Inflammation comprises the immune response to infection or injury and is characterized by activation of a multistep cascade leading to the accumulation of leukocytes in involved tissues (Medzhitov, 2008). In response to a range of insults, vertebrates have evolved a “modular” immune system whereby distinct inflammatory programs are engaged depending on the nature of the perturbation. Although the cellular constituents of these inflammatory modules are largely defined, a detailed understanding of how specific modules are engaged and reinforced is lacking. Clarifying these checkpoints will enhance our understanding of immune responses in host defense and injury and across the spectrum of chronic inflammatory diseases.

Allergic inflammation represents such a module, with signature features of antigen-specific IgE and tissue eosinophilia, although the cellular and molecular circuitry coupling these responses remains unclear. Here, we use genetic and imaging approaches in models of IgE-dependent eosinophilic dermatitis to demonstrate a requisite role for basophils. After antigenic inflammation, basophils initiate transmigration like other granulocytes but, upon activation via their high-affinity IgE receptor, alter their migratory kinetics to persist at the endothelium. Prolonged basophil–endothelial interactions, in part dependent on activation of focal adhesion kinases, promote delivery of basophil-derived IL-4 to the endothelium and subsequent induction of endothelial vascular cell adhesion molecule-1 (VCAM-1), which is required for eosinophil accumulation. Thus, basophils are gatekeepers that link adaptive immunity with innate effector programs by altering access to tissue sites by activation-induced interactions with the endothelium.

IgE-activated basophils regulate eosinophil tissue entry by modulating endothelial function

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Vertebrate immunity has evolved a modular architecture in response to perturbations. Allergic inflammation represents such a module, with signature features of antigen-specific IgE and tissue eosinophilia, although the cellular and molecular circuitry coupling these responses remains unclear. Here, we use genetic and imaging approaches in models of IgE-dependent eosinophilic dermatitis to demonstrate a requisite role for basophils. After antigenic inflammation, basophils initiate transmigration like other granulocytes but, upon activation via their high-affinity IgE receptor, alter their migratory kinetics to persist at the endothelium. Prolonged basophil–endothelial interactions, in part dependent on activation of focal adhesion kinases, promote delivery of basophil-derived IL-4 to the endothelium and subsequent induction of endothelial vascular cell adhesion molecule-1 (VCAM-1), which is required for eosinophil accumulation. Thus, basophils are gatekeepers that link adaptive immunity with innate effector programs by altering access to tissue sites by activation-induced interactions with the endothelium.
effector T cells in response to allergens that promoted eosinophil ingress into tissues (Liu et al., 2011). However, recent studies in a variety of models suggest an unexpected contribution of circulating basophils to allergic inflammatory responses, including the accumulation of eosinophils in target tissues (Mukai et al., 2005; Ohnmacht et al., 2010; Jin et al., 2012; Matsuoka et al., 2013). How circulating basophils influence localized eosinophil recruitment is unclear, but elucidation of this pathway could uncover new strategies for regulating allergic inflammation.

We used models of IgE-dependent eosinophilic skin inflammation that allowed us to establish the hierarchical relationships between IgE and tissue eosinophilia. Through a combination of genetic and imaging approaches, we define a role for IgE-activated basophils in regulating eosinophil accumulation. Basophils exert this effect through a three-step process. First, injury attracts rare, circulating basophils through up-regulation and activation of local vascular adhesion molecules by a process similar to that for other granulocytes. Second, activation of basophil FceRI by antigen leads to secretion of IL-4, a necessary component of the allergic phenotype. Finally, activated basophils arrest their migration into tissues and engage in prolonged endothelial interactions, thus enabling the development of IL-4–induced endothelial vascular cell adhesion molecule-1 (VCAM-1), which is required for the arrest and recruitment of circulating eosinophils. The establishment of enhanced endothelial interactions induced by FceRI engagement during basophil transendothelial migration into tissues explains how a rare circulating cell can establish portals of entry for eosinophils, thus uniting these canonical adaptive and innate components of allergic immunity.

RESULTS

IgE–basophil interactions act as an inflammatory switch to promote allergic inflammation

