

Paired immunoglobulin-like receptor A is an intrinsic, self-limiting suppressor of IL-5-induced eosinophil development

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Eosinophilia is a hallmark characteristic of T helper type 2 (T_H2) cell-associated diseases and is critically regulated by the central eosinophil growth factor interleukin 5 (IL-5). Here we demonstrate that IL-5 activity in eosinophils was regulated by paired immunoglobulin-like receptors PIR-A and PIR-B. Upon self-recognition of β_2 -microglobulin (β_2 M) molecules, PIR-B served as a permissive checkpoint for IL-5-induced development of eosinophils by suppressing the proapoptotic activities of PIR-A, which were mediated by the Grb2-Erk-Bim pathway. PIR-B-deficient bone marrow eosinophils underwent compartmentalized apoptosis, resulting in decreased blood eosinophilia in naive mice and in mice challenged with IL-5. Subsequently, *Pirb*^{-/-} mice displayed impaired aeroallergen-induced lung eosinophilia and induction of lung T_H2 cell responses. Collectively, these data uncover an intrinsic, self-limiting pathway regulating IL-5-induced expansion of eosinophils, which has broad implications for eosinophil-associated diseases.

Eosinophils are bone marrow (BM)-derived myeloid cells that differentiate under the control of the transcription factors GATA-1, PU.1, C/EBP, and the common β chain (β c)-signaling cytokines IL-5, IL-3 and GM-CSF¹. IL-5 is the most potent and specific cytokine for the eosinophil lineage and is responsible for cellular expansion², release from the BM into the peripheral blood (PB)³ and survival⁴ following a variety of triggers, typically T_H2 cell stimuli. At baseline, eosinophils mainly reside in the gastrointestinal tract, fat pads, spleen, lymph nodes and thymus^{5,6} where they function to maintain homeostasis^{6–8}. Yet in settings of allergic inflammation such as those found in asthma, eosinophils expand in the BM and infiltrate the lung, where their accumulation is a characteristic hallmark of disease^{5,7}. Whereas the extrinsic pathways (e.g., IL-5) that regulate eosinophil expansion are relatively well characterized, the presence of intrinsic, self-limiting, molecular checkpoints regulating IL-5-induced eosinophilia is largely unknown and remains to be defined.

A major checkpoint in curtailing cytotoxic T cells and natural killer (NK) cells is inhibitory signaling driven by major histocompatibility complex (MHC)-restricted self-recognition, mediated by receptors that can recognize self via binding of MHC class I molecules. The T cell receptor (TCR) enables positive and negative selection, which is critical for adaptive immunity and avoids autoreactivity⁹. NK cell inhibitory receptors (KIRs) govern cytotoxicity toward virally infected or transformed cells¹⁰.

An additional receptor system that mediates self-recognition by binding to classical and nonclassical MHC class I molecules has

drawn considerably less attention. This receptor system is comprised of PIR-A and PIR-B¹¹. Paired immunoglobulin-like receptors (PIRs) are predominantly expressed by myeloid cells, which endow them with the potential to discriminate self from non-self¹². Comprehensive structural and biochemical analyses have shown that the amino acid sequences of the PIR-A and PIR-B ectodomains are over 92% identical and bind the same MHC class I ligands^{12,13}. PIR-B is a type I transmembrane glycoprotein with six extracellular immunoglobulin-like domains, a hydrophobic transmembrane segment and an intracellular polypeptide with four immunoreceptor tyrosine-based inhibitory motifs (ITIM) or ITIM-like sequences¹². In contrast, PIR-A lacks the extended intracellular motif and requires assistance of adaptor molecules, such as the γ -chain of Fc receptors for efficient expression and function^{12,14}. Subsequent binding of MHC class I molecules by PIRs leads to the recruitment of cytosolic phosphatases by PIR-B, which ultimately results in inhibition of yet undefined signaling cascades triggered by PIR-A^{14,15}. PIR-B can suppress cellular responses elicited by the β c signaling cytokines, IL-3 and GM-CSF^{16,17} as well as by Flt-3L (ref. 18), thus suggesting a role for PIR-B in myeloid cell hematopoiesis. The recent demonstration that eosinophils express PIRs¹⁹, together with the suggested hematopoietic functions of PIR-B, raised the hypothesis that PIR-B may regulate IL-5-induced responses in eosinophils.

In this study, we demonstrate a critical role for PIR-B and PIR-A in eosinophil development. PIR-B suppressed PIR-A-induced apoptosis of eosinophils, thereby counterbalancing survival and growth signals selectively driven by IL-5. Expression of PIR-A and PIR-B was

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regulated by IL-5 and increased during distinct eosinophil maturation stages. Eosinophils maintained a dominant expression of PIR-B over PIR-A under homeostatic conditions. Thus, despite abundant availability of MHC class I ligand, which may trigger PIR-A-induced apoptosis, development of eosinophils was intact because of inhibitory signals driven by PIR-B. Furthermore, we found that PIR-A bound the adaptor protein Grb2 and that apoptotic eosinophils displayed increased activation of Erk and expression of Bim. These data established a new model defining the intrinsic molecular pathways regulating IL-5-induced eosinophilia and highlight self-recognition via PIRs as a key molecular checkpoint in expansion of eosinophils.

RESULTS

PIR-B is required for differentiation of eosinophils *in vitro*

To begin to assess the function of PIR-B in eosinophils, we used the recently described BM-derived eosinophil cell culture system²⁰. Cultures obtained from the low-density fraction of wild-type BM (LDBM) cells by day 14 displayed >95% eosinophils of uniform morphological appearance, including stereotypical bilobed nuclei (Fig. 1a). In contrast, starting from day 10, cultures of *Pirb*^{-/-} LDBM cells showed the appearance of myeloid morphology and by day 14, the entire *Pirb*^{-/-} culture was composed of large, often multinucleated cells lacking eosinophil morphology (Fig. 1a). Total cell counts obtained from the *Pirb*^{-/-} LDBM cell cultures were lower than those obtained from wild-type cultures (Fig. 1b). At day 14, cells retrieved from wild-type

but not *Pirb*^{-/-} cultures expressed CCR3 and major basic protein (MBP), both of which are classical markers of eosinophils (Fig. 1c,d). *Pirb*^{-/-} cells exhibited distinct physical parameters (i.e., granularity and size; Fig. 1e) and had increased expression of myeloid-associated adhesion molecules and activation markers including CD11b, CD18, α_5 integrin, CD103, CD62L and CD69 (Supplementary Fig. 1). *Pirb*^{-/-} cells derived from LDBM cell culture displayed markedly decreased amount of mRNA encoding the eosinophil-associated transcription factors GATA-1 and GATA-2 (Fig. 1f,g), though expression of mRNAs encoding C/EBP, PU.1 and Fog-1 was comparable to that in wild-type controls (data not shown). By the end of the cell cultures, total eosinophil counts in *Pirb*^{-/-} LDBM cell cultures were reduced by $97.3 \pm 0.06\%$ (Average $\pm$ s.e.m) compared to wild-type cultures (Fig. 1h). Decreased eosinophil generation was not due to alterations in the numbers of eosinophil progenitors (defined as Sca1⁺CD34⁺Lin⁻c-Kit^{int}IL-5R α ⁺; Fig. 1i,j)²¹ or alteration in IL-5R α surface expression in eosinophil progenitors (Fig. 1k). The inability to generate mature eosinophils *in vitro* was an intrinsic defect of *Pirb*^{-/-} cells because growing *Pirb*^{-/-} cells in supernatants obtained from wild-type LDBM-derived eosinophil cultures did not render the *Pirb*^{-/-} cells into eosinophils. Moreover, the generation of eosinophils was not impaired when we cultured cells in supernatants harvested from *Pirb*^{-/-} cultures (data not shown). The requirement of PIR-B in eosinophil development was PIR-B-specific and eosinophil-specific because we could generate mature eosinophils from LDBM cells of mice deficient in other

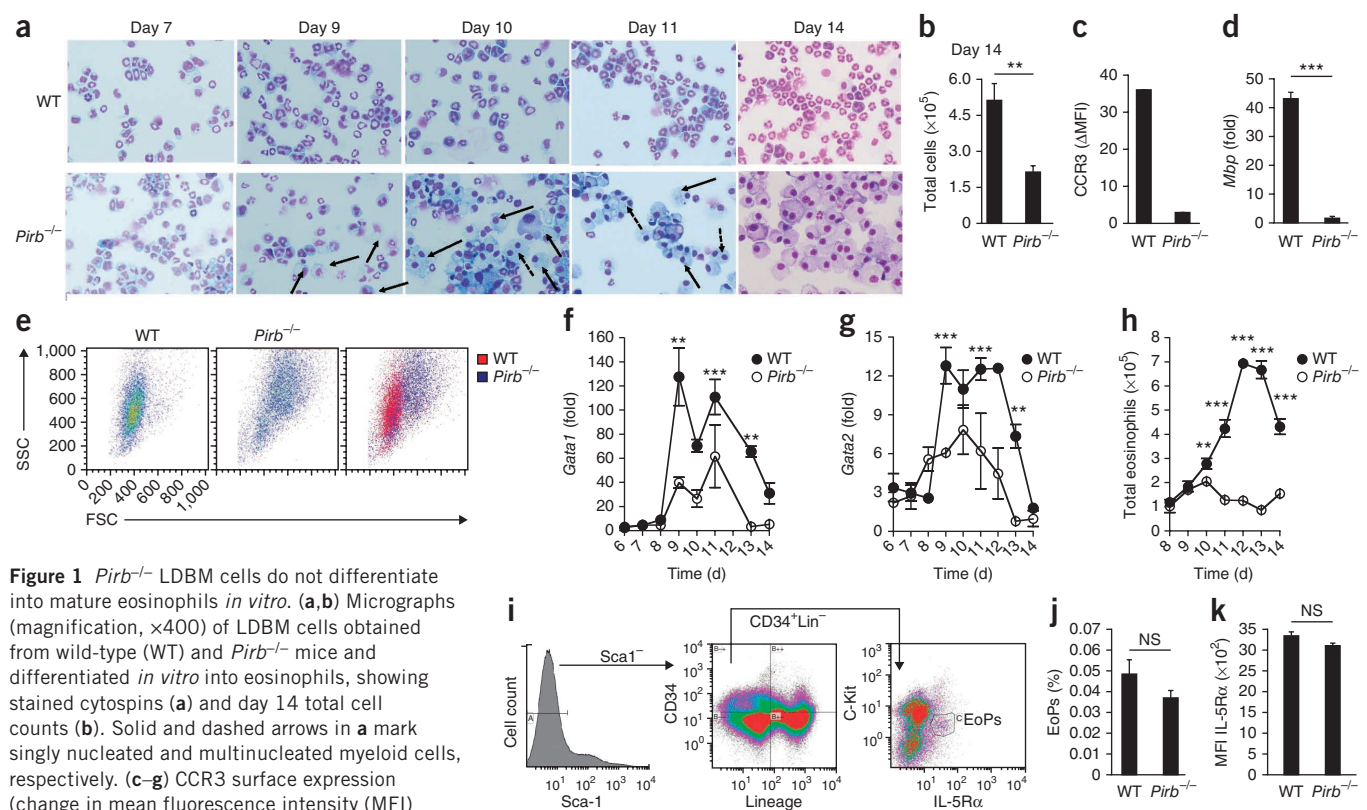


Figure 1 *Pirb*^{-/-} LDBM cells do not differentiate into mature eosinophils *in vitro*. (a,b) Micrographs (magnification, $\times 400$) of LDBM cells obtained from wild-type (WT) and *Pirb*^{-/-} mice and differentiated *in vitro* into eosinophils, showing stained cytopins (a) and day 14 total cell counts (b). Solid and dashed arrows in a mark singly nucleated and multinucleated myeloid cells, respectively. (c–g) CCR3 surface expression (change in mean fluorescence intensity (MFI) of anti-CCR3 stain – isotype control; c), mRNA encoding eosinophil major basic protein (*Mbp*, d), cell size (forward scatter, FSC) and granularity (side scatter, SSC) (e), and expression of *Gata1* (f) and *Gata2* (g) in LDBM WT and *Pirb*^{-/-} cells. All quantitative PCR data were normalized to the expression of housekeeping gene *Hprt*. (h) Total eosinophil numbers as determined by CCR3⁺Siglec-F⁺ cells. (i–k) Assessment of CD45⁺CD34⁺Lin⁻Sca-1⁻c-Kit^{int}IL-5R α ⁺ eosinophil progenitors (EoPs) (i,j) and IL-5 receptor α (IL-5R α) expression in EoPs (k). NS, not significant; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (Student's *t*-test). Data are representative of five independent cell cultures obtained from different mice (a,e,i), representative of three independent experiments (c), pooled from three cultures obtained from three different mice per genotype and conducted in triplicate (b,d,f–h, error bars, s.e.m.; $n = 3$ cell cultures) or pooled from two experiments with five mice per genotype (j,k; error bars s.e.m.; $n = 10$ mice).

