



Research Article

Murine eosinophil development and allergic lung eosinophilia are largely dependent on the signaling adaptor GRB2

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Eosinophils are innate effector cells associated with allergic inflammation. Their development and survival is largely dependent on IL-5 and the common beta chain (β_c) of the IL-5 receptor that serves as docking site for several proteins that mediate down-stream signaling cascades including JAK/STAT, PI3 kinase, NF κ B, and RAS-MAP kinase pathways. The relative contribution of these signaling pathways for eosinophil development and homeostasis in vivo are poorly understood. Here, we investigated the role of GRB2, an adaptor protein that binds to β_c and other proteins and elicits the RAS-MAP kinase pathway. By using GRB2 inhibitors and inducible deletion of the *Grb2* gene in mouse eosinophils we demonstrate that GRB2 plays a critical role for development of eosinophils from bone marrow precursors. Furthermore, *Aspergillus fumigatus*-induced allergic lung eosinophilia was significantly reduced in mice with induced genetic deletion of *Grb2*. Our results indicate that GRB2 is important for eosinophil development in steady-state conditions and during allergic inflammation. Based on these findings pharmacologic GRB2 inhibitors may have the potential to dampen tissue eosinophilia in various eosinophil-associated diseases.

Keywords: Eosinophilia · Allergy · *Aspergillus fumigatus* · IL-5 · GRB2



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Eosinophils are effector cells of the innate immune system and generally associated with type 2 immune responses directed against allergens and helminths. In addition, eosinophils have

various immunomodulatory functions [1]. They have been shown to promote survival of plasma cells in the bone marrow [2], IgA production in the gut [3], polarization of M2 macrophages in adipose tissue [4], repair of muscle and liver tissue [5, 6], thermogenesis [7], protection against tumors [8, 9], and blood

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coagulation [10] among other functions. IL-5 is the most important cytokine that drives eosinophilia by promoting de novo production in the bone marrow but also by inhibiting apoptosis of mature eosinophils [11–13].

The IL-5 receptor is composed of an IL-5-specific alpha chain and the common beta chain (β_c) that is also part of the receptors for IL-3 and GM-CSF. Mice that lack IL-5 or the β_c chain show reduced eosinophil levels demonstrating the important role of IL-5 and the β_c chain for eosinophil development [13–15]. The β_c chain contains a long cytoplasmic tail with several signaling motifs that can be phosphorylated to serve as docking sites for proteins that mediate downstream signaling events. Signaling pathways that have been described include JAK/STAT, RAS-MAP kinase, PI3 kinase, and NF- κ B pathways [16]. By generating mice that lack I κ B α specifically in eosinophils we could recently show that constitutive activation of the NF- κ B pathway in eosinophils promotes their survival in vivo [17]. The MAPK signaling pathway can be initiated by JAK2-mediated phosphorylation of Tyr 577 of the human β_c chain leading to recruitment of the adaptor protein SHC [18] that interacts with the growth factor receptor binding 2 (GRB2) adaptor protein [19]. GRB2 contains one SH2 domain flanked by two SH3 domains. The SH2 domain binds to phosphorylated tyrosines while the SH3 domains mediate the interaction with other proteins including the guanine nucleotide exchange factor SOS resulting in activation of the RAS-MAPK pathway. GRB2 has two related family members, GRAB and GRAB2, and is expressed in many different cell types. Genetic deletion of *Grb2* in mouse embryonic stem cells results in impaired embryonic development [20]. Although GRB2 is generally considered to be a signaling adaptor protein there is also evidence for a function in RNA biogenesis [21]. GRB2 can be located in the nucleus of human cell lines and transfected COS-7 cells but the role of nuclear GRB2 remains unclear [21, 22].

In addition to its interaction with SOS at the β_c chain GRB2 has been shown to bind the phosphatase PTPN11 (also named SHP-2) in IL-5-stimulated human eosinophils in vitro and inhibition or deletion of SHP-2 prevented activation of the RAS-MAPK pathway resulting in cell death [23, 24]. This suggests that GRB2 could play a critical role for promoting eosinophil development and survival in vivo. However, GRB2 may also be involved in signaling downstream of the PIR-A receptor resulting in activation of the proapoptotic molecule BIM [25]. Furthermore, GRB2-mediated activation of the RAS-MAPK pathway was shown to be involved in necrosis downstream of the SIGLEC-8 receptor in IL-5-stimulated human eosinophils [26]. Based on these observations it remains unclear whether GRB2 promotes or rather inhibits eosinophil development and survival under physiologic conditions in vivo.

To investigate the role of GRB2 in eosinophil development we inhibited the SH2 domain or genetically deleted *Grb2*. GRB2 inhibition or deletion caused increased rates of cell death and reduced proliferation in murine bone marrow cultures resulting in impaired eosinophil development. Tamoxifen-induced Cre-mediated deletion of *Grb2* in conditional knock-out mice further demonstrated that GRB2 is required for normal eosinophil development and for allergen-induced lung eosinophilia. These results

clearly demonstrate that GRB2 promotes eosinophil development in vivo suggesting that pharmacologic inhibitors targeting the SH2 domain of GRB2 might be useful for therapeutic intervention in eosinophil-associated diseases.

Results

GRB2 is highly expressed in cytoplasm and nucleus of eosinophils

To investigate the role of GRB2 in regulation of eosinophil differentiation and survival we first analyzed the expression level of GRB2 in mature eosinophils isolated from the peritoneal cavity of IL-5-transgenic mice. Intracellular staining and flow cytometric analysis revealed a high expression level of GRB2 protein in eosinophils (Fig. 1A). Based on previous reports showing GRB2 localization in the cytoplasm as well as the nucleus of human cell lines [21, 22], we performed Western blot analysis with fractionated cytoplasmic and nuclear material of eosinophils to determine the relative abundance of GRB2 in the cytoplasm (with GAPDH as reference marker) and nucleus (with LaminB2 as reference marker). Unexpectedly, comparable amounts of GRB2 were observed in cytoplasmic and nuclear fractions indicating that GRB2 protein in primary eosinophils may not only be involved in signal transduction downstream of surface receptors but also have a yet to be characterized function in the nucleus (Fig. 1B).

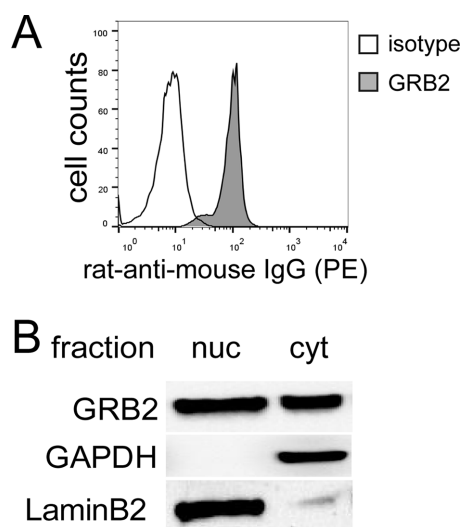


Figure 1. Expression of GRB2 in cytoplasm and nucleus of eosinophils. (A) Intracellular staining of GRB2 in murine eosinophils. Eosinophils were fixed, permeabilized, and stained with anti-GRB2 or a Mouse IgG isotype control. GRB2 expression was measured by flow cytometry. Expression level is displayed as modal. (B) Western blot for GRB2 from ex vivo isolated eosinophils. Cytosolic and nuclear fractions of eosinophils were blotted for detection of GRB2 protein. GAPDH was used as a cytosolic, Lamin B2 as a nuclear reference protein. Data are representative of three independent experiments with a total of six donor mice.

