this increase was abolished by anti-IL-5, indicating that IL-5 upregulates $\alpha_L\beta_2$ *in vivo* [100]. α_M level (varying up to about 20-fold among subjects [112]) is upregulated by the IL-5 family cytokines or various other cytokines, chemoattractants and mediators (up to about fourfold by fluorescence intensity) (Table 1) [68, 101, 112, 113, 123,

160, 162–169]. It was reported to be higher (about 1.5-fold) during the allergy season [170]. Like α_L, α_M is modestly increased (1.2-fold by CF) on blood eosinophils after segmental antigen challenge in a manner prevented by anti-IL-5 [100]. It is upregulated to a greater degree (1.5–1.8-fold by CF or about three-fold by intensity) on BAL [63, 100, 117, 118, 164, 171] and sputum (up to 18-fold) [119, 120] eosinophils. However, blood eosinophil α_M level is not significantly different among patients with mild and severe asthma, and normal subjects [77, 112, 119, 125, 161]. α_X is increased (1.8-fold by intensity) on BAL [118] and (about three-fold) on sputum [120] eosinophils but was found not to be different between subjects with asthma or airway allergy and normal subjects [77]. α_D is expressed at a low level on eosinophils in blood [100] and is induced (about four-fold by intensity) on blood eosinophils in response to IL-5 [172], as well as is induced (12-fold by CF, two-fold by intensity) on BAL eosinophils [63, 66, 100, 172]. β_2 is upregulated by the IL-5 family cytokines and other factors (about 1.5-fold by intensity) (Table 1) [68, 114, 123, 163]. Like α_L and α_M, it is modestly upregulated after segmental antigen challenge and upregulated to a somewhat greater degree on BAL eosinophils (1.5-fold) [63, 100]. Blood eosinophil β_2 expression is not different between subjects with asthma or allergy and normals [77].

Whether an integrin mediates adhesion to and migration on a particular ligand depends on the activation state of the integrin [50, 173–175]; this is at least as important as the expression level. Integrins exist in three major conformations, one inactive, one intermediate-activity, and one high-activity conformation [47, 48, 50–52]. The activation states of integrins can be monitored by conformation-specific monoclonal antibodies (mAbs) [41, 176, 177]. Integrins are activated by so-called “inside-out” signaling triggered through other receptors, including G-protein coupled receptors (GPCRs), and mediated by proteins including talin and kindlins that bind the cytoplasmic tail of integrin β subunits [47, 48, 50, 178, 179].

Eosinophils in blood, on the average, express the epitopes for mAbs N29 and 8E3 [63, 64, 130, 180], which recognize intermediate- and high-activity β_1 [41, 177, 181–184], but have no or very low expression of epitopes for mAbs HUTS-21 and 9EG7, which recognize only high-activity β_1 [41, 177, 185–187], indicating that their β_1 integrins, including $\alpha_4\beta_1$, are in the intermediate conformation. However, N29 and 8E3 reactivities are variable among subjects, ranging from subjects with no signal and thus inactive β_1 integrins over some with low but detectable N29 signal (i.e, a number or a fraction of β_1 integrin molecules on each cell in the intermediate-activity state) to some with high N29 signal (i.e., presumably most molecules on each cell having the intermediate conformation), with N29 varying from a geometric mean CF of 0 to about 700 and positivity up to about 80%) [41, 63, 64, 130], presumably conferring greatly variable capacity to arrest on VCAM-1 on activated endothelium [41] (Fig. 1). N29 reactivity of eosinophils in whole blood can be increased by P-selectin, but not by IL-5, *in vitro* (up to about 1.5-fold by CF, to a greater degree in normal than in subjects with asthma and/or allergy) [64]. The N29 signal correlates with eosinophil-bound [64, 130] or platelet-surface P-selectin [130] *in vivo*. Thus, *in vivo* it is most likely the P-selectin on the surface of activated platelets that is responsible for inducing the intermediate-activity conformation of β_1 integrins on blood eosinophils [41, 130], even though a proportion of soluble plasma P-selectin in asthma appears to be derived from

