Dissection of 3 Developmental Stages of Eosinophils in Mouse Using *Gata1* and *Gata2* Expression

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ABSTRACT

Eosinophils are derived from granulocyte-monocyte progenitors and play important roles in allergic reactions. However, it is difficult to fractionate living eosinophils by flow cytometry, because these granulocytes express multiple cell surface markers that are shared by other cells of hematopoietic or non-hematopoietic origin. Here, we attempted to define the stepwise differentiation of eosinophils *in vivo* by monitoring the profiles of *Gata1* and *Gata2* expression. We found that the development of bone marrow eosinophils is divided into 3 stages. *Gata2* expression was highly detected in eosinophils in early developmental stage while fully differentiated eosinophils do not express *Gata2*. *Gata2*-expressing eosinophils were scarce in peripheral blood and tissue. When mice were subjected to ovalbumin (OVA)-induced asthma, fully differentiated eosinophils markedly increased in number at bone marrow and peripheral blood and migrate into lung. Therefore this study proposes a useful means for the research of allergic diseases caused by eosinophils.

INTRODUCTION

Eosinophils have been assumed as effecter cells in allergic diseases. Roles eosinophils play *in vivo* have been examined and clarified (Lee et al. 2004; Humbles et al. 2004). However, in contrast to the other hematopoietic cell lineages, cell surface markers for the detection of eosinophil lineage and its differentiation stages are still insufficient. Consequently, it has been difficult to isolate eosinophils at specific differentiation stages (Fukushima et al. 2009; Gartner 1980; Hansel et al. 1991; Munoz and Leff 2006), so that eosinophil differentiation pathway remains to be elucidated. In order to delineate the eosinophil differentiation pathway, we need to establish a new method for isolating each developmental stage of eosinophils.

Zinc finger-type transcription factors GATA-1, GATA-2 and GATA-3 are known as hematopoietic GATA factors (Yamamoto et al. 1990). These GATA factors have been shown to be essential for hematopoiesis (reviewed in Ohneda K and Yamamoto M 2002). Knockout of *Gata1* or *Gata2* genes resulted in embryonic lethality because of the lack of primitive (embryonic) hematopoiesis (Fujiwara et al. 1996; Takahashi et al. 1997; Tsai et al. 1994). Both in mouse and human GATA-1 is mainly expressed in erythroid cells, megakaryocytes, must cells, dendritic cells and eosinophils (Gutierrez et al. 2007; Harigae et al. 1998; Shimizu et al. 2008; Takemoto et al. 2008; Weiss and Orkin 1995), whilst GATA-2 is mainly expressed in hematopoietic progenitors, mast cells and eosinophils within the hematopoietic system (Tsai and Orkin 1997; Hirasawa et al. 2002). In this regard, while GATA-1 and GATA-2 are expressed in

eosinophils (Hirasawa et al. 2002), it is not clear at present whether GATA-1 and GATA-2 expressed concomitantly in the same cell or separately in eosinophils at distinct differentiation stages. In the erythroid lineage it has been shown that *Gata2* is expressed in early erythroid progenitors, but the *Gata2* expression decreases and *Gata1* expression increases along with the erythroid differentiation. This phenomenon is known as the GATA switching (Grass et al. 2003, Ohneda K. and Yamamoto M. 2002; Suzuki et al. 2003).

It has been reported that eosinophils are depleted in the ΔdblGATA mouse, which lacks double GATA motifs in the *Gata1* gene promoter (Yu et al. 2002). In the ΔdblGATA mouse GATA-1 expression markedly decreased in eosinophils. On the other hand, in a genetically rescued line of mice expressing *Gata2* transgene in replacing the endogenous *Gata1* (*Gata1-/-:G1-HRD-Gata2*; Hirasawa et al. 2002), eosinophils can be found even in the absence of GATA-1, indicating that GATA-2 is able to complement the GATA-1-deficiency in eosinophils as is the case for erythroid and megakaryocytic cells (Takahashi et al. 2000). These results somehow contradict the hitherto held belief that eosinophils express *Gata1* and *Gata2* concomitantly during differentiation (Zon et al. 1993; Yamaguchi et al. 1998).

IL-5 is a key mediator for differentiation, proliferation and activation of eosinophils (Clutterbuck and Sanderson 1988; Clutterbuck et al. 1989). When mice are challenged with aerosolized ovalbumin (OVA), eosinophil population in the bone marrow, peripheral blood and various tissues increases in an IL-5-dependent manner (Shen et al. 2003; Kopf et al. 1996). However, *IL-5* gene knockout mice have basal-level population of eosinophils (Kopf et al. 1996; Fallon et al. 2002). These observations suggest that eosinophilic progenitors at certain stages can differentiate in an IL-5-independent manner *in vivo*.

In this study we aimed to characterize the eosinophil differentiation pathway exploiting reporter mice that express red or green fluorescent protein (RFP and GFP, respectively) under the regulatory influences for the *Gata1* and *Gata2* genes. To monitor the expression of *Gata1* and *Gata2* genes simultaneously, we also generated a compound reporter mouse line (G1Red:G2GFP mouse line) that expresses red and green fluorescent proteins under the control of the *Gata1* and *Gata2* regulatory domains, respectively. The G1Red:G2GFP mice were subjected to intranasal OVA challenge to monitor the reactivity of eosinophils during stages of development.

MATERIALS AND METHODS

Mice

In constructing the *G1Red* transgene, *Gata1-HRD* (Onodera et al. 1997; Suzuki et al. 2003) was ligated to *DsRed2* cDNA (Clontech, Mountain View, CA). We established 4 lines of G1Red transgenic mouse, and the highest RFP-expressing line was mainly used in this

study. For screening G1Red transgenic mice, the tail DNA was extracted and the *DsRed2* transgene was detected by polymerase chain reaction (PCR) using a pair of primers, Red-S (5'-ACGGCTCCAAGGTGTACGTG-3') and Red-AS (5'-CTCCCAGCCCATGGTCTTC-3'). Both G1GFP (*Gata1-HRD-GFP*) mice and G2GFP (*Gata2* EIS-KI) mice have been described previously (Suzuki et al. 2003 and 2006). ΔdblGATA mice (Yu et al. 2002) were purchased from Jackson Laboratory (Bar Harbor, ME). To generate G1Red:G2GFP mice, G1Red mouse and G2GFP mouse were mated. The ΔdblGATA mice were mated with G2GFP mice to generate ΔdblGATA:G2GFP mice. All mice were treated according to the regulations of *the Standards for Human Care and Use of Laboratory Animals of the University of Tsukuba*, and analyzed at 9 to 16 weeks of age.

Flow cytometry and cell sorting

Cell sorting and marker analysis were performed using FACS Vantage SE and Cell Quest software (Becton Dickinson, San Jose, CA). Mononuclear cell suspensions from bone marrow, peripheral blood, and bronchoalveolar lavage fluid (BALF) of the transgenic mice were prepared and incubated with biotinylated monoclonal antibodies recognizing Ter119, B220, CD4, CD8 (for the lymphoid and erythroid markers, LyE), and CD71. For G1Red:G2GFP mice, cells negative for both markers ($LyE^-/CD71^-$ cells) were enriched by magnetic negative selection using streptavidin-conjugated magnetic beads (BioMag; Polysciences, Warrington, PA), followed by staining with allophycocyanin (APC)-conjugated anti-c-Kit antibodies, streptavidin-conjugated phycoerythrin (PE)-Texas Red and propidium iodide (PI; Sigma, St Louis, MO, USA). For G1GFP, G2GFP, and ΔdblGATA:G2GFP mice, enriched LyE^- cells were stained with PE-conjugated anti-CD71 antibody, APC-conjugated anti-c-Kit antibody, streptavidin-conjugated PE-Texas Red, and PI. For G1Red mice, enriched LyE^- cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD71 antibody, APC-conjugated anti-c-Kit antibody, streptavidin-conjugated PE-Texas Red, and PI. All antibodies and streptavidin-conjugated fluorochromes were purchased from BD Pharmingen (San Diego, CA).

Cytochemistry

Flow-sorted cells were subjected to cytospin and stained by reagents for Wright-Giemsa (MUTO, Tokyo) and EoProbe (BioFX, Owings Mills, MD) staining. Cells representative of each fraction were selected and aligned. Original magnification ×1000 (Scale bar is 10 μm). Cells positive for EoProbe were observed under the fluorescent microscope (Leica Microsystems, Wetzlar, Germany) after exposed to the 561-nm wavelength excitation for 10 seconds to quenching auto fluorescence.

Colony assay

Sorted cells were cultured in 1 ml of 0.8% methylcellulose medium containing 30% fetal bovine serum (FBS). For detection of

eosinophil colony-forming units, medium was supplemented with 100 ng/ml of IL-5 (R&D Systems, Minneapolis, MN) and 100 ng/ml of stem cell factor (SCF) (provided by Kirin Brewery, Takasaki, Japan). Single-cell derived colonies were counted after 9 days of culturing, and some of growing colonies were taken photos everyday during the 14-day cultivation by multi-color and fixed point observation system of BZ-8000 fluorescent microscope (Keyence, Osaka, Japan).

Quantitative RT-PCR

Total RNA were extracted from 5,000 flow-sorted cells using RNeasy (Qiagen, Basel, Switzerland) and reverse-transcribed by Sensicript RT Kit (Qiagen) with random hexamers. For quantitative PCR, samples were analyzed by either SYBR Green or fluorescent probe systems (Eurogentec, Seraing, Belgium) with gene-specific oligo-DNAs listed in supplemental table 1, using ABI PRISM 7700 Sequence Detector (Applied Biosystems, Foster City, CA). Glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA levels were used as internal controls.