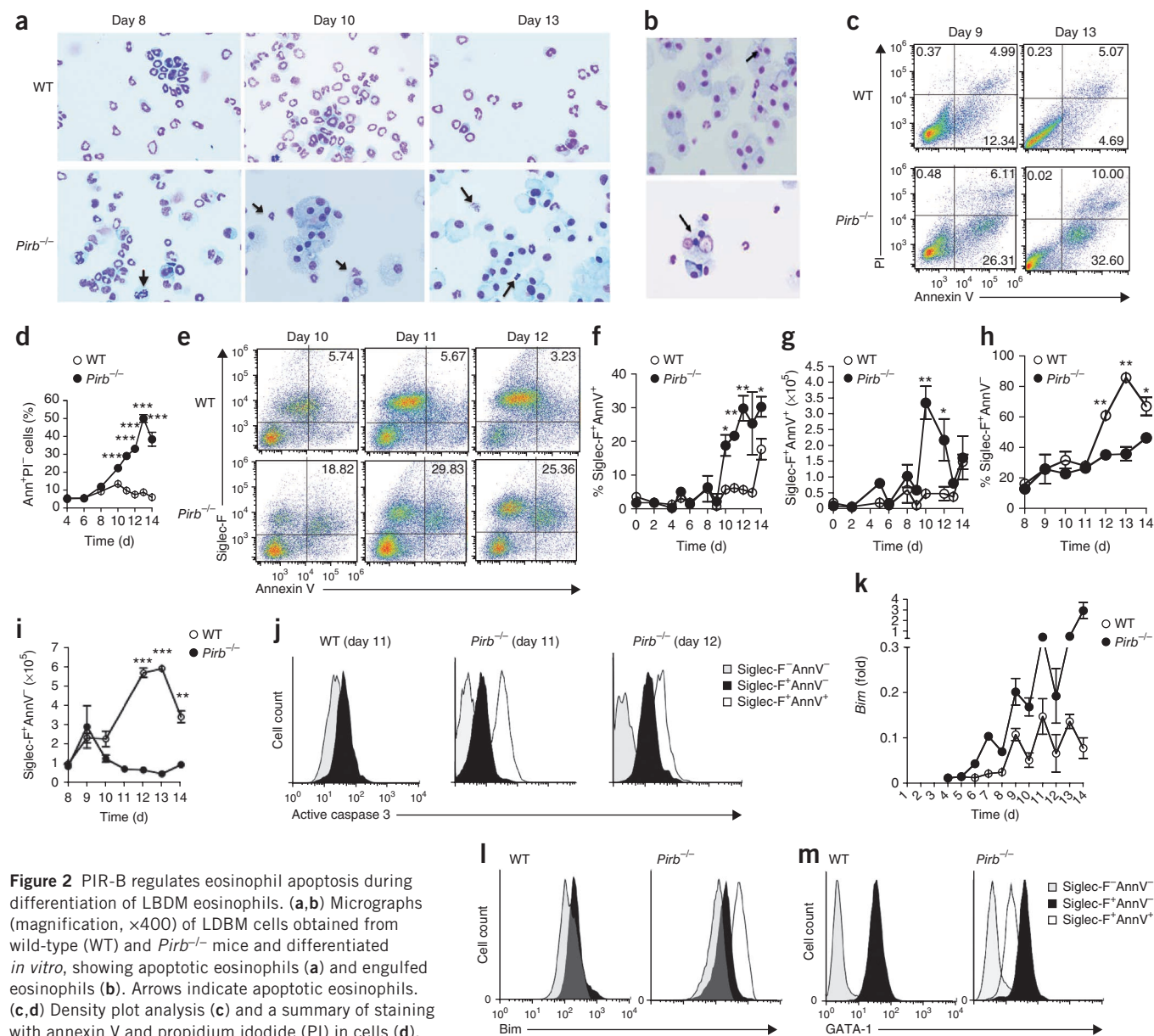


Figure 2 PIR-B regulates eosinophil apoptosis during differentiation of LDBM eosinophils. (**a,b**) Micrographs (magnification, ×400) of LDBM cells obtained from wild-type (WT) and *Pirb*^{-/-} mice and differentiated *in vitro*, showing apoptotic eosinophils (**a**) and engulfed eosinophils (**b**). Arrows indicate apoptotic eosinophils. (**c,d**) Density plot analysis (**c**) and a summary of staining with annexin V and propidium iodide (PI) in cells (**d**). Numbers in quadrants indicate percentages of cells corresponding to this quadrant. (**e–i**) Density plots of anti-Siglec-F⁺ and annexin V (AnnV)-stained cells (**e**), and analysis of the percentage and total cell counts of Siglec-F⁺AnnV⁺ cells and Siglec-F⁺AnnV⁻ cells (**f–i**). (**j,l,m**) Flow cytometry analyses of LDBM cells stained with anti-Siglec-F, annexin V and anti-active caspase 3 (**j**), anti-Bim (**l**) or anti-GATA-1 (**m**). (**k**) Quantitative PCR analysis of *Bcl2l1* (*Bim*) in cDNA from LDBM cells (normalized to expression of the housekeeping gene *Hprt*). **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Students *t*-test). Data are representative of five independent cell cultures obtained from different mice (**a,c,e**), pooled from three cultures obtained from three different mice per genotype, (**d,f–i**; error bars, s.e.m.; *n* = 3 cell cultures), representative of three independent experiments conducted in cell cultures obtained from different mice (**j,l,m**) and pooled from three cultures obtained from three different mice per genotype (**k**; error bars, s.e.m.; *n* = 3 cell cultures).

ITIM-bearing receptors (e.g., CMRF35-like molecule (CLM)-1; ref. 22) and *Pirb*^{-/-} BM yielded normal numbers of macrophages, dendritic cells (DCs), neutrophils and mast cells in the respective cytokine-driven cultures (data not shown and refs. 16,23,24). Collectively, these data indicate that PIR-B regulates an intrinsic pathway, which is specifically required for eosinophilopoiesis.

PIR-B regulates apoptosis of eosinophils

Impaired development of eosinophils in *Pirb*^{-/-} LDBM cell cultures could be either due to a decreased proliferative ability or to increased apoptosis. Assessment of cellular proliferation in response to a

combination SCF plus Flt-3L during the first 2 d of LDBM cell culture revealed no difference between the proliferative activity of wild-type and *Pirb*^{-/-} eosinophil progenitors (Supplementary Fig. 2a). Because of the loss of the distinct eosinophil progenitor cell-surface markers (i.e., c-Kit and IL-5Rα) after 1–2 d in culture, we subsequently assessed cell proliferation in the general LDBM cell cultures and observed no proliferative difference between wild-type and *Pirb*^{-/-} cells (Supplementary Fig. 2b).

Morphological assessment of *Pirb*^{-/-} LDBM cell cultures revealed the presence of apoptotic eosinophils, as identified by nuclear fragmentation and cell blebbing (Fig. 2a). Moreover, apoptotic

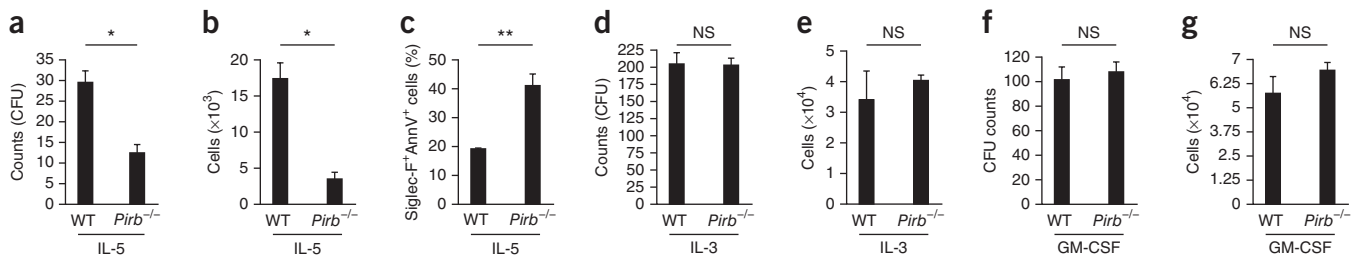


Figure 3 PIR-B regulates IL-5-induced but not IL-3-induced or GM-CSF-induced colony formation. (**a–g**) CFU assays of LDBM-derived cells of wild-type (WT) and *Pirb*^{-/-} mice treated with IL-5 (**a–c**), IL-3 (**d,e**) and GM-CSF (**f,g**). Total CFU counts (**a,d,f**) and relative cell counts per CFU (**b,e,g**) were assessed. Cells from IL-5-induced CFUs from WT and *Pirb*^{-/-} cultures stained with anti-Siglec-F and annexin V followed by analysis of Siglec-F⁺ cells for annexin V expression (**c**). NS, not significant; **P* < 0.01, ***P* < 0.001 (Student's *t*-test). Data are pooled from three independent CFU assays using different mice and conducted in triplicate (error bars, s.e.m.; *n* = 3 CFU assays).

eosinophils and/or apoptotic bodies in *Pirb*^{-/-} cultures were engulfed by the abundant mononuclear myeloid cells in these cultures (Fig. 2b). In accordance with these findings, assessment of early and late apoptosis using annexin V staining combined with propidium iodide (PI) staining, revealed that *Pirb*^{-/-} LDBM cell cultures contained a prominent fraction of annexin V⁺PI⁻ cells (Fig. 2c,d). Costaining for Siglec-F, a surface marker of early eosinophil development²⁵, and annexin V demonstrated that starting at day 10 of the culture, nearly 20% of the *Pirb*^{-/-} LDBM cells were Siglec-F⁺annexin V⁺ comprising approximately half of all *Pirb*^{-/-} Siglec-F⁺ cells (Fig. 2e); in contrast, wild-type eosinophils showed good viability and exhibited only 3–5% Siglec-F⁺annexin V⁺ cells. The greater percentage of Siglec-F⁺annexin V⁺ cells in *Pirb*^{-/-} cultures was reflected by a greater number of total dead eosinophils (Fig. 2f,g). Eventually, the numbers of total dead eosinophils declined because of the overall decrease in total cell counts, which we observed in the *Pirb*^{-/-} LDBM cell culture (Fig. 1b). Analysis of the percentage and total Siglec-F⁺annexin V⁻ cells demonstrated markedly increased percentage and total viable eosinophils in the wild-type cell cultures in comparison with the *Pirb*^{-/-} cell cultures (Fig. 2h,i).

Consistent with their apoptotic appearance, Siglec-F⁺annexin V⁺ cells in *Pirb*^{-/-} LDBM cell cultures expressed increased expression of active caspase 3 (Fig. 2j). Moreover, *Pirb*^{-/-} LDBM cell cultures displayed increased expression of mRNA encoding the proapoptotic molecule Bcl-interacting molecule (Bim)²⁶ in comparison with wild-type cells (Fig. 2k). We observed no differences in expression of mRNA encoding additional pro- or antiapoptotic molecules (Supplementary Fig. 3). *Pirb*^{-/-} Siglec-F⁺annexin V⁺ cells displayed substantially increased amounts of Bim compared with Siglec-F⁺annexin V⁻ wild-type or *Pirb*^{-/-} cells (Fig. 2l). Intracellular staining assessing GATA-1 abundance demonstrated that *Pirb*^{-/-} Siglec-F⁺annexin V⁺ cells displayed less GATA-1 protein compared with Siglec-F⁺annexin V⁻ wild-type or *Pirb*^{-/-} cells (Fig. 2m). GATA-1 and Bim protein expression was similar between wild-type and mutant Siglec-F⁺annexin V⁻ and Siglec-F⁻annexin V⁻ cells (Fig. 2l,m). Thus, *Pirb*^{-/-} cells do not mature into eosinophils *in vitro* likely owing to Bim-mediated apoptosis.