activated endothelial cells [188]. The results on platelet P-selectin are compatible with data showing that activated platelets promote eosinophil recruitment to the airway in mice in a P-selectin-dependent manner [150, 152] and an *in vitro* study that observed increased complex formation between activated P-selectin-bearing platelets and human blood eosinophils from subjects with allergic asthma and indicated that platelet association contributes to the enhanced tethering of such eosinophils to activated endothelium in a P-selectin-dependent manner [189]. As a group, patients with asthma or non-severe asthma, but not severe asthma, have a higher N29 reactivity than normal subjects (2.2-fold for non-severe asthma) [130]. In dual responders, the N29 signal is increased 48 h after segmental antigen challenge (about 1.6-fold) [63]. After whole-lung antigen challenge, N29 reactivity decreases at 8 h (to about an 0.5-fold level) and recovers at 48 h [130], indicating that eosinophils with the highest proportion of activated β_1 integrins are the ones that extravasate. We have suggested that a similar phenomenon, i.e., that the eosinophils with the most activated $\alpha_4\beta_1$ are efficiently removed from the circulation, occurs continuously in severe asthma [130] (Fig. 1). One possible reason for such efficient extravasation may be the greater lung endothelial VCAM-1 expression in severe asthma, as observed in bronchial biopsies [190]. *In vitro*, the proportion of eosinophils that do not attach to VCAM-1 have lower N29 signal [64], also supporting the idea that the cells with the most activated $\alpha_4\beta_1$ are the ones that preferentially adhere. BAL eosinophils have β_1 integrins in the high-activity conformation, judged by their reactivity with mAbs HUTS-21 and 9EG7 [100].

Eosinophils in blood have a low but detectable reactivity with mAb KIM-127 [100], which recognizes intermediate- and high-activity β_2 integrins [41, 47, 177, 191, 192], but very low reactivity with mAb24 [63], which recognizes only high-activity β_2 integrins [41, 47, 177, 193–195]. The KIM-127 signal is decreased after anti-IL-5 administration [100]. In addition, blood eosinophils have no or very low reactivity with activation-sensitive anti-α_M mAb CBRM1/5 [66, 100], which reports the high-activity state of $\alpha_M\beta_2$ integrin [41, 177, 196]. Together, these data indicate that blood eosinophils have a fraction of their β_2 integrins, including $\alpha_M\beta_2$, in the intermediate-activity conformation [41] (Fig. 1), as a result of *in vivo* exposure to either the low concentrations of IL-5 present in blood [75–79] or higher concentrations in, e.g., the bone marrow [7, 84]. *In vitro*, high (ng/ml) doses of IL-5, but not P-selectin, induces mAb24 (1.6-fold by CF, four-fold by intensity) [64, 68] or CBRM1/5 (up to about three-fold by intensity) [66, 68, 160] reactivity of blood eosinophils. In addition, CBRM1/5 reactivity is induced by various, but not all, chemoattractants, e.g., C5a and others but not eotaxin-1 or IL-8 [197, 198]. BAL eosinophils recognize mAb24 [63, 100] and CBRM1/5 [66, 100] and thus display high-activity $\alpha_M\beta_2$. As with CD44 and PSGL-1, activated $\alpha_M\beta_2$ becomes localized to the nucleopod in IL-5-polarized blood eosinophils [68].

In summary, α_M, α_D, and β_2, are upregulated by IL-5 *in vitro* and α_M and β_2 also by other mediators; α_L, α_M, and β_2 are modestly upregulated after segmental antigen challenge in an apparently IL-5-dependent manner; and α_M, α_X, α_D, and β_2 are upregulated on BAL eosinophils. β_1 and β_2 integrins are in the intermediate-activity state on blood eosinophils to varying degree among subjects, β_1 likely as a result of interaction with P-selectin, primarily on activated platelets, and β_2 as a result of low levels of IL-5 (Fig. 1). The high-activity state

of $\alpha_M\beta_2$ can be induced *in vitro* by IL-5 or some chemoattractants. β_1 and $\alpha_M\beta_2$ are in high-activity states on BAL eosinophils.