Allergen sensitization and challenge

Age-matched (9- to 16-week old) G1Red:G2GFP mice (BDF) were sensitized and challenged with chicken ovalbumin (OVA) as described previously (Sakai et al. 1999) with some modifications. Briefly, on days 0 and 7, mice were intraperitoneally injected with 100 μg of OVA (crude grade IV; Sigma, St. Louis, MO), which was emulsified in 1 mg of aluminum hydroxide/magnesium hydroxide (Pierce, Rockford, IL). The sensitized mice were intranasally challenged with 15 μg of OVA in 30 μl of saline on days 14, 15, and 16. On day18, the peripheral blood cells were collected through the retro-orbital plexus after anesthesia, and the BALF-derived cells were obtained from the tracheae by lavaging the lungs 3 times with 0.5 ml of ice-cold PBS containing 2% FBS.

RESULTS

Isolation of eosinophils using Gata1 expression

In order to characterize the differentiation pathway of eosinophils, we utilized expression of *Gata1* and *Gata2* genes. We first attempted isolation of the eosinophils from the bone marrow of G1GFP transgenic mice by using GFP reporter expression, as the G1GFP mice express GFP under the *GATA-1-hematopoietic regulatory domain* (*G1-HRD*; Suzuki et al. 2003). We isolated $LyE^-/CD71^-/c\text{-}Kit^-/G1GFP^+$ cells (G1-R1 fraction; Fig. 1A) and examined morphology of the cells by Wright-Giemsa staining. Cells in the G1-R1 fraction contained segmented nucleus and pale-pink granules in the cytoplasm (Fig. 1B). EoProbe stains eosinophil-specific basic cytoplasm, one of the most characteristic properties of eosinophils, by red fluorescence. The red fluorescence was observed markedly in the G1-R1 fraction cells (Fig. 1B). We also examined expression profiles several eosinophil-related genes plus

Gata1 and *Gata2* genes by quantitative RT-PCR (Fig. 1C). To our expectation *Gata1* mRNA was expressed, albeit moderately, in the G1-R1 fraction. High-level expression of IL-5 receptor α (*IL-5Rα*), major basic protein (*MBP*), eosinophil peroxidase (*EPX*) and *Gata2* mRNAs was identified specifically in the G1-R1 fraction, while Friend of GATA-1 (*Fog1*) mRNA was practically not detected in the fraction. Instead, *Gata1* and *Fog1* mRNAs were expressed abundantly in the G1-R2 fraction. FOG-1 is one of the well-studied cofactors of GATA-1 (Hong et al. 2005). These results thus demonstrate that the G1GFP is a useful marker for the eosinophil isolation and cells in the eosinophil lineage are highly enriched in the G1-R1 fraction. It is interesting to note that in agreement with previous reports on eosinophils (Zon et al. 1993; Querfurth et al. 2000; McNagny and Graf 2002) both *Gata1* and *Gata2* mRNAs were expressed in the G1-R1 fraction.

Gata2-expressing eosinophils were different from Gata1-expressing eosinophils

To ascertain whether *Gata1* and *Gata2* are expressed simultaneously or separately in distinct stages of eosinophil differentiation, we then attempted isolation of eosinophils using a fluorescent protein reporter expressed under the regulatory influence of the *Gata2* gene. For this purpose, we utilized G2GFP mice in which the *GFP* gene is knocked in the *Gata2* gene IS exon (Suzuki et al. 2006). We isolated GFP-positive cells from bone marrow of G2GFP mice and found that population of $LyE^{-}/CD71^{-}/c\text{-}Kit^{-}/G2GFP^{+}$ cells was smaller in G2GFP mouse bone marrow (G2-R1, 0.7%; Fig. 2A) than that in G1-R1 mouse bone marrow (G1-R1, 3.2%; Fig. 1A). We also examined morphology of the G2-R1 fraction cells by Wright-Giemsa staining and EoProbe staining (Fig. 2B). Cells in the G2-R1 fraction contained pale-pink granules in the cytoplasm and showed EoProbe red fluorescence, albeit the number of the granules and intensity of the fluorescence were much less and weaker, respectively, than the cells in the G1-R1 fraction. Similarly, although *C/EBPε* and the eosinophil-specific genes, *IL-5Ra*, *EPX*, *MBP* were expressed in the G2-R1 fraction, the expression level was low compared with the G1-R1 fraction (Fig. 2C). Meanwhile, it was reported that *C/EBPα* gene is expressed mainly in immature hematopoietic cells (Iwasaki et al. 2006), and we found that *C/EBPα* was expressed higher in the G2-R1 fraction than in the G1-R1 fraction. These results taken together suggest that the cells in the G2-R1 fraction may also belong to the eosinophil lineage, but the results further suggest that *Gata2* is expressed in eosinophilic progenitors, while *Gata1* is expressed in mature eosinophils.

Dissection of eosinophil differentiation stages using Gata1- and Gata2-based reporters

To monitor expression of the *Gata1* and *Gata2* genes simultaneously in individual mouse, we generated a transgenic mouse line that expresses red fluorescent protein under the regulatory influence of *G1-HRD* (G1Red

mouse line). Cells in the G1-R1 fraction of G1Red mouse showed similar properties to those of G1GFP mice, indicating that mature eosinophils were enriched in the fraction (Supplemental Fig. 1). We crossed the G1Red mice with G2GFP mice and obtained G1Red:G2GFP mice. To visualize the expression of *Gata1* and *Gata2* genes during eosinophil differentiation, we examined fluorescence change of a single eosinophil colony. When LyE^{-}/CD71^{-}/c-Kit^{+} cells from the bone marrow of G1Red:G2GFP mouse were cultured (Fig. 3A) in the presence of IL-5 and SCF, eosinophil colonies were emerged (Fig. 3B). These colonies started emitting green fluorescence on Day 7. Two days later red fluorescence also started, and the green fluorescence was diminished while red fluorescence still persisted at Day 14. These results suggest that eosinophils during differentiation can be divided, at least, into three stages, *i.e.* G1Red^{-}/G2GFP^{+}, G1Red^{+}/G2GFP^{+} and G1Red^{+}/G2GFP^{-}.

To further characterize hematopoietic cells in these stages, we conducted FACS analyses of LyE^{-}/CD71^{-} cells from the G1Red:G2GFP mouse bone marrow. We fractionated LyE^{-}/CD71^{-}/c-Kit^{-} cells utilizing G1Red and G2GFP (Fig. 3C; G1G2-R1 to G1G2-R4 fractions), and identified three fractions that contained eosinophils. Firstly, showing very good agreement with the G1-R1 analysis, we found cells possessing segmented nucleus and abundant pale-pink granules in the G1G2-R3 (G1Red^{+}/G2GFP^{-}) fraction (Fig. 3D). These cells were densely stained with EoProbe (Fig. 3E), suggesting that this G1G2-R3 fraction contains fully developed eosinophils, as is the case for the G1-R1 fraction. Secondly, as has been noticed in the G2-R1 analysis, cells in G1G2-R1 (G1Red^{-}/G2GFP^{+}) fraction contain much less number of the granules in the cytoplasm (Fig. 3D) than in the G1G2-R3 fraction and were stained only weakly with EoProbe (Fig. 3E), indicating that this fraction contains eosinophilic progenitors. Thirdly and most importantly, cells in the G1G2-R2 fraction possessed less number of pale-pink granules than in G1G2-R3 cells did, but the number is more than that in the G1G2-R1 cells (Fig. 3D). The G1G2-R2 cells showed stronger fluorescence with EoProbe than G1G2-R1 cells did, but the fluorescence was weaker than that of G1G2-R3 (Fig. 3E). These results thus suggest that the R2 fraction (G1Red^{+}/G2GFP^{+}) harbors eosinophils whose differentiation stage is between G1G2-R1 and G1G2-R3 cells.

To further evaluate the differentiation stage of G1G2-R2 cells, we performed quantitative RT-PCR analysis of eosinophil-specific mRNAs (Fig. 3F). The results showed that the G1G2-R3 (G1Red^{+}/G2GFP^{-}) cells exhibited the highest expression of eosinophil-specific genes. Importantly, expression levels of eosinophil-specific genes in the G1G2-R2 (G1Red^{+}/G2GFP^{+}) cells were lower than those of the G1G2-R3 cells, but higher than G1G2-R1 cells. In contrast, expression levels of *C/EBPα*, *C/EBPβ,* and *CD34* genes that are usually activated in immature hematopoietic cells were high in the G1G2-R1 (G1Red^{-}/G2GFP^{+}) cells.

To evaluate the colony-forming potential, we performed a colony-forming assay (Supplemental Fig. 2). We found that none of the LyE$^-$/CD71$^-$/c-Kit$^-$ cells formed eosinophil colonies, indicating these cells are differentiated further from the colony-forming progenitor stage. When we examined colony-forming potential of the cells in LyE$^-$/CD71$^-$/c-Kit$^+$ cells, both G1Red$^-$/G2GFP$^-$ and G1Red$^-$/G2GFP$^+$ cells formed eosinophilic and granulocyte-macrophage colonies (Supplemental Fig. 2). Taken together, these results demonstrate that there are at least three flow-sorted fractions corresponding to relatively differentiated eosinophils after the colony-forming stages. Although we could not purify eosinophil progenitors (EoP), these three eosinophil fractions are detectible by using the expression pattern of *Gata1* and *Gata2* genes.

