A Reporter Mouse Reveals Lineage-Specific and Heterogeneous Expression of IRF8 during Lymphoid and Myeloid Cell Differentiation

Hongsheng Wang, Ming Yan, Jiafang Sun, Shweta Jain, Ryusuke Yoshimi, Sanaz Momben Abolfath, Keiko Ozato, William G. Coleman, Jr., Ashley P. Ng, Donald Metcalf, Ladina DiRago, Stephen L. Nutt and Herbert C. Morse III

*J Immunol* published online 14 July 2014
http://www.jimmunol.org/content/early/2014/07/13/jimmunol.1301939

---

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2014/07/13/jimmunol.1301939.DCSupplemental

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

**Errata**
An erratum has been published regarding this article. Please see next page or:
/content/193/9/4749.full.pdf
A Reporter Mouse Reveals Lineage-Specific and Heterogeneous Expression of IRF8 during Lymphoid and Myeloid Cell Differentiation

Hongsheng Wang,* Ming Yan,† Jiafang Sun,* Shweta Jain,* Ryusuke Yoshimi,§ Sanaz Momben Abolfath,* Keiko Ozato,§ William G. Coleman, Jr., † Ashley P. Ng,†,‖ Donald Metcalf,§‖ Ladina DiRago,§ Stephen L. Nutt,†,‖ and Herbert C. Morse, III*

The IFN regulatory factor family member 8 (IRF8) regulates differentiation of lymphoid and myeloid lineage cells by promoting or suppressing lineage-specific genes. How IRF8 promotes hematopoietic progenitors to commit to one lineage while preventing the development of alternative lineages is not known. In this study, we report an IRF8–EGFP fusion protein reporter mouse that revealed previously unrecognized patterns of IRF8 expression. Differentiation of hematopoietic stem cells into oligopotent progenitors is associated with progressive increases in IRF8-EGFP expression. However, significant induction of IRF8-EGFP is found in granulocyte–myeloid progenitors and the common lymphoid progenitors but not the megakaryocyte–erythroid progenitors. Surprisingly, IRF8-EGFP identifies three subsets of the seemingly homogeneous granulocyte–myeloid progenitors with an intermediate level of expression of EGFP defining bipotent progenitors that differentiation into either EGFPint monocytic progenitors or EGFPab granulocytic progenitors. Also surprisingly, IRF8-EGFP revealed a highly heterogeneous pre–pro-B population with a fluorescence intensity ranging from background to 4 orders above background. Interestingly, IRF8–EGFP readily distinguishes true B cell committed (EGFPint) from those that are noncommitted. Moreover, dendritic cell progenitors expressed extremely high levels of IRF8-EGFP. Taken together, the IRF8-EGFP reporter revealed previously unrecognized subsets with distinct developmental potentials in phenotypically well-defined oligopotent progenitors, providing new insights into the dynamic heterogeneity of developing hematopoietic progenitors. The Journal of Immunology, 2014, 193: 000–000.

Hematopoietic stem cells (HSCs) constantly differentiate into all blood cell lineages via distinct differentiation programs. Lineage specification and commitment are marked by timely activation of one set of transcription factors associated with downregulation of other set(s) of transcription factors important for alternate cell lineage potential. Although early studies led to the proposal that the flow of intermediate cells within each lineage is fixed (1, 2), recent evidence suggests otherwise—that oligopotent progenitor differentiation is very “plastic,” especially when the host is stressed, by infection for example. This causes reprogramming of early lymphoid and myeloid progenitors leading to enhanced development of myeloid lineage cells but curbed production of lymphoid lineage cells (3–6).

The plasticity of hematopoietic differentiation has long been known and recently was confirmed at single-cell level by Naik et al. (7) using a novel “cellular barcoding” technique. The developmental heterogeneity of lineage progenitor cells has led not only to inconsistencies in identifying phenotypes of intermediate stage cells but also to difficulties in positioning the newly found precursors in the orderly progression of lineage differentiation pathways. For example, macrophages are thought to derive from myeloid progenitors, whereas dendritic cells (DCs) are thought to develop from separate pathways originating from either common lymphoid progenitors (CLPs) or common myeloid progenitor (CMPs) (1, 8–11). However, it was recently found that macrophage–DC progenitors (MDPs) with a phenotype of CD117+ were also detected in granulocyte–macrophage (GMP) and monocyte–granulocyte–erythroid–megakaryocyte (MEP) progenitors in granulocyte–myeloid progenitors and the common lymphoid progenitors but not the megakaryocyte–erythroid progenitors. However, significant induction of IRF8-EGFP is found in granulocyte–myeloid progenitors and the common lymphoid progenitors but not the megakaryocyte–erythroid progenitors.

