FLIP: a novel regulator of macrophage differentiation and granulocyte homeostasis

Running head: FLIP in myeloid cells

Qi-Quan Huang1, Harris Perlman1, Zan Huang2, Robert Birkett1, Lixin Kan3, Hemant Agrawal1, Alexander Misharin1, Sandeep Gurbuxani4, John D. Crispino2, Richard M. Pope1

1Divisions of Rheumatology and 2Hematology/Oncology, Department of Medicine, 3Department of Neurology, Northwestern University Feinberg School of Medicine, Chicago, IL, 60691, USA; 4Department of Pathology, University of Chicago. Chicago, IL, 60637 USA

Corresponding author: Richard M. Pope, MD

240 E Huron, Suite M-300

Chicago, IL 60611

312/503-8003; Fax 312/503-0994

Email: rmp158@northwestern.edu
ABSTRACT: FLIP is a well established suppressor of death receptor-mediated apoptosis. To define its essential in vivo role in myeloid cells, we generated and characterized mice with Flip conditionally deleted in the myeloid lineage. Myeloid specific Flip deficient mice exhibited growth retardation, premature death, and splenomegaly with altered architecture and extramedullary hematopoiesis. They also displayed a dramatic increase of circulating neutrophils and multi-organ neutrophil infiltration. In contrast, although circulating inflammatory monocytes were also significantly increased, macrophages in the spleen, lymph nodes and the peritoneal cavity were reduced. In ex vivo cultures, bone marrow progenitor cells failed to differentiate into macrophages when Flip was deleted. Mixed bone marrow chimera experiments employing cells from Flip deficient and wild type mice did not demonstrate an inflammatory phenotype. These observations demonstrate that FLIP is necessary for macrophage differentiation and the homeostatic regulation of granulopoiesis.
INTRODUCTION: Cell surface death receptors, including Fas, TRAIL and TNFR1, are capable of mediating programmed cell death through the activation of caspases 8 or 10. Cellular Flice-like inhibitory protein (FLIP) is critical in the protection against death receptor-mediated apoptosis. Following death receptor ligation caspases 8/10 are recruited to the receptor through adaptor molecules, FADD or TRADD, resulting in homotypic interactions and autocatalytic caspase activation, which is suppressed by FLIP. FLIP is highly expressed in a variety of tumors, and the forced reduction of FLIP is capable of sensitizing certain tumors to death receptor-mediated apoptosis. Our earlier studies with human monocytes demonstrated that FLIP was induced during monocyte to macrophage differentiation, which protected macrophages from Fas-FasL mediated apoptosis. Additionally, FLIP is highly expressed in the macrophages within the synovial tissue of patients with rheumatoid arthritis and the forced reduction of FLIP sensitizes macrophages to Fas-mediated apoptosis.

In addition to protecting against apoptosis, a variety of other functions of FLIP have been characterized. At low levels compared to caspase 8, FLIP may be pro-apoptotic by contributing to caspase 8 activation, promoting Fas-mediated apoptosis. The role of FLIP in T lymphocytes is complex, since it is necessary for the maturation of thymocytes by suppressing apoptosis and is capable of protecting against the extrinsic cell death of mature lymphocytes, but may also promote apoptosis and the activation of NF-κB (reviewed in). In dendritic cells an N-terminal p22 fragment of FLIP induces NF-κB activation. The deletion of FLIP in B lymphocytes results in decreased B lymphocytes and increased sensitivity to Fas-mediated apoptosis and the increased activation of JNK and p38. Other non-apoptotic functions of FLIP include the suppression of TNFα-induced JNK activation, and the impairment of the ubiquitin-proteasome system, resulting in the increased expression of genes regulated by β-catenin. However, the potential non-apoptotic functions of FLIP in myeloid cells are unknown.
To elucidate the *in vivo* role of FLIP in macrophages and neutrophils, we developed mice deficient in FLIP in myeloid cells. These mice had significantly reduced body weight and died prematurely. There was a dramatic increase in circulating neutrophils and monocytes (>10 fold), which was accompanied by multi-organ neutrophil infiltration. Isolated neutrophils deficient in *Flip* demonstrated a mature morphology and underwent normal apoptosis, oxidative burst, and degranulation. In contrast to monocytes, macrophages were greatly reduced in the spleen, lymph nodes, peritoneal cavity. *Ex vivo* cultures, the deletion of FLIP prevented macrophage differentiation, and mixed bone marrow chimeric mice failed to recapitulate the phenotype of the mice deficient in *Flip* in myeloid cells. Together, these observations identify a novel role for FLIP in myeloid homeostasis.
METHODS:

Generation of *Flip* conditional knockout in myeloid lineage: The *Flip* targeting vector, containing three *loxP* sequences inserted in tandem orientation and a neomycin resistance (*neo*) cassette, was transfected into the B6/Blu ES cells (ES Cell Core at Washington University, St. Louis, MO). After removing the *neo* cassette, chimeras were generated by blastocyst injection and embryos re-implantation in C57BL/6 mouse establishing floxed *Flip* (*Fliph/+*) mice, which were breed with the Zp3-cre mice to generate mice with one allele of *Flip* deleted (*Flipdh/+*). After further crosses, myeloid cells deficient in FLIP were generated by crossing with LysM c/+ mice (Jackson Lab, 21), resulting in the genotypes identified in Table 1. Mating *Fliph/+*, LysM c/+ and *Fliph+/d*, LysM c/+ mice resulted in *Fliph/dh*, LysM c/+ mice which were deficient in FLIP in myeloid cells.

Phenotypic analysis: Complete blood counts and differentials were performed employing a Hemavet 950 (Drew Scientific Inc) 22. FLIP expression was determined by immunoblotting with an antibody to the N-terminal peptide of FLIP (Cell Signaling). For cultivation of microorganisms, tissues was collected under sterile conditions and cultured in Brain Heart Infusion Broth (Remel) at 37°C overnight. Circulating cytokines/chemokines were quantified by the Premixed 32 Plex kit (Millipore), and the data was acquired by LUMINEX 200.

Histology and immunohistochemistry: Organs were dissected and fixed in 10% neutral formalin, embedded in paraffin, and then 5 μm sections were stained with hematoxylin and erasin. Cryosections were processed for immunofluorescence staining of macrophages as described 23, then incubated with primary antibodies to mouse F4/80 (B8, eBioscience), CD169 (AbD Serotec), or isotype control IgG at 4°C overnight, and subsequently with Alexa Fluor 594 conjugated anti-rat secondary antibody (Invitrogen) for 2 hours and counterstained with DAPI.
**Immunophenotyping:** Cells were preincubated with anti-mouse CD16/CD32 antibody (BD Phamingen) to block cell surface Fc III/II receptors before antibody staining. Immunophenotyping was performed by multi-color fluorochrome-conjugated antibody cocktails, including antibodies to CD 45, CD11b, F4/80, Gr 1, CD115, CD62L, CD4, CD8, CD3, CD19, B220 and CD11c (ebioscience or BD Pharamingen). Data were acquired on a BD LSR II flow cytometer (BD FACSDIVA software) and analyzed by Flowjo (TreeStar, Inc.). Single cell populations were sorted by Moflo High Speed Sorter.