We established a model of IgE-dependent eosinophilic skin inflammation by infusing C57BL/6 mice with 2 μg dinitrophenol (DNP)–specific monoclonal IgE. After 24–36 h, by which time free IgE is cleared from the blood (Cheng et al., 2010), mice were challenged with the hapten dinitrofluorobenzene (DNFB), applied topically to the ear in a solution of acetone and dibutylphthalate, and ears were harvested for analysis at designated times. On day 3 after challenge, we observed a sixfold increase in the influx of CD11b+Siglec–F−Gr−1b/−/− eosinophils only in presensitized mice (Fig. 1 A and Fig. S1). Gating of eosinophils by this strategy was further verified by expression of GFP in these cells as assessed using IngR mice, which contain an IRES–GFP cassette immediately 3′ of the native IL-4 gene (Mohrs et al., 2001). Eosinophils, basophils, and mast cells from these mice constitutively express GFP at steady-state (Mohrs et al., 2001). The data also indicated that almost all Siglec–F+/− cells in the skin were eosinophils (Fig. S1).
Basophils express DTA constitutively in mast cells or basophils and delete more than 95% of ear skin mast cells and 99% of circulating basophils, respectively, with no impact on the other cell population (Scholten et al., 2008; Sullivan et al., 2011); neither mouse strain alters numbers of circulating eosinophils (not depicted). After sensitization of the respective mice, eosinophil accumulation was ablated in basophil-deleted but not mast cell–deleted mice (Fig. 1, D and E).

**Basophils are required for eosinophilic inflammation after active sensitization and in a mouse model of eczema**

The DNFB passive sensitization system provides a system to explore the role of basophils in eosinophilic inflammation, but does not test whether basophils regulate eosinophilic inflammation in response to physiological levels of native IgE generated in response to soluble antigen. To this end, we immunized mice and tested the role of basophils in eosinophil infiltration after cutaneous antigen challenge (Fig. 2 A). Groups of basophil-sufficient and –deleter mice were immunized with DNP-conjugated to OVA (DNP-OVA). The production of DNP-specific IgE was confirmed at day 8 after immunization in both strains of mice (not depicted), and at day 10, DNFB was applied to the ears. At 3 d after challenge, the Basoph8 mice immunized with DNP-OVA developed greater eosinophilic inflammation as compared with OVA-immunized controls, and as in the passive sensitization model, basophil-deleter mice had significantly decreased eosinophilic inflammation that was similar to OVA-immunized animals (Fig. 2 A).

To more closely mimic the inflammatory response observed in patients with atopic dermatitis, we examined whether basophils regulate eosinophilic infiltration after mechanical injury. Local mechanical injury in atopic dermatitis leads to the “itch–scratch” cycle in patients with atopic dermatitis and perpetuates disease activity (Boguniewicz and Leung, 2010). To induce mechanical injury, we performed tape stripping on mouse ears followed by epicutaneous application with soluble antigen in IgE-sensitized mice, in a manner akin to published protocols (Oyoshi et al., 2010). After tape stripping and antigen application, the absence of basophils significantly curtailed the magnitude of the subsequent eosinophil infiltrate.

**Figure 2. Basophils regulate eosinophil accumulation after vaccination and in models of human atopic dermatitis.** (A) Basophil-deficient (Basoph8 × DTA) or -sufficient mice (Basoph8) were immunized with either DNP-OVA or OVA in alum and challenged with topical DNFB 10 d later. Eosinophil accumulation was examined on day 3 after challenge. Results were pooled from three separate experiments with at least eight mice per group using littermates. (B) Basophil-sufficient (Basoph8) and –deficient (Basoph8 × DTA) mice were sensitized with anti-TNP IgE through intravenous injection and challenged 1 d later by tape stripping and epicutaneous application of TNP-OVA. Eosinophil accumulation was then examined 3 d after antigen challenge. Results were pooled from two separate experiments with at least six mice per group. (A and B) Horizontal bars denote mean. **P-values were determined using ANOVA:** ***, P < 0.001.”**
To examine which cells in the skin express IL-4 after sensitization and challenge, we used KN2 mice, in which a non-signaling human CD2 (hCD2) allele is targeted to the Il4 start site, thus allowing IL-4–producing cells to be assayed directly from tissue without the need for restimulation (Sullivan et al., 2011). As noted above, this is a null IL-4 allele. On average, nearly 25% of skin basophils from heterozygous KN2 mice expressed the surrogate IL-4 reporter on the cell surface 1 d after challenge (Fig. 3, B and C). Moreover, hCD2 expression required prior sensitization with antigen-specific IgE. The rarity of IL-4–expressing cells precluded an unbiased assessment of IL-4 production, but direct examination of cells that can produce IL-4, including CD4+ T cells, eosinophils, and mast cells, revealed that none of these cell types expressed the reporter (Fig. 2 B). Together, the data from these three different IgE-dependent models demonstrate that cutaneous eosinophil accumulation is controlled by basophils during both passive and active sensitization, as well as in mouse models of human atopic skin disease.