PIR-B is required for IL-5-induced colony formation

To assess the specificity of PIR-B to IL-5-induced differentiation of eosinophils, we subjected wild-type and *Pirb*^{-/-} LDBM cells to colony forming unit (CFU) assays using IL-5, GM-CSF or IL-3. *Pirb*^{-/-} cells displayed a specific decrease in CFUs induced by IL-5 but not by IL-3- and/or GM-CSF (Fig. 3). Assessment of the relative cell counts per colony demonstrated that the amount of cells per colony was lower in colonies from *Pirb*^{-/-} CFUs induced by IL-5 (but not IL-3 or GM-CSF; Fig. 3). Furthermore, *Pirb*^{-/-} CFUs displayed a twofold

increase in Siglec-F⁺annexin V⁺ cells specifically in the IL-5-driven colonies (Fig. 3c). Thus, in response to IL-5 (specifically), *Pirb*^{-/-} BM produced fewer colonies, which were smaller and displayed increased apoptosis in comparison with wild-type cells.

PIR-B regulates eosinophil apoptosis *in vivo*

The requirement for PIR-B in IL-5-induced development of eosinophils *in vitro* led us to assess eosinophil numbers in the BM of naive *Pirb*^{-/-} mice. Flow cytometric analysis of BM eosinophils revealed two distinct eosinophil populations in the BM; namely a Siglec-F⁺CCR3^{int} population and a Siglec-F⁺CCR3^{hi} population (likely representing immature and mature eosinophils, respectively)²⁵, both of which displayed eosinophil physical parameters (Fig. 4a,b), morphology and eosinophil granule proteins (for example, MBP; Supplementary Fig. 4). Both eosinophil populations retrieved from naive *Pirb*^{-/-} mice displayed more annexin V⁺ staining, as compared to wild-type controls (Fig. 4b,c). Analysis of other BM-resident myeloid cell populations such as monocytes (gated as Ly6G⁻Ly6B.2⁺) or neutrophils (gated as Ly6G⁺Ly6B.2^{int}) revealed that increased baseline apoptosis in *Pirb*^{-/-} mice was specific to eosinophils (Fig. 4d). Assessment of PB of naive wild-type and mutant mice revealed that the *Pirb* deficiency resulted in 30% fewer blood eosinophils in the steady state (Fig. 4e,f). Eosinophil viability was similar between wild-type and PB *Pirb*^{-/-} eosinophils (data not shown), suggesting that reduction is due to a compartmentalized apoptotic fate in the BM.

Previous studies using *Il5*^{-/-} mice as well as anti-IL-5 neutralization *in vivo* revealed that under homeostatic conditions, BM eosinophils are only partially regulated by an IL-5-dependent pathway^{27,28}. Rather, IL-5 is critical in eosinophil expansion under various disease settings²⁹. To define whether PIR-B regulates *in vivo* steady-state eosinophilia in an IL-5-dependent fashion, we neutralized IL-5 in naive wild-type and *Pirb*^{-/-} mice using the rat anti-mouse IL-5 antibody (clone TRFK5)³⁰. IL-5 neutralization caused an evident (but not complete) decrease in BM and PB eosinophils of wild-type but not *Pirb*^{-/-} mice (Fig. 4g–i). Moreover, treatment with anti-IL-5 caused a specific reduction in the expression of PIR-B but not PIR-A in mature BM eosinophils (i.e., Siglec-F⁺CCR3^{hi}) but not PB eosinophils (Fig. 4j). Collectively, these data demonstrate that PIR-B regulates the IL-5-dependent but not IL-5-independent baseline frequencies of eosinophils.

Consistently, increased apoptosis of *Pirb*^{-/-} BM eosinophils persisted even when we artificially elevated availability of IL-5 by exogenous delivery of recombinant IL-5. Thus, although this regimen decreased the apoptotic fate of some *Pirb*^{-/-} Siglec-F⁺CCR3^{int} and Siglec-F⁺CCR3^{hi} cells, the frequencies of apoptotic eosinophils were still higher in *Pirb*^{-/-} mice in comparison to wild-type controls (Fig. 4k,l).

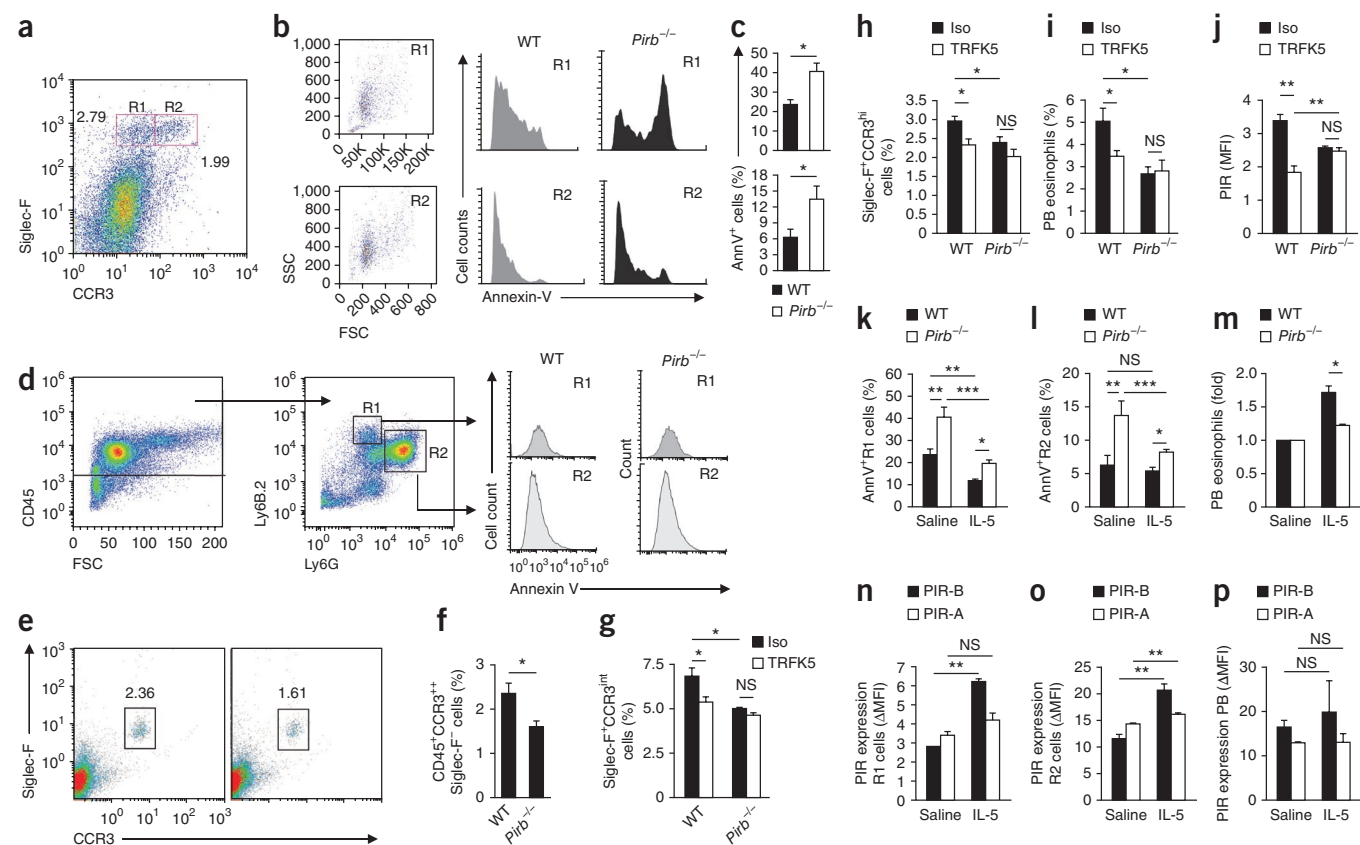


Figure 4 PIR-B regulates eosinophil apoptosis *in vivo* even in the presence of increased IL-5. (a–c) Flow cytometry analysis of Siglec-F- and CCR3-stained BM cells from naive wild-type (WT) and *Pirb*^{−/−} mice showing annexin V expression in Siglec-F⁺CCR3^{int} (R1, a) and Siglec-F⁺CCR3^{hi} (R2, a) cells (b,c). (d) Annexin V expression in BM monocytes and neutrophils. (e,f) Analysis of circulating PB eosinophils in naive WT and *Pirb*^{−/−} mice. (g–i) Frequencies of BM Siglec-F⁺CCR3^{int} cells (g) and Siglec-F⁺CCR3^{hi} cells (h) and PB eosinophils (i) after treatment with anti-IL-5 (TRFK5) or isotype control antibody (Iso). (j) Expression of PIRs on the surface of PB eosinophils after treatment with anti-IL-5 (TRFK5) or isotype control antibody (Iso). (k–m) Assessment of annexin V staining in BM Siglec-F⁺CCR3^{int} (R1, k) and Siglec-F⁺CCR3^{hi} (R2, l) cells and the induction of PB eosinophils after treatment with IL-5 (m). (n–p) Expression of PIRs in BM (n,o) and PB (p) eosinophils after treatment with IL-5. NS, not significant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's *t*-test (c,f,m) and one-way ANOVA followed by Tukey *post hoc* test (g–l,n–p). Data are representative of three experiments using five mice per genotype (a,b,d,e), pooled from three experiments using five mice per genotype (c,f; error bars, s.e.m.; *n* = 15 mice) or pooled from two independent experiments with five and seven mice per genotype per experiment (g–p; error bars, s.e.m.; *n* = 13 mice).

The frequency of eosinophils in the blood of IL-5-treated wild-type mice increased 1.76 ± 0.17 -fold, whereas IL-5-treated *Pirb*^{−/−} mice displayed only a 1.2 ± 0.025 -fold increase (Fig. 4m). Moreover, treatment with IL-5 increased the expression of PIR-B in BM but not in PB eosinophils (Fig. 4n–p). These results establish an intricate relationship between IL-5 and PIR expression.

Self-recognition regulates eosinophil expansion

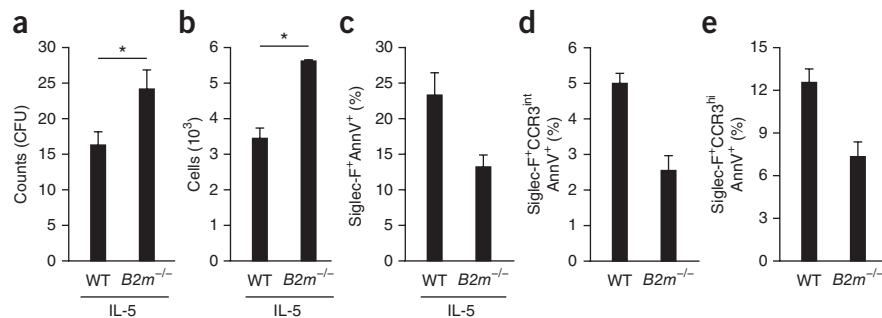
The β_2 microglobulin (β_2 M) subunit of MHC class I molecules serves as a ligand for PIR-B¹¹. Thus, we hypothesized that loss of β_2 M will result in similar eosinophil apoptosis as observed in *Pirb*^{−/−} cell cultures and mice. However, CFU counts obtained from *B2m*^{−/−} LDBM cells demonstrated increased numbers of colonies in response to IL-5 (Fig. 5a). Furthermore, assessment of relative cell counts per colony revealed more cells from the *B2m*^{−/−} CFUs (Fig. 5b) and less Siglec-F⁺annexin V⁺ cells compared with wild-type cells (Fig. 5c). Consistently, both Siglec-F⁺CCR3^{int} and Siglec-F⁺CCR3^{hi} BM eosinophils obtained from naive *B2m*^{−/−} mice were less apoptotic than naive wild-type BM eosinophils (Fig. 5d,e). The finding that loss of MHC class I recognition resulted in decreased eosinophil apoptosis suggested the involvement of an additional receptor that can bind MHC class I molecules and is suppressed by PIR-B, such as PIR-A¹³.

Absence of PIR-B enables proapoptotic signals by PIR-A

To test the hypothesis that PIR-A delivers proapoptotic signaling in eosinophils, we first analyzed the expression of PIRs throughout the LDBM cell culture system. Both PIRs were progressively upregulated on the cultured cells (Fig. 6a). Expression of PIR-A preceded that of PIR-B by 2 d, with PIR-A expression increasing at day 5 (Fig. 6a). The induction of PIR-A (but not of PIR-B) expression thus correlated with the induction of the proapoptotic factor Bim (Fig. 6b and Supplementary Fig. 5). To assess whether PIR-A is responsible for the apoptotic phenotype that we observed in *Pirb*^{−/−} LDBM cell cultures, we neutralized PIR-A in the *Pirb*^{−/−} LDBM eosinophil cell cultures using the anti-PIR-A/B antibody (clone 6C1), which recognizes both PIR-A and PIR-B. We chose this antibody because it recognizes ectodomains 1 and 2 of PIR molecules, which are the major MHC class I molecule-recognizing ectodomains in PIRs³¹.