**Bone marrow reconstitution:** C57BL/6 recipients CD45.1+ congenic (B6.SJL-Ptprca Pepcb/BoyJ, 6 week old females, Jackson Laboratory) were lethally irradiated (1,000 rads), followed by the retroorbital administration of 5x10^6 donor whole bone marrow cells. The donor bone marrow was collected from CD45.1+ (wild type) and CD45.2+ (Flipf/d, LysM c/+ ) mice. The bone marrow was transferred to the recipients containing 0%, 25%, 50%, 75%, and 100% of cells from Flipf/d, LysM c/+ mice. Recipients received sulfamethoxazole (50mg/ml) and trimethoprim (8 mg/ml) in the drinking water, and were subjected to phenotypic analysis 8 weeks post transplantation.

**Programmed cell death:** Mitochondrial transmembrane potential (ΔΨμ) was assessed by retention of Rh123 as described 24. Cell membrane integrity was assessed by exclusion of DAPI. Apoptosis was assessed by detecting the expression of Annexin V (BD Pharmingen) 12,24.

**Assessment of neutrophil function:** Mouse peripheral blood cells were incubated with dihydrorhodamine 123 (DHR, 0.5μM), with or without phorbol 12-myristate 13-acetate (PMA, 10 nM) at 37°C for 30 min. Thereafter, the samples were chilled on ice and incubated with fluorochrome-conjugated anti-CD11b and -Gr1, followed by erythrocyte lysis. Oxidative burst was determined by measuring the conversion of non-florescent dihydrorhodamine 123 into fluorescent rhodamine123 25, and degranulation by the increased expression of cell...
surface CD11b. Myeloperoxidase staining was performed by incubating slides in a solution of 3.3’-diaminobenzidine and hydrogen peroxide.

**Colony formation:** Spleen (1 x 10⁴) or bone marrow cells (0.5 x 10⁵) combined from femurs and tibias, were seeded in methylcellulose media (Methocult, StemCell Tech.) in the presence of IL-3 (10 ng/ml), IL-6 (10 ng/ml), SCF (50 ng/ml), and GM-CSF (10 ng/ml) for 7 days. CFU-GM colonies were counted under light microscopy. For CFU-Mk, cells (1 x 10⁵) were seeded in a collagen-based media (Megacult-C, StemCell Tech.) supplemented with IL-3 (10 ng/ml), IL-6 (10 ng/ml), IL-11 (10 ng/ml), TPO (10 ng/ml) for 7 days. The Megacult-C cultures were dehydrated and stained for acetylcholinesterase activity to determine the CFU-MK colonies.

**Macrophage survival during differentiation:** CD117⁺ (c-Kit) hematopoietic stem cells from the bone marrow of 8-12 week old Flip⁺/⁻ or Flip⁻/⁻ mice were isolated employing antibody to the stem cell receptor CD117 (StemCell Tech.) according to the manufacturer’s instructions. These freshly isolated cKit + cells were cultured in the presence of SCF (10 ng/ml), IL-3 (10 ng/ml), and IL-6 (10 ng/ml) overnight, followed by culture with recombinant retroviral vectors expressing GFP alone or GFP plus Cre for an additional 24 hours. The cells were then incubated with 20% L929 conditioned medium containing M-CSF to allow in vitro macrophage differentiation for 5 days.

**Data analysis:** All quantitative data are presented as mean ± SEM. Statistical analysis between groups was done with 2-tailed Student’s t test. Nonparametric data was analyzed by the Mann-Whitney rank sum test. One way ANOVA (Tukey pairwise mean comparison) was performed for multi-group analysis. Significance levels were set at 0.05.
RESULTS:

**Flip conditional knock out in myeloid lineage.** In order to generate mice with FLIP deleted in myeloid cells, loxP sequences were inserted flanking exons 2 and 3 of *Flip* (Figure 1A), which was employed to generate *Flip* floxed (*Flip*′) embryonic stem cells (Supplemental Figure 1A). Deletion of one floxed allele in the whole genome (*Flip*′/+) was obtained by crossing heterozygous *Flip*′/+ mice with *Zp3-Cre* mice that express Cre in the female germ line (Supplemental Figure 1B). Myeloid lineage specific deletion of the *flipf* was carried out by crossing *Flip*′/+ or *Flip*′/d mice with those expressing Cre driven by the LysM promoter (*LysM c/+*) (Figure 1B). When *Flip*′/+ mice were crossed with the *LysM c/+* mice, the floxed allele was deleted in granulocytes and monocytes, demonstrating the efficiency of Cre in these cells. Crossing *Flip*′/d mice with *LysM c/+* mice, resulted in the deletion of both alleles of *Flip* in bone marrow and peripheral blood granulocytes (Figure 1B). However, complete deletion of the floxed *Flip* allele was not observed in monocyte bone marrow precursors or peripheral blood monocytes (Figure 1B). As expected, neither allele of *Flip* was deleted in circulating T or B lymphocytes when *Flip*′/d or *Flip*′/+ mice were crossed with *LysM c/+* mice (Figure 1B).

**Disruption of Flip in myeloid lineage results in severe postnatal growth retardation and premature death.**

The deletion of *Flip* in myeloid cells resulted in the expected Mendalian birth rate with no indication of embryonic lethality (data not shown). However, the *Flip*′/d, *LysM c/+* mice displayed a runted appearance and a 50% reduction in size (Figure 1C). None of the littermates of the *Flip*′/d, *LysM c/+* mice (defined in Table 1) demonstrated reduced size (Figure 1C) or increased mortality (data not shown) and were therefore collectively employed as the littermate controls in further experiments. Approximately 50% of the *Flip*′/d, *LysM c/+* died before 32 weeks of age, while > 80% of *Flip*′/d, *LysM c/cc* mice died by 3-5 weeks of age, most likely due to a more complete deletion of *Flip* (Figure 1D), and not due to the deletion of both *LysM* alleles, since there was no effect on phenotype, including body weight (Figure 1C) or blood count (Table 1), when one or both alleles of *Flip* were still present. These observations demonstrate that the reduction of FLIP in myeloid cells is critical for postnatal survival.
Deletion of *Flip in the myeloid lineage results in leukocytosis, multi-organ neutrophil infiltration, and systemic infection.* Examination of peripheral blood demonstrated a 5-fold increase of leukocytes in the *Flip*<sup>fl/d</sup>, LysM<sup>c/c</sup> mice compared to the littermate controls (58.8±5.9 vs. 11.0±0.7 x10<sup>3</sup>/μl, p< 0.001). Circulating neutrophils and monocytes were increased > 10-fold compared to the controls (p < 0.001, Figure 2A, Table 1). Eosinophilia, thrombocytosis and anemia, were also present in the *Flip*<sup>fl/d</sup>, LysM<sup>c/c</sup> mice, but not in the littermate controls (Table 1). Examination of stained peripheral blood smears from the *Flip*<sup>fl/d</sup>, LysM<sup>c/c</sup> mice demonstrated mature neutrophils with appropriate nuclear morphology and cytoplasmic granularity. The neutrophils from the *Flip*<sup>fl/d</sup>, LysM<sup>c/c</sup> mice were CD45<sup>+</sup>,CD11b<sup>+</sup>, CD115<sup>-</sup>, Gr1<sup>hi</sup>, CD62L<sup>hi</sup>, similar to the littermate controls (Figure 2C). The circulating monocytes appeared normal by morphology, however, the *Flip*<sup>fl/d</sup>, LysM<sup>c/c</sup> mice demonstrated an expanded population of monocytes that was CD45<sup>+</sup>, CD11b<sup>+</sup>, CD115<sup>+</sup>, Gr1<sup>+</sup>, CD62L<sup>+</sup>, identifying them as inflammatory monocytes (Figure 2C). In contrast, the expected distribution of resident (Gr-1<sup>-</sup>, CD62L<sup>-</sup>) and inflammatory (Gr-1<sup>+</sup>, CD62L<sup>+</sup>) monocytes were observed in the controls (Figure 2C).