**Basophil-derived IL-4 regulates eosinophil entry into the skin**

Basophils produce large amounts of IL-4 after FcεRI ligation (Sullivan et al., 2011), and overexpression of IL-4 is sufficient to drive eosinophilic skin inflammation (Chan et al., 2001). Although sensitized and DNFB-challenged C57BL/6 IL-4+/− mice developed tissue eosinophilia comparable with wild-type mice, IL-4−/− mice (KN2/KN2) had no eosinophil accumulation (Fig. 3 A). To examine which cells in the skin express IL-4 after sensitization and challenge, we used KN2 mice, in which a non-signaling human CD2 (hCD2) allele is targeted to the Il4 start site, thus allowing IL-4–producing cells to be assayed directly from tissue without the need for restimulation (Sullivan et al., 2011). As noted above, this is a null IL-4 allele. On average, nearly 25% of skin basophils from heterozygous KN2 mice expressed the surrogate IL-4 reporter on the cell surface 1 d after challenge (Fig. 3, B and C). Moreover, hCD2 expression required prior sensitization with antigen-specific IgE. The rarity of IL-4–expressing cells precluded an unbiased assessment of IL-4 production, but direct examination of cells that can produce IL-4, including CD4+ T cells, eosinophils, and mast cells, revealed that none of these cell types expressed the reporter (Fig. 2 B). Together, the data from these three different IgE-dependent models demonstrate that cutaneous eosinophil accumulation is controlled by basophils during both passive and active sensitization, as well as in mouse models of human atopic skin disease.

**Basophil-derived IL-4 is required for eosinophilic inflammation.** (A) IL-4−/− (KN2/KN2) and IL-4+/− mice on a C57BL/6 background were sensitized with anti-DNP IgE and challenged with topical application of DNFB 1 d later. 3 d after challenge, ear skin was assessed for eosinophil (left) and basophil (right) infiltration. The experiment is representative of three separate experiments with four to five mice per challenged group. The two mouse lines were bred independently to produce the indicated genotypes. (B) Basophils from Basoph8 × KN2 reporter mice were sensitized and challenged as in A. 1 d after challenge, basophils were examined for hCD2 expression at baseline (left), after IgE sensitization and challenge (middle), and after challenge alone (right). Gates were set against a KN2− control sensitized and challenged in parallel. (C) Pooled results from two separate experiments performed as in A and B with four to four mice per group depicting hCD2 expression on basophils (Basoph8−CD49b−FSClo SSClo), eosinophils (Siglec-F−CD11b−FSClo SSClo), CD4 T cells (CD4+FSClo SSClo), and mast cells (c-kit+h CD44+CD11a−, FSClo SSClo). Results from mice challenged with DNFB only (open circles) or challenge with DNFB after sensitization (closed squares) are shown. (D) Mice lacking IL-4 only in basophils (Basoph8−IL-4flox/−) and littermate controls (Basoph8−IL-4flox/+ ) were sensitized and challenged as in A. Eosinophil (left) and basophil (right) accumulation in the skin was assessed at day 3 after challenge. The data are pooled from two separate experiments with six mice per treatment group in aggregate. (A, C, and D) Horizontal bars denote mean. P-values were determined by ANOVA: *, P < 0.05; **, P < 0.01; ****, P < 0.0001.
We next crossed Basophil mice, KN2 mice, and Il-4/Il-13flox/flox mice (Sullivan et al., 2011) to eliminate IL-4 specifically from basophils. Il-4/Il-13flox/flox mice contain two loxp sites, separated by ~20 kb, that, when recombined, delete functional copies of both genes. As compared with littermate controls, mice

IL-4 surrogate marker (Fig. 3 C), thus identifying basophils as the primary source of IL-4 in this model. NKT cells have also been demonstrated to make IL-4, but NKT cells, as assessed by CD1d tetramer binding, were not recovered in cell populations isolated from involved ears (not depicted).

Figure 4. Basophil-derived IL-4 modulates endothelial cell expression of VCAM-1. (A) Basophil mice were sensitized with anti-DNP IgE, and 1 d later, the mice received anti-α4 antibody by intraperitoneal injection. Immediately after antibody administration, DNP was applied to the ear skin of sensitized mice. Accumulation of eosinophils (left) and basophils (right) in ear skin was assessed 3 d after challenge. Results were pooled from three independent experiments with at least six mice in each challenged group. (B) Basophil mice were sensitized with anti-DNP IgE, and 1 d later, the mice were challenged by topical application of DNP. 2 d after challenge, surface VCAM-1 expression on ear endothelial cells (CD45-CD34-ESAM-1) from basophil-sufficient (middle) and -deficient (right) mice was examined. The left panel depicts baseline VCAM-1 staining in unchallenged mice. Gates were set against an isotype control-stained sample. The graph on the right represents results pooled from three independent experiments with at least six mice in each challenged group. Littermate basophil-sufficient and -deficient animals were used in this experiment. (C) Basophil x IL-4flox/+ (middle) and Basophil x IL-4flox/- (right) littermates were sensitized, challenged, and analyzed as in B. The contour plots are representative of the results, with the graph on the right depicting results pooled from two independent experiments with at least six mice in each challenged group. (D) WT and basophil-deficient (Basophil x DTA) mice were sensitized and challenged as in B. 2 d later, mice received an i.v. injection of PE-conjugated anti-VCAM-1 and APC-conjugated anti-CD31 antibody, 10 min after antibody injection, whole mount micrographs were obtained using laser-scanning confocal microscopy. Each image stack was then transformed into a maximum intensity projection with VCAM-1 – and CD31-stained endothelium. The images were arranged as follows (from left to right): unchallenged WT mice, IgE-sensitized/challenged WT mice, nonsensitized/challenged WT mice, or sensitized/challenged basophil-deficient mice. Arrowheads denote VCAM-1-positive patches. The WT mice were bred independently of the basophil-deficient mice. The image is representative of two separate experiments with at least two mice in each group. Bar, 200 µm. (A–C) Horizontal bars denote mean. P-values were determined by ANOVA: **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
with basophil-specific deletion of IL-4 had a significant attenuation of eosinophils after challenge (Fig. 3 D). The effect was less than that observed in basophil-deleter mice or IL-4–deficient animals (Figs. 1 D and 3 A), but was consistent with the degree of cre-mediated recombination and deletion at the IL-4/13 locus (not depicted). Together, these data show that basophils are the primary source of IL-4 and that basophil IL-4 is necessary for optimal eosinophil tissue accumulation.