Neutralization of PIR-A markedly attenuated the apoptotic phenotype, which we observed in *Pirb*^{−/−} eosinophils (Fig. 6c,d) and noticeably increased the numbers of mature eosinophils (Fig. 6e,f). *Pirb*^{−/−} cultures that received isotype control antibodies displayed a similar number of apoptotic cells and total eosinophils as untreated *Pirb*^{−/−} cultures (Fig. 6e). Morphologically, neutralization of PIR-A partially restored the typical eosinophil granular appearance in *Pirb*^{−/−} cell

Figure 5 Increased eosinophil development and decreased apoptosis in the absence of MHC class I expression. (**a–c**) Total CFU counts (**a**) and relative cell counts per CFU (**b**) for IL-5-treated LDBM-derived cells of wild-type (WT) and *B2m*^{−/−} mice. (**c**) Assessment of apoptosis in IL-5-induced colonies from WT and *B2m*^{−/−} cell cultures by staining with anti-Siglec-F and annexin V. (**d,e**) BM cells from naive WT and *B2m*^{−/−} mice stained with anti-Siglec-F, anti-CCR3 and annexin V, and then Siglec-F⁺CCR3^{int} and Siglec-F⁺CCR3^{hi} cells were gated and assessed for annexin V expression. **P* < 0.05 (Student's *t*-test). Data are pooled from three independent experiments using three cultures from three independent mice (**a–c**; error bars, s.e.m.; *n* = 3 CFU assays) and pooled from one experiment using seven mice per genotype (**d,e**; error bars, s.e.m.; *n* = 7 mice).



cultures (**Fig. 6f**). Collectively, these data demonstrate that unless counteracted by PIR-B, PIR-A induces eosinophil apoptosis, an activity that is revealed in the absence of PIR-B.

To gain mechanistic insight into the functional activities driven by PIR-A, we used a mini-phosphoproteomics approach: we immunoprecipitated PIR-A from the surface of sorted viable *Pirb*^{−/−} LDBM cells and incubated it with a membrane that had been precoated with antibodies against various signaling molecules (**Supplementary Fig. 6a,b**). After incubation, we blotted the membrane with anti-phosphotyrosine to detect interactions between PIR-A and functional downstream signaling intermediates. Given the abundant availability of MHC class I ligands to PIR-A, we hypothesized that the kinases and/or adaptor molecules interacting with PIR-A will be active as well. We found that PIR-A was strongly associated with the adaptor Grb2 (**Fig. 6g**). Given the association between Grb2 and Erk activation³², we hypothesized that apoptotic *Pirb*^{−/−} eosinophils will display increased Erk activation in comparison with viable cells. Assessment of phosphorylated Erk-1 and Erk-2,

in Siglec-F⁺annexin V[−] and Siglec-F⁺annexin V⁺ cells revealed an evident increase in phosphorylation of Erk-1 and Erk-2 in Siglec-F⁺annexin V⁺ cells in comparison with Siglec-F⁺annexin V[−] cells (**Fig. 6h,i**). This effect was specific to Erk activation because activation of Jnk and p38 was not changed between viable and dead eosinophils (**Supplementary Fig. 6**).

Distinct expression for PIRs during eosinophil maturation

Given the proapoptotic role of PIR-A, we sought to understand why peripheral eosinophils survive in the presence of abundant MHC class I expression. To this end, we analyzed the relative expression of PIR-A and PIR-B in various eosinophil populations in the BM (i.e., eosinophil progenitors, Siglec-F⁺CCR3^{int} cells and Siglec-F⁺CCR3^{hi} cells), PB and tissue (i.e., peritoneal cavity). Eosinophil progenitors expressed very low and equivalent amounts of PIR-A and PIR-B (respectively, 1.11-fold ± 0.02-fold and 1.14-fold ± 0.04-fold increase (±s.e.m.) over isotype control, *P* = 0.26, Student's *t*-test). The expression of PIR-A and PIR-B gradually increased as eosinophils developed,

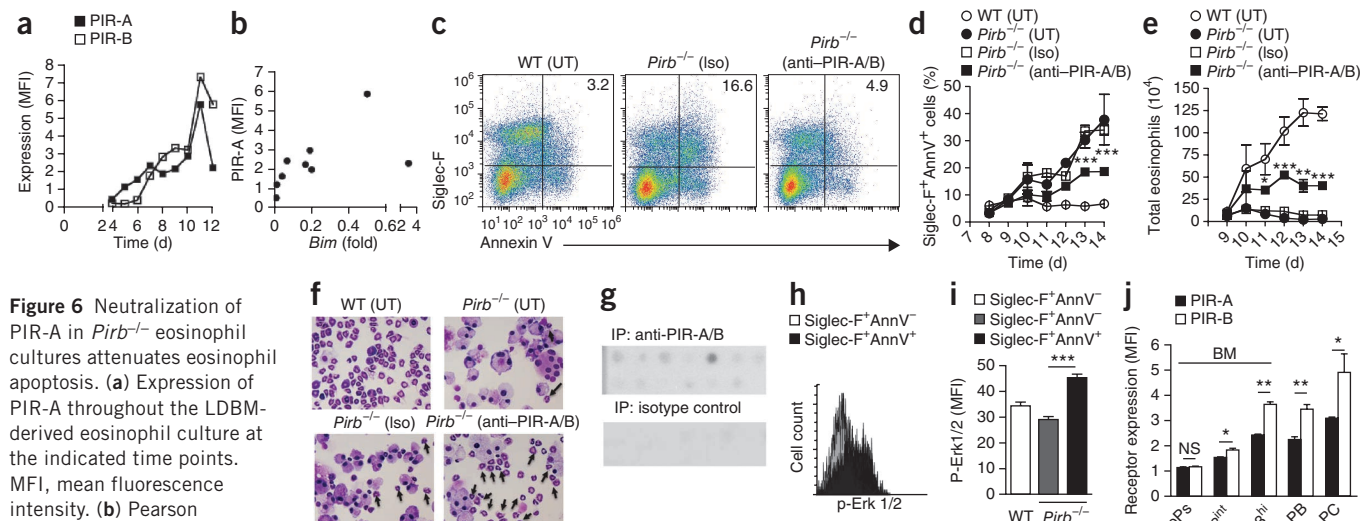


Figure 6 Neutralization of PIR-A in *Pirb*^{−/−} eosinophil cultures attenuates eosinophil apoptosis. (**a**) Expression of PIR-A throughout the LDBM-derived eosinophil culture at the indicated time points. MFI, mean fluorescence intensity. (**b**) Pearson correlation between PIR-A

and Bim mRNA expression (*r* = 0.943, *P* = 0.0004). (**c–e**) Dot plots (**c**) and a summary of the flow cytometric analysis (**d,e**) of eosinophils, from LDBM-derived cells of wild-type (WT) and *Pirb*^{−/−} mice, grown untreated (UT), in the presence of anti-PIR-A/B or isotype control antibodies (Iso), and analyzed for anti-Siglec-F and annexin V staining. (**f**) Micrographs (magnification, ×400) of indicated cells stained with modified Wright Giemsa stain. (**g**) Mini phosphoproteomic analysis of PIR-A: protein interactions in sorted viable (Siglec-F⁺annexin V[−]) *Pirb*^{−/−} cells. (**h,i**) Flow cytometry histogram plot (**h**) and quantitative analysis of phospho-Erk 1/2 (p-Erk1/2) expression in Siglec-F⁺annexin V[−] and Siglec-F⁺annexin V⁺ cells from WT and *Pirb*^{−/−} cells (**i**). (**j**) Expression of PIR-A and PIR-B on the surface of eosinophils from various *in vivo* sources. EoPs, eosinophil progenitors; PC, peritoneal cavity. NS, not significant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (Student's *t*-test). Data are representative of three independent cell cultures obtained from different mice (**a–c,f,g**), representative of three independent experiments (**h**), pooled from five independent cell cultures obtained from different mice (**d,e**; error bars, s.e.m., *n* = 5 cultures) and pooled from two different experiments using three mice per genotype (**i,j**; error bars, s.e.m., *n* = 6 mice).

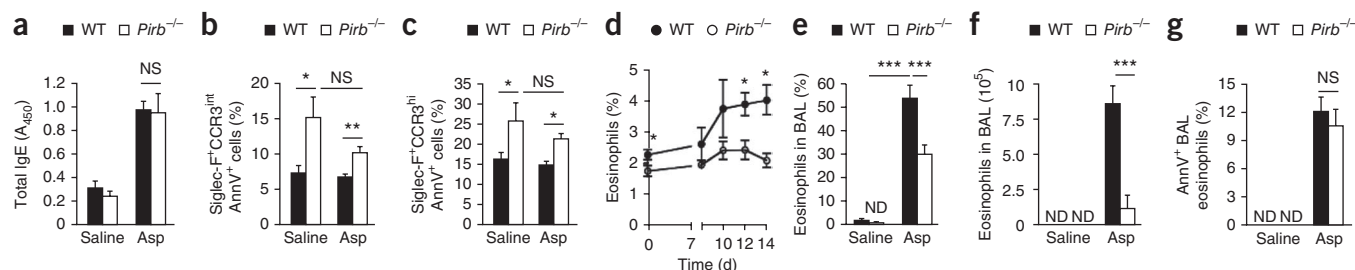


Figure 7 *Pirb*^{-/-} mice display increased BM eosinophil apoptosis as well as decreased PB and tissue eosinophilia after mucosal aeroallergen challenge. (a) Total serum IgE in peripheral blood of wild-type (WT) and *Pirb*^{-/-} mice challenged six times with saline or *A. fumigatus* (Asp). (b,c) Frequencies of annexin V⁺ (AnnV) cells in cells obtained from BM of indicated mice and stained with anti-Siglec-F, anti-CCR3 antibodies and annexin V. Siglec-F⁺CCR3^{int} and Siglec-F⁺CCR3^{hi} cells were gated. (d-f) Saline- and Asp-challenged WT and *Pirb*^{-/-} mice assessed for allergen-induced PB eosinophils (d), bronchoalveolar lavage fluid (BALF) eosinophil percentages (e) and total BALF eosinophils (f). (g) Eosinophils from BALF of saline and Asp-challenged mice assessed for annexin V. ND, not detected. NS, not significant; **P* < 0.05, ***P* < 0.01, ****P* < 0.001 (one-way ANOVA followed by Tukey *post hoc* test (a–c,e) and Student's *t*-test (d,f,g)). Data are pooled from five experiments using three mice per saline treatment and seven mice per allergen treatment for each genotype (error bars, s.e.m.; *n* = 5 experiments).

and eosinophils displayed higher amounts of PIR-B in comparison with PIR-A (Fig. 6j). Therefore, although PIRs compete and bind the same ligands¹³, proapoptotic signaling by PIR-A in eosinophils was constitutively balanced by inhibitory signals mediated by PIR-B, owing to its relatively higher expression.

PIR-B is required for allergic airway inflammation

Regulation of eosinophil development by PIRs suggested a role for PIRs in allergic eosinophilic airway inflammation. Therefore, we sensitized and exposed wild-type and *Pirb*^{-/-} mice to extracts of the aeroallergens *A. fumigatus* or house dust mite. We chose these models because mucosal aeroallergen-challenged *Pirb*^{-/-} mice displayed similar induction of IgE compared to wild-type mice (Fig. 7a). In contrast, systemic chicken egg ovalbumin and alum sensitization of *Pirb*^{-/-} mice results in increased total production of IgE, which is likely due to the dysregulation of DC functions¹⁶ (Supplementary Fig. 7a). Mucosal aeroallergen challenge had no effect on the apoptotic frequency of wild-type BM eosinophils (Fig. 7b,c). However, it was not sufficient to fully rescue BM *Pirb*^{-/-} eosinophils from apoptosis (Fig. 7b,c). Subsequently, aeroallergen-challenged *Pirb*^{-/-} mice displayed a significant reduction in aeroallergen-induced PB eosinophilia and had less eosinophilic infiltrates in bronchoalveolar lavage fluid (Fig. 7d–f). This phenotype was not due to increased local eosinophil cell death in the lungs as *Pirb*^{-/-} mice displayed similar frequencies of annexin V⁺ eosinophils as wild-type mice (Fig. 7g). Decreased lung eosinophilia in aeroallergen-challenged *Pirb*^{-/-} mice was also associated with a minor but significant decrease in accumulation of CD4⁺ T cells (*P* < 0.01, one-way ANOVA followed by Tukey *post hoc* test) and decreased expression of T_H2 cytokines and chemokines, including IL-4, IL-13 and CCL17 (Supplementary Fig. 7b–e). Decreased eosinophilia and T_H2 cell-associated allergic airway disease was not an allergen-specific phenomenon because *Pirb*^{-/-} mice challenged with house dust mites also exhibited less lung eosinophil infiltration (Supplementary Fig. 7f,g).

