Different Roles of G protein subunits $\beta_1$ and $\beta_2$ in Neutrophil Function Revealed by Gene Expression Silencing in Primary Mouse Neutrophils

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ABSTRACT

Neutrophils play important roles in host innate immunity and various inflammation-related diseases. In addition, neutrophils represent an excellent system for studying directional cell migration. However, neutrophils are terminally differentiated cells that are short lived and refractory to transfection; thus, they are not amenable for existing gene silencing techniques. Here we describe the development of a method to silence gene expression efficiently in primary mouse neutrophils. A mouse stem cell virus (MSCV)-based retroviral vector was modified to express shRNAs and fluorescent marker protein at high levels in hematopoietic cells and used to infect mouse bone marrow cells prior to reconstitution of the hematopoietic system in lethally irradiated mice. This method was used successfully to silence the expression of G$\beta_1$ and/or G$\beta_2$ in mouse neutrophils. Knockdown of G$\beta_2$ appeared to affect primarily the directionality of neutrophil chemotaxis rather than motility, whereas knockdown of G$\beta_1$ had no significant effect. However, knockdown of both G$\beta_1$/G$\beta_2$ led to significant reduction in motility and responsiveness. In addition, knockdown of G$\beta_1$, but not G$\beta_2$, inhibited the ability of neutrophils to kill ingested bacteria, and only double knockdown resulted in significant reduction in bacterial phagocytosis. Therefore, we have developed an shRNA-based method to effectively silence gene expression in mouse neutrophils for the first time, which allowed us to uncover divergent roles of G$\beta_1$ and G$\beta_2$ in the regulation of neutrophil functions.

INTRODUCTION

Neutrophils are the most abundant leukocytes in the blood and play an essential role in the early stages of the innate immune responses by ingesting and killing invading pathogens. In response to inflammatory stimuli, neutrophils first adhere to and extravasate through blood vessels, and then migrate through the interstitial tissue toward the site of inflammation. While neutrophils play an important role in the host defense, uncontrolled inflammatory reactions are associated for a variety of pathological conditions, including ischemia-reperfusion injury during heart attack and strokes, arteriosclerosis, rheumatoid arthritis and allergic reactions (1-3). Therefore understanding how neutrophil recruitment and function is regulated is critical for developing potential treatments for a number of disorders.
Neutrophils are also a fine model system to study directional cell migration and chemotaxis, because of their ability to migrate rapidly and directionally under a shallow gradient of chemoattractants. Chemotaxis is a fundamental biological process used by a variety of cell types and underlies a wide range of developmental, physiological, and pathophysiological events. It consists of two basic components, directionality and motility (4-8). Most of the neutrophil chemoattractants, including formyl methionine-leucine-phenylalanine (fMLP), bind to their specific cell surface receptors that are coupled to heterotrimeric G proteins, which upon activation regulate numerous downstream effectors. Studies in Dictyostelium cells demonstrated that the Gβ1 subunit plays a critical role in signal transduction to chemotaxis regulation (9,10). However, the importance of Gβ subunits in neutrophil chemotaxis has yet been investigated.

Despite basic scientific and clinical importance of neutrophil research, it has been hindered by the fact that these cells are terminally differentiated, short-lived, and thus not amenable to in vitro manipulations, including transfection. Targeted gene inactivation in mice has been the only approach to provide primary neutrophils for loss of function studies. Although these studies have provided novel insights into neutrophil biology, the approach is costly and time consuming. Therefore, it is imperative to develop a more efficient approach to study neutrophils.

RNA interference (RNAi) has been shown to be a rapid and powerful tool for knocking down gene expression in a sequence-specific fashion. Compared with chemically synthesized small interfering RNA (siRNA), short hairpin RNAs (shRNA) can be stably expressed in hard-to-transfect primary cells and in whole organisms (11). Recently, the miR30-shRNA cassette has been reported to yield a higher level of shRNA and more efficient knockdown than a simple shRNA design (12). Using the same miR30-shRNA cassette, Zhu and colleagues have silenced multiple target genes simultaneously to overcome isoform redundancy issue (13,14), demonstrating the powerful capabilities of this microRNA based shRNA design. Although shRNA-mediated gene silencing was successful in neutrophil-like cell lines, such as differentiated HL-60, these cells may not faithfully recapitulate the biology of primary neutrophils. In addition, these cell lines can not be used for in vivo studies. By taking advantage of pluripotent differentiation potentials of hematopoietic stem cells, we describe the development of a novel method to silence gene expression in primary neutrophils in mice using a retrovirus expressing shRNAs. Using this new method, we demonstrated that Gβ2 is primarily involved in regulating directionality rather than motility of neutrophil chemotaxis and Gβ1 is involved in bacterial killing by neutrophils.
MATERIALS AND METHODS