Gata2-expressing eosinophils were scarce in peripheral blood

We next analyzed eosinophils in peripheral blood. LyE$^-$/CD71$^-$ hematopoietic mononuclear cells from peripheral blood of G2GFP:G1Red mice were subjected to FACS analysis (Fig. 4A). LyE$^-$/CD71$^-$/c-Kit$^-$ cells were analyzed using G1Red and G2GFP fluorescence, respectively. We also examined LyE$^-$/CD71$^-$/c-Kit$^+$ cells in peripheral blood, but the population was much smaller than those in bone marrow (data not shown). As is the case for bone marrow cells, the LyE$^-$/CD71$^-$/c-Kit$^-$ cells in peripheral blood were divided into four fractions (G1G2-R1 to G1G2-R4) by using G1Red and G2GFP fluorescence. Isolated cells from each fraction were stained with Wright-Giemsa (Fig. 4B). Unlike bone marrow, eosinophils were scarcely identified in G1G2-R1 and G1G2-R2. More than 95% of cells in G1G2-R1 and G1G2-R2 appear to be neutrophils and lymphocytes, which may be contaminated during FACS separation. Similarly, we could not enrich eosinophils efficiently in the G1G2-R3 fraction. The percentage of eosinophils in the G1G2-R3 fraction was between 20% and 40% (Fig. 4B). Lymphoid cells and neutrophils were also included in the G1G2-R3 fraction. Similar results were observed with EoProbe staining; only cells in G1G2-R3 showed red fluorescence (Fig. 4C). These data imply that fully differentiated eosinophils (G2GFP$^-$/G1Red$^+$) tend to migrate from bone marrow to peripheral blood.

Mature eosinophils increased in number in response to allergen challenge

To investigate how eosinophils react to allergen, we generated mouse model of asthma. Eosinophil populations in each tissue were increased in number in mice model of asthma (Fig. 5A). In bone marrow the fully differentiated eosinophil population in the G1G2-R3 (G2GFP$^-$/G1Red$^+$) fraction significantly increased in number as a result of OVA challenge (Fig. 5B). In contrast, we could not find substantial increase in number of immature eosinophils in G1G2-R1 (G2GFP$^+$/G1Red$^-$) fraction (Fig. 5A) nor multipotential hematopoietic progenitor cells (LyE$^-$/CD71$^-$/c-Kit$^+$/G2GFP$^-$/G1Red$^-$ and

LyE$^-$/CD71$^-$/c-Kit$^+$/G2GFP$^+$/G1Red$^-$) that contain EoP (Supplemental Fig. 3).

We also examined eosinophils in peripheral blood and BALF. The eosinophils in peripheral blood significantly increased in number after OVA challenge (Fig. 5B). Although we could not enrich eosinophils efficiently from peripheral blood, the increase in cell population in peripheral blood G1G2-R3 fraction most plausibly reflects increment in the number of eosinophils, because the rate of eosinophil concentration in the G1G2-R3 fraction was elevated after OVA challenge (data not shown). The number of eosinophils in BALF also significantly increased (Fig. 5B). Wright-Giemsa staining confirmed that the cells in BALF G1G2-R3 fraction were mostly eosinophils (data not shown), while there were few eosinophils in BALF in normal conditions. Thus, these results support our contention that in response to allergen challenge fully differentiated eosinophils (G2GFP$^-$/G1Red$^+$) markedly increase in number and migrate into lung.

DISCUSSION

Since eosinophils were identified as effector cells in the etiology of asthma, they have been the focus of research study as potential therapeutic target cells of several immune diseases (Lee et al. 2004; Humbles et al. 2004; Tomaki et al. 2000; Ying et al. 1999; Phillips et al. 2003). Better understanding for the differentiation pathway of eosinophils is directly relevant to the development of novel therapy for several immune diseases caused by eosinophils. Using gene expression profiles is effective way to analyze differentiation of eosinophils. G1Red:G2GFP mice are a useful tool, can purify eosinophils in each developmental stages *in vivo* (Fig. 6A).

In bone marrow, immature eosinophils (G1Red$^-$/G2GFP$^+$) expressed low levels of *Gata1*, whilst fully differentiated eosinophils (G1Red$^+$/G2GFP$^-$) did not express *Gata2* (Fig. 3E). These data indicate that G2GFP faithfully mimics endogenous *Gata2* expression. However, G1Red is not sufficient for recapitulation of endogenous *Gata1* expression in the early developmental stages of eosinophils as well as of erythroid cells. Since *Gata1-HRD* is not active in early progenitor stage of erythroid lineage cells (Suzuki et al. 2003), this 8 kb of gene regulatory domain may be not sufficient for regulation of the *Gata1* gene expression in immature eosinophils.

When we monitored the expression pattern of G1Red and G2GFP for a single colony during eosinophil colony formation, GATA switching also occurred in eosinophils as well as erythroid cells (Fig. 3B). In bone marrow, fully differentiated eosinophils (more than 70% of total eosinophil populations) did not expressed *Gata2*. In hematopoietic development, GATA-2 expression is first identified in hematopoietic stem cells, and persists in multi-potential progenitors (Minegishi et al. 1998; Iwasaki et al. 2005; Suzuki et al. 2006). GATA-2 up-regulates transcription of the *Gata1* gene in early stage of erythroid differentiation (Kobayashi-Osaki et al. 2005), and the

increased GATA-1 finally represses the *Gata2* gene transcription in later stage of erythroid cells (Grass et al. 2003; Johnson et al. 2007). In eosinophil differentiation, it is plausible to explain that the *Gata2* gene expression is diminished by GATA-1 in a similar way with erythroid cells. Although FOG-1 is essential for repression of the *Gata2* gene expression by GATA-1 in erythroid lineage (Johnson et al. 2007; Martowicz et al. 2005; Pal et al. 2004; Querfurth et al. 2000), *Fog1* mRNA is not expressed in eosinophils (Fig.1C). Therefore, we expect that GATA-1 might repress the *Gata2* gene expression during maturation of eosinophils in a FOG-1-independent fashion.

Because *Gata2* can replace *Gata1* in eosinophils (Hirasawa et al. 2002), disruption of *Gata1* expression in eosinophils was considered as block development to fully differentiated eosinophils, which express not *Gata2* but *Gata1*. To confirm this hypothesis we tested whether ΔdblGATA mice (Yu et al. 2002) possess *Gata2*-expressing eosinophils. On this purpose we generated ΔdblGATA:G2GFP mice by mating ΔdblGATA mice with G2GFP mice. We found *Gata2*-expressing eosinophils in the bone marrow of ΔdblGATA:G2GFP as well as in that of G2GFP mice (Supplemental Fig. 4). These results suggest that GATA-1 is an essential transcription factor for development of fully differentiated eosinophils.

Differentiation of eosinophils *in vivo* is the subject of controversy. Some groups reported that CD34 positive eosinophils that have a proliferation potential migrated into tissue (Gauvreau et al. 2000; Southam et al. 2005), whilst others reported that only mature eosinophils migrated into tissue (Wills-Karp and Karp 2004). When we identified the 3 developmental stages of eosinophils, fully differentiated eosinophils rarely expressed *CD34* (Fig. 3F). These eosinophils did not have the potential to form colonies *in vitro* (Supplemental Fig. 2). Also, there were few *Gata2*-expressing eosinophils in peripheral blood and BALF (Fig. 4B, C, and Fig. 5A). These results indicate that EoPs develop into fully differentiated eosinophils in bone marrow and then these eosinophils migrate into peripheral blood (Fig. 6B left).

To test how eosinophils react to allergen, G1Red:G2GFP mice were challenged with OVA. While G1Red expressing mature eosinophils ($G1Red^{+}/G2GFP^{-}$ and $G1Red^{+}/G2GFP^{+}$) markedly increased in number in OVA-treated G1Red:G2GFP mice, we could not detect significant increase of cell population of immature eosinophils ($G1Red^{-}/G2GFP^{+}$) nor multipotential hematopoietic progenitor cells contain EoP ($LyE^{-}/CD71^{-}/c\text{-}Kit^{+}/G2GFP^{-}/G1Red^{-}$ and $LyE^{-}/CD71^{-}/c\text{-}Kit^{+}/G2GFP^{+}/G1Red^{-}$) (Fig. 5B and Supplemental Fig. 3). Furthermore these immature eosinophils or multipotential hematopoietic progenitor cells do not express eosinophil-specific genes (*IL-5Rα, MBP,* and *EPX*) (Fig. 3F). In contrast to our results, previously reported murine and human EoPs significantly increased in number in emergency granulopoiesis and expressed eosinophil-specific genes *IL-5Rα, MBP,* and

EPX (Iwasaki et al. 2005; mori et al. 2009). These discrepancies were probably caused by contamination of mature eosinophils in previously reported EoPs.

Although IL-5 is an essential cytokine in stimulating eosinophil differentiation (Yamaguchi et al. 1988; Clutterbuck et al. 1989), IL-5 knock-out mice still possess a basal-level population of eosinophils, which do not increase drastically in number against allergen challenge or parasitic infection (Shen et al. 2003; Fallon et al. 2002; and Foster et al; 1996). Here, we showed that immature eosinophils (G1Red$^-$/G2GFP$^+$) do not express *IL-5Rα* (Fig. 3F). Moreover, as the basal-level population of eosinophils in *IL-5* KO mice, we could not identify significant increase of these immature eosinophils nor multipotential hematopoietic progenitor cells in OVA-treated G1Red:G2GFP mice (Fig. 5 and Supplemental Fig. 3). Therefore the basal-level population of eosinophils in *IL-5* KO mice seems to be composed of EoPs and immature eosinophils. EoPs might be able to differentiate to immature eosinophils in an IL-5-independent manner and the IL-5 signal pathway is critical for the development of mature eosinophils.