However, while the absolute number of inflammatory monocytes was increased in the *Flip*<sup>fl/d</sup>, LysM<sup>c/c</sup> mice, the number of resident monocytes was not different from the controls (Figure 2D). Analysis of peripheral blood also demonstrated a decreased percentage of B cells and CD4<sup>+</sup> and CD8<sup>+</sup> T cells which was due to the marked increase of granulocytes. However, the absolute number of CD8<sup>+</sup> cells was actually increased in the *Flip*<sup>fl/d</sup>, LysM<sup>c/c</sup> mice, while B cells and CD4<sup>+</sup> lymphocytes were not different from the controls (Supplemental Figure 2A-C).

Because of the changes in leukocytes, lymphoid organs were subjected to pathologic analysis. A dramatic infiltration of neutrophils was observed not only in the bone marrow, but also in spleen, lymph nodes, and thymus (Figure 3). Within the spleen and lymph nodes the architecture was disrupted, with loss of lymphoid follicles. The thymus demonstrated loss of a distinct medulla and cortex, an increase of neutrophils and a
reduction of thymocytes (Figure 3). Morphologically, the bone marrow revealed an expansion of neutrophils, and a reduction of erythropoiesis, without morphologic evidence of myeloid dysplasia (Figure 3). No histologic abnormalities of bone were noted in the Flip deficient mice. The spleen demonstrated a reduction in the percentage of B lymphocytes, and of CD8\(^+\) and CD4\(^+\) lymphocytes in the Flip\(^{\text{fl}}\), LysM \(^{c/}\) mice, although only the absolute number of B cells was reduced (p < 0.01) (Supplemental Figure 3). By immunophenotyping, a significant (p < 0.05-0.01) increase in the absolute number of Gr-1\(^+\) neutrophils was identified in the spleen and lymph nodes, but not the bone marrow or thymus, even though the percentage of neutrophils was increased in each of the organs, from the Flip\(^{\text{fl}}\), LysM \(^{c/}\) mice (Supplemental Figure 4). Non-lymphoid tissues were also affected. Accumulation of neutrophils was demonstrated in the lungs in the inter-alveolar spaces and around large blood vessels and in the small intestine, especially the jejunum (Supplemental Figure 5). Neutrophilic infiltration was less severe in the liver and heart (Supplemental Figure 5), as well as the stomach, large intestine, kidney, and brain (data not shown). Overall, pathologic analysis demonstrated an expansion and a multi-organ infiltration of neutrophils.

To determine if the increased neutrophils might be due to an underlying infection, organs from Flip\(^{\text{fl}}\), LysM \(^{c/}\) mice were cultured for microorganisms. Cultures of the spleen, liver, lymph nodes or peritoneal fluid were positive for bacteria in 30% of the mice, while none of the littermate controls was positive. There was no difference in the neutrophil counts between the Flip\(^{\text{fl}}\), LysM \(^{c/}\) mice that were culture positive and negative, although the culture positive mice were smaller (< 10 gms) compared with those that were culture negative (Supplemental Figure 6A, B). The cultures revealed a number of commensal intestinal organisms including Streptococcus sanguinis, Enterococcus faecalis, Proteus mirabilis and Escherichia coli. Based on susceptibility testing, Flip\(^{\text{fl}}\), LysM \(^{c/}\) mice were treated with antibiotics employing ampicillin in drinking water and/or gentamicin subcutaneously. Treatment reduced the positive cultures by 50% but there was no reduction of circulating neutrophils (Supplemental Figure 6C) and no increase of weight. These results suggest that infection was not the cause of the neutrophil expansion or the increased mortality.
Normal apoptosis, oxidative burst and degranulation in Flip deficient neutrophils: Because of the increased numbers of neutrophils and infections, studies were performed to determine if there was an abnormality of survival or function of neutrophils. Control peripheral blood neutrophils exhibited spontaneous apoptosis with the loss of mitochondrial transmembrane potential and uptake of DAPI that was limited immediately after isolation and increased at 4 hours, with the marked loss of membrane integrity (DAPI+) by 24 hours (Figure 4A). The percentage of cells that had lost mitochondrial transmembrane potential or membrane integrity at 0 and 4 hours was somewhat less in the Flip$^{fid}$, LysM$^{cf/+}$ mice (Figure 4A). However, neutrophil cell death was essentially complete by 24 hours employing circulating granulocytes from the Flip$^{fid}$, LysM$^{cf/+}$ mice, similar to the controls (Figure 4B). Since the Flip$^{fid}$, LysM$^{cf/+}$ mice experienced infections, studies were performed to examine neutrophil function. Peripheral blood neutrophils from Flip$^{fid}$, LysM$^{cf/+}$ mice demonstrated normal oxidative burst (Figure 4C) and degranulation following activation with PMA (Figure 4D). In response to the TLR4 ligand LPS, after 1 hour no neutrophil oxidative burst was detected, although degranulation was observed and was comparable in the Flip deficient and control mice (data not shown). Additionally, myeloperoxidase activity of peripheral blood neutrophils in the Flip$^{fid}$, LysM$^{cf/+}$ mice was comparable to the controls (Figure 4E). These observations do not suggest that an intrinsic abnormality of apoptosis or function was responsible for the neutrophilia or infection.

Flip deletion results in increased extramedullary myelopoiesis: Since there were increased granulocytes, monocytes, and platelets, studies were performed to determine the effect of Flip deletion on myelopoiesis. Examination of the bone marrow revealed no difference of Granulocyte-Monocyte-Macrophage (GM)-Colony Forming Units (CFU) between the Flip$^{fid}$, LysM$^{cf/+}$ and the littermate controls (Table 2). In contrast, employing cells from the spleen, a significant (p < 0.01) increase of CFU-GM was observed in the Flip$^{fid}$, LysM$^{cf/+}$ mice (Table 2). The changes were not restricted to the GM, since the megakaryocyte-CFUs (CFU-Mk) were also increased (p < 0.001) in the spleens of the Flip$^{fid}$, LysM$^{cf/+}$ mice, but not the bone marrow (Table 2). Serum from
the peripheral blood was employed to determine if growth factors might contribute to the extramedullary hematopoiesis. G-CSF, GM-CSF, M-CSF, IL-6, and IL-17 were all increased in the \( \text{Flip}^{\text{fl}} \), \( \text{LysM}^{\text{c/+}} \), compared to the control, mice (Table 3). These observations document a marked increase of splenic extramedullary hematopoiesis, which was associated with an increase of myeloid growth factors.