The Journal of Immunology, 2014, 193: 000–000.

*Virology and Cellular Immunology Section, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD 20852; †Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892; ‡Department of Internal Medicine and Clinical Immunology, Yokohama City University Graduate School of Medicine, Yokohama 236-0004, Japan; Program in Genomics of Differentiation, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892; §Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia; and ‖Department of Medical Biology, University of Melbourne, Parkville, Victoria 3010, Australia

Received for publication July 22, 2013. Accepted for publication June 10, 2014.

This work was supported by the Intramural Research Program of the National Institutes of Health’s National Institute of Allergy and Infectious Diseases (to H.W., J.S., S.J., S.M.A., and H.C.M.), the National Institute of Child Health and Human Development (to R.Y. and K.O.), and the National Institute of Diabetes and Digestive and Kidney Diseases (to M.Y. and W.G.C.). A.P.N., L.D., and D.M. were supported by Program Grant 1016647 and Independent Research Institutes Infrastructure Support Scheme Grant 361646 from the Australian National Health and Medical Research Council. This work was also supported by the Cancer Council (to D.M.) from the Cancer Council of Victoria, a Cure Cancer Australia/Leukaemia Foundation Australia postdoctoral fellowship and a Lions fellowship from the Cancer Council of Victoria (to A.P.N.), and the Victorian State Government Operational Infrastructure. S.L.N. was supported by an Australian Research Council Future fellowship.

H.W. designed, directed and performed experiments, analyzed data, and wrote the paper; M.Y., J.S., S.J., R.Y., S.M.A., A.P.N., D.M., and L.D. designed and performed experiments; A.P.N. wrote the paper; K.O., W.G.C., S.L.N. and H.C.M. directed the research, and H.C.M. steered the research, analyzed data, and wrote the paper. Address correspondence and reprint requests to Dr. Herbert C. Morse, III, and Dr. Hongsheng Wang, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 5640 Fishers Lane, Rockville, MD 20852. E-mail addresses: hmorse@niaid.nih.gov (H.C.M.) and wanghongs@niaid.nih.gov (H.W.)

The online version of this article contains supplemental material.

Abbreviations used in this article: 7AAD, 7-aminoactinomycin D; B6, C57BL/6J; BM, bone marrow; CDP, common DC progenitor; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; DC, dendritic cell; Fii3L, Flk3 ligand; Fr., fraction; GMP, granulocyte–monocyte progenitor; HSC, hematopoietic stem cell; IRF8, IFN regulatory factor family member 8; MDP, macrophage–DC progenitor; MEP, megakaryocyte–erythroid progenitor; pDC, plasmacytoid DC; PreGM, pre–granulocytic–macrophage; qPCR, quantitative real-time PCR; SCF, stem cell factor; WT, wild-type.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1301939
and CD103+ nonlymphoid tissue DCs are also greatly diminished in Hrc and AxioVision 3.1 image acquisition software. Diabetes and Digestive and Kidney Diseases Animal Care and Use Committee, a protocol (K052-LBG-07) approved by the National Institute of the use of mice in this study followed a protocol (LIG-16E) approved by the National Institute of genic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). CX3CR1+ can give rise to both macrophages and DCs (12). These findings suggest that most, if not all, well-characterized progenitor populations are heterogeneous at the clonal level, even though they appear to have a homogeneous phenotype by certain criteria. Most of our current knowledge of how blood cells are made came from studies of transcription factors. One of a series of transcription factors modulating hematopoietic fate determination is IFN regulatory factor family member 8 (IRF8), also known as IFN consensus sequence binding protein. IRF8 is expressed mostly in cells of the hematopoietic system. Microglial cells with a hematopoietic origin also express IRF8 (13, 14). Functional analyses revealed broad contributions of IRF8 to the regulation of myeloid and lymphoid lineage development. The levels of IRF8 transcripts are low in HSCs, but increased in yet poorly defined CLPs, MPs, and common DC progenitors (CDPs) (15, 16). IRF8 deficiency in mice causes disrupted development of monocytes and macrophages but increased differentiation of neutrophils (17). The numbers of several subtypes of DCs including plasmacytoid DCs (pDCs), CD8α+ DCs, and CD103+ nonlymphoid tissue DCs are also greatly diminished in IRF8−/− mice (15, 18–23). In humans, a loss of function mutation of IRF8 also causes a monocytic and DC immunodeficiency (24). Although IRF8 expression is upregulated in both myeloid and lymphoid progenitors, as determined by conventional PCR methods on sorted bulk populations, little is known about how IRF8 participates in the distinct transcriptional programs that control lineage specification and commitment. In this study, we created an IRF8-EGFP reporter mouse by a knockin of the EGFP sequence into the IRF8 stop codon that results in transcription and translation of an IRF8-EGFP fusion protein under the regulation of endogenous IRF8 regulatory elements. Our data revealed previously unappreciated expression patterns of IRF8 that help to explain the functions of IRF8 in distinct lineages of hematopoietic cells and better understand the heterogeneity of early progenitors.