**Basophils regulate endothelial VCAM-1**

We next investigated whether IL-4 from basophils could promote eosinophil accumulation by modulating endothelial function. VCAM-1 is a STAT6-induced gene implicated in the trafficking of eosinophils to several tissues, including the skin (Tozawa et al., 2011). Antibody blockade of α4 integrin eliminated eosinophil accumulation after sensitization and challenge (Miyake et al., 1991), consistent with a role for VCAM-1 (Fig. 4 A). VCAM-1 was not required for basophil entry into the tissue, which reflects the lack of α4-integrins on mouse basophils (Voehringer et al., 2004). To assess the role of basophils in modulating endothelial VCAM-1, we analyzed VCAM-1 expression by flow cytometry on ear endothelial cells (CD45−CD34−ESAM−1+) from sensitized ears 40 h after challenge (Baumhueter et al., 1994; Hirata et al., 2001). VCAM-1+ endothelial cells were present in these mice, and expression of endothelial VCAM-1 was reduced when basophils were deleted using Basoph8 × Rosa-DTA mice (Fig. 4 B). IL-4 from basophils was required for optimal up-regulation of VCAM-1 because basophil-specific deletion of IL-4 similarly diminished VCAM-1 up-regulation on endothelial cells (Fig. 4 C). Finally, we injected anti–VCAM-1 antibody to visualize VCAM-1 on CD31+ endothelial cells in situ (Fig. 4 D). Although VCAM-1+ endothelial “patches” were readily apparent in sensitized and challenged mice, these patches were lost in the absence of either IgE sensitization or basophils, as assessed using Basoph8 × Rosa-DTA mice.

We verified that CD45−CD34−ESAM−1+ skin endothelial cells uniformly express the type II IL-4 receptor, consisting of the IL-4Rα and IL-13Rα1 subunits, by flow cytometry (Fig. 5 A). We also confirmed that IL-4 alone was sufficient to mediate eosinophil accumulation and VCAM-1 expression on the endothelium by examining eosinophil accumulation in the skin after intradermal IL-4 injection (Fig. 5, B and C). Intradermal injection of IL-4 into basophil-deleter mice led to a similar degree of eosinophil infiltration compared with wild-type mice, indicating that basophil-deleter mice have no intrinsic defect in mediating eosinophil flux into the skin (Fig. 5 D). Together, these data indicate that endothelial cells can respond to IL-4 and that IL-4 is both necessary and sufficient to mediate eosinophil skin infiltration in vivo.

Although basophil IL-4 was implicated in modulating endothelial adhesion ligands, it remained puzzling how this could be mediated by such rare numbers of circulating cells that would presumably be in relatively brief contact with endothelial cells. As assessed using direct visualization of fluorescent basophils in Basoph8 mice together with intravascular VCAM-1 labeling, basophils clustered at VCAM+ endothelial patches with 2.8-fold higher density as compared with VCAM− regions (Fig. 6 A). Intriguingly, a kinetic analysis of early time points after antigen challenge revealed that accumulation of basophils in the skin of IgE-sensitized mice was delayed as compared with nonsensitized control animals (Fig. 6 B). This
was specific to basophils because Gr-1+ cells accumulated comparably in both sensitized and nonsensitized animals over this period (Fig. 6 C).