**Deletion of \( \text{Flip} \) results in reduction of macrophages in the spleen and lymph nodes:** Splenomegaly (Figure 5A) with loss of lymphoid follicles and infiltration with granulocytes (Figure 3) was observed in the \( \text{Flip}^{\text{fl}} \), \( \text{LysM}^{\text{c/+}} \) mice. However, the total number of cells in the \( \text{Flip}^{\text{fl}} \), \( \text{LysM}^{\text{c/+}} \) and control spleens was not different (Figure 5B). This is most likely due to an increase of immature and myeloid cells which are larger than lymphoid cells, which were reduced. An increased size of cells in the spleens for the \( \text{Flip}^{\text{fl}} \), \( \text{LysM}^{\text{c/+}} \) mice was documented by the right shift in forward scatter determined by flow cytometry (Figure 5B). No RBC sequestration was apparent (Figure 3). Immunofluorescence staining revealed a marked reduction of macrophages in both the red pulp (anti-F4/80, Figure 5C) and the marginal zone (anti-CD169, Figure 5D) in the \( \text{Flip}^{\text{fl}} \), \( \text{LysM}^{\text{c/+}} \) mice. Examination of lymph nodes also showed a marked reduction of F4/80\(^+\) macrophages (Figure 5E). In contrast there was no reduction of macrophages in the liver (data not shown). By immunoblot analysis, FLIP was reduced, but still present, in lysates of the spleen (Figure 5F) and lymph nodes (Figure 5G) of the \( \text{Flip}^{\text{fl}} \), \( \text{LysM}^{\text{c/+}} \) mice, most likely due to a marked increase of neutrophils which do not express Flip, and a marked reduction of B cells in spleen (Supplemental Figure 3). Therefore, the deletion of \( \text{Flip} \) in myeloid cells results in a reduction of macrophages in the spleen and lymph nodes, with the loss of lymphoid follicles.

**Deletion of \( \text{Flip} \) in the myeloid lineage results in increased neutrophils and the absence of mature macrophages in the peritoneal cavity.** F4/80\(^+\), Gr1\(^-\) resident macrophages were abundant in the peritoneal cavity of the littermate control mice expressing FLIP (Figure 6A). However, in the \( \text{Flip}^{\text{fl}} \), \( \text{LysM}^{\text{c/+}} \) mice, the increased total cell count (Figure 6A, B) was due to a marked (\( p < 0.01 \)) increase of Gr1\(^+\), F4/80\(^-\) neutrophils, while F4/80\(^+\),Gr1\(^-\) macrophages were significantly (\( p < 0.001 \)) reduced (Figure 6A, B). There was a highly
significant inverse relationship between the percentage of macrophages and granulocytes ($r = -0.75$, $p < 0.001$), that was more clearly described by the trend line (Figure 6C). Analysis of the peritoneal space of wild type and the $Flip^{f/d}$, LysM $c^{+/+}$ mice revealed that the percentage of granulocytes increased to $> 10\%$ only when the percentage of macrophages was reduced to $< 10\%$ of the total peritoneal cells (Figure 6C). The macrophages that could be isolated from the peritoneum of $Flip^{f/d}$, LysM $c^{+/+}$ mice did not demonstrate deletion of $Flip$ (Figure 6D). In addition, the $Flip^{f/d}$, LysM $c^{+/+}$ mice demonstrated an increase percentage of cells expressing intermediate levels of F4/80 and Gr1 (F4/80$^{mid}$, Gr1$^{mid}$), which were also CD62L$^+$ (data not shown), and may represent inflammatory monocytes. Further, there was a reduction of resident CD19$^+$ B cells but not CD3$^+$ T cells in the $Flip^{f/d}$, LysM $c^{+/+}$ mice (Supplemental Figure 7). To examine the role of FLIP in elicited macrophages, thioglycollate was injected into the peritoneum. At 20 hours, there was a marked increase of neutrophils in the controls, while neutrophil dominance persisted in the $Flip^{f/d}$, LysM $c^{+/+}$ mice (Figure 6E). In both the $Flip^{f/d}$, LysM $c^{+/+}$ mice and the controls there was a population of F4/80$^{mid}$, Gr1$^{mid}$ cells at 24 hours (Figure 6E). At 72 hours, the controls demonstrated a marked increase of F4/80$^{mid}$, Gr1$^-$ elicited macrophages. In contrast, in the $Flip^{f/d}$, LysM $c^{+/+}$ mice, there was no increase of elicited macrophages, however, the marked increase of neutrophils persisted (Figure 6E, F). Therefore, the deletion of $Flip$ in myeloid cells, not only results in an increase of peritoneal neutrophils, but also a marked reduction of resident and elicited macrophages.

**Flip is necessary for macrophage differentiation:** Experiments were performed to determine if the reduction of macrophages upon $Flip$ excision was due to a defect in differentiation or to cell death following differentiation. c-Kit$^+$ hematopoietic stem cells from floxed $Flip$ mice were infected with a recombinant GFP-Cre retrovirus to promote $Flip$ excision in vitro. Following infection, the cells were differentiated into macrophages for 5 days. There was no difference in the numbers of GFP$^+$ cells (Figure 7A) or GFP$^+$ cells that were CD11b$^-$, F4/80$^-$, or CD11b$^+$, F4/80$^-$ or CD11b$^+$, F4/80$^+$ mature macrophages (Figure 7B) when $Flip^{f/+}$ and $Flip^{f/d}$ c-Kit$^+$ cells were infected with a control GFP expressing retrovirus. In contrast, there was a reduction of GFP$^+$ cells (Figure 7C) and GFP$^+$ cells that were CD11b$^-$ F4/80$^-$, or CD11b$^+$, F4/80$^-$, or CD11b$^+$, F4/80$^+$ mature macrophages.
macrophages (p < 0.01-0.001, Figure 7D) on days 1, 2 and 5 following infection of $Flip^{fad}$ c-Kit+ cells with the GFP, Cre expressing retrovirus. This reduction does not appear to be due to increased apoptosis in response to $Flip$ deletion, because there was no difference in the percentage of annexin V positivity for any of the cell-types examined (data not shown). Further, when total bone marrow cells were employed to generate macrophages, the caspase inhibitors IETD-fmk or zVAD.fmk did not rescue the GFP+, F4/80+ macrophages generated from the $Flip^{fad}$ mice ex vivo following the deletion of $Flip$ (data not shown). Together these observations suggest that FLIP is necessary for macrophage differentiation.

