

# Generation of Eosinophils from Unselected Bone Marrow Progenitors: Wild-Type, TLR- and Eosinophil-Deficient Mice

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**Abstract:** We have recently devised a culture method that generates large numbers of eosinophils at high purity from unselected BALB/c mouse bone marrow progenitors [Dyer *et al.*, 2008. *J. Immunol.* 181: 4004-9]. Here we present the extended scope of this approach, as we have used this method successfully to generate eosinophil cultures of virtually 100% purity from bone marrow from C57BL/6 mice, and from TLR2, TLR3, TLR7 and TLR9-gene-deleted mouse strains on the C57BL/6 background. Both wild-type and TLR3 gene-deleted bone marrow eosinophils (bmEos) are functional, releasing peroxidase in response to the secretagogue, platelet activating factor. We have also used this method to re-evaluate production of eosinophils in bone marrow cultures from  $\Delta$ dblGATA mice, a strain that is eosinophil-deficient *in vivo*. Interestingly, bmEos can be detected in the  $\Delta$ dblGATA cultures (5% of total cells at day 10), although ~80-fold fewer bmEos are detected in  $\Delta$ dblGATA than in parallel wild-type (BALB/c) bone marrow cultures. Overall, we find that generation of large numbers of eosinophils at high purity from unselected bone marrow progenitors proceeds efficiently in a variety of wild-type and gene-deleted strains, and as such this approach shows promise as a universal method for the study of eosinophil structure and function (199 words).

**Keywords:** Cytokines, interleukin-5, toll-like receptors, mouse, hematopoiesis.

## INTRODUCTION

Eosinophils are granulocytic leukocytes that develop from pluripotent bone marrow progenitors in response to Th2 cytokine stimulation, most notably in response to elevated levels of interleukin-5 (reviewed in [1, 2]). Although eosinophils are readily recognized by their distinct morphology, the biological role and functions of these cells remain uncertain and controversial. For example, although eosinophilia is a prominent component of the host response to helminthic parasite infection, the presumed role of eosinophils in anti-parasite host defense is not at all clear [3]; in fact, Fabre and colleagues [4] recently presented data suggesting that eosinophils serve to promote parasite, rather than host survival in a chronic infection model. Likewise, consensus opinion holds that eosinophils play a prominent role in pathogenesis of allergic asthma, yet large-scale clinical studies lead to conflicting conclusions [5-7].

In order to explore eosinophil development, and ultimately the function of individual genes and gene-products within eosinophils, we developed a culture system which permits us to generate large numbers of eosinophils at high purity (> 95%) from unselected BALB/c bone marrow progenitors [8]. We found that BALB/c bone marrow-derived eosinophils (bmEos) expressed relevant transcripts and lineage-specific cell surface proteins and underwent chemotaxis in response to eotaxin-1. As only wild-type BALB/c mice were evaluated in this first study, it was

important to determine whether our findings were unique to this one strain of mice, or whether this method might be applied more universally.

Here, we explore the potential of this method as a means to generate functional bmEos from the C57BL/6 mice, as well as from bone marrow cells from specific gene-deleted mice on the C57BL/6 background. We also use this improved culture system to revisit our earlier findings [9], comparing the production of bmEos *ex vivo* from wild-type and eosinophil-deficient  $\Delta$ dblGATA mice.

## MATERIALS AND METHODOLOGY

**Mice.** Six to eight-week old wild-type BALB/c and C57BL/6 mice were purchased from Taconic Farms (Rockville, MD). Eosinophil-deficient  $\Delta$ dblGATA mice on the BALB/c background [10] were bred and maintained on site. TLR2, TLR7, and TLR9 gene-deleted mice (C57BL/6 background) [11-13] are used with written permission of Dr. Shizuo Akira and were graciously provided by Dr. Rachel Caspi (NEI/NIH) and Dr. Jennifer Wang (U. Mass. Medical). TLR3 gene-deleted mice [14] were graciously provided by Dr. Ivett Jelinek (NCI/NIH). All methods herein were reviewed and approved *via* NIAID Animal Study Proposal LAD 7E.

**Bone Marrow Isolation and Culture.** The method for eosinophil production from unselected bone marrow progenitors is described in detail in reference 8. Briefly, bone marrow cells were collected from the long bones by flushing with Iscove's Modified Dulbecco's Medium (Invitrogen), and red blood cells were lysed in dH<sub>2</sub>O followed by the addition of 10X PBS to 1X final concentration. After centrifugation, the cells were washed once in PBS containing 0.1% bovine serum albumin (BSA).