We next performed confocal imaging of ear tissue whole mounts 24 h after challenge to assess the localization of basophils (Fig. 6 C). Basophils in nonsensitized but challenged mice were distributed widely throughout the tissues. In contrast, basophils in sensitized and challenged mice accumulated in proximity to blood vessels and were only sparsely distributed deeper in tissues (Fig. 6 C). Quantification of 2,865 basophils from nonsensitized mice and 1,494 from sensitized mice confirmed an enhanced association of basophils with the vasculature in sensitized animals (Fig. 6 D). Consistent with IL-4 being downstream of basophil clustering, we observed basophil clustering in global and basophil-specific IL-4−/− animals that was comparable with that in controls (not depicted). We also localized vascular-associated basophils to endothelial junctions (Fig. 6 E), which are enriched for vascular-associated versus nonassociated basophils at 24 h after challenge. Each dot represents the percentage of vascular-associated basophils from a single imaging volume for the given condition. (E) Basoph8 mice were treated as in B, and a maximum intensity projection (large panel) of an ear skin whole mount from sensitized Basoph8 mice 24 h after challenge depicting basophils associated with CD31+ endothelial cell junctions is shown. The white arrowhead denotes a representative basophil associated with CD31 on the endothelium with the individual z-section and corresponding channels highlighted at the right. Image is representative of two independent experiments with at least two mice in each group. Bars: (A) 50 µm; (C) 200 µm; (E) 20 µm. (A, B, and D) Horizontal bars denote mean. P-values were derived from analysis using a two-tailed Student’s t test: *, P < 0.05; ***, P < 0.0001. 

Figure 6. Activated basophils interact with the endothelium. (A, left) Basoph8 mice were sensitized with anti-DNP IgE and, 1 d later, challenged with topical application of DNFB on the ear skin. Approximately 1.5 d after challenge, the mice received conjugated anti-CD31 and anti–VCAM-1 antibody. 10 min after injection, ear skin tissue was harvested and imaged using laser-scanning confocal microscopy. A representative maximum intensity projection of basophils near VCAM-1 patches in CD31-stained endothelium from sensitized, challenged Basoph8 mice is depicted. (right) Quantification of segmented blood vessels is depicted comparing the density of basophils in VCAM-1+ vessel segments with VCAM-1− segments. Each dot represents the density of basophils in segments derived from a single z-stack. Data are pooled from three mice and a total of 11 z-stacks, with a total 10-mm length of blood vessels examined with 55% of the length VCAM+ and 45% VCAM−. (B) Basoph8 mice were sensitized and challenged as in A with anti-DNP IgE. Basophil (left) and Gr-1+ cell (right) accumulation in ear skin was then assessed at the indicated time points. IgE-sensitized mice (squares) were compared with nonsensitized mice (circles) before challenge. Error bars represent SD. Data are representative of two independent experiments with three mice at each time point. (C) Sensitized and nonsensitized Basoph8 mice were challenged with DNFB, and 24 h later, the mice received anti-CD31 conjugated to APC by i.v. injection. Mouse skin was then harvested and mounted 10 min later and imaged as in A. The images represent maximum intensity projections with basophils and CD31+ blood vessels from both sensitized (top) and nonsensitized (bottom) mice. The inset depicts a zoomed-in view of the dashed box. Images are representative of three individual mice for each group with three to four z-stacks taken from each mouse. (D) The images derived from C were further analyzed for vascular-associated versus nonassociated basophils at 24 h after challenge.
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Basophils demonstrate activation-dependent interactions with endothelial cells

We used in vitro basophil transendothelial migration assays to explore further the nature of interactions between activated basophils and the endothelium. A prior study has demonstrated that leukotriene B4 (LTB4) acts as a migratory stimulus for basophils in vivo (Reese et al., 2007), and we confirmed that LTB4 contributes to basophil migration in our passive sensitization model using mice unable to produce (Alox5⁻/⁻) or respond (LTB4R⁻/⁻) to LTB4 (Fig. 7 A). Thus, we used LTB4 as a chemotactic agent for basophils expanded using IL-3 complexes in vivo and sensitized by passive transfer of anti-trinitrophenol (TNP) IgE. Basophil migration was then examined across gelatinized transwell inserts overlaid with confluent bEnd.3 endothelial cells in the presence or absence of TNP-OVA added to the bottom well to mimic the distribution of the DNFB antigen in the skin. Basophil migration across the endothelium in response to LTB4 required integrin-mediated interactions with the endothelial monolayer as antibody blockade of αL-integrin eliminated basophil migration (not depicted). Consistent with an activation-dependent interaction with the endothelium, basophil migration to the bottom well was decreased in the presence of TNP-OVA (Fig. 7 B). This decrease in migration was dependent on the presence of the endothelial monolayer because activated basophils migrated similarly in response to LTB4 in the absence of endothelial cells (Fig. 7 C). The decrease in migration caused by interactions between antigen-activated basophils and endothelial cells led us to test whether signaling pathways coincidently engaged by activated basophils and integrin-mediated adhesion altered migration kinetics. Consistent with this hypothesis, inhibition of integrin signaling with the dual Pyk2/FAK inhibitor PF 431396 (D) or the selective FAK inhibitor PF 573228 (E). The concentration of each inhibitor is also noted. Data are pooled from two independent experiments each with duplicate transwells at each time point. [A–E] Student's t tests were performed for statistical analysis: *, P < 0.05; **, P < 0.01.