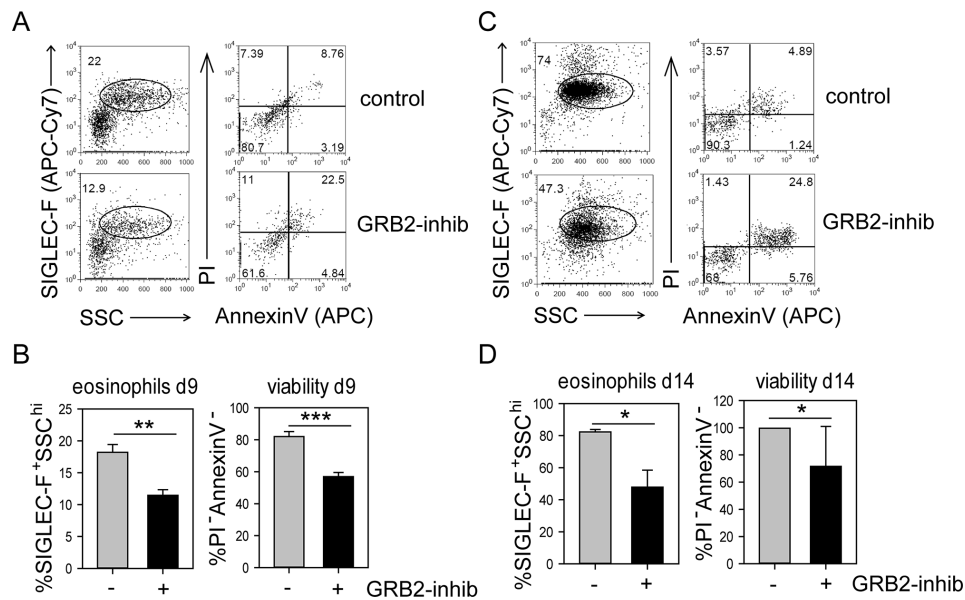


Figure 2. GRB2 inhibition affects eosinophil development. BMDE cultures were treated with the GRB2-SHC inhibitor sclerotiorin from d4 on and analyzed on d9 and d14 of the eosinophil *in vitro* differentiation protocol. (A, C) Assessment of apoptosis and cell death during eosinophil development by Annexin V/PI staining after sclerotiorin treatment. Displayed are representative flow cytometry plots of Annexin V/PI stainings from BMDE culture on d9 (A) and d14 (C), with a parental gate for eosinophils, defined as SIGLEC-F⁺ and SSC^{high}. (B) and (D) average percentage of fully differentiated eosinophils from bone marrow cultures and the percentage of nonapoptotic eosinophils on day 9 (B) and day 14 (D) treated with sclerotiorin or DMSO as a control. Bars show the mean + SEM with four samples from two experiments. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 by Student's *t*-test.

GRB2 inhibition promotes cell death of developing but not mature eosinophils

GRB2 is known to associate with the β_c chain of the IL-5 receptor and could therefore be involved in early development of eosinophils from precursor cells in the bone marrow. This assumption could be tested by culturing bone marrow cells in IL-5 containing medium in the presence or absence of the GRB2 inhibitor sclerotiorin that blocks the SH2 domain of GRB2 [27]. In these cultures, bone marrow cells were placed for 4 days in FTL3-L- and SCF-containing medium to increase the frequency of hematopoietic stem cells. On day 4 the culture conditions were changed to IL-5-containing medium to promote the development of eosinophils as previously described [28]. The GRB2 inhibitor or DMSO solvent (control) was added from day 4 onwards and samples were analyzed on day 9 and 14. Flow cytometric analysis on day 9 revealed lower frequencies of eosinophils (SIGLEC-F⁺SSC^{hi}) and less viable (Annexin V⁻/PI⁻) eosinophils in GRB2 inhibitor-treated samples as compared to controls at this timepoint (Fig. 2A, B). Eosinophils continued to develop in these cultures during the next five days so that on day 14 control cultures contained about 80% eosinophils with close to 100% viability while GRB2 inhibitor-treated cultures contained about 47% eosinophils with 65% viability (Fig. 2C, D).

To further investigate whether GRB2 is required for survival of mature eosinophils we cultured mature bone marrow-derived eosinophils with IL-5 in the presence or absence of the GRB2 inhibitor and analyzed their survival 48 hours later. Only 3% of eosinophils survived in the absence of IL-5 while about 90%

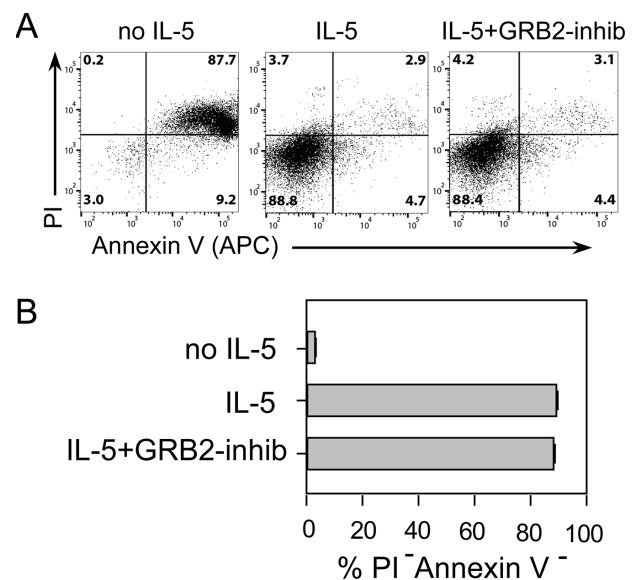


Figure 3. GRB2 inhibition does not affect viability in mature eosinophils. Fully differentiated BMDE were cultured with sclerotiorin or DMSO. As a positive control for apoptotic cells, differentiated eosinophils were cultured without IL-5 for the same time. (A) Representative Annexin V/PI dot plots from one of two independent experiments. (B) The average percentage of viable eosinophils of (A) is shown. Bars show the mean + SEM from one of two independent experiments with three samples.

viability of eosinophils was observed in cultures containing IL-5 irrespective of the presence or absence of the GRB2 inhibitor (Fig. 3).