Plasmids

The initial retroviral vectors MIGR1 (MSCV-IRES-GFP-retrovirus-1) and the packaging vector pCL-ECO are generous gifts from Dr. Diane Krause at Yale University. For the LTR-YFP-shGβ2 vector, we replaced GFP in MIGR1 with YFP-miR-shGβ2, which was amplified by PCR from pSLIK-miR-shGβ2 and carries a targeting sequence of TGCTCATGTATTCCCACGACAA (14,15). For the CMV-YFP-shRNA vectors, the CMV promoter was amplified from pAAV-MCS vector (Stratagene, Cedar Creek, Texas) by PCR and inserted between LTR and YFP-miR-shRNA. MiR-shRNA sequences for Gβ1 knockdown were designed by using the RNAi Codex algorithm (http://codex.cshl.org/RNAi_central/RNAi.cgi?type=shRNA). Four targeting sequences were tested. They are: A: GCGACTCTTTCTCAGATCACAA; B: ATCTGGGACAGTTATACCACAA; C: AACATTATCTGTGGTATCACAT; D: GGCCGAGCAACTGAAGAACCAA. The D sequence was used for the knockdown studies in neutrophils. To generate plasmid containing tandem shRNAs, miR30-shGβ2 was amplified by PCR and inserted downstream of miR-Gβ1.

Td-Tomato was amplified and inserted into pGEX-3X (GE Healthcare, Piscataway, NJ) for the bacterial killing assay. All constructs were verified by sequencing.

Cell culture

We use PHE (Phoenix Ecotropic) cells, which are capable of producing viral gag-pol and envelope proteins, as the packaging cell line (16). PHE and NIH 3T3 cells were maintained in 90% DMEM, 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37°C under 5% CO2 in humidified air.

Retrovirus Production and infection

One day prior to transfection, PHE cells were seeded at a density of 3×10^5 cells/T 75 flask. Retroviral vector and pCL-ECO were co-transfected into cells using Lipofectamine Plus (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Two days later, the medium containing viruses was concentrated using Amicon Ultra columns (Millipore, Billerica, Massachusetts). For viral titer determination, serial dilutions of virus supernatants were added to NIH 3T3 cells in a 24-well plate, followed by centrifugation at room temperature to enhance the transduction efficiency. Twenty-four hours later, cells were analyzed by a flowcytometer.

Flow cytometry analysis

APC-CD11b, PerCP-B220 and APC-CD3ε antibodies were purchased from eBioscience (San Diego, CA). APC-Gr1 and Perp-Ly-6G antibodies were from BD (San J ose, CA). The single color flow analysis was done in the Guava EasyCyte Mini Base System (Millipore, Billerica, Massachusetts) and the multiple color flow analysis was performed in a LSR II FACS analyzer (BD, San J ose, CA). Cell sorting was performed by a FACS Aria sorter (BD, San J ose, CA). Results were analyzed by FlowJo software (Treestar, Ashland, OR).

Western Blotting

Western blotting was performed with the following antibodies: Anti-pSer473-Akt antibody (Cell Signaling Technology, Beverly, MA), anti-Gβ2 and anti-GFP
(Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-actin (Sigma, St. Louis, MO) and anti-β-tubulin antibody (collected from the supernatant of a hybridoma cell line). The proteins were detected by using the SuperSignal West Pico chemiluminescence substrate (Thermo Fisher Scientific, Rockford, IL).

**Generation of neutrophils from virus-infected bone marrow cells.**

C57BL/6 mice, aged 8-12 weeks, were obtained from Taconic (Germantown, NY). The donor mice were treated with 5-fluorouracil (Sigma, St. Louis, MO) at 150 mg/kg to enrich hematopoietic stem cells in the bone marrow. Three days later, bone marrow cells were harvested from femurs and tibias and cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) containing 20% endotoxin-free fetal bovine serum (Invitrogen, Carlsbad, CA), 50 ng/ml recombinant murine stem cell factor, 10 ng/ml recombinant murine interleukin-3, and 10 ng/ml recombinant human interleukin-6 (PeproTech, Rocky Hill, NJ) for two days. Cells were then infected twice with viruses in the presence of 4 µg/ml polybrene (Millipore, Billerica, Massachusetts). The transduced bone marrow cells were transplanted back into lethally irradiated recipient mice (9.5 Gy; γ-rays) by retro-orbital injection. Eight weeks later, the transplanted mice were euthanized for the further analysis. The use and care of animals were approved by the Institutional Animal Care and Use Committee at Yale University.

**Neutrophil preparation and Dunn chamber chemotaxis assay**

Neutrophils were isolated from bone marrows by using discontinuous percoll gradient as previously described (17). The purity of the preparation was verified by Ly6G staining, which is above 95% pure for neutrophils. Formyl-Met-Leu-Phe (fMLP) was purchased from Sigma (St. Louis, MO). The chemotaxis assay using a Dunn chamber was carried out as previously described (18) with some modifications. To minimize inconsistency between assays, we monitored chemoattractant gradients using free fluorescein isothiocyanate dye. Only cells under certain gradient characteristics were analyzed and included in our statistical analysis (Fig. S1A). Time-lapsed image series acquired in the aforementioned chemotaxis experiments were analyzed using the MetaMorph image analysis software. The software calculates the x,y coordinate of the centroid of a cell that is designated for tracking in each of the images. Several parameters that reflect the chemotactic behaviors of a cell are obtained from these timed coordinates series. Because the cells in general do not move with a constant velocity, they often move a distance which is less than the noise level of the cell tracking algorithm in the short time interval between two consecutive frames. To exclude those meaningless tracking results from the data analysis, we set a cutoff of 7.5 µm, which represent one half of an average body length of a polarized neutrophil, as the minimal distance that a cell has to travel in order for the coordinate to be included in the following analyses.