Others

Various other cell-surface proteins are potential eosinophil activation markers (Table 1) [4, 40, 60, 110]. These include CD9, whose expression on blood eosinophils from subjects with allergic rhinitis and occasional asthma was found to be higher during a high-pollen load season (1.2-fold by fluorescence intensity) [170] but was in another study not different between subjects with asthma or airway allergy and normal subjects [77]. CD45RO expression on blood eosinophils is higher in patients with mild-moderate asthma than in normals (about 65% positive cells versus 5%) [125]. CD48 on blood eosinophils is upregulated by IL-3 (about two-fold by intensity), but not by IL-5 or GM-CSF, and is higher in patients with asthma (two-fold) than in normal subjects [199]. CD66e is modestly upregulated (about 1.3-fold by CF) on blood eosinophils after segmental antigen challenge and more highly upregulated (three-fold) on BAL eosinophils [110]. Galectin-3 expression on blood eosinophils is higher in allergic than in normal subjects (about 12% versus 4% positive cells) [200], compatible with the ability of galectin-3 to interact with $\alpha_4\beta_1$ integrin and contribute to eosinophil rolling and arrest on VCAM-1 or activated endothelium *in vitro* [200] and to eosinophil recruitment to the airway *in vivo* [158, 201, 202]. Neuropeptide S receptor expression is higher in patients with severe asthma than in patients with mild asthma or normal subjects (two-to-three-fold by intensity) [203].

Some final remarks on activation markers

As described above, multiple eosinophil-surface proteins are potential markers of or have been proposed to report cell activation, some of which are altered in asthma, upon antigen challenge, or on BAL or sputum eosinophils (Table 1). There are various aspects or patterns of eosinophil activation *in vivo* and *in vitro*. Many proteins are upregulated on airway eosinophils, while a few, like L-selectin or IL-5Rα, are downregulated. One group of proteins, including α_L, α_M, and β_2, integrins, PSGL-1, CD44, CD66e, and IL-3Rα, are modestly upregulated on blood eosinophils after segmental antigen challenge [100, 110], at least the first four of these in an apparently IL-5-dependent fashion [100], and more highly upregulated on BAL eosinophils [100, 110]. Many but not all proteins mentioned are upregulated or activated *in vitro* by IL-5 family cytokines or chemoattractants. For instance, $\alpha_M\beta_2$ integrin is upregulated or activated by IL-5 but not P-selectin; whereas β_1 integrins are activated by P-selectin and not IL-5 [41, 64]. IL-13Rα1 is upregulated by various cytokines including IL-5 or GM-CSF (Table 1) but downregulated by IL-4 or IL-13 [139]. IL-2Rα is upregulated by GM-CSF but downregulated by IFN-γ [134]. Further, there are different time frames of activation *in vitro*, ranging from minutes over hours to days. Activation of integrins or FcγRII occurs within minutes to an hour [64, 66, 68, 112, 146, 160, 197, 198]. One group, including α_M integrin, CD16, and CD63, are rapidly mobilized to the cell surface [40, 144], presumably due to transport of preformed proteins from granules [204]. Another group, including CD48, α_M integrin, aminopeptidase N (CD13), ICAM-1, and semaphorin 7A, appear to be upregulated to a greater degree in response to IL-3 than to other cytokines over an approximately 16–24 h period [101, 123, 136, 199]. In summary, the

potential activation markers reflect exposure to partly similar and partly different stimuli and time frames.

Associations with features of asthma

Although many surface proteins have been proposed as eosinophil activation markers or biomarkers in asthma, for only a few of those described above have their expression level or activation state been shown to correlate with features of asthma (Table 2). For these studies, unfractionated, whole blood has been used for flow cytometry as described above [63, 100, 112, 119, 130, 146, 180].

Activated FcγRII (CD32) on blood eosinophils has been reported to correlate with fraction of exhaled nitric oxide (FENO), an indicator of airway inflammation, in asthma (Table 2) [40].

The expression level of α_M integrin has been shown to correlate with airway hyperresponsiveness in moderate to severe asthma [119] or in patients with a dual-response asthma phenotype [112] (Table 2).