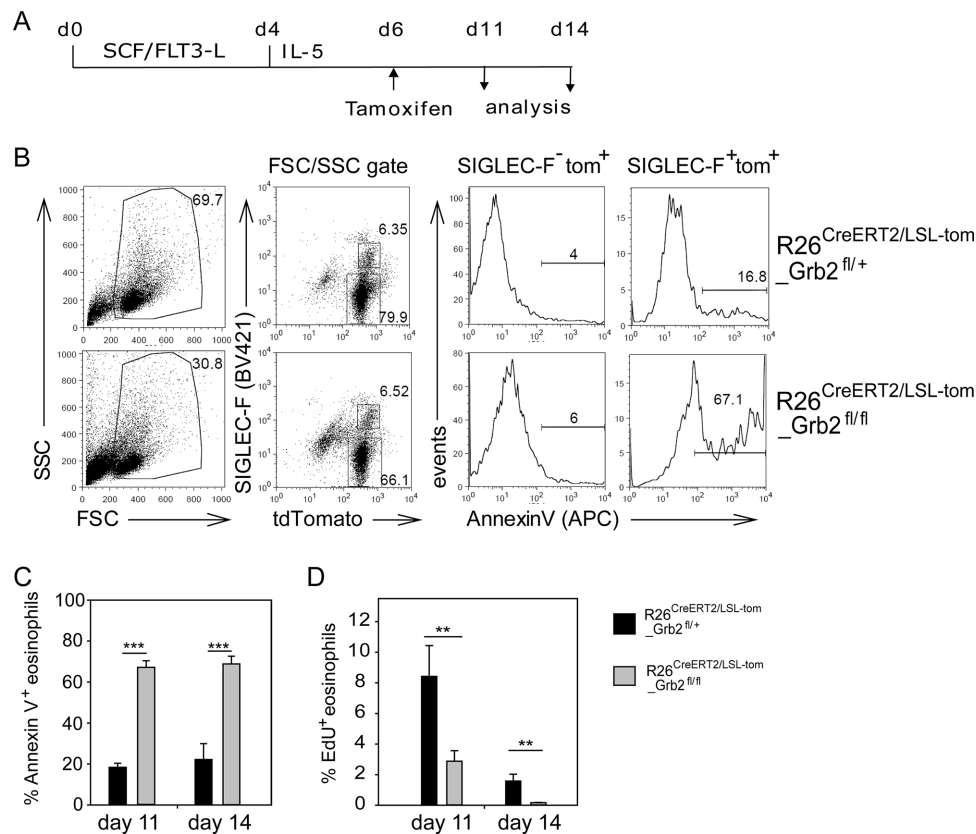


Figure 4. Cre-mediated GRB2 deletion promotes death and reduced proliferation of developing eosinophils in vitro. (A) Eosinophil differentiation protocol for tamoxifen-induced activation of the Cre recombinase in developing eosinophils derived from R26^{CreERT2/LSL-tom}_Grb2^{fl/+} and R26^{CreERT2/LSL-tom}_Grb2^{fl/fl} mice. Cells received tamoxifen on d6 and were analyzed on d11 and d14. (B) Annexin V staining of developing eosinophils treated with tamoxifen. Gates were set for tdTomato⁺ and SIGLEC-F⁺ and nonviable cells were determined by Annexin V staining. The same was done with SIGLEC-F⁻ cells as a control. Data are representative for two independent experiments. (C) Frequencies of Annexin V⁺ BMDE treated with tamoxifen. Bars show the mean + SEM from pooled data of two experiments with total $n = 7$ samples per group. *** $P < 0.001$ by Student's *t*-test. (D) Frequencies of EdU⁺ BMDE treated with tamoxifen. Bars show the mean + SD of one experiment with two biological replicates performed in triplicates, so $n = 6$ samples per group. ** $P < 0.01$ by Student's *t*-test.

Taken together, these results demonstrate that inhibition of GRB2 results in impaired eosinophil development while it had no impact on viability of mature eosinophils.

Cre-mediated deletion of Grb2 induces apoptosis in vitro

Since the GRB2 inhibitor sclerotiorin may also inhibit other proteins we decided to use a genetic *Grb2* deletion approach to further investigate the role of GRB2 for eosinophil development. Therefore we generated R26^{CreERT2/LSL-tom}_Grb2^{fl/fl} mice in which the tamoxifen-inducible Cre recombinase (CreERT2) is expressed from one allele of the Rosa26 locus while the Cre-inducible red fluorescent protein tdTomato (LSL-tom) is expressed from the other allele. In addition, these mice contain two conditional knock-out alleles for *Grb2*. Tamoxifen administration leads to activation of Cre, marks Cre-expression cells with tdTomato and deletes *Grb2* in tdTomato-positive cells. Control mice (R26^{CreERT2/LSL-tom}_Grb2^{fl/+}) contained one wild-type allele of *Grb2* that could not be removed by Cre activity. Bone marrow cells from both strains of mice

were cultured as described for Fig. 2 and tamoxifen was added on day 6 of the cultures, which were then analyzed by flow cytometry on days 11 and 14 (Fig. 4A). At the early timepoint (day 11) most of the cells in the leukocyte FSC/SSC gate expressed tdTomato and the same frequency of eosinophils (SIGLEC-F⁺) was present in both cultures (Fig. 4B). However, further analysis for cell viability revealed that the majority of eosinophils from R26^{CreERT2/LSL-tom}_Grb2^{fl/fl} mice were undergoing cell death (AnnexinV⁺) while this was not the case for eosinophils of control mice (Fig. 4B, C). Cell death could not be reduced by adding the pan-caspase inhibitor Z-VAD-FMK to the cultures (Supporting Information Fig. 1). Furthermore, increased cell death was not observed in the noneosinophil (SIGLEC-F⁻) population (Fig. 4B). Analysis of eosinophil proliferation by EdU incorporation revealed a significant reduction in eosinophil proliferation in cultures from R26^{CreERT2/LSL-tom}_Grb2^{fl/fl} mice as compared to control mice (Fig. 4D).

These results demonstrate that genetic deletion of *Grb2* during in vitro differentiation of eosinophils from bone marrow precursor cells results in enhanced apoptosis and reduced proliferation and basically confirm the findings obtained with the GRB2 inhibitor.

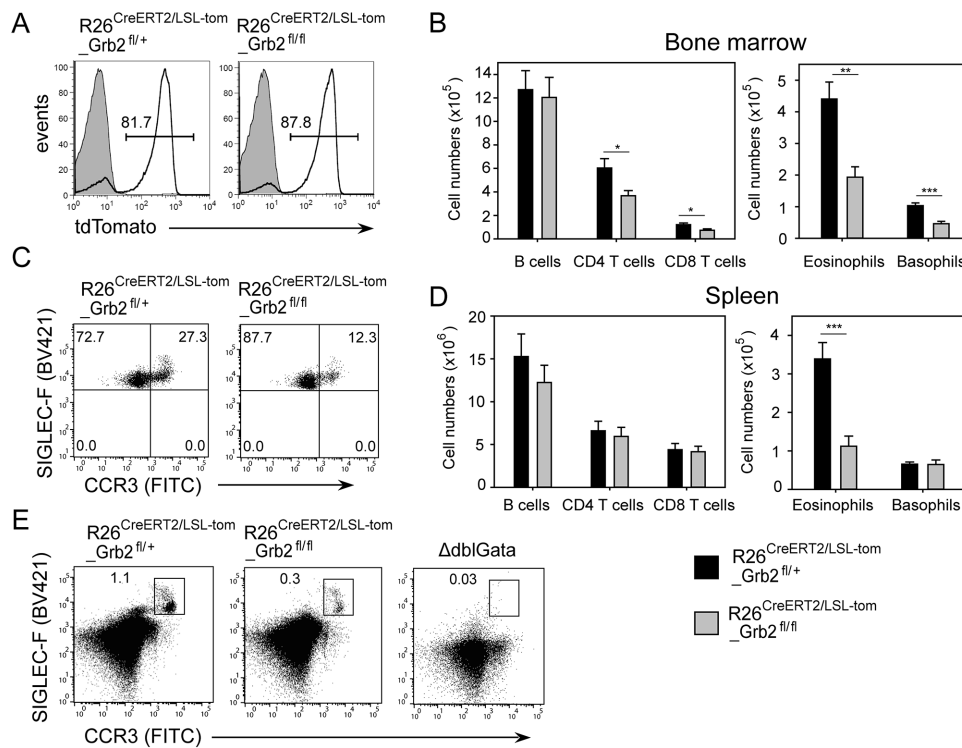


Figure 5. Eosinophil homeostasis requires *Grb2* expression under steady-state conditions. (A) The percentage of tdTomato⁺ cells and thus cells with active Cre in blood of indicated mice after tamoxifen treatment was analyzed by flow cytometry and histograms show the expression of tdTomato in total leukocytes. Open histograms are from tamoxifen-treated mice and filled histograms are from control mice that were left untreated. (B) Absolute numbers of B cells, CD4 and CD8 T cells, basophils and eosinophils in the bone marrow of indicated mice after tamoxifen treatment. Data of two independent experiments were pooled and include 7–9 mice per group. (C) Representative dot plots of bone marrow eosinophils from indicated mice after tamoxifen treatment. Cells were pregated on SIGLEC-F⁺ and SSC^{high} to visualize the ratio of immature to mature eosinophils via CCR3 expression. (D) Absolute numbers of B cells, CD4 and CD8 T cells, basophils and eosinophils in the spleen of indicated mice after tamoxifen treatment. Data of two independent experiments were pooled and include 7–9 mice per group. (E) Representative dot plots of eosinophils from the spleen defined as SIGLEC-F⁺ and CCR3⁺ from indicated mice after tamoxifen treatment are shown. See Supplemental Figure 2 for the full gating strategy. For validation of our eosinophil population definition, an eosinophil-deficient Δ dblGata spleen is displayed as control. Bars show the mean+SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by Student's t-test.