In order to determine if the neutrophilia was intrinsic to the loss of $Flip$ in myeloid cells, bone marrow reconstitution experiments were performed, employing CD45.1+ wild type and CD45.2+$^+$, $Flip^{fad}$, LysM $^{c/+}$ bone marrow cells, injected into CD45.1+$^+$ lethally irradiated hosts, all on a C57BL/6 background. In mixed chimera experiments, when 25%, 50% or 75% of the bone marrow cells injected into the lethally irradiated recipients were from the $Flip^{fad}$, LysM $^{c/+}$ mice, no increase of circulating neutrophils or monocytes was observed (Table 4). Further, decreased body weight, increased spleen size, and decreased peritoneal macrophages were not observed in the mixed chimeras. However, the CD45.2+$^+$ $Flip^{fad}$, LysM $^{c/+}$ bone marrow cells were unable to compete with the wild type CD45.1+$^+$ cells (Supplemental Figure 8). It was only when 100% of the bone marrow cells were from the $Flip^{fad}$, LysM $^{c/+}$ mice that the inflammatory phenotype, including increased circulating neutrophils (Table 4), was observed. All of the mice that received 100% $Flip^{fad}$, LysM $^{c/+}$ bone marrow cells demonstrated an increase of CD45.2+$^+$ neutrophils compared to all the other groups (Supplemental Figure 8C). Of interest, the one mouse with the greatest increase of CD45.2+$^+$ neutrophils, also demonstrated an increase of recipient CD45.1+$^+$ neutrophils. G-CSF, GM-CSF and IL-6 were increased only in this mouse and the two other mice with the greatest increase of neutrophils (data not shown), supporting the role of growth factors/cytokines in the neutrophilia. These observations suggest that the neutrophilia observed in the $Flip^{fad}$, LysM $^{c/+}$ mice is due to a cell autonomous defect in the bone marrow progenitors which results in increased cytokines/growth factors which contribute to the granulopoiesis.
DISCUSSION: The cellular mechanisms responsible for maintaining homeostatic balance in myeloid cells has not been fully characterized. Here, we demonstrate that the deletion of \(\text{Flip}\) in myeloid cells resulted in a marked increase of circulating neutrophils, associated with multi-organ neutrophil infiltration, including spleen, lymph nodes, thymus, intestine and lung. There was no evidence of myeloid dysplasia in the bone marrow, spleen or other organs. Increased granulopoiesis with an increase of CFU-GM was observed in the spleens of the \(\text{Flip}^{\text{fl}}\), \(\text{LysM}^{c/+}\) mice, although not in the bone marrow. Under homeostatic conditions, the half life of circulating neutrophils is 8 hours or less, and neutrophilia may occur by increased survival. Although there was a slight reduction of neutrophil apoptosis at 0 and 4 hours, which may be due to the increased circulating growth factors, there appeared to be no intrinsic defect in neutrophil apoptosis, since at 24 hours neutrophil apoptosis \textit{in vitro} was nearly complete employing neutrophils from the \(\text{Flip}^{\text{fl}}\), \(\text{LysM}^{c/+}\) mice. Since FLIP protects against death receptor-mediated apoptosis, a reduction of FLIP might be expected to lead to decreased cell survival. However, \(\text{Flip}\) mRNA was not expressed in circulating wild type neutrophils (data not shown), further supporting our observations that suggest FLIP does not play a role in neutrophil survival. This interpretation is consistent with known role of the intrinsic mitrochondrial pathway involving \(\text{Mcl-1, A1, Bcl-xL}\) in neutrophil apoptosis\(^27\). In contrast to the results observed with FLIP, the deletion of \(\text{Mcl-1}\) employing \(\text{LysM-Cre}\), results in the neutropenia, with no reduction of macrophages\(^{28,29}\).

Neutrophilia may also occur by increased production or increased release from the bone marrow in response to stress such as infection\(^30\). The \(\text{Flip}^{\text{fl}}\), \(\text{LysM}^{c/+}\) mice demonstrated extramedullary hematopoiesis associated with an increase of growth factors in the circulation that promote myelopoiesis. However, the data do not support the underlying infection as the cause for the increased neutrophils since 70% of the \(\text{Flip}^{\text{fl}}\), \(\text{LysM}^{c/+}\) mice were culture negative. Further, although antibiotic treatment reduced the rate of infections, it had no effect on the circulating neutrophils or the phenotype. These observations suggest that the persistent acute inflammation contributed to the stunted growth and premature death in the \(\text{Flip}^{\text{fl}}\), \(\text{LysM}^{c/+}\) mice.
The marked increase of circulating neutrophils and the multi-organ infiltration with neutrophils was not expected. Prior studies have demonstrated that mice lacking the adhesion molecule CD18, exhibit neutrophilia, with increased IL-17 and G-CSF, which contributed to the increased neutrophil counts. Further, in these mice, the phagocytosis of apoptotic neutrophils by macrophages and dendritic cells suppressed granulopoiesis, which was mediated by a reduction of IL-23, and subsequently IL-17 and G-CSF. However, the \( \text{Flip}^{\text{fla}}, \text{LysM}^{\text{c/c+}} \) mice demonstrated a reduction of splenic, lymph node and peritoneal macrophages, suggesting that increased IL-23 was not responsible. In fact, circulating p40 which is common to both IL-12 and IL-23, was not elevated (data not shown). In contrast IL-17 and G-CSF, in addition to IL-6, GM-CSF and M-CSF, were all increased in the \( \text{Flip}^{\text{fla}}, \text{LysM}^{\text{c/c+}} \) mice, suggesting that these growth factors may have contributed to the increased granulopoiesis. However, the results of the bone marrow transfer experiments suggest that the primary defect was a cell autonomous defect in myeloid development of FLIP deficient bone marrow derived cells, since these cells were capable to transferring the disease, in the absence of competitor wild type cells. The defect in myeloid development resulted in increased growth factors and cytokines which resulted in the neutrophilia.

Further, the mixed chimera experiments demonstrate that \( \text{Flip}^{\text{fla}}, \text{LysM}^{\text{c/c+}} \) bone marrow cells were not able to compete with the wild type cells, suggesting an impaired hematopoietic stem/progenitor cell phenotype. This may be due to excessive cytokines and growth factor production in the \( \text{Flip}^{\text{fla}}, \text{LysM}^{\text{c/c+}} \) mice, which may change their ability in homing, repopulating or potentially exhaust the stem/progenitor cells. It is unlikely that FLIP deletion in the hematopoietic stem cells was directly responsible because LysM is weakly expressed and only in a minority of these cells.