Variation in integrin activation states is likely more important than variation in expression levels. N29 reactivity of blood eosinophils, indicative of the intermediate-activity state of β_1 integrins, correlated inversely with forced expiratory volume in 1 s (FEV_1, as percentage of baseline) after inhaled corticosteroid (ICS) withdrawal or across all visits during a double-blind placebo-controlled, two-period crossover study in patients with mild asthma (Table 2) [180]. Receiver-operator characteristic (ROC) curve analysis demonstrated that the N29 signal predicted decreased FEV_1 and performed better than did the established asthma markers sputum eosinophil percentage or FENO [180]. Further, N29 correlated with FENO after ICS withdrawal [180]. In another study, blood eosinophil N29 reactivity 48 h after segmental antigen challenge in subjects with mild allergic asthma correlated with the late-phase fall in FEV_1 3–8 h after the whole-lung antigen challenge performed during screening [63]. The ICS withdrawal and antigen challenge studies were on subjects with non-severe asthma who were young (mean 21 years) [63, 180]. In an observational study that was part of the Severe Asthma Research Program (SARP) [205] on a population with asthma of varying severity and a higher mean age, N29 reactivity correlated with FEV_1/FVC (FVC = forced vital capacity) in young subjects (under 30 years old) with non-severe asthma but did not correlate in severe asthma [130]. The subjects in that study belonged to a population that had been classified using cluster analysis [22, 206]. N29 correlated best and significantly with FEV_1/FVC in cluster 1 [207], which consists of subjects with mild allergic asthma [22, 205, 206]. Blood eosinophil reactivity with mAb KIM-127, indicative of the intermediate-activity state of β_2 integrins, at baseline (the time of segmental antigen challenge in subjects with mild allergic asthma) correlated with BAL eosinophil percentage 48 h later (Table 2) [100]. Unfortunately, KIM-127 was not assayed in the earlier studies.

A possible explanation for the lack of correlation between the N29 signal and lung function in severe asthma and older patients is high degree of ongoing extravasation of eosinophils with the most activated integrins, as discussed above. An additional possible explanation is that a subpopulation of subjects with asthma, likely particularly patients with severe asthma

or older patients, do not have a persistent predominantly eosinophilic airway inflammatory phenotype but rather an intermittent or persistent mixed eosinophilic-neutrophilic, neutrophilic, or non-eosinophilic paucigranulocytic phenotype [18, 21, 23–27, 29–32], which may contribute to the weakening of the association between eosinophil activation and lung function. Future studies would be needed to address the question whether activation of, e.g, neutrophil integrins is associated with such less eosinophilic phenotypes.

Conclusions

This review has described multiple eosinophil-surface proteins that have been proposed to report or potentially report cell activation. Although many of these are upregulated or otherwise altered in response to cytokines, chemoattractants, or other stimuli *in vitro*, or after antigen challenge, in asthma, or on airway eosinophils *in vivo*, for only a few of those suggested have associations been found between their level or activation state and features of asthma.

These include greater degree of the intermediate-activity state of β_1 integrins on blood eosinophils, reported by mAb N29, which is associated with decreased pulmonary function, late-phase response, and airway inflammation in subjects with non-severe asthma but not in severe asthma. In addition, intermediate β_2 integrin activation, assessed by mAb KIM-127, is associated with airway eosinophilia in non-severe asthma.

The results from these studies indicate that the activation state of blood eosinophils varies among subjects, with normal subjects and some subjects with asthma having non-activated β_1 integrins or only a small number of their β_1 integrins in the intermediate-activity state, over subjects with non-severe asthma with a variable number of their β_1 and β_2 integrins in the intermediate-activity or partly activated state, to subjects with severe asthma that have a lower degree of intermediate-activity β_1 integrins, presumably because the most activated cells have marginated on activated endothelium or extravasated [41, 130] (Fig. 1). Together with other data mentioned above, this indicates that in severe or uncontrolled asthma, the circulation becomes depleted of eosinophils that have the greatest degree of integrin activation, and possibly the highest levels of PSGL-1 and CD44. A similar model of FcγRII activation on circulating eosinophils was recently presented, in which activation first increases with increasing degree of systemic inflammation and then decreases at the highest level of systemic inflammation [40]. Fully activated eosinophils, characterized by integrins in the high-activity state and highly upregulated $\alpha_M\beta_2$ integrin and other proteins, as is the case on airway eosinophils (Table 1), are not or seldom seen in a blood sample, possibly because such cells may transiently be present immediately before arrest, or may only occur on arrested eosinophils before extravasation, or may occur only in the tissue and not in the circulation (Fig. 1).