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FIGURE LEGENDS

Figure. 1. Isolation of eosinophils using *Gata1* expression.

(A) Flow cytometry for CD71 and GFP expression (G1GFP) of LyE^-/c-Kit^- hematopoietic mononuclear cells from the bone marrow of G1GFP mice. The percentage in each quadrangle is shown. (B) Morphology of cells in each fraction (G1-R1, G1-R2, and G1-R3). Cells from each fraction were isolated and subjected to Wright-Giemsa (top row) and EoProbe staining (bottom row). (C) Relative mRNA expression levels of indicated genes in cells from each region were measured by quantitative RT-PCR. The results are normalized to the level of GAPDH mRNA

Figure. 2. Isolation of eosinophils using *Gata2* expression.

(A) Flow cytometry for CD71 and GFP expression (G2GFP) of LyE^-/c-Kit^- hematopoietic mononuclear cells from the bone marrow of G2GFP mice. The percentage in quadrangle is shown. (B) Morphology of *Gata2*-expressing eosinophils (G2-R1). Isolated *Gata2*-expressing eosinophils were subjected to Wright-Giemsa (top) and EoProbe staining (bottom). (C) The gene expression profile of *Gata2*-expressing eosinophils (G2-R1) was compared with *Gata1*-expressing eosinophils (Fig. 1A, G1-R1). Relative mRNA expression levels of indicated genes in cells from each region were measured by quantitative RT-PCR. The results are normalized to the level of GAPDH mRNA

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Figure. 3. Isolation of 3 developmental stages of eosinophils using *Gata1* and *Gata2* expression.

(A) LyE^-/$CD71^-$/c-Kit^+ hematopoietic mononuclear cells from the bone marrow of G1Red:G2GFP mice were cultured with IL-5 and SCF in semi-solid medium. (B) Time-lapse images of a growing single colony were taken by bright field (top), RFP (G1Red, middle) and GFP (G2GFP, bottom) images on indicated days. (C) LyE^-/$CD71^-$/c-Kit^- hematopoietic mononuclear cells from the G1Red:G2GFP mouse bone marrow were divided into 4 sub-fractions with RFP (G1Red) and GFP (G2GFP) expression. The percentage of cells in each quadrangle of the data from flow cytometry (left) is represented in the right boxes with the name and position of each region. (D, E) Flow-sorted cells in each region were subjected to Wright-Giemsa (D) and EoProbe (E) staining. Cells in G1G2-R1 (lower right), -R2 (upper right), -R3 (upper left) and -R4 (lower left) are shown. The position of each panel is represented in (C). Scale bars are 10 μm. (F) Relative mRNA expression levels of indicated genes in cells from each region were measured by quantitative RT-PCR. The results are normalized to the level of GAPDH mRNA

Figure. 4. Isolation of eosinophils from peripheral blood.

(A) Flow cytometry for RFP (G1Red) and GFP (G2GFP) expression of LyE^-/$CD71^-$/c-Kit^- hematopoietic mononuclear cells from peripheral blood of G1Red:G2GFP mice. The percentage of

cells in each quadrangle (left) is represented in the right boxes as well as Fig. 3C. (B, C) Flow-sorted cells in each region were subjected to Wright-Giemsa (B) and EoProbe (C) staining. The position of each panel is represented each cell fraction as indicated by boxes in (A) (same with Fig. 3B). Scale bars represent 10 μm.

Figure. 5. The reactivity of eosinophils in each development stages to allergen challenge.
(A) Flow cytometry for RFP (G1Red) and GFP (G2GFP) expression of $LyE^{-}/CD71^{-}/c\text{-}Kit^{-}$ hematopoietic mononuclear cells from bone marrow (BM), peripheral blood (PB) and bronchoalveolar lavage fluid (BALF) of G1Red:G2GFP mice before (Control; top row) or after (bottom row) OVA-induced asthma. The percentage of cells in each quadrangle is shown. (B) The changes in cell populations are represented by bar graphs with mean ± SD from more than 3 independent experiments (**, $P < 0.01$; *, $P < 0.05$ compared with the control).

Figure. 6. Model for the differentiation and developmental pathways of eosinophils *in vivo*.
(A) Eosinophils differentiate from HSC (Hematopoietic Stem Cell) through CMP (common myeloid progenitor), GMP (granulo-macrophage progenitor) and EoP (eosinophil progenitor). As the differentiation progressed, expression level of *Gata2* decreased while that of *Gata1* increased. Finally, *Gata2* expression diminished and expression level of *MBP* and *EPX* increased. (B) In bone marrow, EoPs developed into fully differentiated eosinophils ($G1Red^{+}/G2GFP^{-}$). Fully differentiated eosinophils migrate into peripheral blood but detected scarcely in lung under normal conditions. These fully differentiated eosinophils markedly increased in number in bone marrow by OVA challenge, and then migrate into peripheral blood and lung.

Supplemental Figure. 1. Isolation of eosinophils from bone marrow of G1Red transgenic mice.
(A) Construction of reporter gene: individual exons are depicted as solid boxes. G1Red mice express the DsRed2 reporter gene under the control of *G1-HRD*. (B) Flow cytometry for CD71 and RFP expression (G2Red) of $LyE^{-}/c\text{-}Kit^{-}$ hematopoietic mononuclear cells from the bone marrow of G1Red mice. The percentage in each quadrangle is shown. (C) Morphology of cells in fraction G1-R1. Cells from G1-R1 fraction were isolated and subjected to Wright-Giemsa staining.

Supplemental Figure. 2. EoPs were included in $LyE^{-}/CD71^{-}/c\text{-}Kit^{+}$ fractions.
The results of the methylcellulose colony assay with $LyE^{-}/CD71^{-}$ hematopoietic mononuclear cells from the bone marrow of G1Red:G2GFP (Fig. 3C, S.Fig.3 control). Cells in $LyE^{-}/CD71^{-}/c\text{-}Kit^{+}/G2GFP^{-}/G1Red^{-}$ and $LyE^{-}/CD71^{-}/c\text{-}Kit^{+}/G2GFP^{+}/G1Red^{-}$ had the potential to form eosinophil and granulocyte-macrophage colonies. Cytokine mixture used in this study consisted of SCF, IL-3, IL-6, GM-CSF, Epo, and Tpo. (cK:c-Kit; G2:G2GFP; G1:G1Red)

<u>Supplemental Figure. 3.</u> The reactivity of multipotential hematopoietic progenitor cells that contain EoPs to allergen challenge.

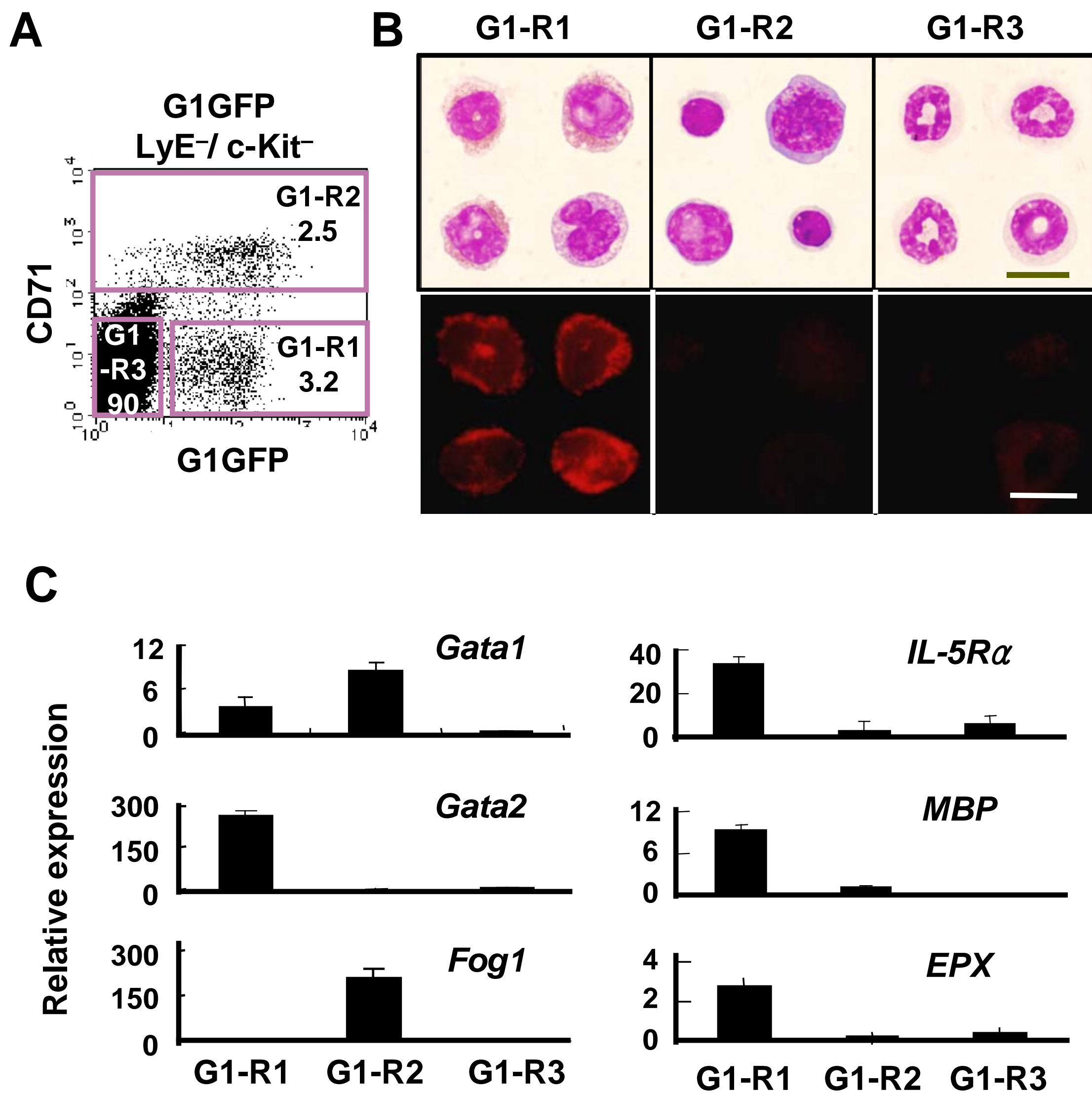
Flow cytometry for G1Red and G2GFP expression of LyE$^-$/CD71$^-$/c-Kit$^+$ hematopoietic mononuclear cells from the bone marrow of G1Red:G2GFPmice. LyE$^-$/CD71$^-$/c-Kit$^+$ cells were analyzed using G1Red and G2GFP in normal (left) and OVA-induced asthma conditions (right). The percentage of cells in each box is shown