As PIR-B is also expressed in myeloid cells other than eosinophils, impaired lung eosinophilia in aeroallergen-challenged *Pirb*^{-/-} mice could result from deficiency in these cells. To specifically probe for the involvement of CD11c⁺ myeloid cells in the impaired response of the *Pirb*^{-/-} mice to the aeroallergen challenge, we generated mice that harbor a specific PIR-B deficiency in their CD11c⁺ cell compartment (including lung DCs and alveolar macrophages). To this end, we generated mixed BM chimeras^{33,34} using BM obtained from mice expressing the diphtheria toxin receptor under the regulation

of the *Itgax* promoter (*CD11c-DTR*) and *Pirb*^{-/-} BM cells. This resulted in mice that, upon treatment with diphtheria toxin, retained a *Pirb*-deficient DC and macrophage compartment, whereas other hematopoietic cell populations retained their mixed wild-type and *Pirb*^{-/-} composition (Supplementary Fig. 7h–j). Restricted absence of PIR-B from CD11c⁺ myeloid cells did not affect the ability of the mice to respond to *A. fumigatus* as *CD11c-DTR Pirb*^{-/-} chimeric mice displayed expression of IL-4 and CCL17 similar to that in *CD11c-DTR*-wild-type control chimeras (Supplementary Fig. 7k,l). Taken together, these data demonstrate that decreased eosinophil accumulation and T_H2 cell-associated inflammation is not due to the expression of PIR-B in CD11c⁺ cells; rather it is likely an eosinophil-dependent phenomenon.

DISCUSSION

The ability to discriminate 'self' from 'non-self' via binding of MHC class I molecules is a major immunological concept leading to tolerance of cytotoxic T cells and NK cells³⁵. However, myeloid cells are also capable of self-recognition through PIR-A and PIR-B, which elicit activating and inhibitory signaling, respectively¹¹. In this study, we dissected the function of the MHC class I binding receptors PIR-A and PIR-B in eosinophil maturation and subsequent allergic airway responses, and demonstrated key roles for PIRs in IL-5-induced expansion of eosinophils.

IL-5 has an exclusive requirement for the rapid expansion of eosinophils especially in disease settings such as asthma²⁷. Our findings demonstrate that eosinophilia regulated by IL-5 is governed by a constant 'tugging war' with PIR-A. T_H2 settings (such as those observed in asthma) substantially increase the ability of IL-5 to induce eosinophilia by strengthening the 'positive' signals for eosinophil development. Eosinophils likely escape their apoptotic fate, which is regulated by PIR-A, exit the BM and enter the PB, consequently migrating into the inflamed tissue. PIR-B, which is hierarchically dominant to PIR-A, serves as a 'permissive' checkpoint for IL-5-induced development of eosinophils by suppressing the activities of PIR-A. Thus, a constant cross-talk between PIR-A (acting as a proapoptotic 'vector') and IL-5 and PIR-B (acting as anti-apoptotic 'vectors') exists in eosinophils. Collectively, our model demonstrates that upon self-recognition, PIR-B is a negative regulator of PIR-A-induced apoptosis, which counterbalances expansion signals mediated by IL-5 (Supplementary Fig. 8).

Various candidate 'self'-recognizing molecules are widely (and sometime exclusively) expressed by myeloid cells including eosinophils. These include PIRs^{19,36}, CD200 molecules, Siglec-family

receptors and CD47, which interacts with signal regulatory protein- α (SIRP- α , also known as CD172)³⁷. Despite this, the requirement for self-recognition in eosinophil activities is unknown. Our data demonstrate that inhibitory signaling by PIR-B is dominant over PIR-A-induced eosinophil apoptosis and raises the question of how this can occur if they both bind similar ligands? One evident explanation for the relative dominance of PIR-B over PIR-A is the finding that eosinophils express higher amounts of PIR-B than PIR-A and therefore MHC class I binding will lead to more inhibitory signals than proapoptotic signaling, consequently maintaining a high eosinophil survival rate. IL-5 neutralization caused a specific decrease in expression of PIR-B and shifted the balance toward dominant expression of PIR-A relative to PIR-B. Conversely, administration of IL-5 resulted in a compartmentalized increase in expression of PIR-B (and to lesser extent PIR-A) in BM eosinophils. These data suggest that PIRs constitute an additional 'fine-tuning' mechanism that regulates IL-5-driven eosinophilia. Hence, increased amount of IL-5 will result in decreased apoptosis owing to increased prosurvival signals that are directly mediated by the IL-5 receptor and decreased apoptosis owing to increased expression of PIR-B, which suppresses PIR-A-induced apoptosis. Similarly, the absence of IL-5 renders eosinophils apoptotic owing to a lack of survival signals directly mediated by the IL-5 receptor and increased apoptotic signaling by PIR-A owing to decreased expression of PIR-B and hence lack of inhibition.

We have recently established that *Pirb*^{-/-} mice display increased tissue eosinophilia in the gastrointestinal tract¹⁹ and adipose tissue (data not shown). Thus, despite increased apoptosis of eosinophils in the BM of *Pirb*^{-/-} mice, steady-state eosinophil numbers in the gastrointestinal compartment are elevated. This is likely due to the fact that the regulation of eosinophil expansion by PIRs is restricted to the BM compartment. Once eosinophils 'escape' the developmental regulation by PIRs and enter the blood, PIR-B can suppress eosinophil CCR3 signaling and subsequent eosinophil migration¹⁹. Therefore, tissues that display a constant eotaxin gradient, such as the gastrointestinal tract and adipose tissue, will display heightened eosinophilia in the absence of PIR-B^{6,38}. Indeed, intratracheal administration of IL-13 that generates a strong chemotactic gradient for eosinophil recruitment (but not IL-5-dependent generation of eosinophils in the BM) results in increased lung eosinophil accumulation in *Pirb*^{-/-} mice¹⁹. In contrast to the case with administration of IL-13, under typical T_H2 settings rapid IL-5-induced generation of eosinophils in the BM occurs, and PIR-B is required for eosinophil generation and subsequent lung infiltration. Although we cannot entirely exclude the involvement of additional mechanisms governed by PIR-B in T_H2 settings, the finding that the expression of PIR-B in the CD11c⁺ myeloid cell compartment is dispensable for decreased T_H2 cell-associated disease in *Pirb*^{-/-} mice and the finding that mucosal sensitization was similar between wild-type and *Pirb*^{-/-} mice strongly suggest that decreased CD4⁺ T cell accumulation and expression of T_H2 cell-associated cytokines and chemokines is eosinophil-dependent. In support of this notion, numerous studies demonstrate a key contribution for eosinophils in the development of allergic asthma partially by their ability to regulate local lung responses of T_H2 cells and DCs^{5,39-41}.

Our data suggest that PIR-B does not regulate IL-5-induced expansion of eosinophils by direct interactions with IL-5 receptor signaling components (similar to the way PIR-B regulates GM-CSF and/or IL-3-induced responses^{16,17}). This finding is of particular interest because it is different than the current reported interactions of other ITIM-bearing receptors with IL-5 receptor. For example, IL-5 primes the proapoptotic effects of Siglec-8 (ref. 42), and increased expression

of IL-5 potentiates the suppression of IL-5-induced survival by CD300a⁴³. The aforementioned data suggest direct biochemical interactions of Siglec-8 and CD300a with the IL-5 receptor. In addition, we demonstrated that PIR-A associated with the adaptor molecule Grb2, an upstream regulator of the Mek-Erk pathway³². This interaction is likely mediated via the γ -chain of Fc receptors, which is required for surface expression of PIR-A¹², has been shown to interact with Grb2 (ref. 44) and can mediate proapoptotic signaling⁴⁵. Similar to our findings that suggest a role for Erk in PIR-A-mediated apoptosis of eosinophils, Erk activation has been identified as critical in delivering proapoptotic signals via Siglec-8 in IL-5-activated eosinophils⁴².

In summary, we provided evidence that self-recognition of MHC class I molecules by PIRs has a critical role in development of eosinophils and consequent expansion in settings of allergic airway disease. These data led us to a new model of the molecular pathways regulating eosinophilia and have broad implications for eosinophilic diseases.

METHODS

Methods and any associated references are available in the [online version of the paper](#).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

N.B.B.-M., D.S., I.M., M.L., D.K.-A., C.B., P.C.F., D.R. and A.M. did *in vitro* and *in vivo* experiments and analyzed data. M.E.R. and S.J. provided critical reagents and analyzed data. A.M. supervised the study, and wrote and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. Male and female 6–8-week-old *Pirb*^{-/-} mice (backcrossed for more than nine generations to C57BL/6)¹⁶, CC10-*Il13*^{Tg} and MHC class I-deficient (*B2m*^{-/-}) mice have been described elsewhere^{46,47}. C57BL/6 wild-type (WT) mice were obtained from Harlan Laboratories. 6-week-old CD11c-diphtheria toxin receptor (DTR) transgenic mice (B6.FVB-Tg(Itgax-DTR/GFP)57Lan/J) were used for generation of BM chimeras³³. In all experiments, age-matched and gender-matched mice were housed under specific pathogen-free conditions according to Tel-Aviv University and/or Weizmann Institute Animal Care Committee approved protocols. Animal studies were conducted using experimental protocols which were approved by the Tel Aviv University's Animal Care and Use Committee (Protocol number M-13-030). Sample size was chosen according to previous experience conducting similar experiments. Animals were randomly chosen for each experimental group, and no blinding was used.

Eosinophil bone marrow culture. Eosinophils were grown from the BM of WT and *Pirb*^{-/-} mice with modifications based on a prior report²⁰. Briefly, BM cells were harvested and loaded on a histopaque gradient (Sigma). LDBM cells were collected and cultured in the presence of SCF and Flt3L for 4 d. Thereafter, the medium was replaced with IL-5 for the rest of the culture (up to day 14). All cytokines were purchased from Peprotech.

Cell proliferation. Cell proliferation was assessed using the Click-iT EdU kit (Invitrogen) according to the manufacturer's instructions.

Allergen sensitization and challenge. Allergic eosinophilic airway inflammation was induced by challenging mice intranasally with either *A. fumigatus* or house dust mite (Bayer Pharmaceuticals), three times a week for 2–3 weeks as previously described¹⁹. In brief, mice were lightly anesthetized by isoflurane inhalation, and 10 µg of total *A. fumigatus* protein extract in 50 µl of saline was applied to the nasal cavity using a micropipette with the mouse held in the supine position. After instillation, mice were held upright until they were alert. Mice were killed 24–48 h after the last challenge, and bronchoalveolar lavage was performed and fluid was assessed for differential cell counts and mediator content as described¹⁹. The concentration of lipopolysaccharide in the *A. fumigatus* extracts was less than 2 pg/ml as detected by the Limulus assay.

IL-5 administration and neutralization. WT and *Pirb*^{-/-} mice were treated with recombinant mouse IL-5 (5 µg/mouse in 200 µl saline) or anti-IL-5 (Biolegend, TRFK5 antibody, 50 µg/mouse in 200 µl saline) for five consecutive days. Thereafter, the mice were killed, and BM and blood were obtained for flow cytometry analyses.