The classical paradigm for leukocyte extravasation [208], which has been applied to eosinophils [43], depict circulating cells as having inactive integrins that become activated when rolling cells are exposed to chemokines associated with the surface of activated endothelium. This paradigm now needs to be modified to include *in vivo* pre-activation or “priming” [40, 91, 92], mediated by P-selectin (primarily on activated platelets) and IL-5,

causing eosinophils to display integrins in partially activated conformations [41] (Fig. 1). The modified paradigm is in accord with other recent evidence that subsets of leukocytes have a fraction of their integrins in an intermediate-activity state [209].

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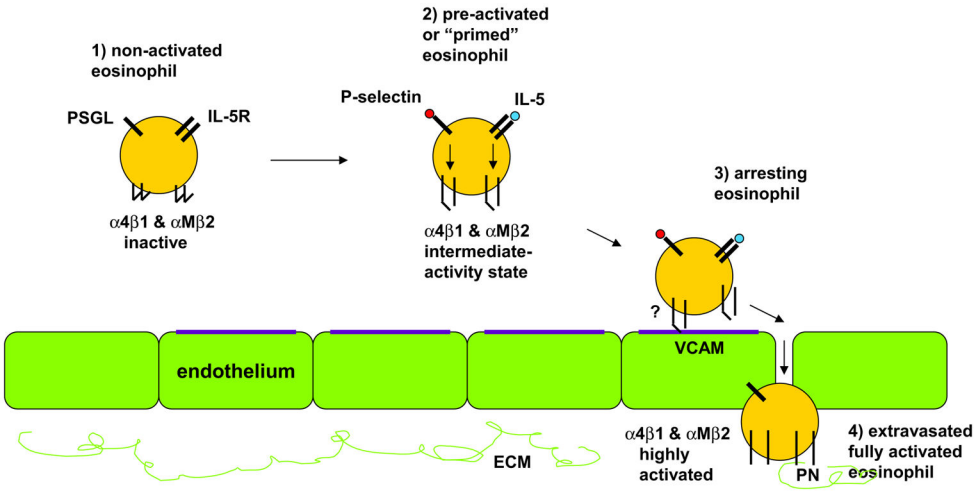

Fig. 1. Model of activation states of eosinophils in circulation and during arrest and extravasation in asthma

1. Circulating non-activated eosinophil with $\alpha_4\beta_1$ and $\alpha_M\beta_2$ in the inactive integrin conformation, as found in normal subjects, some subjects with non-severe asthma, or in severe asthma; sampled in severe asthma due to great extravasation of activated cells.
2. Pre-activated or "primed" circulating eosinophil with (variable numbers of) $\alpha_4\beta_1$ and $\alpha_M\beta_2$ in the intermediate-activity integrin conformation, as a result of P-selectin- and IL-5-triggered signaling, respectively, as found to varying degree primarily in some subjects with non-severe asthma (*in vivo* it is most likely P-selectin on the surface of activated platelets that is responsible for this activation of $\alpha_4\beta_1$).
3. Eosinophil arresting on activated endothelium in asthma with $\alpha_4\beta_1$ and $\alpha_M\beta_2$ in unknown state, likely in the intermediate-activity integrin conformation, with $\alpha_4\beta_1$ primarily mediating arrest on VCAM-1 with a possible minor contribution of $\alpha_M\beta_2$.
4. Extravasated or tissue eosinophil in asthma with $\alpha_4\beta_1$ and $\alpha_M\beta_2$ in the high-activity integrin conformation (and with down-regulated IL-5 receptor), and $\alpha_M\beta_2$ interacting with periostin in the extracellular matrix.