(Woodfin et al., 2011). Fully 90% of basophils (n = 110) were in direct contact with areas of enhanced CD31 staining. Overall, these data suggest an antigen-mediated activation event facilitates prolonged basophil interactions with the endothelium.

Figure 7. Basophil activation inhibits transendothelial migration. (A) WT C57BL/6, Alox5⁻/⁻, or Ltb4r1⁻/⁻ mice were sensitized with anti-DNP IgE and challenged with DNFB by topical application 1 d later. Basophil accumulation was analyzed 3 d after challenge. Each of these lines was bred independently, and the data were pooled from two separate experiments with at least six mice per group. Horizontal bars denote mean. (B and C) Basoph8 mice received IL-3 complex through an intravenous injection, and 3 d later, the spleens were harvested from these animals and placed in a transwell migration assay in the presence (B) or absence (C) of endothelial cells. Data are representative of four independent experiments with duplicate transwells performed each time. (D and E) Basoph8 mice were treated as in B, and the transendothelial cell migration assay was performed in the presence of the dual Pyk2/FAK inhibitor PF 431396 (D) or the selective FAK inhibitor PF 573228 (E). The concentration of each inhibitor is also noted. Data are pooled from two independent experiments each with duplicate transwells at each time point. [A–E] Student’s t tests were performed for statistical analysis: *, P < 0.05; **, P < 0.01.
DISCUSSION
The association between eosinophilia and elevated IgE in atopy has been long known, but the mechanisms underlying this linkage have remained unclear. Using a model of IgE-dependent skin inflammation, we demonstrate that basophils enter sites of endothelial activation as with other blood-borne granulocytes but, after FcεRI ligation by antigen, alter their migratory kinetics such that prolonged interactions occur with endothelial cells. Activated basophils secrete IL-4, which induces VCAM-1 expression on the vascular endothelium necessary for entry of eosinophils, which constitutively express α4-integrins. These data reveal a novel paradigm by which rare circulating basophils can alter their migratory behavior in response to tissue-derived antigens and thereby become gatekeepers capable of changing the characteristics of the localized inflammatory response through modulation of endothelial ligands. In this way, deposition of local signals can account for this, our data could be consistent with canonical pathways of rolling, (b) integrin-mediated firm adhesion, (c) intravascular crawling to sites amenable for diapedesis, and (d) endothelial transmigration (Kolaczkowska and Kubes, 2013). Although basophils follow canonical pathways of rolling and firm adhesion (Saeki et al., 2013; unpublished data), the enhanced interactions at the endothelial interface after IgE activation suggest unique antigen-dependent regulation of the last two steps. Our data support a model in which activation during diapedesis alters basophil motility and promotes the “arrest” of basophils within or along the endothelial basement membrane where antigen might be encountered. This change in transmigration is in part dependent on signaling through the FAKs activated during integrin and FcεRI coengagement.

The focal adhesion kinases FAK and Pyk2 are activated by cell surface receptors in response to diverse stimuli, including extracellular matrix constituents, integrin ligation, growth factors, antigen receptor clustering, and chemokines (Mitra et al., 2005). During integrin-mediated activation, FAK/Pyk2 does not directly bind the cytoplasmic tails of integrins in vivo. Instead, FAK/Pyk2 interacts with the proteins paxillin and talin, which in turn link integrins to the actin cytoskeleton. Most studies suggest that inhibition or loss of FAK leads to loss of migration (Mitra et al., 2005), and we observed a similar loss of basophil migration with or without antigen at high concentrations of inhibitor (unpublished data). These data suggest that basophil adherence to endothelium can be “tuned” by concomitant engagement of FcεRI and integrins. Although several models could account for this, our data could be consistent with impaired degranulation as the absence of FAK leads to impaired mast cell degranulation (Vial et al., 2000). This would abrogate the subsequent release or cell surface display of molecules that could promote adhesion to endothelial cells (Kannan et al., 1996). These kinases may have a more general role in myeloid cell degranulation as neutrophils lacking Pyk2 also show defective degranulation (Kamen et al., 2011).