Real-time quantitative PCR. RNA samples from the whole lung were subjected to reverse transcription analysis using SuperScript II reverse transcriptase (Invitrogen) according to manufacturer's instructions. Quantitative PCR analysis was performed using the CFX96 system (Bio-Rad laboratories) in conjunction with the ready-to-use fast-start SYBR Green I Master reaction kit (Roche Diagnostic Systems). Results were normalized to *Hprt* cDNA as previously described²⁴. The primers that were used in this study were: *Il4* forward (Fwd), TCAACCCCCAGCTAGTTGTC and reverse (Rev), TGTTCTTCGTTGCTGTGAGG; *Mbp* Fwd, CAAAGCTCAGTCAGTTGCC and Rev, ATCGAAAGCGTTTGCATCGG; *Gata1* Fwd, TCAAGTCCATCAGGTGAACC and Rev, TCCCTTTGCCAGATGCCTT; *Sp1* (*Pu.1*) Fwd, CCCGGATGTGCTTCCCTTAT and Rev, TCCAAGCCATCAGCTTCTCC; *Fog1* Fwd, AGCTGTGACCTGATGGTGG and Rev, GGCCTCATCTCTCTGTAGATCT; *Gata2* Fwd, AGACGACAACCACACCTTA and Rev, TCCTTCTTCATGGTCAGTGG; *Cebpa* Fwd, GTTAGCCATGTGGTAGGA GACA and Rev, CCCAGCCGTTAGTGAAGAGT; *Bcl2* Fwd, GTCCCGCC TCTTACCTTTTTCAG and Rev, GATTCTGGTGTTCCTCCCGTTGG; *Bax* Fwd, GCGTGGTTGCCCTCTTCTACTTTG and Rev, AGTCCAGTGTC CAGCCCATGATG; *Bim* Fwd, CGACAGTCTCAGGAGGAACC and Rev, CCTTCTCCATACCAGACGGA; *Bid* Fwd, TGGAAAGACCTTGGCCTGA AGACA and Rev, AAGGTACATGGTGTGGATGCAGGA; *Bad* Fwd, GAGTATGTTCCAGATCCAG and Rev, GTCCTCGAAAGGGCTAAGC; *Pirb* Fwd, GGTGACAGGACACTACTGGAACCC and Rev, CCACGAG

AGCTTCTGTGGTCTCT; *Il5ra* Fwd, ACACTTTTCCAGCATTCGGCT and Rev, TGCCTTTGCTCTTGGTCAGG; *Hprt* Fwd, GTAATGATCAGTCAA CCGGGGAC and Rev, CCAGCAAGCTTGCAACCTTAACCA.

Flow cytometry. Flow cytometry analysis of LDBM cells and enzymatically digested lung or bronchoalveolar lavage cells was conducted using the following antibodies (all purchased from eBioscience unless stated otherwise): anti-PIR-A/B (10-1-PIR, 1:660), anti-CD62L (MEL-14, 1:100), anti-β7-FITC (FIB27, Biolegend, 1:200), anti-α₅-FITC (HMA5-1, 1:50), anti-CD48 (HM-48-1, 1:100), anti-CD18 (M18/2, 1:200), anti-CD69 (H1.2F3, 1:100), anti-CD103 (2E7, 1:80), anti-F4/80 (BM8, 1:200), anti-CD86 (GL1, 1:160), anti-MHC class II (NIMR-4, 1:660), anti-PIR-A/B (6C1, BD Bioscience, 1:100) anti-CD45 (30-F11, 1:50), anti-CD11c (N418, 1:200), anti-CD11b (M1/70, 1:140), anti-B220 (RA3-6B2, 1:100), anti-Gr-1 (RB6-8C5, 1:200), anti-CD3 (145-2C11, 1:100), anti-CD3 (17A2, 1:800), anti-CD4 (GK1.5, 1:100), anti-CD8 (SK1, 1:80), anti-Siglec-F (E50-2440, BD Bioscience, 1:100) and anti-CCR3 (83101, R&D Systems, 1:100). Events were acquired by a Gallios flow cytometer system (Beckman Coulter) or FACScalibur (BD Bioscience), and data were analyzed using the Kaluza (Beckman Coulter) or FlowJo (TreeStar) software on at least 10,000–50,000 events. Surface molecule expression was calculated by defining the delta mean fluorescence intensity between the specific antibody stain and the isotype-matched control antibody. All sorting experiments were performed using FACSaria III (BD Bioscience) in the flow cytometer core of the Tel Aviv University. PIR-A and PIR-B expression was calculated by defining the delta mean fluorescence intensity between anti-PIR-A/B stain and isotype control. The expression of PIR-A was deduced by staining WT and *Pirb*^{-/-} cells with anti-PIR-A/B, which enabled us to determine the relative expression of PIR-A (present on the surface of *Pirb*^{-/-} cells versus PIR-A and PIR-B on the surface of WT cells as described previously^{19,24}).

Immunoprecipitation and phosphorylation assays. Siglec-F⁺ annexin V⁻ cells were sorted from LDBM *Pirb*^{-/-} cell cultures and lysed in M-PER lysis buffer (Pierce Endogen) supplemented with protease inhibitor cocktail (Sigma-Aldrich) on ice for 30 min. The cell lysate was precleared and PIR-A was immunoprecipitated using Protein A/G-conjugated anti-PIR-A/B antibodies or isotype-matched control (Dynabeads, Invitrogen). Custom-designed membranes coated with various antibodies were purchased from Hydropromatrix. The immunoprecipitated PIR-A:protein complex was diluted in 2 ml of PBS and incubated with the precoated membrane. After washing, the membrane was incubated with anti-mouse phosphotyrosine conjugated to horseradish peroxidase (pY99; Santa Cruz Biotechnology)¹⁹. The membrane was developed using enhanced chemiluminescence (ECL)-plus (GE Healthcare).

Colony formation assays. A total of 1 × 10⁵ BM-nucleated cells in a volume of 0.1 ml was plated in 0.9 ml methylcellulose medium (MethoCult 3234, Stem Cell Technologies), supplemented with 20 ng/ml mouse rIL-5 (50 ng/ml), rIL-3 (20 ng/ml) or rGM-CSF (10 ng/ml) (Peprotech), and placed in a humidified incubator with 5% CO₂ at 37 °C. Colonies containing at least 50 cells were counted 10 d after incubation. The number of colonies/10⁵-nucleated bone marrow cells was calculated. Relative cell counts were determined using flow cytometry by obtaining a fixed amount of colonies and acquiring events for 120 s as described⁴³.

Annexin V apoptotic assay. Apoptosis was detected using an Annexin V apoptosis detection kit (eBioscience), according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay. Bronchoalveolar lavage fluid CCL17, IL-4 and IL-13 were assessed using a commercial ELISA kit (R&D Systems Duo Set, Lower detection limits: 15.62, 3.91 and 62.5 pg/ml, respectively). Serum IgE was measured using a commercial kit purchased from BD Bioscience according to the manufacturer's instructions. Lower detection limit for IgE was 15 pg/ml.

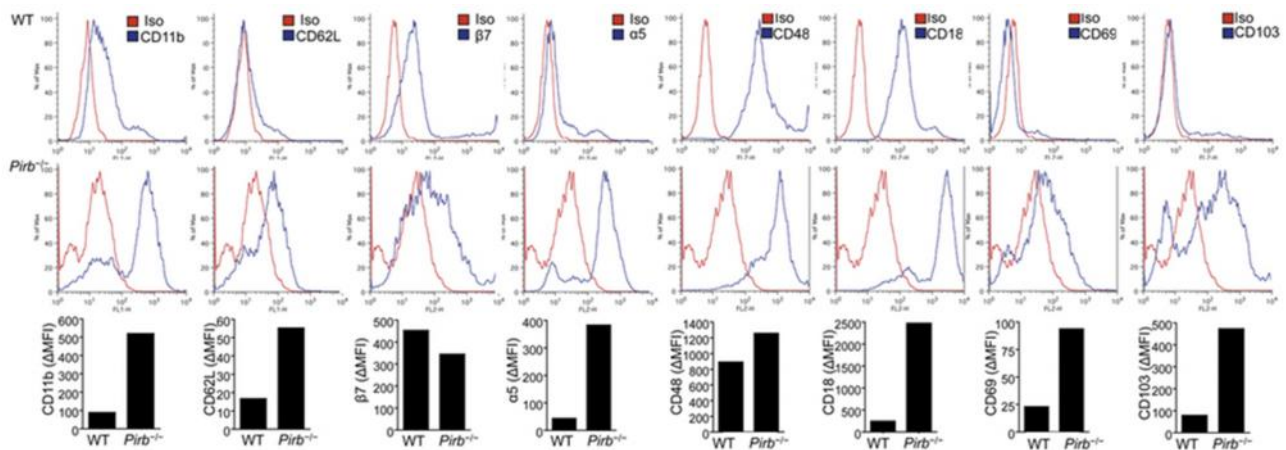
Bone marrow-derived macrophage and dendritic cell generation. Macrophages and dendritic cells were grown from the BM of WT and *Pirb*^{-/-} mice. Briefly, BM cells were harvested and cultured in the presence of M-CSF or GM-CSF (20 ng/ml, Peprotech), respectively, for 7–9 d.

Bone marrow chimera mice. Syngeneic BM chimeras were generated as described^{33,48}. Briefly, WT C57BL/6 mice were exposed to a single lethal total body irradiation of 950 rad. One day after irradiation, a 1:1 mixture of 5×10^6 BM cells obtained from CD11c-DTR mice (CD45.1⁺) and WT or *Pirb*^{-/-} BM cells (CD45.2⁺) was injected into the irradiated mice, thus generating mixed BM chimeras. Thereafter, the mice were allowed to rest for 10 weeks, and engraftment was validated in PB samples using anti-CD45.1 and anti-CD45.2 staining. After confirmation of engraftment, the mice were challenged with *A. fumigatus* extract as described above. To deplete CD11c⁺ cells, diphtheria toxin (8 ng/g body weight) was injected (intraperitoneally) every other day starting 24 h before the initial allergen challenge. CD11c⁺ cell depletion was monitored by flow cytometry using anti-CD45.1 and anti-CD11c staining.

Statistical analysis. Data were analyzed by ANOVA followed by Tukey *post hoc* test using GraphPad Prism 4. Alternatively, several experiments were analyzed by Student's *t*-test. Data are presented as mean $\pm$ s.e.m., and values of $P < 0.05$ were considered significant.

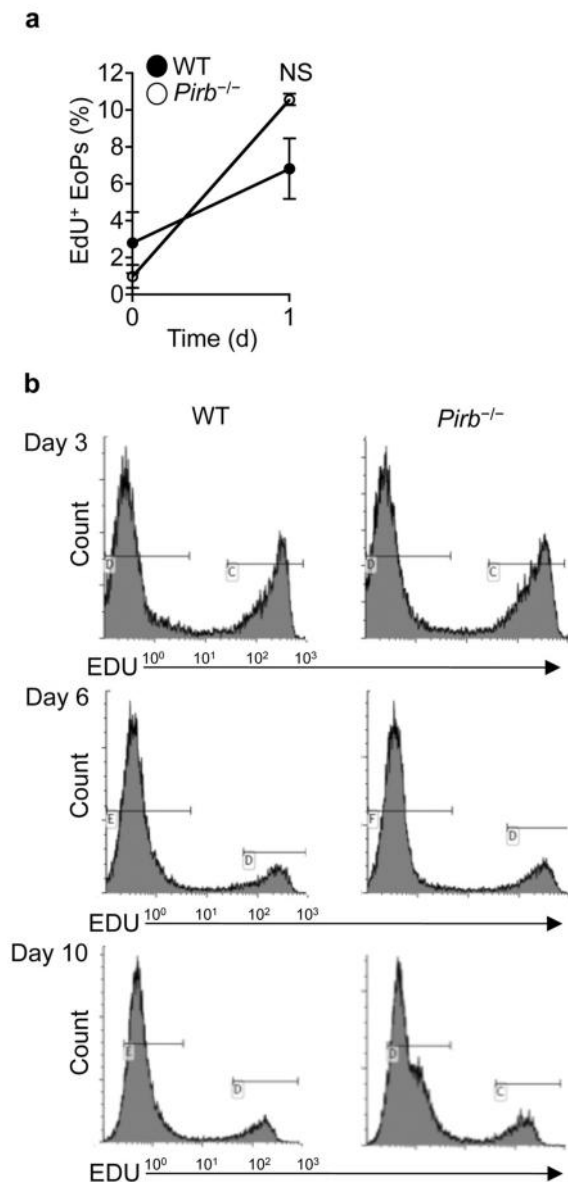
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Supplementary Figure 1: Expression of surface activation markers and adhesion molecules in *Pirb*^{-/-} LDBM cells.