Please see text for references.
ECM, extracellular matrix; IL-5, interleukin-5; IL-5R, interleukin-5 receptor; PN, periostin; PSGL, P-selectin glycoprotein-1; VCAM, vascular cell adhesion molecule-1.

Table 1

Eosinophil-surface proteins potentially reporting cell activation.

Protein	Obervation	Reference
CD4	Upregulated by GM-CSF or IL-3	[135]
CD9	Upregulated during allergy season	[170]
CD35 (CR1)	Upregulated by fMLF, downregulated in BAL	[171, 210]
CD44	Upregulated by IL-5, after segmental antigen challenge, in BAL, or in sputum, higher in well-controlled than poorly controlled asthma	[103, 106, 110, 124]
CD45RO	Upregulated in mild-moderate asthma	[125]
CD48	Upregulated by IL-3 or in asthma	[199]
CD58	Upregulated in BAL	[118]
CD63 (LAMP-3)	Upregulated by IFN-γ or in BAL	[118, 211]
CD66e (CEACAM5)	Upregulated after segmental antigen challenge or in BAL	[110]
CD67	Upregulated by PAF+fMLF or in BAL	[116, 118]
CD69	Upregulated by IL-5, GM-CSF, IL-3, IFN-γ, IL-4, IL-13, TNF-α, or IL-17; after whole-lung antigen challenge; or in BAL	[65, 101–111]
CD81	Upregulated by IL-5, GM-CSF or IL-3	[65]
α_L integrin (CD11a)	Upregulated in asthma or after segmental antigen challenge, decreased by anti-IL-5 after segmental antigen challenge	[100, 161]
α_M integrin (CD11b)	Upregulated by IL-3, GM-CSF, IL-5, eotaxin-1, fMLF, PAF, RANTES, C5a, TNF-α, IL-33, or ATP; during allergy season; after segmental antigen challenge; on cells with platelet satellitism after whole-lung antigen challenge; in BAL; or in sputum; decreased by anti-IL-5 after segmental antigen challenge	[63, 68, 100, 101, 112, 113, 117–120, 123, 151, 160, 162–171]
α_X integrin (CD11c)	Upregulated in BAL or in sputum	[118, 120]
α_D integrin	Upregulated by IL-5 or in BAL	[63, 66, 100, 172]
β_2 integrin (CD18)	Upregulated by IL-5, GM-CSF, IL-3, eotaxin-1 or TSLP; after segmental antigen challenge; on cells with platelet satellitism in AERD; in BAL; decreased by anti-IL-5 after segmental antigen challenge	[63, 68, 100, 114, 123, 149, 163]
Aminopeptidase N (CD13)	Upregulated by IL-3, IL-5, or GM-CSF, or in BAL	[212]
FcαRI (CD89)	Upregulated in asthma or allergy	[141]
FcεRII (CD23)	Upregulated by IL-5, GM-CSF or IL-3	[65]
FcγRI (CD64)	Upregulated by IFN-γ	[142]
FcγRII (CD32)	Upregulated by IFN-γ or IL-3	[142, 143]
FcγRIII (CD16)	Upregulated by C5a, fMLF, PAF, or IFN-γ; in allergy or allergic asthma; or after whole-lung antigen challenge	[141, 142, 144, 145]
Galectin-3	Upregulated in allergy	[200]
HLA-DR	Upregulated by IFN-γ, GM-CSF, or IL-4; in BAL; or in sputum	[118, 120, 122, 171, 213]
ICAM-1 (CD54)	Upregulated by IL-3, GM-CSF, IL-5, IFN-γ, or TSLP; in BAL; or in sputum	[114, 118, 120–123]