Materials and Methods

**Mice**

IRF8-EGFP fusion protein reporter mice were generated by Ozgene using a C57BL/6J (B6) germline targeting strategy illustrated in Fig. 1. Mice were genotyped by PCR analysis of tail DNA using primers wild-type (WT) IRF8 reverse (5′-CGTCTAGCTCGACAGAGTTC-3′), IRF8 forward (5′-TGATACTTACAACAGACGAC-3′), and IRF8 GFP reverse (5′-CGCTAGAATTCTGTGCGATGTT-3′). B6 and B6.SJL-Ptpcα Pepcb (CD45.1) congenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The use of mice in this study followed a protocol (LIG-16E) approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee, a protocol (K052-LBG-07) approved by the National Institute of Diabetes and Digestive and Kidney Diseases Animal Care and Use Committee, and protocols approved by the WEHI animal and ethics committee.

**Flow cytometry**

Cells were prepared and stained as reported previously (25). mAbs specific for cell surface markers are listed in Supplemental Table I. Cells were analyzed using an LSR II analyzer (BD Biosciences) and FlowJo software.

Dead cells were excluded by gating on cells negative for a viability dye (7-AAD, propidium iodide, or fixable viability dye eFluor506). Doublets were excluded electronically by setting a SSC-A versus FCS-W gate. For some experiments, cells were sorted by a FACSAria sorter (BD Biosciences). For intracellular staining, bone marrow (BM) cells were first stained with Abs for defining BM cell subsets, followed by fixation and permeabilization with a Fix-and-Perm kit (Invitrogen). The cells were then stained with a polyclonal anti-IRF8 Ab (C-19X; Santa Cruz Biotechnology) and a secondary Alexa Fluor 546-labeled donkey anti-goat Ab (Invitrogen). The cells were analyzed by flow cytometry.

**Cytomorphology**

Cyto centrifuge preparations were stained with a May-Grünwald-Giemsa stain before microscopic examination. Images were acquired using a Nikon Eclipse E600 microscope, ×100/1.3 NA oil objective with AxioCam Hrc and AxioVision 3.1 image acquisition software.

**Clonogenic colony assays**

Cultures were performed with sorted populations using semisolid agar cultures in 1-ml volumes of 0.3% agar in IMD containing 20% FCS-W newborn calf serum using cytokine stimuli as previously described (26), incubated for 7 d in a fully humidified atmosphere of 5% CO2 in air. Colonies were fixed, dried onto glass slides, and stained for acetylcholinesterase and then with Luxol fast blue and hematoxylin and the number and type of colonies were determined by microscopic examination.

**Immunization**

Mice were immunized i.p. with 100 μg NP-KLH (Biosearch Technologies) in alum. Splenocytes were analyzed 7 d later by flow cytometry.

**Quantitative real-time PCR**

FACS-purified B cell subsets were extracted for RNA using a RNeasy Mini kit (QIAGEN) including a DNA digestion step, according to the manufacturer’s instructions. Approximately 50–100 ng total RNA in 20 μl was reverse transcribed with Superscript III reverse transcriptase (Invitrogen). One nanogram of cDNA was amplified in triplicate using an ABI Prism 7900HT SDS with the SYBR Green PCR Master Mix reagents (Applied Biosystems) and primers (Table I). The housekeeping genes Gapdh and Hprt were amplified as internal controls. The relative RNA levels were calculated by the 2−DDCt algorithm (27).

**In vitro differentiation assay**

Sort-purified B cells were cultured on an OP9 stromal cell layer (purchased from American Type Culture Collection) with cytokines including IL-7 (10 ng/ml), Flt3 ligand (Flt3L; 200 ng/ml), stem cell factor (SCF; 50 ng/ml), GM-CSF (40 ng/ml), IL-4 (20 ng/ml), or IL-15 (10 ng/ml) for different periods of time. The cells were then analyzed by flow cytometry.