<u>Supplemental Figure. 4.</u> ΔdblGATA mice possessed *Gata2*-expressing eosinophils in bone marrow.

(A) Flow cytometry for CD71 and GFP expression (G2GFP) of LyE$^-$/c-Kit$^-$ hematopoietic mononuclear cells from the bone marrow of ΔdblGATA:G2GFP mice. The percentage in quadrangle is shown. (B) The percentage of *Gata2*-expressing eosinophils from bone marrow of G2GFP mice and of ΔdblGATA:G2GFP mice. (Data are mean ± SD).

FIGUERS & TABLES

Figure 1.



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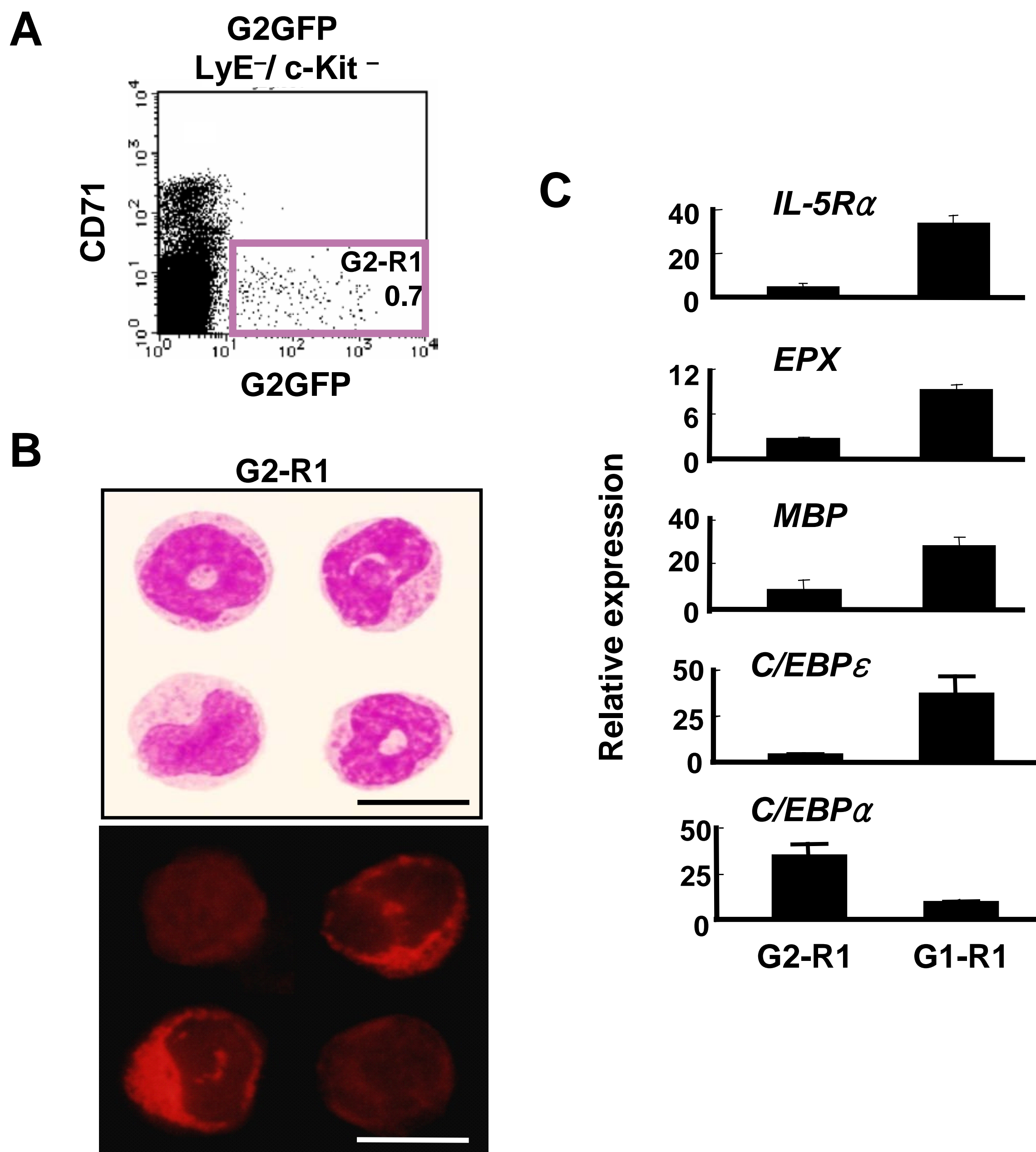
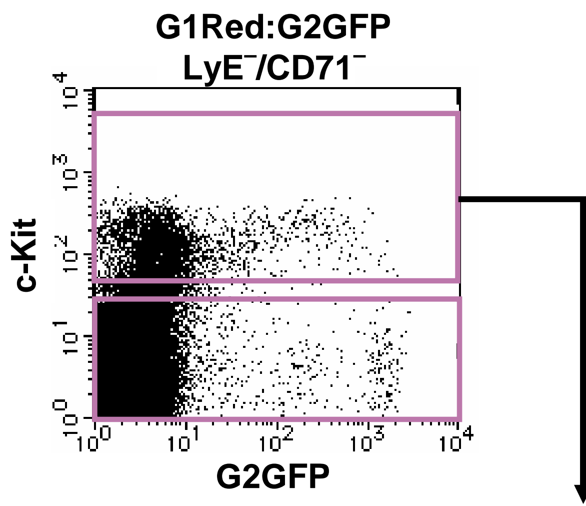


Figure 3A, B.