Resident peritoneal macrophages were markedly reduced in the \( \text{Flip}^{\text{fla}}, \text{LysM}^{\text{c/c+}} \) mice. When peritoneal macrophages were reduced to < 10%, peritoneal granulocytes increased, suggesting that the reduction of macrophages contributes to the increase of granulocytes. The few Gr1\(^-\), F4/80\(^+\) macrophages that could be isolated for the peritoneum of the \( \text{Flip}^{\text{fla}}, \text{LysM}^{\text{c/c+}} \) mice, or differentiated from bone marrow or spleen, were not deleted for \( \text{Flip} \). Therefore, we were unable to examine the non-apoptotic functions of FLIP in macrophages,
since any macrophages that could be isolated expressed FLIP. Prior studies demonstrated that under resting conditions neither Gr1+ nor Gr1− monocytes homed to the peritoneum, while only Gr1+ monocytes migrated to the peritoneum after thioglycollate 36, 37. In the Flip^{fl/d}, LysM^{c/+} mice, resident F4/80^{mid}, Gr1^{mid} cells, consistent with inflammatory monocytes, were increased in the peritoneum. The recruitment of these cells may have been due to the acute inflammation with neutrophils. Following the injection of thioglycollate at 20 hours, the inflammatory monocytes and neutrophils were comparable in the Flip^{fl/d}, LysM^{c/+} and control mice. However, in the Flip^{fl/d}, LysM^{c/+} mice no increase of F4/80+, Gr1− elicited macrophages was observed at 72 hours, consistent with a failure of the inflammatory monocytes to differentiate into macrophages, resulting in a persistent acute inflammatory response. We can not exclude the possibility that following macrophage differentiation, the cells died. However, even when examined at 48 hours (data not shown), no increase of Gr1− macrophages was observed in the Flip^{fl/d}, LysM^{c/+} mice, suggesting that Flip is essential for macrophage differentiation.

Further documenting the importance of FLIP for macrophage differentiation, infection of c-Kit+ bone marrow cells with a recombinant GFP-Cre retrovirus resulted in a reduction of differentiated CD11b+, F4/80+ macrophages derived from bone marrow of Flip^{fl/d} mice. When Flip was deleted, there was also a reduction of CD11b−, F4/80− and CD11b+, F4/80− cells, demonstrating that the defect was not limited to the terminal stage of differentiation. There was no evidence of increased apoptosis to account for the reduction of macrophages. Relevant to this observation, another member of the death receptor pathway, caspase 8, is also necessary for normal macrophage differentiation 38 and the deletion of caspase 8 in myeloid cells prevents macrophage differentiation 39. In contrast, over-expression of FADD, another member of the Fas mediated death pathway, accelerates the differentiation of macrophages, independent of any death receptor 40. Our observations are consistent with those that identified the role of a complex of FADD, caspase 8, RIP1 and FLIP, which suppressed NF-κB activation, which was necessary for M-CSF-mediated macrophage differentiation 40. These
observations are also reminiscent of the observation that the germ line deletion of either FLIP, caspase 8 or FADD is embryonic lethal, suggesting that these molecules also cooperate during embryonic development.

Together, these observations suggest the deletion of Flip in macrophages was necessary for the development of neutrophilia and the inflammatory phenotype. Of interest, a recent study demonstrated that deletion of dendritic cells also resulted in neutrophilia and a multi-organ infiltration with neutrophils. Although these mice also exhibited increased Th17 cells, in contrast to the Flip$^{+/}$, LysM$^{+/}$ mice, they also exhibited an infiltration of CD4$^+$ T cells in intestine and kidney, as well as increased numbers of F4/80$^+$ macrophages. Therefore, an intact mononuclear phagocytic system appears necessary for normal granulopoiesis and that deletion of either macrophages or dendritic cells results in neutrophilia. In summary, this study demonstrates that the expression of FLIP in myeloid cells is critical for the differentiation of macrophages, which is a characteristic feature of chronic inflammation, and that the acute inflammatory phenotype is secondary to the reduction of macrophages and an increase of cytokines and growth factors.
ACKNOWLEDGEMENTS: This work supported in part by NIH grants AR048269 and AR055240 to RMP and AR050250, AR054796, and AI06759 to HP and a Within Our Reach Grant from the American College of Rheumatology to RMP. JDC is a Scholar of the Leukemia and Lymphoma Society. The authors thank Dr. Guang-Yu Yang, Northwestern University, Department of Pathology, for assistance with interpreting the histology of the gastrointestinal tract. We thank Rudina Sobkoviak and Jeff Kraynak for their contributions helping to establish and maintain the Flip conditional knock out mice and Dr. Chiang-Ching Huang, Department of Preventive Medicine Northwestern University for assistance with statistical analysis.

AUTHOR CONTRIBUTIONS: Q.Q.H and R.M.P. conceived the project, designed experiments, performed data analysis and wrote the manuscript preparation. R.M.P. supervised the project. Q.Q.H and R.B generated and maintained the KO mice and performed the major experiments in genotypic and phenotypic analysis and data acquisition. H.P and J.D.C designed experiments, interpreted results and assisted in manuscript preparation. Z.H designed and performed CFU and bone marrow infection experiments. L.K. performed immunohistochemistry analysis. S.G. performed the histological and pathological identification. H.A. and A.M. assisted with the mixed chimeras and their analysis. All authors discussed the results and implications and contributed to the manuscript.

CONFLICT OF INTEREST: None
REFERENCES:

Table 1: Complete blood counts of Flip<sup>fld</sup>, LysM<sup>c/c</sup> and the littermate control mice

<table>
<thead>
<tr>
<th>Genotypes</th>
<th># of Flip alleles in whole genome</th>
<th># of Flip alleles in myeloid lineage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 alleles (Flip&lt;sup&gt;+/+, f/+ or f/f&lt;/sup&gt;, LysM&lt;sup&gt;+/+&lt;/sup&gt;)</td>
<td>1 allele (Flip&lt;sup&gt;+/+, d/+ or f/d&lt;/sup&gt;, LysM&lt;sup&gt;+/+&lt;/sup&gt;)</td>
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<tr>
<td>N =</td>
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### Leukocytes

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<th>Genotypes</th>
<th>(10&lt;sup&gt;3&lt;/sup&gt;/µL)</th>
<th>Genotypes</th>
<th>(10&lt;sup&gt;3&lt;/sup&gt;/µL)</th>
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<th>Genotypes</th>
<th>(10&lt;sup&gt;3&lt;/sup&gt;/µL)</th>
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<td>Neutrophils</td>
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<td>7.4±0.80</td>
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<td>9.0±1.5</td>
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<td>13.9±1.7</td>
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<td>1.9±0.3</td>
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<td>0.2±0.03</td>
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<td>Monocytes</td>
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<td>0.5±0.18***</td>
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<td>0.5±0.2</td>
<td></td>
<td>0.7±0.1***</td>
<td></td>
<td>0.80±0.15**</td>
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<td>8.9±1.2</td>
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<td>Eosinophila</td>
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<td>0.2±0.04*</td>
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<td>0.1±0.07</td>
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<td>0.2±0.03***</td>
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<td>0.02±0.01</td>
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<td>Erythrocytes</td>
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<tr>
<td>Red blood cell</td>
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<td>9.8±0.3***</td>
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<td>9.3±0.3*</td>
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<td>9.7±0.1*</td>
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<td>9.5+0.3***</td>
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<td>9.5±0.55**</td>
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<td>7.4±0.3</td>
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<td>Hemoglobin (g/dL)</td>
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<td>13.5±0.3***</td>
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<td>13.2±0.4**</td>
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<td>13.7±0.3**</td>
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<td>15.1±1.5***</td>
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<td>15.5±3.3**</td>
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<td>9.4±0.3</td>
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<tr>
<td>Hematocrit (%)</td>
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<td>40.8±0.9***</td>
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<td>38.8±1.5**</td>
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<td>41.4±0.7**</td>
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<td>40.5±1.2***</td>
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<td>41.0±2.4***</td>
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<td>30±0.8</td>
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<td>Thrombocytes</td>
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<tr>
<td>Platelet (10&lt;sup&gt;9&lt;/sup&gt;/µL)</td>
<td>974±78</td>
<td>941±62</td>
<td>825±45</td>
<td>902±72 *</td>
<td>1026±131</td>
<td>1389±76</td>
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<tr>
<td>Mean platelet volume (fL)</td>
<td>4.6±0.1</td>
<td>4.5±0.07</td>
<td>4.5±0.07</td>
<td>4.8±0.1</td>
<td>4.3±0.04***</td>
<td>5.0±0.06</td>
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</tbody>
</table>