**In vivo adoptive transfer assay**

A total of 0.4–2 × 103 of sort-purified cells were injected i.v. into sublethally (500 rad) irradiated B6.CD4.1 mice. The mice were given antibiotics.
in their drinking water after irradiation. The splenocytes of recipients were stained and analyzed by flow cytometry 10 d later.

Statistical analysis

Student t test was used to determine the statistical significance of the data. A p value, 0.05 was considered to be statistically significant.

Results

Generation of IRF8-EGFP fusion protein reporter mice

The stop codon at exon 9 of the mouse Irf8 locus was replaced with an EGFP sequence (Fig. 1A). The PGK-Neo selection cassette inserted into exon 9 next to the EGFP sequence was deleted from the mouse germline using Cre recombinase. The targeting strategy resulted in the expression of mRNA encoding an IRF8-EGFP fusion protein under the control of the endogenous Irf8 regulatory elements. A retroviral construct encoding essentially the same IRF8-EGFP fusion protein was previously used to study IRF8 function in infected cells or cell lines and was not found to cause unexpected effects because of the EGFP sequence (28). Genotyping of Irf8-EGFP knockin mice identified heterozygous and homozygous mice (Fig. 1B). As expected, the IRF8–EGFP mice developed normally without differences from WT mice. Moreover, homozygous mice, which expressed twice as much IRF8-EGFP as heterozygous mice, were phenotypically indistinguishable.

Table II. Cellular distribution of hematopoietic cells in WT and IRF8-EGFP mice

<table>
<thead>
<tr>
<th>BM&lt;sup&gt;a&lt;/sup&gt; Genotype</th>
<th>Absolute Cell Numbers (×10&lt;sup&gt;7&lt;/sup&gt;) per Femur (Mean ± SD)</th>
<th>Absolute Cell Numbers (×10&lt;sup&gt;7&lt;/sup&gt;) (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CMPs</td>
<td>GMps</td>
</tr>
<tr>
<td>WT</td>
<td>0.15 ± 0.02</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>IRF8-EGFP&lt;sup&gt;Ep+/+&lt;/sup&gt;</td>
<td>0.18 ± 0.04</td>
<td>0.1 ± 0.02</td>
</tr>
<tr>
<td>IRF8-EGFP&lt;sup&gt;Ep/Ep&lt;/sup&gt;</td>
<td>0.15 ± 0.03</td>
<td>0.1 ± 0.03</td>
</tr>
</tbody>
</table>

<sup>a</sup>BM cells from indicated mice were stained and analyzed by flow cytometry. The gating schemes used for calculating each population were depicted in Figs. 2 and 4. Data represent four mice per group.

<sup>b</sup>Splenocytes from indicated mice were stained and analyzed by flow cytometry. The cells were gated for T cells (CD4<sup>+</sup>), follicular (FO) B cells (CD19<sup>+</sup> IgM<sup>+</sup>CD23<sup>+</sup>CD21<sup>-/-</sup>), marginal zone (MZ) B cells (CD19<sup>+</sup>IgM<sup>+</sup>CD23<sup>-</sup>–CD21<sup>+/+</sup>), DCs (MHCI<sup>+</sup>CD11<sup>+</sup>chi), neutrophils (N) (CD3<sup>-</sup>CD19<sup>-</sup>Gr-1intCD11b<sup>+</sup>), and monocytes (M) (CD3<sup>-</sup>CD19<sup>-</sup>Gr-1intCD11b<sup>+</sup>). Data represent four mice per group.
able from heterozygous and WT mice for the cellular distribution of lymphocytes and myeloid cells among different lymphoid organs (Table II).