The lack of a prominent mast cell contribution is intriguing because mast cells release an array of vasoactive substances and cytokines after IgE-mediated activation (Galli and Tsai, 2010). As shown here, and as corroborated in other models of cutaneous hypersensitivity (Mukai et al., 2005), we found no effects on eosinophil or Gr-1” infiltrates in mast cell–deficient mice (Fig. 1 E...
and not depicted). This and prior data support nonredundant contributions of mast cells and basophils during allergic immune responses, with activated mast cells exerting primary effects on vascular permeability that enhance the influx of serum proteins into the tissue space (Medzhitov, 2008). Basophils might additionally amplify these effects through release of mMCP-11 (Yamagishi et al., 2011). In humans (but not mice), eosinophils have been suggested to express the trimeric form of FceRI (αc5; Gould and Sutton, 2008), but the functional relevance of this in the context of allergic dermatitis remains indeterminate.

Our reporter data and genetic evidence indicate a prominent role for basophil-derived IL-4 in regulating eosinophilic dermatitis. However, the basophil-specific knockouts exhibited a more modest decrease in eosinophil accumulation as compared with either basophil-deficient mice or global IL-4 knockouts. Although incomplete recombination (estimated at 60–75% efficiency) of the 4/13 locus could be responsible, additional basophil-derived factors could also contribute. Activated basophils have been reported to produce TNF (Sokol et al., 2008), which can also promote VCAM-1 up-regulation (Neumann et al., 1996). The relative decrease in eosinophil influx in mice receiving intradermal IL-4 compared with the IgE-mediated response may reflect such contributions. Additionally, although endothelial cells express the type II IL-4R, our data do not formally rule out an intermediate cell type that responds to IL-4 and thus indirectly promotes endothelial cell VCAM-1 expression.

Although many details of the molecular and cellular circuitry governing specification of immune responses have been defined, our data reveal a novel paradigm by which rare circulating basophils can alter their migratory behavior in response to tissue-derived antigens and thereby change the characteristics of the localized inflammatory response through modulation of endothelial ligands. Our data provide a link between eosinophils and IgE through an interaction of antigen-activated basophils and the endothelium, which facilitates prolonged delivery of basophil IL-4 to the endothelium to modulate endothelial adhesions. The identification of basophils as “gatekeepers” for allergic skin diseases could provide new strategies to control allergic disease by interfering with this central pathway for eosinophil accumulation in skin, and perhaps in other tissues.

MATERIALS AND METHODS

Mice. Basoph8, KN2, IL-4/13lox-14, 4get, and Rosa-DTA have been previously described (Mohr et al., 2001, 2005; Voehringer et al., 2008, 2009). C57BL/6 mice were obtained from The Jackson Laboratory. Rag2−/− mice were obtained from Taconic. MCPT5-cre BAC transgenic (provided by A. Roers, University of Technology Dresden, Dresden, Germany), Alox5−/−, Lib4−/−, and Forl1−/− mice have been described previously (Donbrowicz et al., 1993; Chen et al., 1994; Tager et al., 2000; Scholten et al., 2008). All mice were backcrossed to C57BL/6 for at least 10 generations, except for experiments with Basoph8 × IL-4/13lox-14 mice, which were BALB/c × C57BL/6 F1 mice. Mice were housed in specific pathogen-free facilities. Animal use was governed by the Institutional Animal Care and Use Committee.

IgE dermatitis and tissue preparation. Mice were injected via the tail vein with 2 µg DNP-specific monoclonal IgE, Spe-7 (Sigma-Aldrich). 24–36 h later, mice were challenged with a solution of 0.6% DNPB dilitated in a 1:1 mixture of acetone and dibutylphthalate applied onto the dorsal and ventral halves of the ear (Matoula et al., 2010). 10 µl of solution was placed on each side of the ear. At designated times, ears were dissected, separated into ventral and dorsal halves, and finely minced. Tissue was resuspended in HBSS plus 1 Wünsch U/ml Liberase TL (Roche) for 45 min at 37°C with constant agitation. Digestion was quenched with a >2-vol excess of ice-cold PBS with 3% bovine serum and strained through a 70-µm mesh before analysis. For control IgE experiments, anti-OVA (E-G5; Chondrex) was used in place of Spe-7.

Antibodies, cell populations, and flow cytometry. Antibodies against the following antigens were used: CD11b (M1/70; ebioscience), CD49b (DX5, ebioscience), Siglec-F (ES0-2440; BD), Ly6G/C (RB6-8C5; BD), FceRI (Mar-1; BioLegend), CD45 (30-F11; BioLegend), CD34 (RAM34; ebioscience), ESAM-1 (1G8; BioLegend), CD31 (309; ebioscience), c-kit (2B7; BioLegend), CD106 (429; ebioscience), IL-4Rα (mIL-4-M1; BD), and CD231a (13MOKA; ebioscience). Key cell populations were defined as follows: (a) eosinophils, FSC−SSC−CD11b−Siglec-F− or FSC−SSC−Siglec-F−4get−5; (b) basophils, FSC−SSC−CD49b−FceRI−Gr-1−CD4+CD8−CD39− p8−Siglec-F− or FSC−SSC−Basoph8−CD49b−; (c) mast cells, 4get−/c-kit+ or FSC−SSC−c-kit+; (d) endothelial cells, CD45−CD34+ESAM−1−; and (e) PMNs/monocytes, Gr-1−CD11b+ Flow cytometry data acquisition was performed on an LSR-II (BD), using FlowJo to analyze the data.