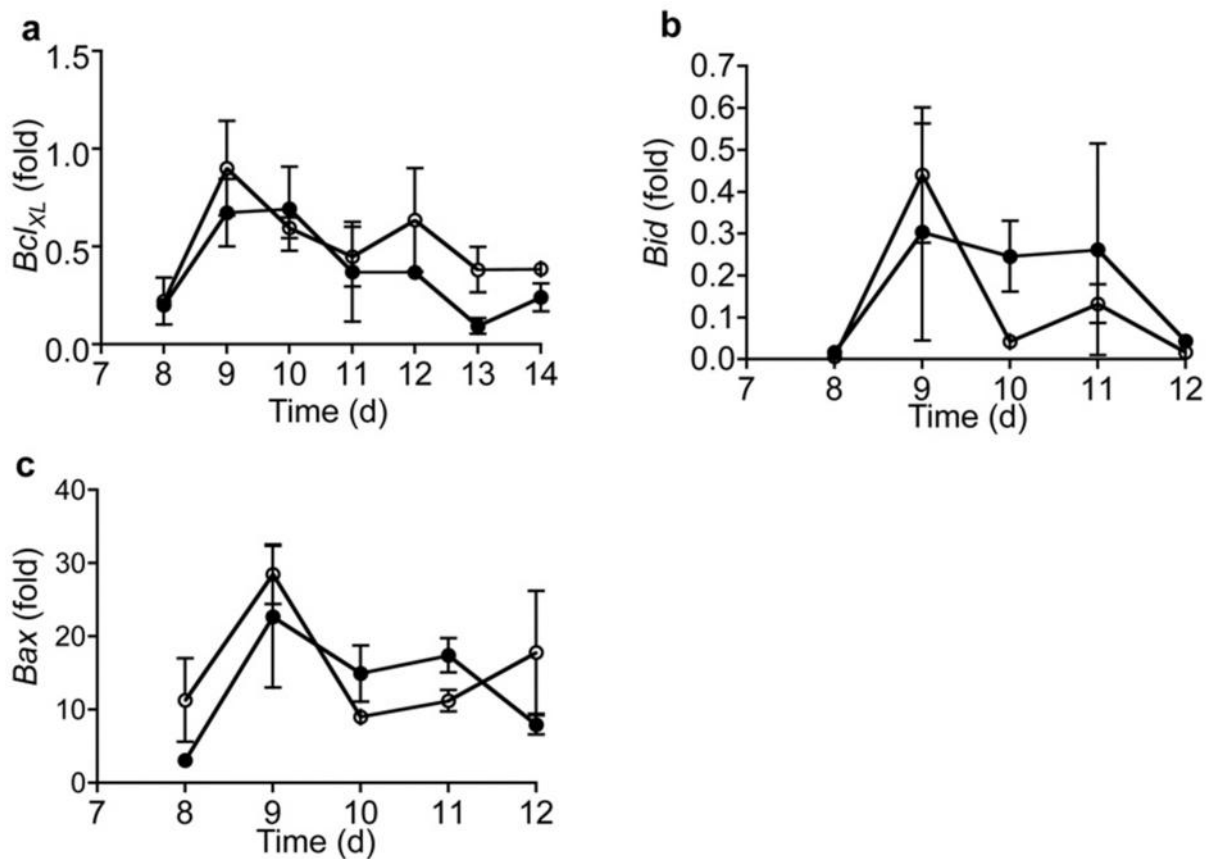
Expression of surface activation markers and adhesion molecules in *Pirb*^{-/-} LDBM cells. LDBM cells were obtained from wild-type (WT) and *Pirb*^{-/-} mice and differentiated in-vitro into eosinophils. At day 14 of the culture, cells were stained with the indicated antibodies and isotype matched control. Assessment of surface expression was conducted by flow cytometric analysis of at least 10,000 events. Delta mean fluorescent intensity (ΔMFI) was calculated by subtracting the background isotype matched control staining. Data are representative histogram plots from 3 independent experiments.

Supplementary Figure 2: Assessment of cell proliferation in *Pirb*^{-/-} LDBM cell cultures.



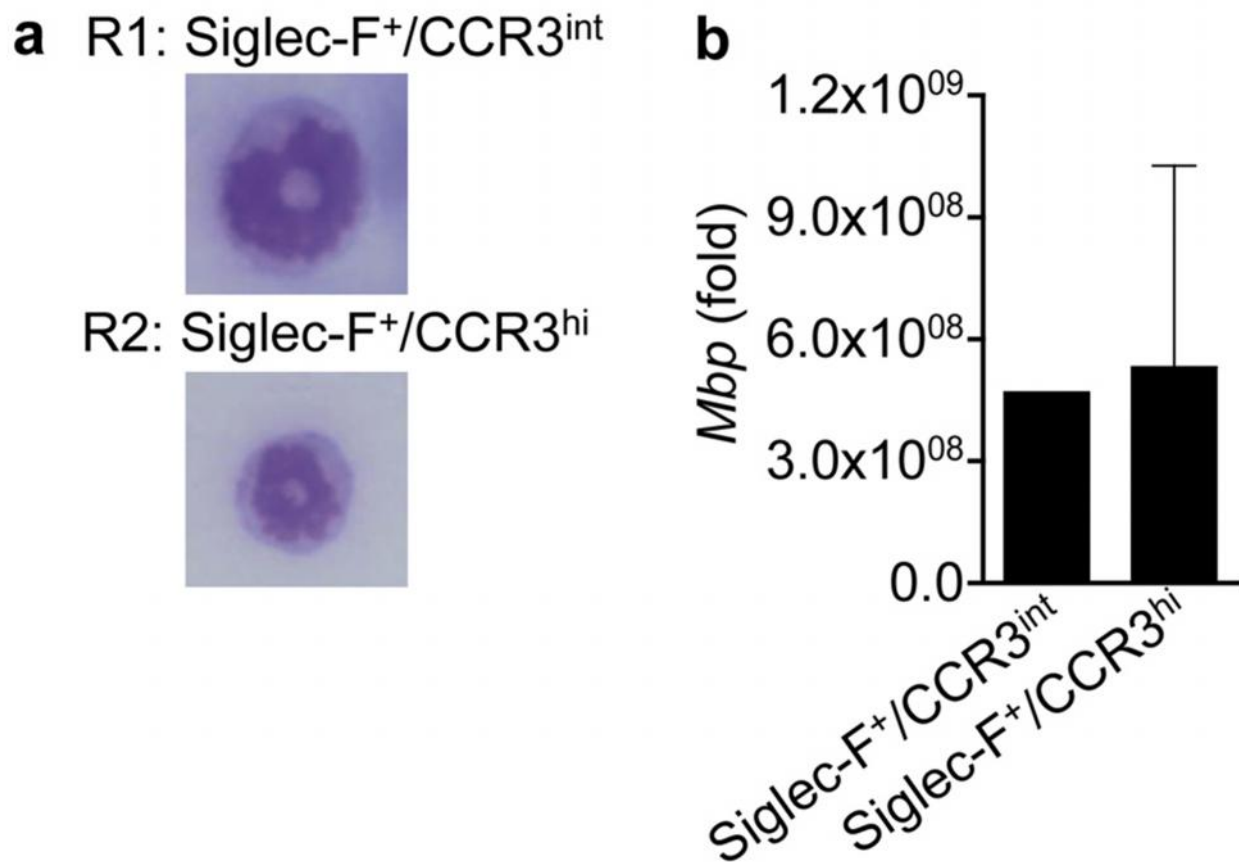
Assessment of cell proliferation in *Pirb*^{-/-} low-density bone marrow cell cultures. (a) Low-density bone marrow (LDBM) cells were obtained from wild-type (WT) and *Pirb*^{-/-} mice and differentiated in vitro into eosinophils. At the indicated time points, cellular proliferation was assessed in eosinophil progenitors EoPs, (defined as Sca1-CD34+Lin-C-KitintIL-5R α + cells) (b) or the general cell population using EdU. Data are representative of at least three independent experiments.

Supplementary Figure 3: Assessment of pro- and anti-apoptotic molecule expression in Pirb^{-/-} LDBM cell culture.



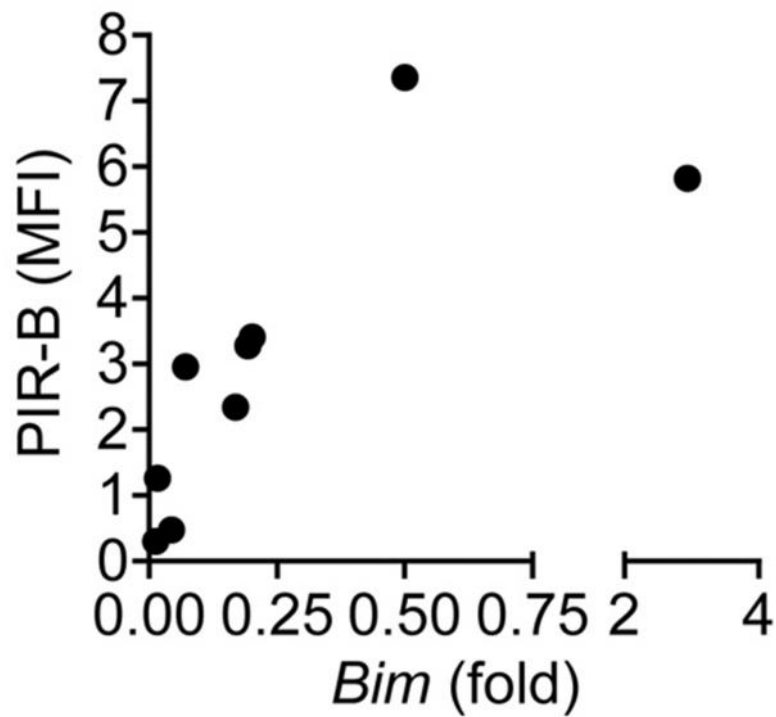
Assessment of pro- and anti-apoptotic molecule expression in Pirb^{-/-} low-density bone marrow cell culture. The expression of (a) BclXL, (b) Bax and (c) Bid was assessed in cDNA obtained from low-density bone marrow (LDBM) cells from wild-type (WT) and Pirb^{-/-} mice by qPCR analysis. Gene expression was normalized to the house keeping gene hypoxanthine-guanine phosphoribosyltransferase (Hprt). Data are representative of LDBM cultures from n=3 mice.

Supplementary Figure 4: Siglec-F+CCR3^{int} and Siglec-F+CCR3^{hi} BM cells display eosinophil morphology and granule proteins.



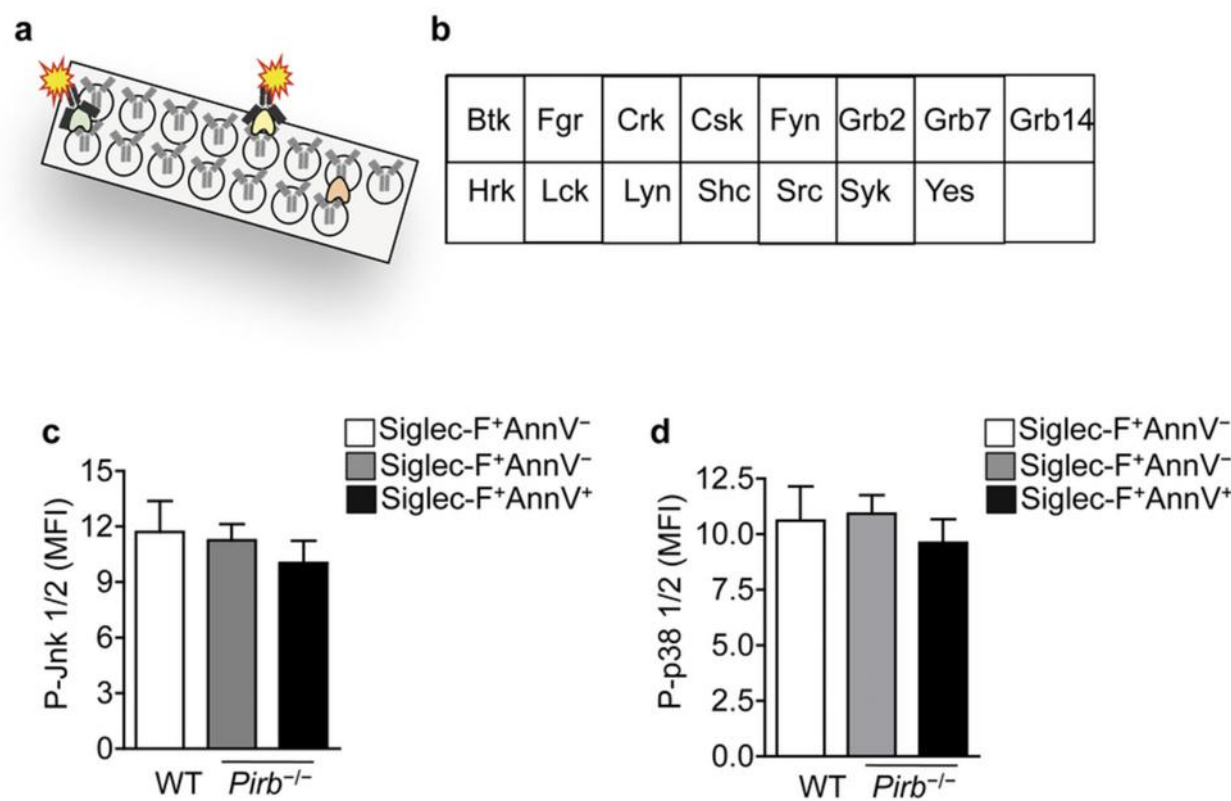
Siglec-F+CCR3^{int} and Siglec-F+CCR3^{hi} bone marrow cells display eosinophil morphology and granule proteins. (a) Bone marrow cells from naïve wild-type mice were obtained and stained with anti-Siglec-F and anti-CCR3. Thereafter, Siglec-F+CCR3^{int} and Siglec-F+CCR3^{hi} cells were sorted, cytopspins prepared and stained with modified Wright Giemsa stain. (b) The expression of eosinophil major basic protein (Mbp) was determined using qPCR. Gene expression was normalized to the house keeping gene hypoxanthine-guanine phosphoribosyltransferase (Hprt). Data are representative of LDBM cultures from n=3.