**IRF8-EGFP expression distinguishes bipotent granulocyte-macrophage progenitors from more differentiated progeny**

Previous analyses of *Irf8* transcripts showed that expression of IRF8 increased after differentiation of HSCs into Lin−IL-7R−Sca-1−cKit+ myeloid progenitors and Lin−IL-7R+Sca-1+cKitLo CLPs (16). By using multicolor flow cytometry and a gating strategy of Pronk et al. (29), we detected IRF8-EGFP expression in early hematopoietic progenitors. As shown in Fig. 2A and 2B, long-term- and short-term-HSCs expressed negligible levels of IRF8-EGFP (Fig. 2B). Differentiation of HSCs into the multipotent progenitor → CMP → granulocyte–myeloid progenitor (GMP) pathway coincided with sequential increases in IRF8-EGFP expression (Fig. 2B). Nearly all Flt3+ CLPs were positive for IRF8-EGFP, whereas megakaryocyte–erythroid progenitors (MEPs) were negative (Fig. 2B). Further characterization of CLPs by using the Hardy criteria (CD3−Gr1−CD11b−Ly6C−Ter119−CD19−CD24−AA4.1hi CD43+B220−cKitloIL7R+) (30) or the Weissman criteria (CD3−Gr1−CD11b−Ly6C−Ter119−CD19−CD24−AA4.1hi CD43+B220−cKitloIL7R+) (31) revealed similar results (Fig. 2C, Supplemental Fig. 1A).

To study this further, we examined myeloid progenitor populations using an expanded range of markers to characterize IRF8-EGFP expression in the granulocyte–macrophage committed Lin−IL-7R−Sca-1−cKit+CD150−FcγRII/III+ pre-granulocyte–macrophage (PreGM) Flt3+ progenitors and multipotential PreGM Flt3− progenitors (Fig. 3A, left panel), both of which lie within the CMP fraction that we had characterized previously (32, 33). Using this approach, we identified IRF8-EGFP expression in Flt3+ PreGMs, which are the first identifiable cells committed to forming GMPs (Fig. 3A, middle panel) but not in the multipotent Flt3− PreGMs that form GMPs as well as bipotential MEPs in vivo (32). These data suggest that IRF8 expression identifies the first point in the myeloid progenitor hierarchy associated with granulocyte–monocyte commitment from CMPs.

Given the range of IRF8-EGFP expression in GMPs (Fig. 2B), we then fractionated GMPs into three subpopulations based on IRF8-EGFPlo, IRF8-EGFPint, and IRF8-EGFPhi expression (Fig. 3A, right panel) and examined their cytomorphology (Fig. 3B) and myeloid colony-forming potential by semisolid agar clonogenic

---

**FIGURE 2.** IRF8-EGFP expression in BM hematopoietic progenitors. (A) Gating schemes shown for the identification of LT-HSC, ST-HSC, multipotent progenitor (MPP), GMP, CMP, MEP, and CLP. Lineage panel Abs included anti-CD3, B220, CD11b, Gr-1, and Ter119. Cells were gated on 7AAD− singlet cells. (B) Overlays are expression of IRF8-EGFP over WT controls. (C) IRF8-EGFP expression in CLPs defined by Rumfelt et al. (30). Data are representative of eight independent experiments.
colony assays using cytokine stimuli associated with specific cytokine receptor signaling, namely G-CSF, M-CSF, GM-CSF, and IL-3 (Fig. 3C).

Morphologically, all GMP populations were composed primarily of promyelocytes, myelocytes, and metamyelocytes but with IRF8-EGFPlo GMPs containing some more differentiated granulocyte ring forms and eosinophil progenitors as well (Fig. 3B). In semisolid agar colony-forming assays, the IRF8-EGFPint GMPs were capable of forming all myeloid colonies in vitro and on a per cell basis possessed the greatest granulocyte colony-forming potential in response to all cytokine stimuli (Fig. 3C). Interestingly, IRF8-EGFPhi GMPs were biased toward macrophage colony formation after exposure to GM-CSF and M-CSF in particular while having virtually no response to G-CSF (Fig. 3C). In contrast, IRF8-EGFPlo GMPs did not possess significant colony forming potential to any of the cytokine stimuli when compared with the other GMP populations, consistent with this population being composed primarily by more differentiated and no longer clonogenic granulocyte progenitors. Eosinophil colony forming potential was minimal and basically seen only in cultures of IRF8-EGFPlo cells.

Taken together, these data demonstrate that GMPs are a heterogeneous collection of myeloid-committed progenitors, with intermediate IRF8 expression identifying GMPs that could contribute to all forms of myeloid colonies in vitro with the greatest potential to form granulocyte colony-forming potential colonies. IRF8-EGFPphi cells have predominantly monocytic lineage potential, whereas IRF8-EGFPlo GMPs appear to have lost most myeloid clonogenic potential.