We computed the following parameters to quantify the chemotactic behaviors of a cell (Fig. S1B). Assuming that a cell migration trace consists of n points ($p_1, p_2, \ldots, p_n$) in that order so that
they have coordinates \((x_1, y_1)_i, \ldots, (x_n, y_n)_i\) respectively. Let \\
\(\Delta x_i = x_{i+1} - x_i, \Delta y_i = y_{i+1} - y_i\) then the

distances between consecutive points are:
\\
\[D_i = |p_{i+1} - p_i| = \sqrt{\Delta x_i^2 + \Delta y_i^2}\]

Directionality error angle \(\alpha_i = \frac{180}{\pi} \arccos \left( \frac{\Delta x_i}{D_i} \right) \)

It measures the angle between the cell migration direction and the gradient direction. We take the average
directionality angle for all \(p_i\)’s. A smaller value indicates that the cell is more closely following the gradient.

\[\text{Motility} = \frac{|p_n - p_2|}{\text{Elapsed Time}}\]

the overall cell migration speed.

**Phagocytosis and bacterial killing assay**

E.coli (DH10B) transformed with pGEX-Td-Tomato plasmid was induced by IPTG to express Td-Tomato, the red fluorescence protein. Bone marrow was isolated from mouse tibia and femur of hind legs then incubated at 37°C in the presence of bacteria expressing red fluorescence at a ratio of 1:10 for 15 minutes. Non-internalized bacteria were then removed by washing three times by centrifugation at 100g for 5 minutes in Hanks buffer with 1% BSA and fluorescence intensity at this time point used as a measurement of phagocytosis. Following this initial bacterial loading cells were incubated at 37°C for the time periods indicated prior to fixation with 2% PFA. Bone marrow cells were then labeled with APC-conjugated anti-mouse Gr1 in order to identify the neutrophil population and fluorescence was then assessed by flow cytometry using a BD LSRII FACS analyzer.

**RESULTS**

To develop an efficient method for loss-of-function study of mouse primary neutrophils, we tested whether the lentiviral miR30-embedded shRNA production system developed by Simon and colleagues (15,20) would be able to silence gene expression in neutrophils in a scheme outlined in Fig. S2. Briefly, lentiviruses expressing GFP were used to infect mouse bone marrow cells, which were subsequently used to generate neutrophils after transplantation into lethally irradiated recipient mice. We found that this lentivirus-based system did not infect mouse bone marrow cells at a high efficiency (data not shown). We decided to switch to a MSCV-based
system because MSCV infects hematopoietic stem/progenitor cells at high efficiencies (21). The insert encoding Venus fluorescence protein (a variant of YFP) and an shRNA targeting the β2 subunit of G protein embedded within the miR30 sequence was subcloned from the lentiviral system into MIGR1, a MCSV-based retroviral vector (Fig. 1A). We refer to this vector as LTR-YFP-shGβ2. The reasons for choosing the Gβ2 shRNA in this study were: 1) the shRNA was shown to be effective and specific (15); 2) Gβ2 is one of the two Gβ abundantly expressed in mouse neutrophils based on our expression microarray analysis (Fig. 1B); and 3) the role of Gβ in neutrophil chemotaxis has not been investigated.

We infected bone marrow cells isolated from C57BL/6 mice with LTR-YFP-shGβ2 virus and transplanted them into lethally irradiated C57BL/6 recipients. After 8 weeks of recovery and repopulation, neutrophils were isolated from the transplanted mice and analyzed for YFP expression. As shown in Fig. 1C, 44.92% of isolated neutrophils were YFP-positive, suggesting that this retroviral vector provides reasonable infection efficiency. We subsequently sorted the YFP-positive neutrophils (Fig. 1D) and analyzed them for shRNA-mediated knockdown efficiency. Because YFP and the shRNA were expressed from the same transcript, we expected the YFP-positive cells to express the shRNA. We observed about a 50% reduction in Gβ2 expression in neutrophils expressing YFP (Fig. 1E). However, little effect on chemoattractant (fMLP)-induced phosphorylation of Akt at Ser-473 was observed. This phosphorylation event is known to depend on Gβγ (14,22). Therefore, we hypothesized that the LTR promoter-based vector may not produce sufficient shRNA to allow effective knockdown of Gβ2 and result in the expected functional defects.