GRB2 is required for eosinophil homeostasis in vivo

To further investigate whether *Grb2* deletion affects eosinophil development in vivo, we injected tamoxifen intraperitoneally into R26^{CreERT2/LSL-tom}_Grb2^{fl/fl} and control mice and analyzed the mice on day 14. The labeling efficiency was about 80–90% in total leukocytes of the blood in both strains of mice (Fig. 5A). Next, we analyzed single cell suspensions by flow cytometry and determined the number of tdTomato-positive eosinophils and other cell types in the bone marrow and spleen. In the bone marrow we observed a reduction of eosinophil numbers by more than 50% in R26^{CreERT2/LSL-tom}_Grb2^{fl/fl} as compared to control mice (Fig. 5B). Basophil and T-cell numbers were also reduced while B cells were not affected (Fig. 5B). We have previously shown that the chemokine receptor CCR3 is a maturation marker on eosinophils that gets upregulated on mature eosinophils in the bone marrow [29]. Double-staining for SIGLEC-F and CCR3 on SIGLEC-F⁺tdTomato⁺ gated cells from the bone marrow revealed that eosinophils in R26^{CreERT2/LSL-tom}_Grb2^{fl/fl} mice contained less CCR3⁺ cells indicating an impaired maturation of eosinophils in these mice as compared to controls (Fig. 5C).

In the spleen we observed a drastic reduction of eosinophils in R26^{CreERT2/LSL-tom}_Grb2^{fl/fl} mice as compared to controls while basophils, T and B cells were not reduced (Fig. 5D, E).

We conclude from these experiments that GRB2 is indeed required for eosinophil development and maturation in vivo. GRB2 also regulates the number of T cells and basophils in the bone marrow but in contrast to the eosinophil population, this effect is not observed in the spleen.

Aspergillus fumigatus-induced lung eosinophilia is GRB2-dependent

Eosinophil numbers can rapidly increase during allergic inflammation and we therefore sought to investigate the role of GRB2 for allergen-induced lung eosinophilia. Therefore, we administered 2×10^6 live conidia of *Aspergillus fumigatus* intranasally every third to fourth day, injected 1 mg tamoxifen i.p. on days 8, 12, and 15 and analyzed the accumulation of eosinophils in the lung on day 17 (Fig. 6A). We generated single-cell suspensions of total lung tissue and determined the frequency and

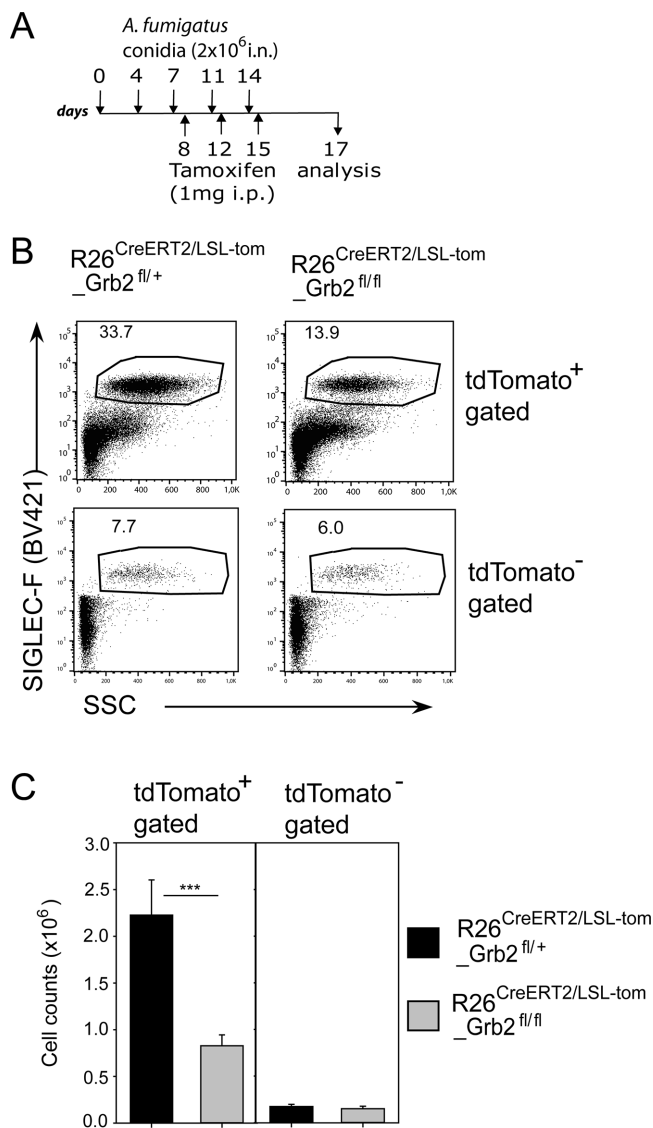


Figure 6. Allergic lung eosinophilia can be prevented by *Grb2* deletion. (A) *Aspergillus fumigatus* infection protocol for induction of allergic airway inflammation. Both groups of mice were infected five times with viable conidia i.n., additionally injected with tamoxifen i.p. three times at the indicated timepoints and analyzed on day 17. (B) Representative dot plots for eosinophils from *Aspergillus*-exposed lungs of indicated mice are displayed. Events were pregated on viable tdTomato⁺ or tdTomato⁻ cells and eosinophils were defined as SIGLEC-F⁺ and SSC^{high} among autofluorescence-negative cells that also excludes SIGLEC-F⁺ alveolar macrophages. (C) Total cell numbers for tdTomato⁺ and tdTomato⁻ lung eosinophils after airway sensitization of indicated mice are shown. Data of three independent experiments were pooled and include 11–13 mice per group. Bars show the mean + SEM. ****P* < 0.001 by Mann–Whitney U-test.

total numbers of eosinophils gated on either tdTomato-positive or tdTomato-negative cells from R26^{CreERT2/LSL-tom} *_Grb2*^{fl/fl} or control mice. Massive accumulation of tdTomato-positive eosinophils was observed in the lungs of control mice but not in R26^{CreERT2/LSL-tom} *_Grb2*^{fl/fl} mice (Fig. 6B and C). Importantly, this difference was not observed among the tdTomato-negative eosinophils in which Cre remained inactive (Fig. 6B and C). There-

fore, impaired lung eosinophilia is due to eosinophil-intrinsic *Grb2* deletion rather than indirect effects that would limit the expansion of both tdTomato-positive and -negative eosinophils. We conclude that intranasal challenge with *A. fumigatus* conidia causes lung eosinophilia that is largely dependent on GRB2.

Discussion

Eosinophil-associated disorders range from allergic inflammation to autoimmunity and hypereosinophilic syndrome that may result in eosinophil-mediated tissue damage including lung damage, ulcerative colitis, esophagitis, and myocarditis [30–32]. A better understanding of eosinophil development, homeostasis and effector functions is critical to develop efficient therapeutic options that lower the number and activity of these cells in different pathologic conditions [32]. Here, we focused on the role of the signaling adaptor molecule GRB2 for mediating eosinophil development under steady-state conditions and during allergic inflammation of the lung. We found that GRB2 is not only expressed in the cytoplasm of eosinophils but also in the nucleus where it may be involved in RNA processing as has been described for other cell types [21]. Classically, GRB2 is thought to mainly function as signaling adaptor down-stream of various cytokine receptors, including the IL-5 receptor, but its potential role in RNA processing should not be ignored and this aspect is certainly worth to investigate further.