Protein	Obervation	Reference
IL-2Rα (CD25)	Upregulated by GM-CSF or IL-3, or in BAL, downregulated by IFN-γ	[110, 134, 135]
IL-3Rα (CD123)	Upregulated by IL-3, IL-5, or GM-CSF, after segmental antigen challenge, or in BAL	[108, 110, 136]
IL-5Rα (CD125)	Downregulated by IL-5, GM-CSF, or IL-3, or in BAL; increased by anti-IL-5	[107, 108, 136–138]
IL-13Rα1 (CD213a1)	Upregulated by TGF-β, IFN-γ, TNF-α, IL-5, or GM-CSF; downregulated by IL-13 or IL-4	[139]
IL-17RA (subunit of IL-25R)	Upregulated in mild allergic asthma	[140]
IL-17RB (subunit of IL-25R)	Upregulated in mild allergic asthma	[140]
L-selectin (CD62L)	Downregulated by IL-5, PAF, fMLF, or TSLP; in BAL; or in sputum	[112–119]
Neuropeptide S receptor	Upregulated in severe asthma	[203]
PSGL-1 (CD162)	Downregulated by PAF, upregulated after segmental antigen challenge, decreased transiently after whole-lung antigen challenge, decreased by anti-IL-5 after segmental antigen challenge	[100, 130, 131]
Semaphorin 7A (CD108)	Upregulated by IL-3, GM-SCF, or IL-5; or in BAL	[136]
TSLP receptor	Upregulated by TNF-α and IL-3	[214]
Activated α_M integrin (CD11b)	High-activity state induced by IL-5, RANTES, MCP-3, or C5a; or in BAL	[66, 68, 100, 160, 197, 198]
Activated β_1 integrin (CD29)	Intermediate-activity state induced by P-selectin, in non-severe but not severe asthma, or after segmental antigen challenge in dual responders; correlates with eosinophil-bound or platelet-surface P-selectin; increased on cells with high level of surface-associated P-selectin; decreased on cells non-adherent to VCAM-1 or transiently after whole-lung antigen challenge	[63, 64, 130]
	High-activity state in BAL	[100]
Activated β_2 integrin (CD18)	Intermediate-activity state decreased by anti-IL-5	[100]
	High-activity state induced by IL-5 or in BAL	[63, 64, 68, 100]
Activated FcγRII (CD32)	Activated state induced by IL-5, GM-CSF, or fMLF; in mild asthma; after whole-lung antigen challenge in dual responders; or in BAL	[112, 146]

Observations refer to cell-surface expression level, usually determined by flow cytometry, and are, if not indicated otherwise, on blood eosinophils.

AERD, aspirin-exacerbated respiratory disease; ATP, adenosine triphosphate; BAL, bronchoalveolar lavage; C, complement (factor); CEACAM, carcinoembryonic antigen-related cell adhesion molecule; Fc, fragment, crystallizable (of immunoglobulin); fMLF, formyl-methionyl-leucyl-phenylalanine; GM-CSF, granulocyte macrophage-colony stimulating factor; HLA, human leukocyte antigen; ICAM, intercellular adhesion molecule; IFN, interferon; IL, interleukin; LAMP, lysosomal-associated membrane protein; MCP, monocyte chemotactic protein; PAF, platelet activating factor; PSGL, P-selectin glycoprotein ligand; R, receptor; RANTES, regulated on activation, normal T cell expressed and secreted; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin; VCAM, vascular cell adhesion molecule.

Table 2

Correlations between activation states of blood eosinophils and features of asthma.

Protein/Activation state	Correlation	Reference
Intermediate-activity β_1 integrin	Inverse with FEV_1 after or during ICS withdrawal in mild asthma, predicts decreased FEV_1 in ROC curve analysis	[180]
	FENO after ICS withdrawal in mild asthma	[180]
	Inverse with FEV_1/FVC in younger subjects with non-severe asthma	[130]
	Inverse with FEV_1/FVC in phenotype cluster 1 (mild atopic asthma)	[207]
	48 h after segmental antigen challenge, correlates with late-phase FEV_1 fall after whole-lung antigen challenge in mild allergic asthma	[63]
Intermediate-activity β_2 integrin	Percentage BAL eosinophils in mild allergic asthma	[100]
α_M integrin level	Inverse with PC_{20}	[112, 119]
Activated FcγRII	FENO	[40]

BAL, bronchoalveolar lavage; FENO, fraction of exhaled nitric oxide; FEV_1, forced expiratory volume in 1 s: FVC, forced vital capacity; ICS, inhaled corticosteroid; PC_{20}, provocative concentration of methacholine or histamine producing a 20% fall in FEV_1; R, receptor; ROC, receiver-operator characteristic.

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