Supplementary Figure 5: Correlation analysis between PIR-B and Bim expression.



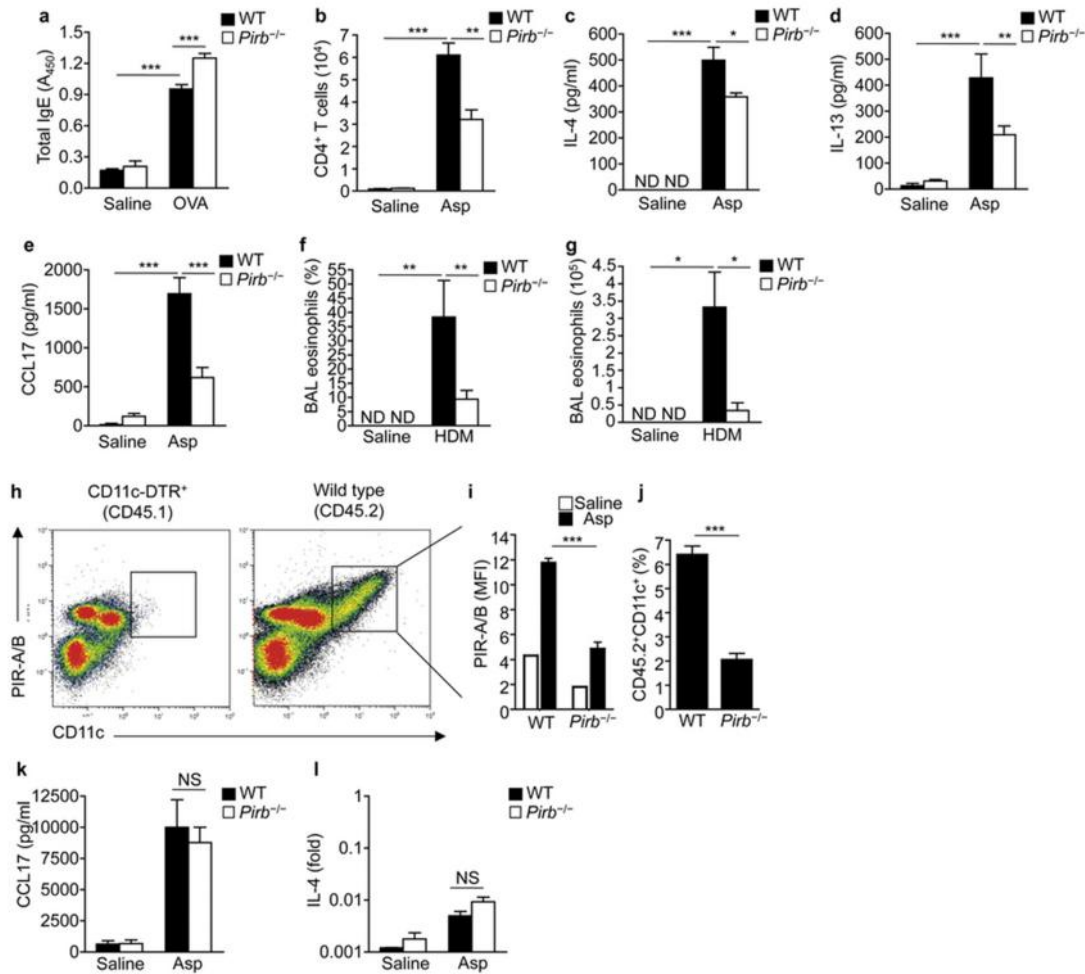
Correlation analysis between PIR-B and Bim expression. The expression of PIR-B was assessed throughout the low-density bone marrow (LDBM)-derived eosinophil culture using flow cytometric analysis at the indicated time points. Pearson correlation between PIR-B and Bim expression is shown ($r = 0.47$, $P = 0.31$).

Supplementary Figure 6: Miniphosphorproteomics and assessment of phospho JNK and p38 in BM eosinophils.



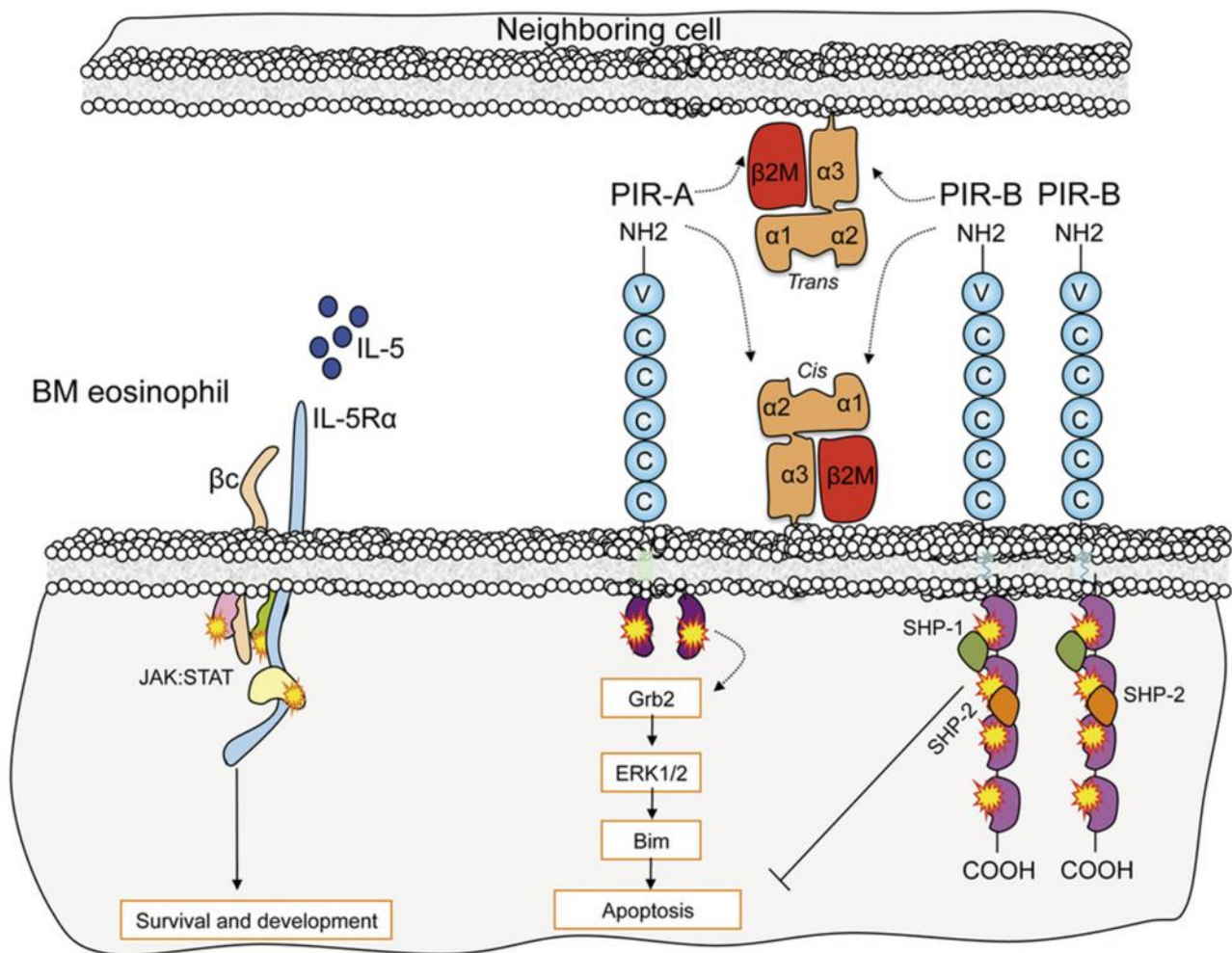
Miniphosphorproteomics and assessment of phospho JNK and p38 in BM eosinophils. (a) A custom-made membrane coated with various antibodies recognizing several kinases and/or adaptor molecules was used to determine the interactions between PIR-A and (b) selected phosphorylated downstream signaling intermediates. (c-d) Wild-type (WT) and *Pirb*^{-/-} BM cells were stained with anti-Siglec-F, Annexin-V and anti-phospho(p)-JNK or anti-phospho(p)-p38. Thereafter, the mean fluorescence intensity of (c) phospho-Jnk 1/2 (P-Jnk 1/2) and (d) phospho-p38 (P-p38) was assessed by flow cytometry.

Supplementary Figure 7: PIR-B is required for development allergic airway inflammation.



PIR-B is required for development allergic airway inflammation. (a) Wild-type (WT) and *Pirb*^{-/-} mice were sensitized with Alum+OVA on days 0 and 14. On day 24, serum was obtained; serially diluted and total IgE was assessed. (b-e) WT and *Pirb*^{-/-} mice were challenged with allergen extracts of *Aspergillus fumigatus* (Asp) (f-g) or house dust mite (HDM). Thereafter, (b) the frequencies of CD3⁺CD4⁺ T cells and expression of (c) IL-4, (d) IL-13 and (e) CCL17 were assessed. Bronchoalveolar lavage fluid was obtained from HDM-challenged mice and (g) eosinophil percentages and (h) total numbers assessed. (i-l) Mixed bone marrow (BM) chimeric mice that harbor a specific deletion of PIR-B in their CD11c⁺ cell component were generated. Following adoptive transfer, engraftment of mixed BM cells from WT, *Pirb*^{-/-} and CD11c-diphtheria toxin receptor (CD11c-DTR) mice were assessed using anti-CD45.1 and anti-CD45.2 staining. Ablation of lung CD11c⁺ cells following diphtheria toxin treatment was examined. Single cell suspensions from the lungs of chimeric BM mice were obtained and (i) PIR-A/B expression as well as (j) frequencies of CD45.2⁺ cells assessed. Chimeric BM mice were intranasally challenged with saline or *Aspergillus fumigatus* (Asp) and (k) CCL17 was measured using ELISA and (l) lung IL-4 expression was assessed using qPCR analysis. Data are representative of at least two independent experiments using seven mice per group; NS-non significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Supplementary Figure 8: Schematic presentation of the proposed function of PIRs in eosinophil expansion.



Schematic presentation of the proposed function of PIRs in eosinophil expansion. Exposure of eosinophils to IL-5 in the bone marrow induces eosinophil growth and expansion by delivering survival signals. In contrast to IL-5, self-recognition via PIR-A induces pro-apoptotic signaling in eosinophils and counter regulates IL-5-driven responses. PIR-B, which is expressed in eosinophils in higher levels than PIR-A, suppresses the pro-apoptotic signaling driven by PIR-A, via a pathway that likely involves activation of Bim and ERK. Thus, PIR-B serves as a permissive checkpoint for IL-5 induced eosinophil expansion.