No difference among groups with 1 or 2 Flip alleles either in whole genome or in myeloid lineage was identified. Significant differences were noted between the identified values compared with the Flip<sup>fld</sup>, LysM<sup>c/c</sup> group, indicated by *, p<0.05, **, p<0.01 and ***, p<0.001.
Table 2: Extramedullary hematopoiesis in the \textit{Flip}^{ld}, \textit{LysM}^{c/+} mice:

<table>
<thead>
<tr>
<th></th>
<th>Littermate controls (# of colonies)</th>
<th>\textit{Flip}^{ld}, \textit{LysM}^{c/+} (# of colonies)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocyte</td>
<td>Spleen</td>
<td>5.8 ± 0.8</td>
<td>270.2 ± 36.1</td>
</tr>
<tr>
<td></td>
<td>Bone marrow</td>
<td>92.3 ± 12.7</td>
<td>74.2 ± 14.4</td>
</tr>
<tr>
<td>Monocyte</td>
<td>Spleen</td>
<td>1.8 ± 0.4</td>
<td>42.1 ± 0.9</td>
</tr>
<tr>
<td>Macrophage</td>
<td>Bone marrow</td>
<td>31.1 ± 4.2</td>
<td>21.4 ± 1.6</td>
</tr>
</tbody>
</table>

Values represented are mean of 3 independent experiments employing gender-matched littermate controls performed in triplicate. * NS, not significant.
Table 3: The *Flip*<sup>fd</sup>, *LysM*<sup>c/+</sup> mice exhibit increased myeloid cytokines:

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Littermate controls [pg/ml]</th>
<th><em>Flip</em>&lt;sup&gt;fd&lt;/sup&gt;, <em>LysM</em>&lt;sup&gt;c/+&lt;/sup&gt; [pg/ml]</th>
<th>Mean fold increase</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-CSF</td>
<td>598.4 ± 213.1</td>
<td>8648.2 ± 1690.9</td>
<td>14.45</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>IL-6</td>
<td>65 ± 39.4</td>
<td>1640 ± 464.4</td>
<td>25.23</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>M-CSF</td>
<td>3.9 ± 0.54</td>
<td>11.4 ± 3.9</td>
<td>2.9</td>
<td>&lt; 0.03</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0.8 ± 0.8</td>
<td>21.7 ± 11.4</td>
<td>26.5</td>
<td>NS*</td>
</tr>
<tr>
<td>IL-17</td>
<td>4.4 ± 1.0</td>
<td>14.9 ± 5.4</td>
<td>3.4</td>
<td>&lt; 0.003</td>
</tr>
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</table>

n=14 in each group; * NS, not significant

Table 4: Mixed bone marrow chimeras do not develop an inflammatory phenotype in the peripheral blood:

<table>
<thead>
<tr>
<th>Ratio of donor BM cells implanted from Control / KO mice (x10&lt;sup&gt;3&lt;/sup&gt;/μl)</th>
<th>100/0</th>
<th>75/25</th>
<th>50/50</th>
<th>25/75</th>
<th>0/100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Granulocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammatory Monocytes</td>
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<td>Resident Monocytes</td>
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<td>B cells</td>
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<td>CD4+ T cells</td>
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<tr>
<td>CD8+ T cells</td>
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</table>

Data presented represents the number of cells x10<sup>3</sup>/μl determined by total cell count and flow cytometry, 8 weeks post bone marrow transplant. N=5-6 in each group.
FIGURES:

Figure 1. Deletion of Flip in myeloid lineage results in postnatal growth retardation and premature death. (A) Schematic of the Flip targeting strategy. A 9.5 kb Flip genomic DNA segment (exon 2 to 4 from a C57BL/6J mouse BAC library) was employed to engineer the Flip conditional mutagenesis construct. Exons are indicated by rectangular boxes and E and B represent the restriction cleavage sites of EcoRI and BamHI. Three loxP sites were inserted as indicated by triangles. The dashed lines represent the flanking region for Southern blot probes. The location of 3 PCR primers (a, b, c) and orientation are indicated by arrows. Amplification employing primers a and b generates a 220 bp fragment for wild type(Flip*) and a 270bp fragment for the floxed (Flipf) allele. Cre induced recombination generates a 150 bp fragment for depleted Flip allele (Flipd) employing primers a and c. All recombinant DNA and animal procedures were approved by the Office of Research Safety and the Institutional Animal Care and Use Committee of Northwestern University. (B) PCR genotyping of the LysM-cre induced cell type specific deletion of Flipf. The representative Flipf+, LysM c/+ littermate control and Flipf/d, LysM c/+ KO mice were genotyped from tail biopsy, and the different cell types were isolated from bone marrow (BM) and peripheral blood were genotyped by 3 PCR primers indicated in panel A. Granulocytes (Gran) were 11b+/Gr1+ F4/80--; peripheral blood monocytes or bone marrow monocyte precursors (Mono) were 11b+, F4/80++; B cells were 11b/CD19+ and T cells were 11b/CD3+. (C) Body weight of Flipf/d, LysM c/+ (n = 53) and littermate controls including: Flip+/+, Flipf/+ or Flipff, LysM +/- (n=9); Flipf/d or Flipf/d, LysM +/- (n= 10); Flip+/+,LysM c/+ (n=5); Flipf/+ or Flipf/d, LysM c/+ (n = 24) and Flip+/+, Flipf/+ or Flipf/d, LysM c/c (n= 12) . All mice are between 6 to 24 weeks of age. *** p < 0.001, compared with indicated groups. (D) Postnatal viability of Flipf/d, LysM c/+ (n = 101) and the Flipf/d, LysM c/c (n = 17) and littermate controls (n = 475) which included: Flip+/+,Flipf/+ or Flipff, LysM +/- (n=112), Flipf/d or Flipf/d, LysM +/- (n= 112), Flipf+/,LysM c/+ (n=47) , Flipf/+ or Flipf/d, LysM c/+ (n= 127) and Flip+/+, Flipf/+ or Flipf/d, LysM c/c (n = 77) mice.
Figure 2. *Flip* deletion in myeloid lineage results in leukocytosis. (A) Peripheral blood from *Flip<sup>fl/fl</sup>, LysM<sup>cre/cre</sup>* mice (n= 56), and their littermate controls (mixed genotypes, n=50) were examined for completely blood count and neutrophils and monocytes are presented. *** p < 0.001 compared to the controls. (B) Representative blood smears from a *Flip<sup>fl/fl</sup>, LysM<sup>cre/cre</sup>* mice and controls stained with Hema-3. Data are representative of smears from 3-4 mice for each group (C) Representative flow cytometric analysis of circulating monocytes and neutrophils of *Flip<sup>fl/fl</sup>, LysM<sup>cre/cre</sup>* and gender-matched littermate controls. Cells are gated by side scatter (SSC) and the expression of CD115, CD62L and Gr1. (D). Absolute cell count of resident and inflammatory monocytes, calculated from the total number of monocytes and the % of each subset employing 5 *Flip<sup>fl/fl</sup>, LysM<sup>cre/cre</sup>* and gender-matched littermate controls.