Previous studies on the role of GRB2 in eosinophils are rather controversial. IL-5 has been described to induce phosphorylation of SHC and GRB2 in human eosinophils [33]. IL-5 was also found to induce binding of GRB2 to the β_c chain of the IL-5 receptor and SHP-2 that activates the RAS-MAPK pathway and thereby inhibits eosinophil death [23, 24]. In contrast, GRB2 has also been linked to induction of eosinophil death downstream of SIGLEC-8 [26] and PIR-A [25]. Therefore, it remained unclear, whether GRB2 promotes or inhibits eosinophil development and survival in vivo.

By using a GRB2 inhibitor or mice with inducible deletion of *Grb2* we could demonstrate that GRB2 is required for IL-5-induced eosinophil differentiation from committed precursors in the bone marrow. GRB2 blockade or genetic deletion resulted in lower frequencies and reduced numbers of viable eosinophils in bone marrow cultures. Furthermore, Tamoxifen-induced GRB2 deletion in vivo resulted in impaired eosinophil generation during steady-state conditions and reduced lung eosinophilia in an allergic asthma model.

Although the β_c chain of the IL-5 receptor is the main signaling unit and provides several docking sites to elicit several down-stream signaling pathways, there is also evidence for an involvement of the IL-5R α chain in mediating mitogenic signals. It was shown that a membrane proximal region of IL-5R α is required for IL-5-induced activation of JAK1 and JAK2 which in turn phosphorylated STAT5 and the β_c chain [34]. Mice deficient in STAT5a or STAT5b showed normal eosinophil frequencies in bone marrow, blood, and peritoneum while IL-5-induced eosinophilia in bone marrow and peritoneum was impaired by

about 50% [35]. Therefore, GRB2 appears to play a more important role as compared to STAT5a or STAT5b during steady-state development of eosinophils or during IL-5-induced eosinophilia. The β_c chain is also part of the receptors for IL-3 and GM-CSF that promote development of basophils and neutrophils, respectively. Interestingly, we observed that GRB2 was also required for normal basophil development in bone marrow, yet basophil numbers in blood and spleen were not affected by the deletion of *Grb2* (Fig. 5 and data not shown).

Two previous reports indicated that GRB2 is involved in induction of cell death in eosinophils by signals downstream of the surface receptors PIR-A [25] or SIGLEC-8 [26]. Although we did not specifically address this function, in our hands the overall phenotype resulting from *Grb2* deletion or inhibition was decreased eosinophil development and survival in steady-state conditions and during allergic inflammation. Further studies on the role of GRB2 in PIR-A and SIGLEC-8 signaling would be important to investigate the potential mechanism(s) by which GRB2 may contribute to induction of cell death when these receptors are cross-linked by antibodies or by binding to their respective ligands.

In conclusion, our results indicate that the use of pharmacologic GRB2 inhibitors might be beneficial for treatment of various eosinophil-associated inflammatory conditions including allergic diseases and hyper-eosinophilic syndromes that are often associated with severe tissue damage.

Materials and methods

Mice

Grb2^{fl/fl} mice have been described [36]. They were bred to *R26^{CreERT2}* (C57BL/6-Gt(ROSA)26Sor^{tm9(Cre/ESR1)}Arte; [37]) and *R26^{LSL-tom}* (B6.Cg-Gt(ROSA)26Sor^{tm9(CAG-tdTomato)}Hze/J; [38]) mice to generate experimental *R26^{CreERT2/LSL-tomato}_Grb2^{fl/fl}* mice and *R26^{CreERT2/LSL-tom}_Grb2^{fl/wt}* control mice. Eosinophils were also generated from bone marrow of BALB/c mice and isolated from spleen and peritoneum of IL-5 transgenic mice [39]. Mice were housed under specific-pathogen free conditions and used between 8–12 weeks of age. Experiments with animals have been approved by the Local Government of Lower Franconia and were performed in accordance with German animal protection law and European Union guidelines 86/809.

Tamoxifen-induced *Grb2* deletion

Mice received 1 mg tamoxifen in 200 μ L sunflower oil (both Sigma-Aldrich, St. Louis, MO) by intraperitoneal injection at the indicated time points. For in vitro studies 4-hydroxytamoxifen (Sigma-Aldrich) was applied at 0.5 μ M to bone marrow cultures at indicated timepoints.

Aspergillus fumigatus-induced lung eosinophilia

A total of 2×10^6 live conidia of *A. fumigatus* (ATCC 46645 [40]) were applied in 50 μ L 0.9% NaCl + 0.0005% Tween 20 (Roth, Karlsruhe, Germany) to the nostrils of isoflurane-anesthetized mice on days 0, 4, 7, 11, and 14.

Generation of single cell suspensions for flow cytometric analysis

Spleens were mechanically dispersed using the plunger of a 5 mL syringe and passed through a 100 μ m cell strainer. Bone marrow was taken from tibia and femur and flushed out with 5 mL FACS buffer (PBS, 2% FCS). Single cell suspensions were generated by passing multiple times through a 20G canule (BD Microlance, Franklin Lakes, NJ). Lungs were first perfused by injection of 9 mL PBS into the right ventricle of the heart and placed in RPMI1640 (PAN-Biotech Inc., Aidenbach, Germany). Tissue was minced with scissors and enzymatically digested by adding LiberaseTM (Roche, Basel, Switzerland) and DNase DN25 (Sigma-Aldrich) both at 100 μ g/mL for 40 min at 37°C with continuous shaking. Suspensions were passed through a 100 μ m cell strainer to remove tissue debris. Red blood cells were removed from all organs by ACK lysis (155 mM NH₄Cl, 9.99 mM KHCO₃, 0.10 mM EDTA). Cell suspensions were washed in FACS buffer and subjected to staining protocols for flow cytometry analysis.

Generation of bone marrow-derived eosinophils

Eosinophils were generated from bone marrow as previously described [28]. In brief, bone marrow was flushed out from femur and tibia to generate single cell suspensions. Erythrocytes were removed by hypotonic lysis. Bone marrow cells were adjusted to 1×10^6 cells/mL in BM medium (RPMI 1640, 20% FCS, 100 IU/mL penicillin, 10 μ g/mL streptomycin, 2 mM glutamine, 25 mM HEPES, $1 \times$ nonessential amino acids, 1 mM sodium pyruvate and 50 μ M 2-mercaptoethanol) supplemented with 100 ng/mL SCF (PeproTech, Rocky Hill, NJ) and 100 ng/mL FLT3 ligand (FLT3-L; PeproTech) from days 0 to 4 in 75 cm³ culture flask or alternatively to 96-well plates. From day 4 on cells were kept in BM medium supplemented with 10 ng/mL recombinant murine IL-5 (R&D Systems, Minneapolis, MN) instead of SCF and FLT3-L. Every second day half of the medium was replaced with fresh medium supplemented with IL-5. At day 14 the cultures contained >90% bone marrow-derived eosinophils (BMDE) indicated by high side scatter profile and expression of SIGLEC-F.

GRB2 inhibition

The inhibitor sclerotiorin inhibits the SH2 domain of GRB2 [27]. The inhibitor was dissolved in DMSO and added at 10 μ M to bone marrow cultures from day 4 onwards. 0.2% vol/vol DMSO

alone served as control. Cells were harvested on day 9 or day 14 and subjected to Annexin V/propidiumiodid (PI) staining to detect dead or dying cells. Mature BMDE were exposed to the same concentration of the inhibitor and cultured in the presence or absence of 10 ng/mL IL-5. Cells were harvested after 48 hours and subjected to Annexin V/PI staining.