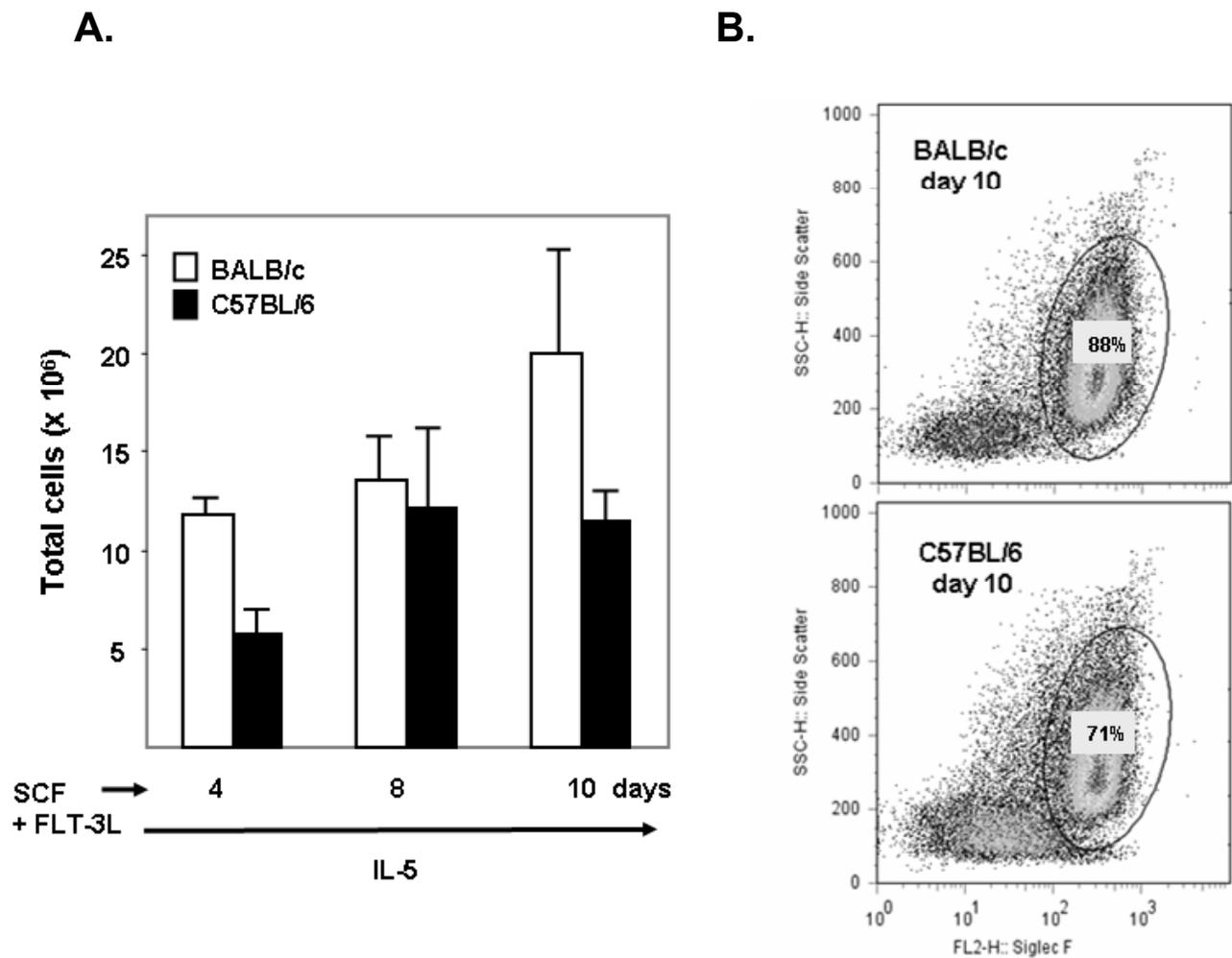
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The bone marrow cells were seeded at  $10^7$ /mL in media containing RPMI 1640 (Invitrogen) with 20% fetal bovine (Cambrex), 100 IU/mL penicillin and 10  $\mu$ g/mL streptomycin (Cellgro), 2 mM glutamine (Invitrogen), 25 mM HEPES and 1x non-essential amino acids and 1 mM sodium pyruvate (Gibco) and 50  $\mu$ M  $\beta$ -mercaptoethanol (Sigma) and supplemented with 100 ng/mL stem-cell factor (SCF; PeproTech) and 100 ng/mL FLT3-Ligand (FLT3-L; PeproTech) from day 0 to day 4. On day 4, the media containing SCF and FLT3-L was replaced with media containing 10 ng/mL recombinant mouse interleukin-5 (rmIL-5; R&D Systems) alone. Viable (trypan blue-excluding) cells were enumerated manually *via* hemocytometer counts and determined on triplicate wells.