FIGURE 3. IRF8-EGFP identifies specific myeloid PreGM and GMP populations. (A) Gating schemes for identification of PreGM and GMP populations with IRF8-EGFP expression shown. (B) Cytomorphology of IRF8-EGFPphi-, EGFPint-, and EGFPlo GMP populations sorted from Lin- BM cells of IRF8-EGFP mice. The cells were stained with a May–Grunwald–Giemsa stain and shown at original magnification ×100. (C) Clonogenic colony-forming assays of IRF8-EGFPphi, EGFPint, and EGFPlo GMP populations cultured with 10^5 U/ml G-CSF, GM-CSF, M-CSF, or IL-3. The numbers are CFUs of granulocyte (G), granulo-monocyte (GM), monocyte (M), and eosinophil (Eo) per 100 cells plated (means ± SDs of three biological replicates).
for this B lineage subset, we combined markers used by the Hardy and Kincaid groups to exclude granulocytes, monocytes, NK cells, and T cells as well as erythroid and megakaryocytic lineage cells. Thus, the pre–pro-B cells were defined as CD19<sup>+</sup>CD43<sup>+</sup>CD24<sup>−</sup>B220<sup>+</sup>AA4.1<sup>+</sup>cKit<sup>+</sup>IL7R<sup>+</sup>DX5<sup>−</sup>CD3<sup>−</sup>Gr-1<sup>−</sup>CD11b<sup>−</sup>CD11c<sup>−</sup>Ly6C<sup>−</sup>Cx3cr1<sup>−</sup>Ter119<sup>−</sup> (Fig. 4A). As shown in Fig. 4B, the expression of IRF8-EGFP in Fr. A cells was strikingly heterogeneous with fluorescence intensity ranging from negative (background) to 10<sup>4</sup> above background. In contrast, cells in Fr. B through Fr. F expressed moderate and relatively homogeneous levels of IRF8-EGFP (Fig. 4B).

Using the Weissman nomenclature, which includes Ly6D to distinguish all-lymphoid progenitors, B cell lineage progenitors, and pre–pro-B cells (31), we observed similar heterogeneity of IRF8-EGFP expression in Ly6D<sup>+</sup> pre–pro-B cells (Supplemental Fig. 1A). Most recently, Medina et al. (36) have shown that PDCA-1 could reduce pDC contamination from the pre–pro-B fraction. By including PDCA-1 as an additional marker, we evaluated the Hardy Fr. A for IRF8-EGFP expression. We found that PDCA-1 expression was heterogeneous in the Fr. A compartment. Excluding PDCA-1+ cells did not change the heterogeneous nature of IRF8-EGFP expression (Supplemental Fig. 1B). From this, we conclude that the pre–pro-B compartment is a mixture of cells with different levels of IRF8-EGFP expression.

Further analyses of peripheral B cells revealed consistently homogeneous and similar levels of IRF8-EGFP expression throughout the transitional to mature stages (Supplemental Fig. 2). In keeping with previous studies of IRF8 transcripts employing microarray and quantitative RT-PCR analyses (37), expression of IRF8-EGFP was significantly increased in germinal center B cells and decreased in plasma cells (Supplemental Fig. 2). In addition, neutrophils were negative for IRF8-EGFP, whereas NK cells and macrophages expressed intermediate levels (Supplemental Fig. 2).

**IRF8-EGFP expression distinguishes B cell committed from noncommitted pre–pro-B cells**

The finding that expression levels of IRF8-EGFP in the pre–pro-B cell compartment spanned 4 logs in fluorescence intensity prompted us to sort-purify EGFP<sup>−</sup>, EGFP<sup>int</sup>, and EGFP<sup>hi</sup> pre–pro-B cells and examine their B cell developmental potentials. First, we examined expression levels of a selected set of 18 genes that are considered to be “signature genes” representing lymphoid, myeloid, and DC lineages as well as signaling activities of cytokine receptor pathways. As shown in Fig. 5A and 5B, EGFP<sup>neg</sup>, int, and hi subsets were well distinguished by expression patterns of these genes. The lymphoid genes Pax5, Rag2, Pu.1, and Flt3 were enriched in the EGFP<sup>hi</sup> subset, whereas the myeloid and dendritic progenitor representative genes Cebpβ, Csf1r, Csf2r, and Cx3cr1 were over-expressed in the EGFP<sup>int</sup> subset. Next, we evaluated the B cell developmental potential of these subsets in vitro under lymphoid permissive conditions. EGFP<sup>int</sup> pre–pro-B cells cultured in the presence of IL-7 gave rise to substantial numbers of CD19<sup>+</sup> B cells within 48 h, whereas EGFP<sup>neg</sup> and EGFP<sup>hi</sup> pre–pro-B cells did so at greatly reduced frequencies (Fig. 5C). After 4 d of culture, the generation of CD19<sup>+</sup> B cells was significantly enriched in all subsets, but the frequency of B cells generated by EGFP<sup>neg</sup> cells was still the highest (90%), followed by EGFP<sup>int</sup> (60%) and EGFP<sup>hi</sup> (31%) (Fig. 5C). Finally, because the EGFP<sup>int</sup> pre–pro-B cells expressed both lymphoid and myeloid progenitor genes (Fig. 5B), we tested the developmental potential of EGFP<sup>int</sup> pre–pro-B cells by an adoptive transfer assay. Although EGFP<sup>int</sup> pre–pro-B cells could yield a small number of B cells in vivo at 10 d following adoptive transfer, most of the progeny belonged to DC and/or monocyte lineages based on expression of markers including B220, CD19, IRF8-EGFP, Gr-1, and CD11b (Fig. 5D). Altogether, we conclude that B cell lineage specification and commitment is primarily associated with intermediate levels of IRF8-EGFP expression.