Immunization. 100 µl of a 0.5 mg/ml solution of DNP-OVA (Biosearch Technologies) was mixed dropwise with agitator with Injeject Alum (Thermo Fisher Scientific) at a 1:1 ratio. The solution was then mixed for 30 min and injected into the peritoneum of index mice.

Epicutaneous antigen application. Mice received 2 µg anti-TNP IgE (C38-2; BD) i.v. 1 d before challenge. For the challenge, mice were anesthetized and then gently tape stripped (six times on the dorsal surface of the ear and six times on the ventral surface) using Scotch Tape (3M). A 1 x 1-cm piece of gauze was then soaked with 50 µl of a 1 µg/ml solution of TNP-OVA in PBS. The gauze was then affixed to each side of the ear for 1 h using a DuoDERM patch (Convatec) as a dressing. The patch was then removed, and the mice were analyzed at the indicated time.

In vivo antibody infusions. For labeling the vasculature in situ, 4 µg anti–CD31-APC (clone 390; ebioscience) was administered i.v. 10 min before analysis. For VCAM-1 experiments, 2 µg anti–VCAM-1 antibody (clone 429; ebioscience) was administered i.v. 10 min before analysis.

Whole mount microscopy. After dissection, the ears were split into dorsal and ventral halves, immobilized in a Petri dish dermal side up, and submerged in ice-cold PBS. Samples were analyzed using a confocal microscope (A1r; Nikon) using a 25× objective (NA = 1.05) or a 20× objective (NA = 0.95). Imaging volumes were analyzed using Imaris software (Bitmap). VCAM-1 segmentation was performed by identifying the borders of the VCAM-1 patches along the long axis of the vessel and then segmenting the vessel based on these borders. 11 z-stacks with a total length of ~10 mm of vessels were analyzed. In total, these z-stacks contained ~0.1 µm3 of VCAM-1 “segments” and 0.08 µm3 VCAM-1–“segments” that were assessed.

Immunofluorescence microscopy. Intact ears were fixed in a 4% paraformaldehyde solution in PBS for 2 h on ice, rinsed with PBS overnight, and then incubated in 30% sucrose for 2 h. Ears were embedded in OCT (Sakura) and cut into 8-μm sections. Sections were stained with anti–Siglec-F-biotin and detected with streptavidin Alexa Fluor 555. DAPI was used as a counterstain. Images were acquired using an Axio Imager 2 microscope using a 20x objective with NA = 0.80 and analyzed using AxioVision software (both Carl Zeiss).

Antibody blockade. 100 µg antagonist antibodies for α4 integrin (PS/2; provided by J. Cyster, UCSF) was administered via intraperitoneal injection 3 h before challenge. Isootype control antibodies were administered in control mice.

Transwell migration assays. To generate a sufficient source of basophils for the assay, we administered IL-3 complexes (10 µg IL-3 [R&D Systems]
complexed to 50 µg anti-IL-3 [MP2-8F8; BioLegend] for 5 min at room temperature) to Basophil mice 3 d before migration assays (Finkelman et al., 1993). Mice were then sensitized with 5 µg monomonal IgE against TNP (C38-2; BD) 24 h before the assay. On the day of the migration assay, spleenocytes were enumerated, and a total of 12,500 basophils were placed in the upper chamber of the transwell apparatus with LTB4 at 5 nM ± 100 ng/ml TNP-OVA (Biosearch Technologies) placed in the lower chamber analogous to published protocols (Ansel et al., 1999). For transendothelial migration, basophils in the lower chamber were assessed by flow cytometry after a 4-h incubation at 37°C. Each condition was performed in duplicate. Inhibitors PF 431396 and PF 573228 (Tocris Bioscience) were diluted to the indicated concentration and placed in both the upper and lower wells of the transwell just before addition of the basophils.

Statistical analysis. One-way ANOVA was used for all in vivo analyses involving three or more groups. Intergroup analyses were then performed using Tukey’s multiple comparisons test. For analyses with only two groups, a two-tailed, unequal Student’s t test was performed. On all graphs, p-values are denoted by the following nomenclature: * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001. All analyses were performed using Prism software version 6.0b (GraphPad Software).

Online supplemental material. The gating scheme for flow cytometry of basophil, eosinophils, and neutrophils is shown in Fig S1. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20141671/DC1.

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