**Flow Cytometric Detection of Eosinophils.** Cells were incubated with either PE-conjugated rat anti-mouse Siglec F or PE-conjugated IgG2A6 isotype control (1 $\mu$ g per  $10^6$  cells; BD Pharmingen) for 30 minutes at 4°C. After staining, the cells were fixed in 4% paraformaldehyde and analyzed by flow cytometry. Data were acquired with a FACSCalibur

flow cytometer (BD Biosciences) and analyzed with FloJo software v 7.1 (Tree Star, Inc). Siglec F-positive cells were identified by comparison to the PE-conjugated IgG2A6 isotype control.

**Degranulation Assay:** Detection of eosinophil peroxidase (EPO) released by platelet-activating factor (PAF)-stimulated bmEos was essentially as described [15]. Cells were collected by centrifugation and resuspended phenol-red-free Roswell Park Memorial Institute (RPMI) medium at a concentration 250,000 cells/mL and 25,000/100 uL were used per well. One uL of platelet activating factor (PAF C16) or 1 uL of vehicle, dimethyl sulfoxide (DMSO), was added to appropriate wells to achieve the indicated concentrations. Cells were incubated at 37°C, 5% CO<sub>2</sub> for 30 minutes. The assay was developed using 100 uL o-phenylene-diamine (OPD) reagent (800 uL 5mM OPD in 4 mL 1M Tris (pH8.0), 5.2 mL H<sub>2</sub>O and 1.25 uL 30% H<sub>2</sub>O<sub>2</sub>) in the presence of H<sub>2</sub>O<sub>2</sub>. The reaction was terminated by the addition of 100  $\mu$ L of 4M H<sub>2</sub>SO<sub>4</sub> to each well. The relative production of EPO was determined by reading the plate at



**Fig. (1).** Eosinophils generated in cultures of unselected bone marrow from both BALB/c and C57BL/6 mice. (A) Bone marrow cultures were initiated at  $10^7$  cells / mL. The number of viable cells was determined at timepoints shown in response to rmSCF (100 ng / mL) and rmFLT-3L (100 ng / mL) for 4 days after isolation, followed by rmIL-5 (10 ng / mL) alone thereafter. (B) Eosinophils were identified at day 10 in culture by side scatter (SSC, y-axis) and reactivity with anti-Siglec F+ antibodies (x-axis).

wavelength 492 nm. Data is reported as mean absorbance +/- standard error of the mean in relation to vehicle only control (1 % DMSO).

**RESULTS**

**Eosinophils (bmEos) are Generated from C57BL/6 Bone Marrow Culture.** Our previous study examined production of functional eosinophils at high purity from unselected bone marrow from BALB/c mice [8]. Here we demonstrate production of bmEos from progenitors from C57BL/6 mice. Using the identical culture conditions (100 ng / mL SCF + 100 ng / mL FLT-3L for 4 days, followed by 10 ng / mL IL-5 alone thereafter), 71% of the cells in C57BL/6 bone marrow culture at day 10 were Siglec F+ bmEos, comparable to the 88% bmEos detected in the BALB/c bone marrow cultures (Fig. 1). Both BALB/c and C57BL/6 bone marrow cultures reach ~100% bmEos by day 14 [8]. BmEos can also be generated from the 129Sv mouse strain (data not shown).

**BmEos from Gene-Deleted Progenitors.** The percent bmEos were determined from cultures of C57BL/6 wild-type bone marrow and bone marrow isolated from TLR gene-deleted mice (Fig. 2). As shown, individual deletions of TLR 2, TLR3, TLR 7 and TLR9 have no impact on the rate at or extent to which bmEos are generated in culture. All cultures reached virtually 100% bmEos at days 16 – 18, including those from C57BL/6 wild-type mice.