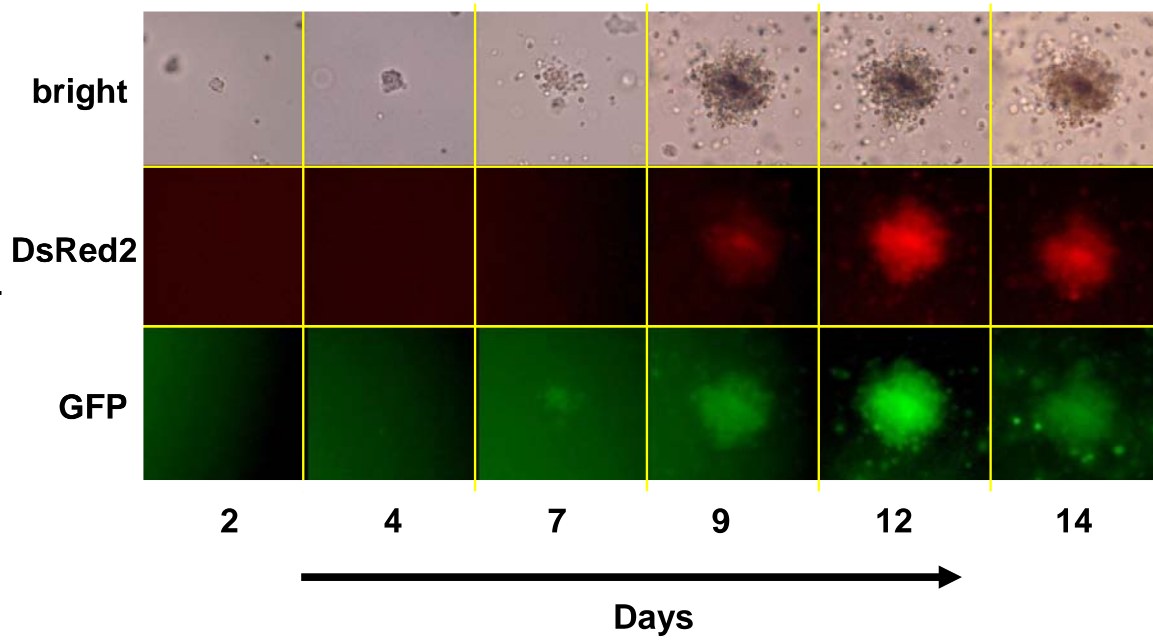
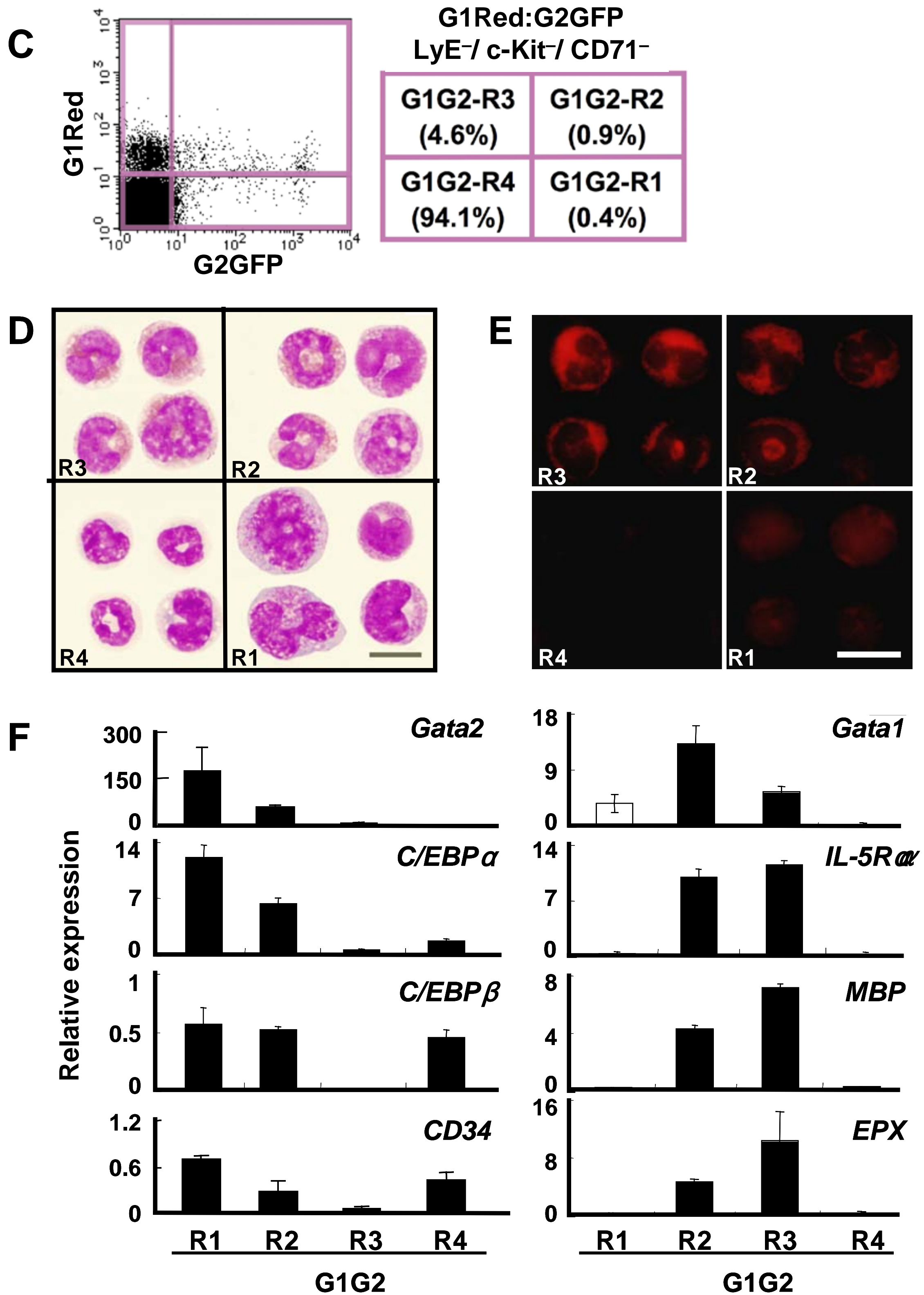


Figure 3C - F.



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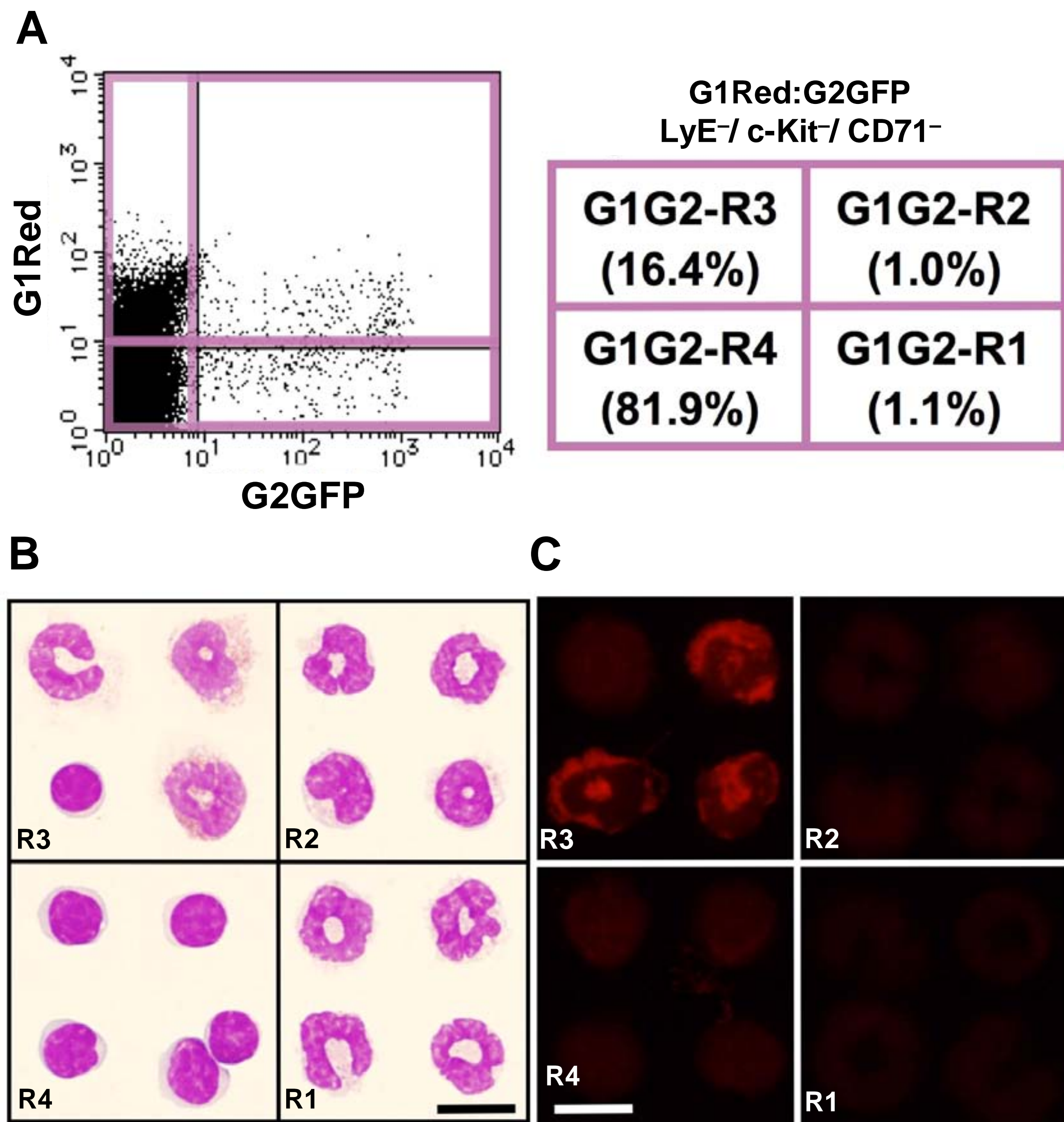

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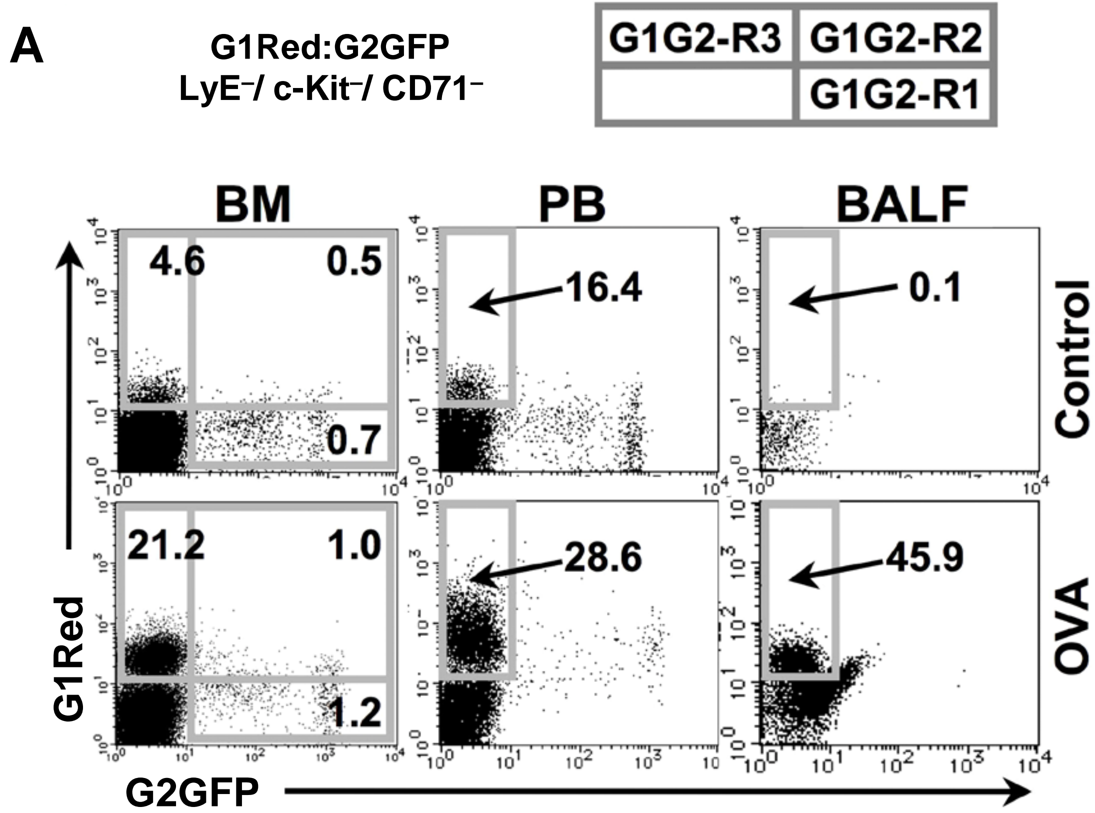