To improve the shRNA production, we decided to add a strong transcriptional promoter/enhancer by inserting a cytomegalovirus (CMV) promoter with a β-globin intron between the 5’ LTR and the YFP-miR30-shRNA expression cassette (Fig. 2A). The β-globin intron enhances transcriptional activities as an enhancer-like element (23). A second construct with the luciferase shRNA embedded in the miR30 backbone in place of the Gβ2 shRNA was made as a control for our studies described below. These two new constructs are referred to as CMV-YFP-shGβ2 and CMV-YFP-shLuc. We first tested these new constructs by infecting NIH 3T3 cells and found that cells infected with viruses generated from the new constructs (CMV-YFP-shLuc or -Gβ2) expressed higher levels of YFP than those generated from LTR-YFP-shGβ2 at the same multiplicity of infection (MOI) (Fig. 2B and 2C). Importantly, despite the same Gβ2 shRNA sequence in both LTR- and CMV-driven vectors, CMV-YFP-shGβ2 yielded much more efficient knockdown of Gβ2 expression than LTR-YFP-shGβ2 (Fig. 2D). These results suggest that the CMV promoter coupled with the β-globin intron sequence leads to higher expression levels than the viral LTR and that higher shRNA expression translates into more effective gene expression knockdown.

Next, we tested whether the modified vector would be more efficient in silencing gene expression in mouse neutrophils. The CMV-YFP-shLuc and
CMV-YFP-shGβ2 viruses were used to infect mouse bone marrow cells, which were subsequently transplanted into lethally irradiated recipient mice. Fig. 3A shows a representative set of flowcytometric analyses of neutrophils isolated from these transplanted mice. In this experiment, 30% of CMV-YFP-shGβ2 neutrophils were YFP-positive, whereas close to 60% of CMV-YFP-shLuc neutrophils were YFP-positive. The YFP positive neutrophils were isolated by fluorescence activated cell sorting (FACS), and their purity is shown in Fig. 3B. The sorted YFP-positive neutrophils were then subjected to Western analysis. As shown in Fig. 3C, the level of Gβ2 was markedly reduced in sorted YFP-positive neutrophils expressing shGβ2 compared with neutrophils from non-transplanted mice or neutrophils expressing shLuc. In all of the experiments performed, there was 70-85% reduction in Gβ2 expression levels in cells expressing shGβ2 compared to the controls. Importantly, there was a clear reduction in fMLP-induced Akt phosphorylation in cells expressing YFP-shGβ2 compared to the controls (Fig. 3C). Of note, silencing Gβ2 expression in neutrophil did not affect the expression of Gβ1 or Gαi2 (Fig. 3C).

Because Gβ1 is also highly expressed in murine neutrophils (Fig. 1B) and it may have redundancy function as Gβ2, we also tried to silence Gβ1 expression using our new vector. Four different shRNAs targeting Gβ1 were designed and the most potent shRNA (shGβ1-D) was identified by significantly suppressing endogenous Gβ1 expression in NIH 3T3 cells (Fig. S3). This shRNA was used in the following study. After bone marrow transplantation, we sorted out the YFP positive neutrophils transduced with Gβ1 shRNA or control luciferase shRNA (Fig. 4A) and performed Western analysis. As shown in Fig. 4B, the level of Gβ1 was significantly reduced in sorted YFP-positive neutrophils expressing shGβ1 compared with control neutrophils from non-transplanted mice or neutrophils expressing shLuc.

One of the main powers of miR30-shRNA system is to simultaneously knockdown of multiple different gene targets (13). To exploit this possibility in our system, we tried to double knockdown Gβ1 and Gβ2 in neutrophils. The second miR-shRNA was inserted downstream of the first miR-shRNA and both shRNAs were driven by the enhanced CMV promoter (Fig. 4C). Similarly, we sorted out the YFP positive neutrophils transduced with Gβ1 shRNA and Gβ2 shRNA (double knockdown) or control luciferase shRNA (Fig. 4D), and we observed the efficient suppression of Gβ1 and Gβ2 expression in neutrophils (Fig. 4E), suggesting the successful knockdown multiple gene targets in vivo.

Next, we performed three functional assays for transduced neutrophils. First, we tested the effect of Gβ knockdown on in vitro neutrophil chemotaxis using a Dunn chamber, in which a shallow fMLP gradient was established and cell migration was tracked using time-lapsed videomicroscopy. Two key parameters were obtained from analysis of the time lapsed image series: directionality error (reflecting how well a cell follows the chemoattractant gradient) and motility (refer to Materials and Methods for details). Significant portions of the neutrophils isolated from transplanted mice were YFP-negative. Fig. S4A shows
bright-field and fluorescence images of neutrophils in Dunn chambers, in which YFP-positive and negative neutrophils can be readily recognized and tracked. These YFP-negative cells have little or no shGβ2 expression, and therefore normal levels of Gβ2 protein (Fig. S4B & S4C). Thus, these YFP-negative cells served as excellent internal controls for the Dunn chamber chemotaxis assay, which are known to be variable. In addition, paired statistical analyses were used to identify small differences in chemotaxis parameters caused by gene silencing. Analysis of chemotactic parameters of YFP-positive cells and negative cells revealed that YFP-positive neutrophils isolated from CMV-YFP-shGβ2 virus-infected mice showed impaired directionality compared to YFP-negative cells (Fig. 5A). Interestingly, no significant directionality defect was detected in Gβ1 silenced neutrophils compared to control cells (Fig. 5A). And no further directionality defect was observed in Gβ1 and Gβ2 double knockdown neutrophils as compared to Gβ2 silenced cells (Fig. 5A), suggesting that Gβ2 is the major isoform responsible for neutrophil directionality. The effect of Gβ1 knockdown or Gβ2 knockdown on motility appeared to be insignificant (Fig. 5B). Neutrophils suppressing both Gβ1 and Gβ2 expression (Fig. 5B) showed a modest, but significant, reduction in motility. In addition, approximately 30% of Gβ1/Gβ2 double knockdown neutrophils failed to respond to fMLP and were immobile, whereas knockdown of either Gβ subunit had no significant effects on the number of responding cells (Fig. 5C). As important controls, there were no differences between YFP-positive and negative neutrophils isolated from mice transplanted with CMV-YFP-shLuc virus-infected bone marrow cells (Fig. 5A & 5B). Therefore, these results together indicate that Gβ2, but not Gβ1, has an important role in neutrophil directionality regulation, whereas both Gβ subunits are involved in motility regulation.