**DC progenitors express very high levels of IRF8-EGFP**

DCs are generated from lineage potential–restricted progenitors including MDPs and CDPs (38, 39). Studies of MDPs (Lin<sup>−</sup>CD11c<sup>−</sup>SiglecH<sup>−</sup>MHCII<sup>−</sup>Flt3<sup>−</sup>cKit<sup>−</sup>CD115<sup>−</sup>) and CDPs (Lin<sup>−</sup>CD11c<sup>−</sup>SiglecH<sup>−</sup>MHCII<sup>−</sup>Flt3<sup>−</sup>cKit<sup>−</sup>CD115<sup>+</sup>) revealed strikingly high and homogeneous expression of IRF8-EGFP (Fig. 6A). BM pDCs (B220<sup>−</sup>SiglecH<sup>−</sup>CD11c<sup>−</sup>MHCII<sup>−</sup>) were also IRF8-EGFP<sup>hi</sup> (Fig. 6B). By contrast, splenic DCs exhibited heterogeneous ex-
expression of IRF8-EGFP (Fig. 6C). We conclude that DC lineage specification and commitment are associated with very high levels of IRF8-EGFP expression.

T lineage cells express negligible levels of IRF8-EGFP

A previous report indicated that IRF8 is dispensable for development of T cells (17). To determine whether IRF8-EGFP was expressed by T cells, we examined thymic and splenic T lineage cells by flow cytometry. The overall levels of IRF8-EGFP expression in thymic T lineage cells including early T cell progenitors were negative to weak positive (Fig. 7A, 7B). Most peripheral naive T cells including CD4 and CD8 T cells were also negative for IRF8-EGFP expression (Fig. 7C). The minimal levels of IRF8-EGFP expression in T lineage cells under steady-state conditions argue that IRF8 is not required for thymic T cell development and homeostasis, consistent with the characteristics of IRF8 knockout mice (17).
IRF8-EGFP expression is upregulated in activated B and T cells

To determine whether expression of IRF8-EGFP is altered in activated lymphocytes, we stimulated purified B and T cells with anti-BCR, -TCR, LPS, CpG, and/or IFN-γ and measured expression levels of IRF8-EGFP by flow cytometry. As shown in Fig. 8A, expression of IRF8-EGFP was significantly upregulated in B cells stimulated with LPS, CpG, and anti-IgM Abs. A synergistic effect on IRF8-EGFP expression was observed between LPS and IFN-γ. Stimulation of T cells by coligation of the TCR and CD28 also dramatically enhanced expression of IRF8-EGFP (Fig. 8B). We therefore conclude that expression of IRF8-EGFP, similar to IRF8 native proteins in stimulated B and T cells (40), is markedly upregulated in activated B and T cells.

Discussion

Previous studies showing that IRF8-deficient mice exhibited broad defects in the development of a variety of cell types led to the concept that IRF8 might regulate gene programs facilitating cellular lineage differentiation. However, except for the evidence that IRF8 may extinguish gene programs for neutrophil fate and promote differentiation of the macrophage and the DC lineages (15, 41), little is known whether and how IRF8 could regulate myeloid versus lymphoid lineage selection at different branch points. We now use an IRF8-EGFP reporter mouse to demonstrate that IRF8-EGFP exhibits different expression patterns in distinct progenitor cells of the myeloid and lymphoid lineages, correlating with previous studies showing that IRF8 is differentially expressed in distinct lineages of hematopoietic cells. Most importantly, IRF8-EGFP reporter mice revealed previously unappreciated heterogeneity in expression of IRF8 in some of the seemingly homogeneous populations of oligopotent progenitors including CMPs, GMPs, and pre-pro-B cells.