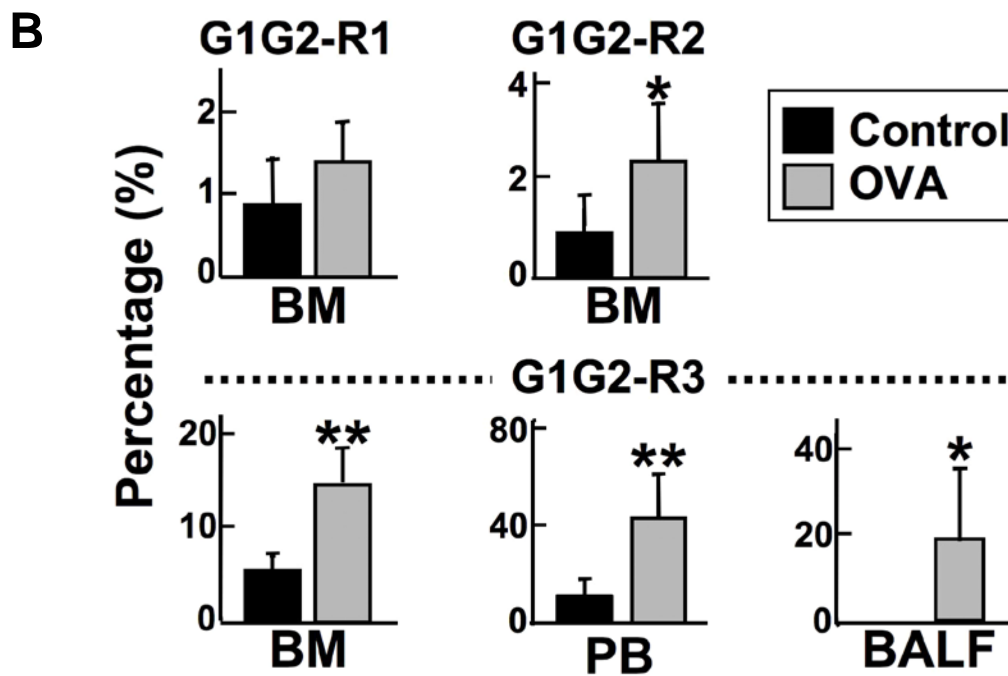

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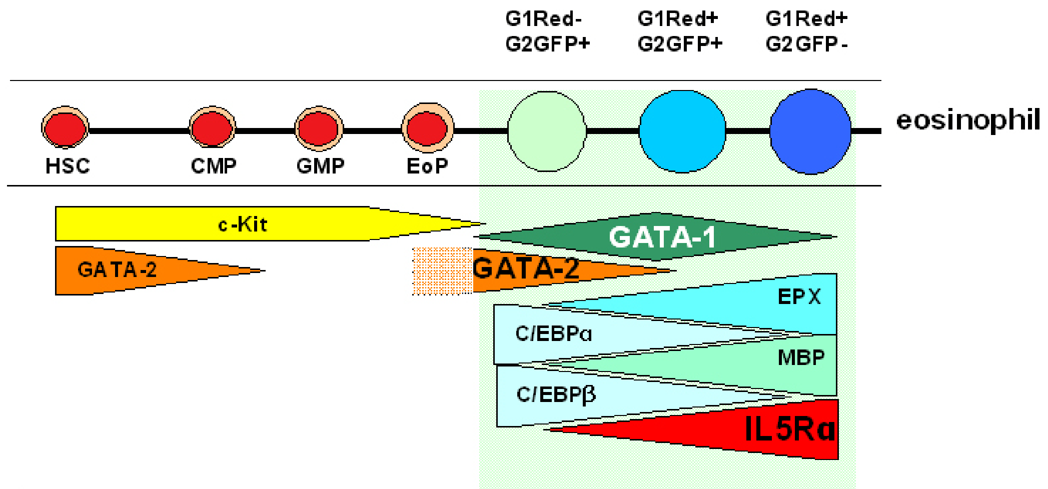
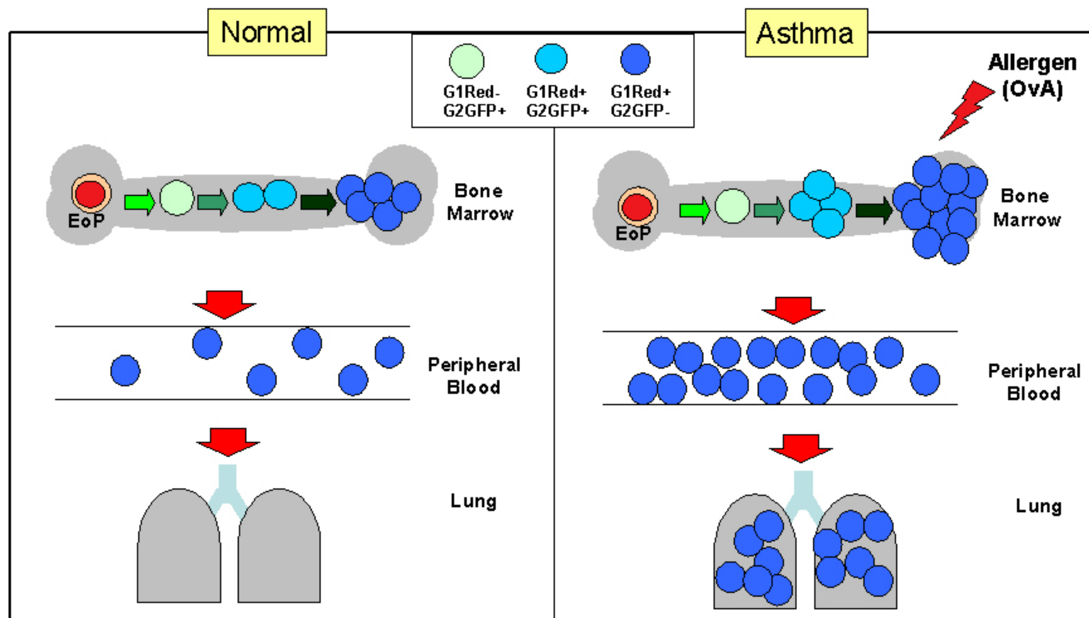
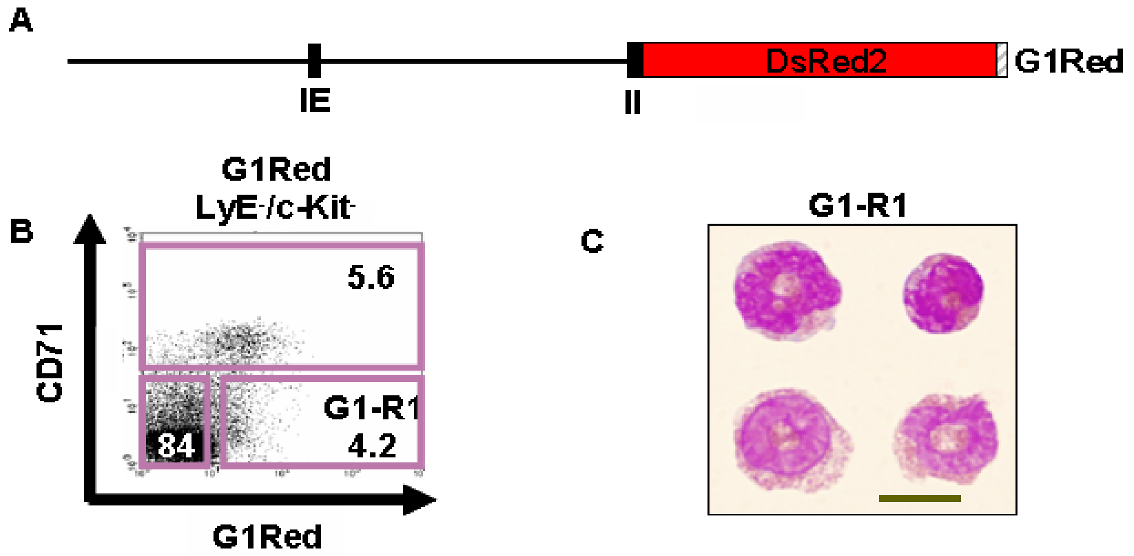