To examine neutrophil recruitment in vivo, we used the air pouch model (24). In this model, subcutaneous injection of air into dorsal surface of mice results in the formation of an air pouch, which has a lining morphologically similar to the synovium. Carrageenan, polysaccharide extracted from red seaweeds, was injected into the pouch to induce robust inflammation, as indicated by an increase in total neutrophil number and higher neutrophil percentage in the pouch exudates compared with those in mice injected with saline (data not shown). Among these neutrophils recruited into the pouch, two or three populations (YFP-low, YFP-medium and YFP-high) were observed based on their YFP expression (Fig. 6A). In our analysis, we refer to YFP-high cells as YFP-positive cells and YFP-low cells as YFP-negative cells. As indicated in Fig. S4, YFP-negative cells also served as internal controls for YFP-positive cells in this assay. For each animal, the ratio between YFP-positive neutrophils and YFP-negative neutrophils in the pouch exudates (Fig. 6A, bottom panel), which reflects the migration ability of transduced neutrophils, was normalized based on the same ratio in the blood (Fig. 6A, top panel). As expected, neutrophils expressing luciferase shRNA (shLuc) migrate normally in vivo (the ratio of YFP+/YFP- is 0.97±0.05, Fig. 6B). However, the infiltration of Gβ2 silenced neutrophils was markedly inhibited (the
ratio of YFP+/YFP− is 0.47±0.12). Although we observed the inhibited migration in Gβ1 silenced neutrophils (the ratio of YFP+/YFP− is 0.72±0.22, Fig. 6B), the difference is not statistically significant compared with that in shLuc group. Importantly, the more severe migration defect was detected in Gβ1 and Gβ2 double knockdown neutrophils (the ratio of YFP+/YFP− is 0.29±0.11, Fig. 6B). These data suggest that both Gβ subunits play important roles in neutrophil recruitment in vivo while Gβ2 appears to be more dominant isoform than Gβ1 in regulating neutrophil migration in vivo.

Finally, we performed phagocytosis and bacterial killing assays using neutrophils isolated from transplanted mice bone marrow. We developed a flowcytometry-basis assay to assess these two processes. We incubated neutrophils with E coli expressing Td-Tomato, a red fluorescence protein (25) at 37°C for 15 minutes to allow phagocytosis to occur. After differential centrifugation to remove the free bacteria, the levels of Td-Tomato were determined by flow cytometry at varying time points. The initial level of red fluorescence reflects endocytosis, while the reduction of red fluorescence reflects bacterial killing. To validate this assay, we compared this new method with the one that count actual bacterial numbers, and we observed the similar results between this flow-based method and conventional colony forming unit (CFU) method (Fig. S5B). In the presence of diphenyliodonium (DPI), an inhibitor of NADPH oxidase complex, no decrease in red fluorescence was observed over the 30 minute experimental time course, while we did detect a significant reduction of red fluorescence in the absence of DPI inhibitor (Fig. S5C). Moreover, we didn’t observe the decrease of red fluorescence when fluorescence labeled bacteria were incubated with neutrophils in ice for 30 minutes (Fig. S5A). These data further demonstrate that the reduction of red fluorescence measured by flow cytometry is due to neutrophil mediated bacterial killing.

Knockdown of Gβ1 did not alter the ability of cells to phagocytose bacteria when the fluorescence intensity of YFP- and YFP+ cells was compared after the 15 minute ingestion period (Fig. 7A,C). Knockdown of Gβ2 caused a slight, but statistically insignificant, decrease (Fig. 7E). However, knockdown of both Gβ subunits led to a significant reduction (Fig. 7G), suggesting that both subunits are involved in the regulation of phagocytosis. With regard to bacterial killing, only knockdown of Gβ1 demonstrated a moderate, but significant, inhibition over the time course indicated (Fig. 7D). In contrast, Gβ2-silenced neutrophils and neutrophils expressing shLuc control showed no significant changes in bacterial killing (Fig. 7B,F). These data suggest that signaling downstream of Gβ1 mediates events may facilitate the efficient destruction of phagocytosed bacteria in neutrophils. Although mechanisms for this Gβ1-mediated bactericidal effect are not known, Gβ1 may regulate this effect via regulating superoxide production or degranulation. The lack of effect of Gβ1/Gβ2 double knockdown on the apparent killing (Fig. 7H) may be due to fewer bacteria that were phagocytosed by the double knockdown cells, obscuring the moderate increase resulted from Gβ2 knockdown. Nevertheless, these results are consistent with the conclusion that the lack of effect of Gβ2 knockdown on
bacterial killing may not be due to the compensation by Gβ1.