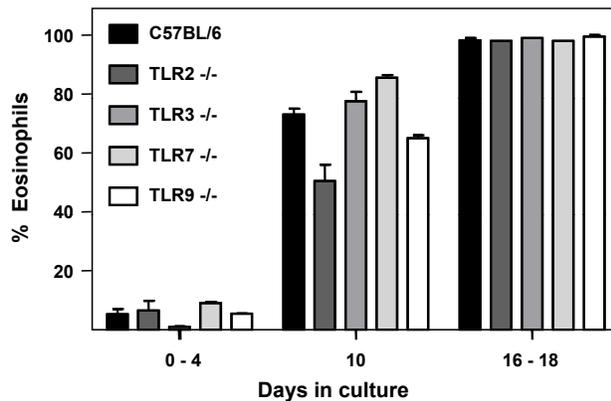


Fig. (2). Eosinophils generated from bone marrow of TLR 2, TLR 3, TLR 7, and TLR 9 gene-deleted mice. Eosinophils were identified by staining with Diff-Quik and are shown as percent of total cell number on days indicated.

**BmEos Degranulate.** In our previous publication [8], we found that bmEos expressed typical proteins and transcripts, and underwent chemotaxis in response to gradients of mouse eotaxin-1 (CCL11). Here, we characterize another functional response, as we demonstrate that mouse bmEos degranulate in a dose-dependent fashion in response to the eosinophil-secretagogue, platelet-activating factor (Fig. (3) PAF; [16, 17]).

**BmEos from Eosinophil-Deficient  $\Delta$ dblGATA Mice.** Interestingly, and in contrast to our previous study [9], with this cytokine regimen, markedly fewer bmEos were detected in bone marrow cultures from eosinophil-deficient  $\Delta$ dblGATA mice when compared to those generated from BALB/c wild-type mice (Fig. 4).

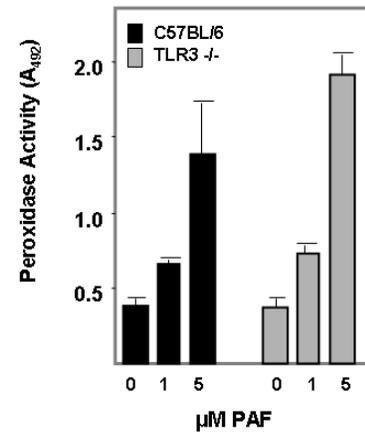
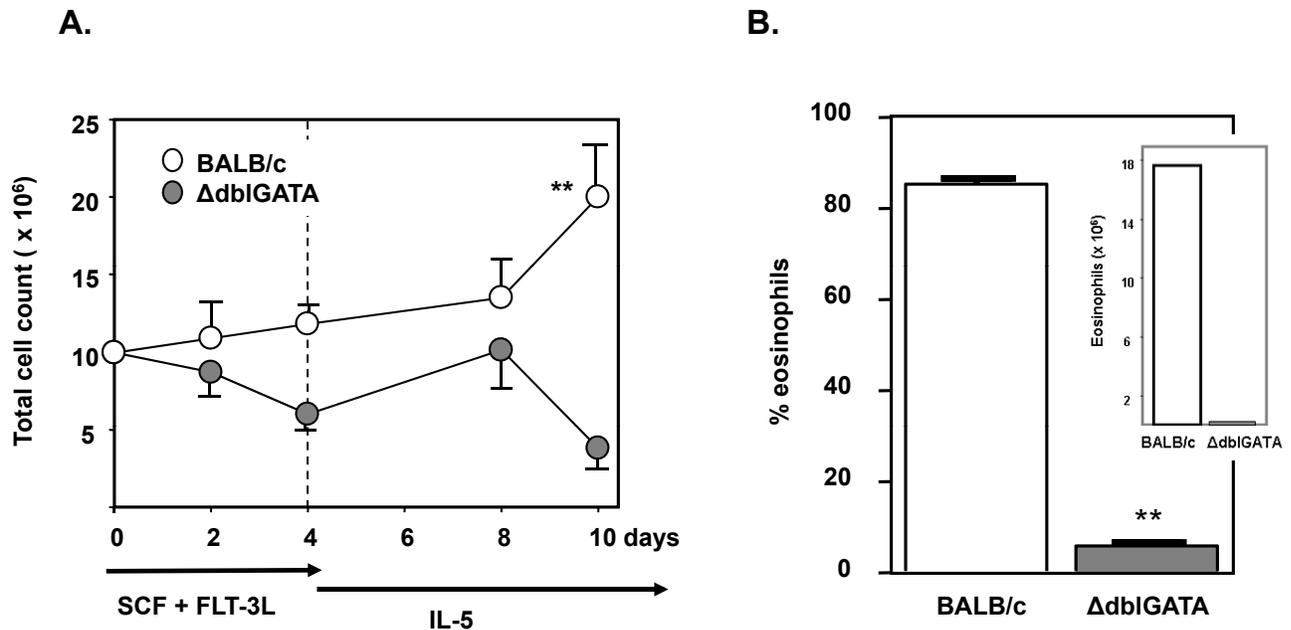


Fig. (3). Eosinophils generated from bone marrow of wild-type and TLR3 gene-deleted mice release eosinophil peroxidase in response to challenge with PAF. Extracellular peroxidase activity was measured as hydrolysis of o-phenylene diamine (OPD) as described [15]; peroxidase activity detected at baseline (zero concentration) represents the response to 1% DMSO vehicle control.