Flow cytometry

Single cell suspensions were firstly incubated with rat anti-mouse CD16/CD32 antibodies (clone 2.4G2, BioXcell, West Lebanon, NH) to avoid unspecific staining. The following antibodies were applied for 20 min at 4°C: FITC-labeled anti-mouse CCR3 (JO73E5), Allophycocyanine (APC)-Cy7-labeled anti-mouse CD4 (RM4-5), APC-Cy7-labeled anti-mouse B220 (RA-3-6B2), PerCP-Cyanine 5.5-labeled anti-mouse CD8 (53-6.7), PerCP-Cyanine 5.5-labeled anti-mouse CD11c (N418), all from eBioscience/Thermo Fisher Scientific (Waltham, MA); Alexa Fluor 488-labeled anti-mouse CD49b (HM α 1) and APC-labeled anti-mouse CD200R3 (Ba103) from BioLegend (San Diego, CA); Alexa Fluor 647, APC-Cy7 and BV421-labeled anti-mouse SIGLEC-F (E50-2440) from BD Biosciences (San Jose, CA).

For intracellular GRB2 detection in BMDE, cells were fixed and permeabilized using the ICF staining kit (eBioscience) according to manufacturer's protocol, stained with purified anti-GRB2 (clone 81/GRB2, BD Biosciences) or anti-mouse IgG isotype control (MOPC-21, BioLegend) followed by PE-conjugated rat anti-mouse IgG (clone RMG1-1, BioLegend).

For analysis of cell death, cells were resuspended in Annexin V binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂). Annexin V (ImmunoTools, Friesoythe, Germany) was added for 20 min. Finally, PI (Thermo Scientific) or DAPI was added to cell suspensions shortly before acquisition by the BD FACS CantoII flow cytometer.

For EdU incorporation analysis cells were treated with 4-hydroxytamoxifen on day 6 as indicated above, exposed to 10 μ M EdU on day 10 or day 13 for 12 h each and analyzed for percentage of EdU⁺ eosinophils on day 11 or 14, respectively. Cells were stained with anti-SIGLEC-F and Fixable Viability Dye eFluor 506 (Thermo Scientific) additionally to the Click-it Plus EdU Alexa Fluor 647 Flow Cytometry Assay Kit (Thermo Scientific).

Data were analyzed with FlowJo software (version 6.4, Treestar, Ashland, OR) and in accordance to published flow cytometry guidelines [41].

Western blotting

Eosinophils were purified from the peritoneal cavity of IL-5 transgenic mice as described [17]. Nuclear and cytoplasmic protein fractions were separated for further Western Blot analysis using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). Ten microgram protein were subjected to SDS-PAGE electrophoresis and blotted on PVDF membranes (Bio-Rad, Munich,

Germany). The following primary antibodies were applied: rabbit anti-mouse Grb2 (Cell Signaling, Danvers, MA), rabbit anti-mouse GAPDH (D16H11, Cell Signaling), rabbit anti-mouse LaminB2 (Proteintech, Rosemont, IL). A HRP-coupled donkey anti-rabbit (Rockland Immunochemicals Inc., Limerick, PA) served as secondary antibody. Membranes were shortly exposed to Signal Fire chemiluminescence substrate (Cell Signaling) and imaged with the ChemoCam-Imager (Intas, Goettingen, Germany).

Statistical analysis

Statistical assessment of the acquired data was performed with Sigmaplot 12.3 software (Systat Software Inc., San Jose, CA). Data were analyzed using two-tailed Student's *t*-test for normally distributed data or Mann-Whitney U-test for nonnormally distributed data as indicated in the figure legends.

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R.W., A.D., and D.V. designed experiments and wrote the manuscript. R.W. and A.D. performed experiments. L.N. and S.K. provided essential organisms.

Conflict of interest: The authors have no financial or commercial conflicts of interests.

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cell sorting in immunological studies. *Eur. J. Immunol.* 2017. **47**: 1584–1797.

Abbreviations: **BMDE**: bone marrow-derived eosinophils · **GRB2**: growth factor receptor binding 2

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Ralf Willebrand, Axel Dietschmann, Lars Nitschke, Sven Krappmann
and David Voehringer

**Murine eosinophil development and allergic lung eosinophilia are largely
dependent on the signaling adaptor GRB2**

Murine eosinophil development and allergic lung eosinophilia are largely dependent on the signaling adaptor GRB2

Ralf Willebrand, Axel Dietschmann, Lars Nitschke, Sven Krappmann and David Voehringer

Correspondence: Prof. David Voehringer, Department of Infection Biology, University Hospital Erlangen and Friedrich-Alexander University Erlangen-Nuremberg (FAU), Germany

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Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision

03-Apr-2018

Dear Prof. Voehringer,

Please accept our sincere apologies for the delay in the decision.

Manuscript ID eji.201847555 entitled "Eosinophil development and allergic lung eosinophilia are largely dependent on the signaling adaptor Grb2" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter.

Both referees have requests/suggestions for improving the manuscript. We encourage to take into account their comments.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. Should you disagree with any of the referees concerns, you should address

this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

You should also pay close attention to the editorial comments included below. **In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. For all data, including the new data generated for the revision of the manuscript, please report the number of independent experiments and number of samples per experiment (or experimental replicates). For the flow cytometry data, please show the full gating strategy. In all the histograms/plots shown please report which fluorochromes were used. Failure to do this will result in delays in the re-review process.**

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Marta Vuerich

On behalf of
Prof. Maria Yazdanbakhsh

Dr. Marta Vuerich
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Reviewer: 1

Comments to the Author

In general, it is unclear as to how the article moves the field forward. IL-5's role in eosinophil development and function has been well established, as has IL-5's signaling through Grb2. That Grb2 would have an impact on eosinophilia in allergy is wholly expected.

Experimental evidence showing that the Grb2 protein is not expressed in the KO and inhibition studies appears to be lacking.

It is unclear why mice genetically modified to lack Grb2 signaling would develop eosinophils at a similar rate to control animals. If it is expected (?), this should be discussed.

In the Grb2 inhibition experiments, the amount of DMSO that is added to the bone marrow cultures is not given. This could have direct deleterious effects on the cells.

Flow cytometry data throughout appears to reflect very poor eosinophil differentiation when following standard published protocols (eg, day-9 Siglec-F+ SSC^{hi} eosinophils are only at ~19% of the population when Dyer's protocol would suggest they should be closer to 90% of the population). A reason is not given.

Flow cytometry gating throughout appears very arbitrary without well defined populations, probably reflecting low numbers of mature cells and potential issues with the differentiation protocols. Flow data appears to be missing for Day 14. How were mature eosinophils identified?

Flow data is missing for Figure 5B. In Figure 5E the result is a 0.3% population. Flow cytometry is notorious for artifact. A population so small will need to be validated some other way.

In Figure 6, the text should reflect that tamoxifen was administered after each IN. Tamoxifen is an anti-fungal agent, so did both groups exhibit similar inflammation? Did tamoxifen get injected into control animals? What was the result if sclerotiorin was used (to validate the in vitro experiment)? How much tamoxifen was used in the in vivo experiments?

How was the dose of sclerotiorin determined (IC₅₀ is 22 micromolar, why use 10)?

Minor comments:

Throughout the entire paper there is inconsistent nomenclature for genes and proteins. Please see: https://academic.oup.com/molehr/pages/Gene_And_Protein_Nomenclatures.

There is inconsistent use of commas in the text. The Oxford comma would be beneficial to clarity in many

instances.

Reviewer: 2

Comments to the Author

Willebrand et al present data demonstrating that pharmacological inhibition and genetic ablation of Grb2 results in strikingly impaired development of eosinophils as well as eosinophil expansion following fungal exposure due to increased death.

Based on the current literature, it remains unclear whether Grb2 promotes survival or death of eosinophils. Data and mechanisms for both activities have been provided.

The present manuscript helps to clarify this question and is therefore an important contribution. The experiments are well-done and conclusive.

Major points:

1. The authors suggest that Grb2 prevents apoptosis, but the death pathway has not been studied. I would suggest to use a pan caspase inhibitor such as Z-VAD and check whether survival of Grb2-deficient eosinophils can be rescued.
2. Also, the experiments do not rule out a requirement of Grb2 for eosinophil proliferation. BrdU/EdU incorporation assay should be performed.
3. Given the requirement of the common beta chain for GM-CSF receptor signaling and its requirement for lung DC development, in particular XCR1+CD103+ DCs, it will be interesting to check whether absence of Grb2 also affects survival of this population.