**DISCUSSION**

In this study, we have developed a method by which gene expression can be effectively silenced in primary mouse neutrophils. This method uses a combination of bone marrow transfer and microRNA-embedded shRNA production to achieve high efficient gene expression knockdown in primary neutrophils. Using this method, we successfully silenced Gβ1, Gβ2 or both Gβ1 and Gβ2 in neutrophils and uncovered unexpected roles of these G protein subunits in regulating neutrophil biology.

Retroviral transduction following transplantation of genetic manipulated hematopoietic stem cells (HSCs) is a powerful tool for studying hematopoietic cells in vivo. This method has been successfully used in not only myeloid cells, such as macrophage (26) and dendritic cells (27), but also lymphoid cells (28-30). However, most of studies applied gain-of-function strategy. Our new method has several distinct advantages. First, it allows loss-of-function studies of neutrophils and will provide an efficient alternative to the time-consuming gene targeting approach, which thus far had been the only way to study loss of function of neutrophils. Advancement in the field of neutrophil biology has been in part limited by the long time frame to generate genetically modified primary neutrophils. In contrast to more than one year needed for generation of a knockout mouse line, our method only takes 3 months to produce the mice for the study. In addition, the method allows studying neutrophil functions in various in vivo models, as suggested in our air pouch model (Fig. 6), which is a clear advantage over knockdown studies in neutrophil-like cell lines.

The second advantage of this method is that cells unaffected by gene knockdown are generated simultaneously serving as excellent internal assay controls. This is particularly beneficial in experiments subjected to individual mouse and systematic variations, including the in vivo studies. In this study, we use YFP-negative neutrophils as internal controls for in vitro chemotaxis assay (Fig. 5), in vitro bacterial killing assay (Fig. 7) and in vivo air pouch model (Fig. 6).

The third advantage is that this method may be adapted to express proteins in neutrophils. By generating MIGR-CMV vectors with different fluorescence proteins with differing emission spectra, the method expands possibilities for multiple labeling neutrophils for both loss-of-function and gain-of-function studies.

Because hematopoietic stem cells can differentiate into multiple-lineage hematopoietic cells, our approach will very likely be applicable to the other hematopoietic lineages as well. To address this question, we did FACS analysis in spleen cells of MIGR-CMV-miR-shLuc transplanted mice and wild type mice. Using B220 as a B cell marker and CD3ε as a T cell marker, we detected a B220^YFP^ double positive population and a CD3ε^YFP^ double positive population in spleen of MIGR-CMV-miR-shLuc transplanted mice but not in wild type mice (Fig. S6), suggesting that the method of retroviral
transduction followed by bone marrow transplantation can transduce myeloid and lymphoid cells as well as other hematopoietic cells. Therefore, our new vectors will provide important tools to study gene functions in primary hematopoietic cells in vivo and in vitro.

This study demonstrates that Gβ₂ knockdown primarily affected directionality of neutrophil chemotaxis in a shallow fMLP gradient, whereas knockdown of Gβ₁ had little effect. On the other hand, both Gβ subunits are involved in motility regulation, because knockdown of either had insignificant effects while knockdown of both resulted in a significant reduction in cell motility and the number of immotile cells. These conclusions were supported by observations in vivo using an air-pouch model and therefore suggest that Gβ₁ and Gβ₂ may regulate differing aspects of some neutrophils biology. This conclusion is further supported by the observation that Gβ₁ knockdown suppressed neutrophil-mediated bacterial killing, whereas Gβ₂ knockdown was ineffective in this regard. Meanwhile, both Gβ subunits appear to be involved in bacterial phagocytosis regulation. Indeed, previous studies in macrophage-like cell line have suggested that the roles of Gβ₁ and Gβ₂ in cell migration differ (14). This study therefore extends the idea that Gβ₁ and Gβ₂ are not entirely redundant in their functions.