**DISCUSSION**

In this manuscript, we demonstrate the general nature of our bone marrow eosinophil culture protocol. Specifically, we document production of bmEos from C57BL/6, a genetically-distinct wild-type strain used extensively for creating gene-deleted mice. We also generated bmEos from four independent gene-deleted mouse strains on the C57BL/6 background. The ability to generate bmEos from gene-deleted strains is a critical feature of this method, as mature eosinophils isolated from mouse blood and spleen are biosynthetically inert and have relatively little mRNA; as such, introduction of RNA-silencing agents are unlikely to have a substantial impact on protein targets. Specifically, we generated substantial numbers of TLR2, TLR3, TLR7 and TLR9 gene-deleted eosinophils that can be used for functional studies. Numerous TLRs have been detected in both human and mouse eosinophils [18-21]; TLR2 has been implicated in interactions of eosinophils with mycobacteria [19], and TLR7 was shown to be crucial for eosinophil-mediated clearance of respiratory viruses *in vivo* [21]. There is also substantial evidence supporting a role for TLRs in hematopoiesis [22]; TLRs are expressed on pluripotent progenitors and they direct differentiation toward the myeloid (granulocyte, macrophage) lineages [23, 24].

We also revisited our findings on the role of the  $\Delta$ dblGATA enhancer of the mouse GATA-1 gene in promoting eosinophil hematopoiesis *ex vivo*.  $\Delta$ dblGATA gene-deleted mice are completely devoid of mature eosinophils, both at baseline or in response to Th2 stimulation [10, 25, 26]; the molecular basis for this finding is not yet clear. In an earlier study, we found that  $\Delta$ dblGATA and wild-type BALB/c mouse bone marrow cultures each yielded ~5 – 7% eosinophils in response to four days with SCF and FLT-3L, followed by various combinations of IL-5, IL-3 and GM-CSF [9]. In this work, when we restrict the secondary stimulus to IL-5 alone, wild-type cultures undergo specific expansion of the eosinophil lineage, with bmEos representing nearly 100% of the cell population by day 14.



**Fig. (4). Eosinophils generated from bone marrow of  $\Delta$ dblGATA eosinophil-deficient mice. (A)** Bone marrow cultures were initiated at  $10^7$  cells / mL. The number of viable cells was determined at timepoints shown in response to the cytokine regimen described in the Legend to Fig. (1). Significant difference between counts detected at day 10, \*\* $p < 0.01$ . **(B)** Eosinophils identified by side scatter and Siglec F expression are shown as percent total cell number on day 10, \*\* $p < 0.01$ . *Inset*: number of eosinophils in bone marrow culture at day 10 as calculated from the data in (A) and (B).

Interestingly, the  $\Delta$ dblGATA bone marrow cultures can still generate eosinophils using IL-5 alone as the secondary stimulus, but the bmEo-population remains at ~5% of the total, representing ~80-fold fewer bmEos than that produced by the parallel wild-type culture. Interestingly, the original cytokine combination, which included IL-5, IL-3 and GM-CSF, skewed the distribution of GATA-1 transcripts, suggesting that the dbiGATA deletion might be circumvented *ex vivo* by use of an alternative promoter [9]; we have not evaluated transcript distribution in response to IL-5 alone. However, we have shown clearly that the  $\Delta$ dblGATA bone marrow progenitors are not capable of a complete response to IL-5. As such, this system can serve as an important tool for evaluating the molecular basis of the dbiGATA deletion and its direct connection to IL-5-mediated expansion of the eosinophil lineage.

## CONCLUSION

In summary, we have shown that generation of eosinophils at high purity from unselected bone marrow progenitors proceeds effectively in a variety of wild-type and gene-deleted strains, and as such is a universal tool for the study of eosinophil structure and function.

## ACKNOWLEDGEMENTS

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$\Delta$ dblGATA colony. This study was funded by NIAID Division of Intramural Research.

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