Figure 3. *Flip* deletion in myeloid lineage results in multi-organ neutrophil infiltration.
Hematoxylin and eosin staining of representative sections of tissues from the indicated organs from *Flip<sup>fl/fl</sup>, LysM<sup>cre/cre</sup>* and control mice. The area in the box is enlarged in the panel below. Data are representative of sections from 3-4 mice for each group.

Figure 4. Normal apoptosis and function in *Flip* deficient circulating neutrophils. (A) Time dependent loss of mitochondrial transmembrane potential (ΔΨ<sub>m</sub>) was assessed by decreased Rh123 fluorescence (x axis) and the loss of membrane integrity assessed by uptake of DAPI (y axis). (B) The percent of live cells is identified as Rh123<sup>+</sup>, DAPI<sup>-</sup> (n = 4 for each group), * represents p<0.05 and ** p < 0.01 between groups. (C) Representative myeloperoxidase staining of peripheral blood smears, observed by light microscopy (400X). Data are representative of 3 *Flip<sup>fl/fl</sup>, LysM<sup>cre/cre</sup>* and gender-matched littermate controls. (D) The ability oxidize non-florescent dihydrorhodamine 123 (DHR) was accessed as increased mean fluorescence intensity (MFI) following PMA activation. (E) Neutrophil degranulation was determined by increased MFI of cell surface CD11b following PMA stimulation. The observations were obtained from 5 *Flip<sup>fl/fl</sup>, LysM<sup>cre/cre</sup>* and gender-matched littermate controls.)
Figure 5. Decreased macrophages and FLIP expression in the spleen and lymph node. Spleen size (n = 31, A) and total number of spleen cells (n = 16, B) in Flip^fd, LysM^c/+ and littermate controls. A representative flow histogram of forward scatter (FSC) from ungated splenocytes of Flip^fd, LysM^c/+ and littermate control mice is presented in the inset of panel B. Immunofluorescence microscopy of spleen was performed to identify red pulp macrophages (anti-F4/80) (C) or marginal zone macrophages (anti-CD169) (D), and of lymph nodes with anti-F4/80 antibodies (E). The data are representative of sections from 3-4 mice of each group. The area in the box is enlarged in the panel below. Randomly selected spleens (panel F) and lymph nodes (panel G) from littermate controls and Flip^fd, LysM^c/+ mice were employed to examine the expression of FLIP determined by immunoblot analysis. The data are representative of > 4 mice for each group.

Figure 6. Flip deletion results in increased neutrophils and decreased macrophages in the peritoneum. Resident (A, B) or thioglycollate elicited (E, F) cells from peritoneal cavities were analyzed by flow cytometry, gating on CD11b+ cells. The macrophages (MΦ) were identified as F4/80+, Gr1−, while granulocytes (Gran) were identified as F4/80−, Gr1+. (B) The total number of cells, resident macrophages and granulocytes from controls (n = 20) and Flip^fd, LysM^c/+ mice (n = 26) are summarized. (C) There was an inverse relationship between the percentage of peritoneal macrophages and granulocytes in the wild type and Flip^fd, LysM^c/+ mice. (D) PCR genotyping of granulocytes and macrophages isolated from peritoneal cavities were performed employing the 3 PCR primers indicated in Figure 1. (F) The total number of thioglycollate elicited macrophages and granulocytes at 72 hours (n=7 for each group) are summarized. * represents p<0.05, ** p<0.01 and *** p<0.001 between groups.

Figure 7. Deletion of Flip suppresses macrophage differentiation. cKit+ hematopoietic stem cells from Flip^+/+ and Flip^fd were isolated from bone marrow and seeded at 2.5x10^5 cells/well followed by infection retroviral vectors expressing GFP alone (A,B) or GFP and Cre (C,D). Following the infection, the cells were
differentiated *in vitro* to macrophages in 20% L929 medium. The total number of GFP+ cells (panels A and C) and the GFP+, CD11b<sup>−</sup>, F4/80<sup>−</sup>, the GFP+, CD11b<sup>+</sup>, F4/80<sup>−</sup>, and the GFP+, CD11b<sup>+</sup>, F4/80<sup>+</sup> cells (panels B and D) were determined after 1, 2 and 5 days of differentiation. Data in panels B and D represents the mean ± SE of 4 independent experiments.
Figure 2

A. Neutrophils and Monocytes cell count (x10^3/μL) for Control, Flip^f/d^ LysM^c/c^-/- and Flip^f/d^ LysM^c/c^+/+.

B. Comparison of Control and Flip^f/d^ LysM^c/c^+/+ monocytes under light microscopy.

C. Flow cytometry analysis of Granulocytes and Monocytes in Control and Flip^f/d^ LysM^c/c^+/+ conditions.

D. Cell count (x10^3/μL) comparison between Resident Monocyte, Inflammatory monocyte in Control and Flip^f/d^ LysM^c/c^+/+ conditions.
Figure 3

Spleen

Lymph node

Thymus

Bone marrow

Control

Flip^{f/d} LysM^{C/+}

Control

Flip^{f/d} LysM^{C/+}
Figure 5

A. Bar graph showing spleen weight (g) for Control and Flip^f/d LysM^c/+ groups. **P < 0.001.

B. Histogram showing percentage of spleenocytes for Control and Flip^f/d LysM^c/+ groups.

C. Immunofluorescence images for Control and Flip^f/d LysM^c/+ groups.

D. Immunofluorescence images for Control and Flip^f/d LysM^c/+ groups.

E. Immunofluorescence images for Control and Flip^f/d LysM^c/+ groups.

F. Western blot images for FLIP and Actin expression in Control and KO groups.

G. Western blot images for FLIP and Actin expression in Control and KO groups.
Figure 6
FLIP: a novel regulator of macrophage differentiation and granulocyte homeostasis

Qi-Quan Huang, Harris Perlman, Zan Huang, Robert Birkett, Lixin Kan, Hemant Agrawal, Alexander Misharin, Sandeep Gurbuxani, John D. Crispino and Richard M. Pope