Genetic studies in Dictyostelium suggest that Gβ is required for phagocytosis and chemotaxis (31). However, our knockdown of two major Gβ subunits in neutrophils led to partial effects on chemotactic responses and phagocytosis. The lack of robust effects in some of the assays may be due to incomplete inactivation of the target genes. Because the expression levels and phenotype outcomes are not linear, an apparent efficient knockdown of the expression of a gene may fail to lead to a robust phenotype. This is an obvious drawback of this type of methods. In addition, other Gβ subunits may also be expressed in neutrophils even though they were not detected at high levels by our microarray analyses, which may not be reliable for estimating protein levels. Nevertheless, the methods outlined in this paper allow quick determination of a given gene for its roles in regulating a wide array of neutrophil activities and lay the groundwork for more thorough future studies.
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FIGURE LEGENDS:

Figure 1. Suppression of Gβ2 expression in Neutrophils using the LTR-YFP-shGβ2 vector
A. Schematic representation of the LTR-YFP-shRNA vector. Gene-specific shRNA is embedded into the transcript of human microRNA 30 (miR30). To facilitate the selection, the vector also contains the YFP marker gene driven by the LTR promoter and the puromycin-resistance gene (Puro) regulated by internal ribosome entry site sequence.
B. Gβ expression in mouse neutrophils. Total RNAs were prepared from isolated bone marrow neutrophils and analyzed by the Affymetrix cDNA expression microarray system. The expression level of five Gβ isoforms was shown as the arbitrary unit (AU) of the microarray signal.
C. Neutrophils from non-transplanted mice or mice transplanted with cells infected with LTR-YFP-shGβ2 viruses were isolated and analyzed by a flowcytometer prior sorting.
D. Flowcytometric analysis of sorted neutrophils from C.
E. Western analysis of sorted YFP-positive neutrophils from D. Neutrophils were stimulated with or without 4 µM fMLP for 1 minute and cell lysates were separated by SDS-PAGE and detected with indicated antibodies. NT, non-transplanted; LshGβ2, LTR-YFP-shGβ2.

Figure 2. Efficient YFP expression and Gβ2 knockdown by the CMV-driven vector in NIH 3T3 cells
A. Schematic diagram of the CMV-YFP-shRNA vector. YFP and shRNA are driven by the CMV promoter with a β-globin intron.
B. NIH 3T3 cells were infected with viruses as indicated in the figure at MOI 10 and observed under a fluorescence microscope with a 20X objective 5 days after infection.
C. Flowcytometric analysis of cells prepared as described in B.
D. Cells prepared as described in B were analyzed by Western blotting with indicated antibodies.

Figure 3. Suppression of Gβ2 expression in primary mouse neutrophils using the CMV-YFP-shGβ2 vector
A. Neutrophils were isolated from non-transplanted mice or mice transplanted with virus-infected bone marrow cells and analyzed by a flowcytometer.
B. Flowcytometric analysis of sorted YFP-positive neutrophils.
C. Sorted YFP-positive neutrophils were stimulated with or without 4 µM fMLP for 1 minute, followed by Western analysis. NT, non-transplanted; shGβ2, CMV-YFP-shGβ2; shLuc, CMV-YFP-shLuc.

Figure 4. Suppression of Gβ1 expression or Gβ1 and Gβ2 expression in primary mouse neutrophils
A. Flowcytometric analysis of sorted YFP-positive neutrophils from mice infected with
CMV-YFP-shGβ1.

B. Sorted YFP-positive neutrophils were stimulated with or without 4 µM fMLP for 1 minute, followed by Western analysis. NT, non-transplanted; shGβ1, YFP negative neutrophils sorted from mice infected with CMV-YFP-shGβ1 virus; shGβ1+, YFP positive neutrophils sorted from mice infected with CMV-YFP-shGβ1 virus; shLuc+, YFP positive neutrophils sorted from mice infected with CMV-YFP-shLuc.

C. Schematic diagram of the CMV-YFP-shRNA1-shRNA2 vector.

D. Flowcytometric analysis of sorted YFP-positive neutrophils from mice infected with CMV-YFP-shGβ1-shGβ2 virus.

E. Sorted YFP-positive neutrophils were stimulated with or without 4 µM fMLP for 1 minute, followed by Western analysis. NT, non-transplanted; shGβ1,Gβ2-, YFP negative neutrophils sorted from mice infected with CMV-YFP-shGβ1-shGβ2 virus; Gβ1,Gβ2+, YFP positive neutrophils sorted from mice infected with CMV-YFP-shGβ1-shGβ2 virus; shLuc+, YFP positive neutrophils sorted from mice infected with CMV-YFP-shLuc.

Figure 5. Chemotaxis assay for neutrophils from transplanted mice

Neutrophils isolated from transplanted mice were assayed for their chemotactic responses to an fMLP gradient in a Dunn chamber. As described in the materials and methods section, neutrophils were stimulated by fMLP and observed under a time-lapse video microscope to monitor the migration of neutrophils using the Metamorph software. The directionality (A) and motility (B) of neutrophils infected with the indicated virus were determined in YFP positive and YFP negative fractions. The percentages of motile YFP+ cells relative to motile YFP− neutrophils are shown in (C). * indicates p < 0.05 (paired Student t-Test) between YFP-positive and YFP-negative group.

Figure 6. In vivo neutrophil infiltration assay for transplanted mice

A. Mice transduced with indicated virus were injected with 5 mg carrageenan into their air pouch to induce the inflammation. 4 hours later, cells from blood (top panel) or pouch exudates (bottom panel) were stained with APC-CD11b and PerCP-Ly-6G antibodies and neutrophils (CD11b+Ly-6G+ cells) were gated out to analyze their YFP expression. YFP-negative and YFP-positive neutrophils were shown in the contour plots.