Minor point

Fig 4C: it appears as if the number of eosinophils is increased in cultures of CreERT2/Grb2^{fl/fl} as compared to -CreERT2/Grb2^{+/fl} in the absence of Tamoxifen, which would not make much sense given that both are Grb2-sufficient. But it may be the difference is not significant or an experimental variation. Please discuss.

First Revision – authors' response

28-Jun-2018

We thank the Editor and Reviewers for their insightful comments on our manuscript. A number of points were raised which we addressed by performing new experiments and by revising the manuscript to

improve the quality. We added new data to Figures 2, 4 and 5. Additional data is shown in Supplemental Figures 1 and 2. We hope that the revised manuscript can now be accepted for publication.

Point-by-point reply:

Reviewer#1:

Comments to the Author

In general, it is unclear as to how the article moves the field forward. IL-5's role in eosinophil development and function has been well established, as has IL-5's signaling through Grb2. That Grb2 would have an impact on eosinophilia in allergy is wholly expected.

Reply: Although Grb2 is known to be involved in signaling through the IL-5 receptor it is not known to what extent Grb2-mediated signaling or other signaling pathways downstream of the IL-5 receptor regulate eosinophil development and survival. In addition, Grb2 is also associated with other receptors (Pir-A, Siglec-8) in eosinophils which were reported to induce apoptosis. We discuss these points in the introduction (page 4). Therefore, we believe that our study moves the field forward by demonstrating that Grb2 is required for eosinophil development and allergen-induced lung eosinophilia.

Experimental evidence showing that the Grb2 protein is not expressed in the KO and inhibition studies appears to be lacking.

Reply: Since Grb2 is required for eosinophil development, its deletion prevents the development of viable eosinophils in which one could determine the efficiency of Grb2 deletion. Therefore, we cannot directly address the efficiency of Grb2 deletion in the conditional KO mice.

It is unclear why mice genetically modified to lack Grb2 signaling would develop eosinophils at a similar rate to control animals. If it is expected (?), this should be discussed.

Reply: This comment probably refers to Figure 5. In Figure 5A we show that the efficiency of Tamoxifen-induced Cre activity (with total tdTomato⁺ leukocytes as read-out) is the same in both mouse strains. Figure 5B demonstrates that mice in which Grb2 was deleted by Tamoxifen-administration develop less eosinophils as compared to identically treated control mice.

In the Grb2 inhibition experiments, the amount of DMSO that is added to the bone marrow cultures is not given. This could have direct deleterious effects on the cells.

Reply: We are aware of the toxic effects of DMSO and therefore added the same amount of DMSO to the control samples to match the DMSO concentration in the experimental samples (0.2% vol/vol). This information is now included in the Materials and Methods section.

Flow cytometry data throughout appears to reflect very poor eosinophil differentiation when following standard published protocols (e.g., day-9 Siglec-F⁺ SSC^{hi} eosinophils are only at ~19% of the population when Dyer's protocol would suggest they should be closer to 90% of the population). A reason is not given.

Reply: We basically use the protocol published by Dyer et al. and achieve about 20% eosinophils on day 9 with a further increase to 90% at day 14. We use this protocol since a few years and published a kinetic in Schwartz et al. (2016) Blood 125:3896. The more rapid differentiation in the Dyer et al. paper could be due to differences in the fetal calf serum or the batch of recombinant cytokines used by both groups.

Flow cytometry gating throughout appears very arbitrary without well defined populations, probably reflecting low numbers of mature cells and potential issues with the differentiation protocols. Flow data appears to be missing for Day 14. How were mature eosinophils identified?

Reply: We revised Figure 2 and added flow cytometry data for day 14. Eosinophils were identified by high expression of Siglec-F and high side scatter (SSC) profile as indicated in Figure 2A and C.

Flow data is missing for Figure 5B. In Figure 5E the result is a 0.3% population. Flow cytometry is notorious for artifact. A population so small will need to be validated some other way.

Reply: We show flow cytometry data for Figure 5 now in the Supplemental Figure 2. We have profound experience for flow cytometric analysis for many years and we are confident that even a rather small population of 0.3% can be reliably identified as eosinophils. As further proof that the small population shown in Figure 5E indeed represents eosinophils we performed flow cytometric analysis with eosinophil-deficient Δ dblGata mice where this population is missing and added this FACS plot to Fig. 5E.

In Figure 6, the text should reflect that tamoxifen was administered after each IN. Tamoxifen is an anti-fungal agent, so did both groups exhibit similar inflammation? Did tamoxifen get injected into control animals? What was the result if sclerotiorin was used (to validate the in vitro experiment)? How much tamoxifen was used in the in vivo experiments?

Reply: We revised the text in the results section on page 8 and the legend to Figure 6 on page 20 to indicate that both groups of mice received the same amount of tamoxifen and the timing of tamoxifen administration. Injection of sclerotiorin solved in DMSO was not performed due to toxic effects of DMSO in vivo. 1 mg tamoxifen was used for each injection as indicated in Figure 6A.

How was the dose of sclerotiorin determined (IC50 is 22 micromolar, why use 10)?

Reply: Our decision to use sclerotiorin at 10 μ M was based on the rationale that we wanted to exclude potential bystander effects and DMSO-mediated effects that could result in toxicity independently of Grb2 inhibition.

Minor comments:

Throughout the entire paper there is inconsistent nomenclature for genes and proteins. Please see: https://academic.oup.com/molehr/pages/Gene_And_Protein_Nomenclatures.

[Reply:](#) We thank the reviewer for this comment. Indeed the official nomenclature for proteins in mice is based on the gene symbol abbreviation but with all letters in upper case. Gene symbols are first letter upper case then lower case and all italicized. We made these corrections for the whole manuscript.

There is inconsistent use of commas in the text. The Oxford comma would be beneficial to clarity in many instances.

[Reply:](#) We tried our best to use commas appropriately. Any mistakes may be corrected during the type setting process.

Reviewer: 2

Comments to the Author

Willebrand et al present data demonstrating that pharmacological inhibition and genetic ablation of Grb2 results in strikingly impaired development of eosinophils as well as eosinophil expansion following fungal exposure due to increased death.

Based on the current literature, it remains unclear whether Grb2 promotes survival or death of eosinophils. Data and mechanisms for both activities have been provided.

The present manuscript helps to clarify this question and is therefore an important contribution. The experiments are well-done and conclusive.

Major points:

1. The authors suggest that Grb2 prevents apoptosis, but the death pathway has not been studied. I would suggest to use a pan caspase inhibitor such as Z-VAD and check whether survival of Grb2-deficient eosinophils can be rescued.

[Reply:](#) This suggestion is well taken and we performed experiments to address this point. We found no inhibition of cell death by Z-VAD-FMK in Grb2-deficient eosinophils as shown in the new Supplemental Figure 1.

2. Also, the experiments do not rule out a requirement of Grb2 for eosinophil proliferation. BrdU/EdU incorporation assay should be performed.

[Reply:](#) We agree that impaired proliferation of developing Grb2-deficient eosinophils could contribute to the phenotype we describe here. To directly investigate this possibility we added EdU to eosinophil cultures and observed reduced EdU incorporation in Grb2-deficient as compared to control eosinophils. We includes this new data in the revised Figure 4D.

3. Given the requirement of the common beta chain for GM-CSF receptor signaling and its requirement for lung DC development, in particular XCR1+CD103+ DCs, it will be interesting to check whether absence of Grb2 also affects survival of this population.

[Reply:](#) Although we agree that the role of Grb2 in lung DC development would be interesting to study, we feel that this aspect goes beyond the scope of this manuscript and should be studied in a separate project including DC subsets in different tissues during steady-state and inflammatory settings.