SUPPLEMENTAL DATA

S. Figure 1.



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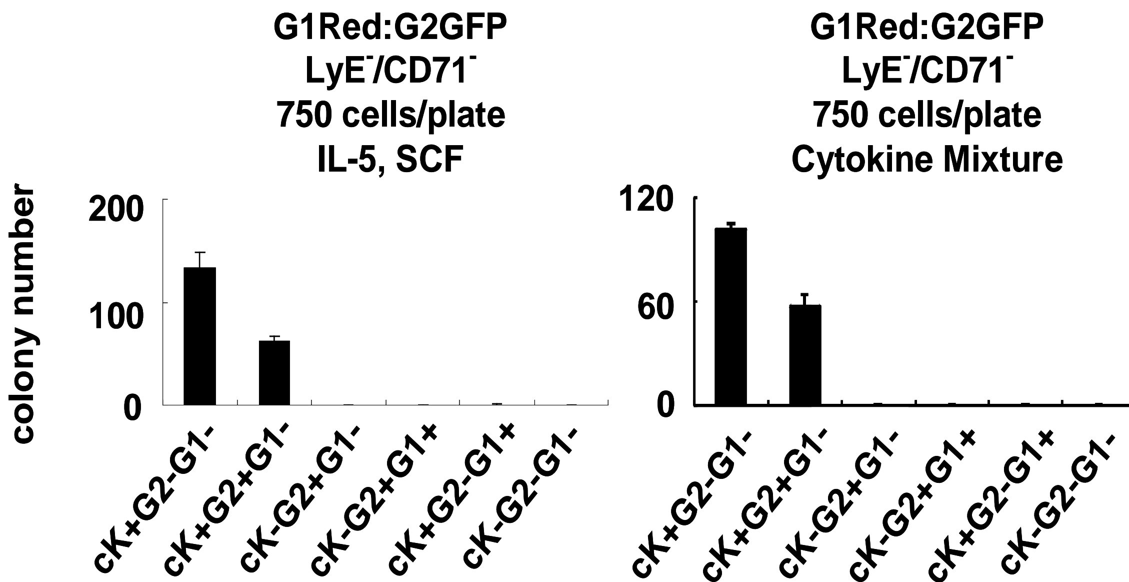

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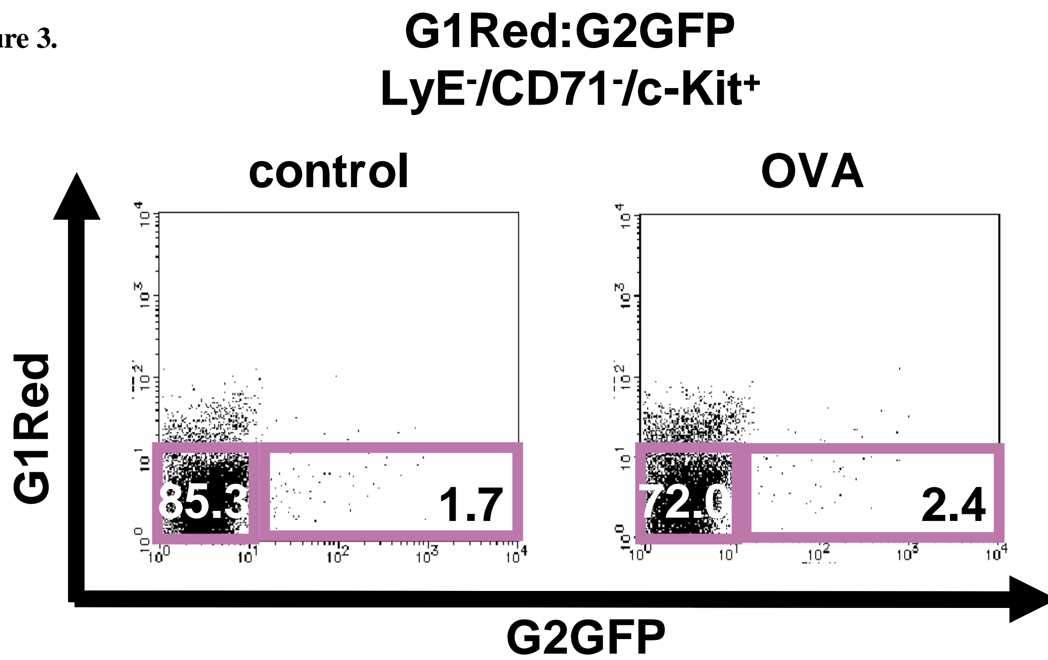

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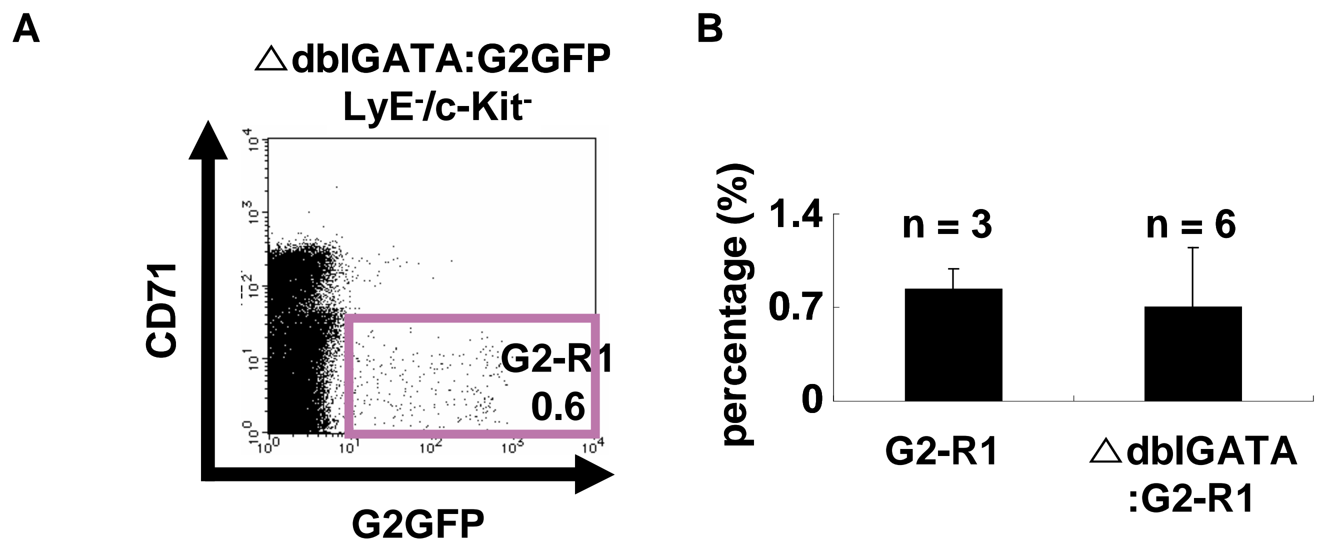

Supplemental table. 1. Primer and Probe set for Real Time PCR.

<table>
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<tr><td colspan="2">VIC-labeled probe, 5'-CCCAAGAAGCGAATGATTGTCAGCAAA</td></tr>
<tr><td rowspan="2">Gata2</td><td>5'-GAATGGACAGAACCGGCC</td><td>5'-AGGTGGTGGTTGTCGTCTGA</td></tr>
<tr><td colspan="2">VIC- labeled probe, 5'-AAGCGGAGGCTGTCTGCTGCCAG</td></tr>
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<tr><td colspan="2">JOE- labeled probe, 5'-CAAGCTTCCCGTTCTCAGCC</td></tr>
<tr><td rowspan="2">MBP</td><td>5'-CCCCTGGAGGACACTCTTCT</td><td>5'-GGAGCGTCTGCTCTTCATCT</td></tr>
<tr><td colspan="2">FAM- labeled probe, 5'-GGACCAGA</td></tr>
<tr><td rowspan="2">EPX</td><td>5'-TCACTTGACCGAGTGTCACC</td><td>5'-CTCCTGACTAACCGCTCTGC</td></tr>
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<tr><td rowspan="2">C/EBPβ</td><td>5'-TGATGCAATCCGGATCAA</td><td>5'-ACACGTGTGTTGCGTAGTCC</td></tr>
<tr><td colspan="2">FAM- labeled probe, 5'-TGGCTGAG</td></tr>
<tr><td rowspan="5">SYBR</td><td>CD34</td><td>5'-GGGTAGCTCTCTGCCTGATG</td><td>5'-TCCGTGGTAGCAGAAGTCAA</td></tr>
<tr><td>Fog1</td><td>5'-CATGGCTAGTCCCTGGAGTG</td><td>5'-CCAGGATAGGCCCTCAGTG</td></tr>
<tr><td>C/EBPϵ</td><td>5'-TCCCCTGCAGTACCAAGTG</td><td>5'-GTGCCTTGAGAAGGGGACT</td></tr>
<tr><td>CCR3</td><td>5'-TCAACTTGGCAATTTCTGACCT</td><td>5'-CAGCATGGACGATAGCCAGG</td></tr>
<tr><td>IL-5Rα</td><td>5'-GGTCCCGGTATGCAGTTCTA</td><td>5'-GGAAGACCCTGGTTAGATCCTT</td></tr>
</table>

Each mRNA expression level was examined by quantitative RT-PCR analyses using listed oligo-DNAs. PCR was performed with fluorescent probe system (a-f) or SYBR Green system (g-k) using ABI7300 sequential detector.