B. The ratio between YFP-positive and YFP-negative neutrophils in the pouch exudates were normalized with the YFP-positive and YFP-negative neutrophil ratio in the blood in the same animal. * indicates p < 0.05 and ** indicates p < 0.01 (Student t-Test) compared with the shLuc group.

Figure 7. Phagocytosis and bacterial killing assay for neutrophils from transplanted mice

A, C, E, G) Mouse bone marrow cells isolated from the indicated mice transplanted with cells infected with shLuc (A,B), ShGβ1 (C,D), ShGβ2 (E,F) or ShGβ1-β2 (G,H) were incubated at 37°C in the presence of bacteria expressing red fluorescence at a ratio of
1:10 for 15 minutes. Unbound and non-internalized bacteria were then washed away and red fluorescence intensity in neutrophils (Gr1\textsuperscript{hi} cells) at this time point used as a measurement of phagocytosis.

Following bacterial loading, cells were incubated for the indicated time periods then fluorescence measured by flow cytometry. The geometric mean of the red fluorescence between YFP-positive and YFP-negative Gr1\textsuperscript{hi} neutrophils was plotted with the time in indicated groups. * indicates p < 0.05 (paired Student t-Test) between YFP-positive and YFP-negative group.
Figure 1

A. Diagram showing the components of shRNA with 5’ LTR, YFP, 5’miR30, 3’miR30, Puro, and 3’ LTR.

B. Table showing gene names and microarray signal intensity (AU):

| Gene name | Microarray signal intensity (AU) |
|-----------|---------------------------------|
| Gnb1      | 1020.05                         |
| Gnb2      | 2800.31                         |
| Gnb3      | 71.17                           |
| Gnb4      | 67.09                           |
| Gnb5      | 58.37                           |

C. Flow cytometry graphs comparing non-transplanted (0.54) and LTR-YFP-shGβ2 (44.92).

D. Flow cytometry graphs comparing non-transplanted and LTR-YFP-shGβ2.

E. Western blot analysis showing effects of fMLP with "+" and "-" conditions and "NT LshGβ2" and "NT LshGβ2" conditions.
Figure 3

A

![Fluorescence histograms for CMV-YFP-shG2 and CMV-YFP-shLuc](image)

- **Non-transplanted**: Count
  - 40
  - 30
  - 20
  - 10
  - 0.28

- **CMV-YFP-shG2**: Count
  - 30.23

- **CMV-YFP-shLuc**: Count
  - 54.97

B

![Bar graph comparing fluorescence levels](image)

- **Non-transplanted**
- **CMV-YFP-shLuc**
- **CMV-YFP-shG2**

C

| fMLP: | - | + |
|-------|---|---|
|       | NT | shGβ2 | shLuc |
| NT    | Gβ2- |  |  |
|       | pS473-Akt- |  |  |
|       | Gαi2- |  |  |
|       | Gβ1- |  |  |
|       | β-actin- |  |  |
Figure 4

A

Non-transplanted
YFP + CMV-shLuc
YFP + CMV-shGβ1
YFP - CMV-shGβ1

B

| fMLP: | - | + |
|-------|---|---|
| Gβ1-  |  |  |
| Gβ2-  |  |  |
| β-actin- |  |  |

Non-transplanted
YFP - CMV-shGβ1, β2
YFP + CMV-shLuc
YFP - CMV-shGβ1, β2

C

shRNA1

shRNA2

5' LTR → CMV → YFP
5' miR30
3' miR30
5' LTR

D

E

| fMLP: | - | + |
|-------|---|---|
| Gβ1-  |  |  |
| Gβ2-  |  |  |
| pS473-AKT- |  |  |
| β-actin- |  |  |
Figure 5

A

Directional Error (Degree)

- YFP-
- YFP+

shLuc shGβ1 shGβ2 shGβ1,β2

B

Translocation (μM/30 Min)

- YFP-
- YFP+

shLuc shGβ1 shGβ2 shGβ1,β2

C

Responding cells (%)

shLuc shGβ1 shGβ2 shGβ1,β2
**Figure 6**

A

| shLuc | shGβ2 | shGβ1,β2 |
|-------|-------|----------|
| ![Blood Exudates](image1) | ![Blood Exudates](image2) | ![Blood Exudates](image3) |
| ![Blood Exudates](image4) | ![Blood Exudates](image5) | ![Blood Exudates](image6) |

B

| Normalized YFP+/YFP- Neutrophils in Pouch |
|------------------------------------------|
| ![Normalized YFP+/YFP- Neutrophils in Pouch](chart) |

* **p < 0.05**
** **p < 0.005
Different roles of Gβ1 and Gβ2 in neutrophil function revealed by gene expression silencing in primary mouse neutrophils
Yong Zhang, Wenwen Tang, Matthew C. Jones, Wenwen Xu, Stephanie Halene and Dianqing Wu

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