Minor point

Fig 4C: it appears as if the number of eosinophils is increased in cultures of CreERT2/Grb2^{fl/fl} as compared to -CreERT2/Grb2^{+/fl} in the absence of Tamoxifen, which would not make much sense given that both are Grb2-sufficient. But it may be the difference is not significant or an experimental variation. Please discuss.

[Reply:](#) This difference is indeed not significant and probably due to mouse-to-mouse differences in the number of progenitor cells in the bone marrow samples that can give rise to eosinophils. However, we repeated this experiment and show now frequencies with statistics from pooled data of two independent experiments in the revised Figure 4C.

Editorial comments

Word limits, Figures and legends, flow cytometry analysis, graphs, Western blots, number of independent experimental repeats, statistics.

[Reply:](#) We payed close attention to all points and believe the manuscript is consistent with the formal requirements.

Second Editorial Decision

27-Jul-2018

Dear Prof. Voehringer,

It is a pleasure to provisionally accept your manuscript entitled "Eosinophil development and allergic lung eosinophilia are largely dependent on the signaling adaptor GRB2" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: <https://onlinelibrary.wiley.com/toc/15214141/0/ja>). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Nadja Bakocevic

on behalf of
Prof. Maria Yazdanbakhsh

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and David Voehringer

**Murine eosinophil development and allergic lung eosinophilia are largely
dependent on the signaling adaptor GRB2**

Supplemental Figure 1

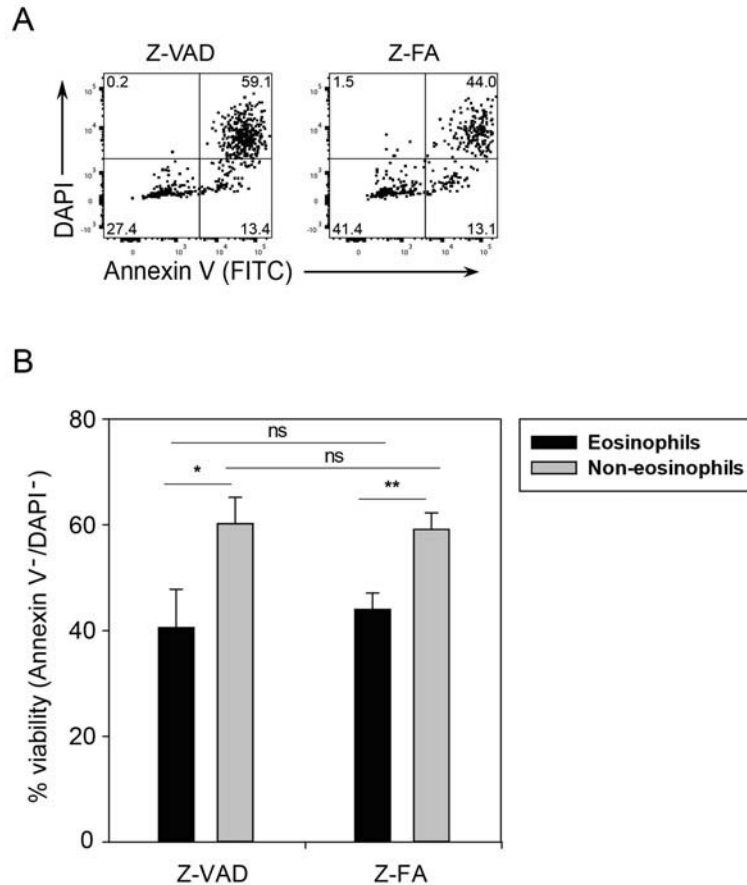


Figure S1: Pan-caspase inhibition of developing BMDE. BMDE cultures from $R26^{CreERT2/LSL-tom_Grb2^{fl/fl}}$ mice were treated once with 5 μ M 4-Hydroxy tamoxifen or ethanol as solvent control on day 6. Simultaneously 20 μ M Z-VAD-FMK was added to rescue from possibly caspase-mediated cell death. Z-FA-FMK was used as corresponding control. Inhibitors were refreshed with every medium exchange of the culture. Cells were analyzed via Annexin V/DAPI staining using flow cytometry on day 11. (A) Exemplary dot plots are shown. Cells were pre-gated on tdTomato⁺ and SIGLEC-F⁺ as displayed in Figure 4. (B) Statistical analysis of eosinophil viability upon caspase inhibition. Displayed are pooled percentages of Annexin V⁻/DAPI⁻ eosinophils and non-eosinophils from two independent experiments (total n=8 per group).

Supplemental Figure 2

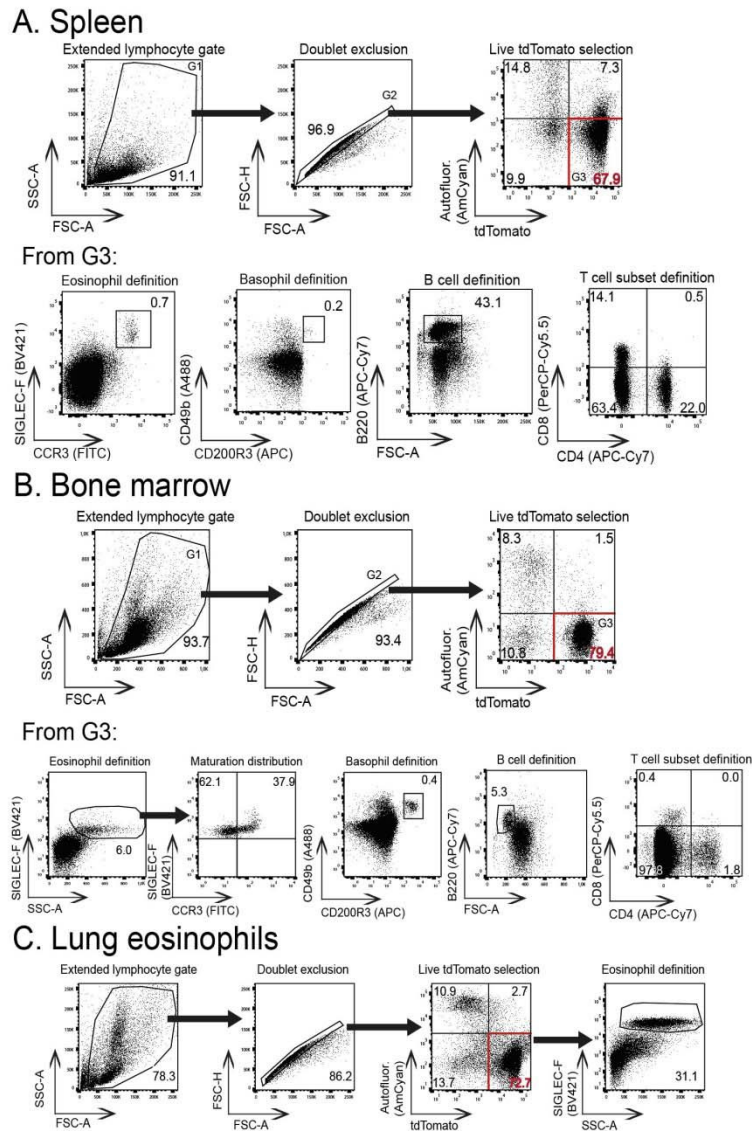


Figure S2: Gating strategy. Total cells were gated for single, AmCyan-autofluorescence⁻ and tdTomato⁺ cells. Afterwards eosinophils were defined via α SIGLEC-F and α CCR-3, basophils via α CD49b and α CD200R3, B cells via α B220 and T cells via α CD4 and α CD8. Clones and fluorochromes of all antibodies are given in Materials and Methods. Gating is displayed for spleen (A), bone marrow (B) and lung eosinophils (C), which were defined by SIGLEC-F and high SSC profile.