The most prominent finding of this study is that the IRF8-EGFP reporter allele made it possible to accurately measure the levels of IRF8 protein expression, on a single-cell level, in all hematopoietic cell types from the earliest adult progenitors to their terminally differentiated progeny. The levels of IRF8-EGFP expression were initially low in HSCs but progressively increased along with HSC differentiation into two major branches of the hematopoietic tree, the myeloid and lymphoid lineages. The lack of IRF8-EGFP expression in the third branch, the megakaryocytic and erythroid lineages, is consistent with the idea that IRF8 has no role in promoting differentiation along these pathways. Surprisingly, expression of IRF8-EGFP was extremely heterogeneous in the well-defined and seemingly homogeneous GMPs, with ∼40% of these cells being negative for IRF8-EGFP expression. By using additional markers, we were able to define the IRF8-EGFP-positive and -negative subpopulation as being Flt3+ PreGMs with restricted GMP potential and Flt3− PreGMs with GMP, megakaryocytic, and erythroid potential, respectively. We further identified three subsets of GMPs with varying levels of IRF8-EGFP expression and differentiation potential. Perhaps counterintuitively, the IRF8-EGFPint GMPs represented the true bipotent GMPs because they are readily primed for the granulocyte and macrophage lineages. In contrast, the IRF8-EGFPhigh GMPs significantly enriched for monocytic lineage forming progenitors, whereas the IRF8-EGFPneg GMPs were more mature granulocytic and eosinophilic progenitors that had lost significant CFU potential. Although the driving force for IRF8 expression in GMP subsets is currently unknown, our finding that IRF8-EGFP subsets of GMPs responded differently to cytokine-driven differentiation in vitro (Fig. 3) supports the hypothesis that cytokines may play more selective (42) than instructive (43) roles in development of oligopotent progenitors.

Another striking finding of the IRF8-EGFP reporter is the heterogeneity of the pre-pro-B cell compartment. Since the first description of B220+CD19− pre-pro-B cells (Fr. A) by Hardy et al. (34), the B cell developmental potential of this population has been assessed by both in vitro and in vivo assays. The inconsistent observations in the literature have been largely attrib-
uted to contamination by other types of cells, such as DCs that express Ly6C (44) or PDCA-1 (36). In this study, we used stringent gating strategies revised by Hardy and colleagues (30) and identified three subsets with low, intermediate, and high levels of IRF8-EGFP in this well-defined Fr. A compartment (Fig. 4). When tested under lymphoid permissible conditions, EGFPint Fr. A cells quickly differentiated into CD19+ B cells, suggesting that IRF8-EGFPint pre–pro-B cells are readily specified to the B cell lineage. Although IRF8-EGFPneg Fr. A cells could be precursors of IRF8-EGFPint and -EGFPhi pre–pro-B cells, as suggested by studies of cells cultured with Flt3L, SCF, IL-7, and IL-15 (Supplemental Fig. 3A), most of IRF8-EGFPhi Fr. A cells appeared to be specified for the DC and/or monocyte lineages (Fig. 5 D). Because pDCs express B220 and very high levels (10^4 above background) of IRF8-EGFP (Fig. 6), it was possible that IRF8-EGFPhi pre–pro-B cells could be contaminated by a small number of pDCs that expressed low levels of Ly6C and were not excluded efficiently by gating. To exclude this possibility, we assessed PDCA-1 expression in Fr. A cells. PDCA-1+ pDCs recently have been shown to contaminate the analysis of pre–pro-B cells, as suggested by studies of cells cultured with Flt3L, SCF, IL-7, and IL-15 (Supplemental Fig. 3A), most of IRF8-EGFPPhi Fr. A cells appeared to be specified for the DC and/or monocyte lineages (Fig. 5 D). Because pDCs express B220 and very high levels (10^4 above background) of IRF8-EGFP (Fig. 6), it was possible that IRF8-EGFPhi pre–pro-B cells could be contaminated by a small number of pDCs that expressed low levels of Ly6C and were not excluded efficiently by gating. To exclude this possibility, we assessed PDCA-1 expression in Fr. A cells. PDCA-1+ pDCs recently have been shown to contaminate the analysis of pre–pro-B cells (36). Consistent with the results from using the Hardy and the Weissman gating strategies, gating on PDCA-1+ Fr. A cells still yielded heterogeneous expression levels of IRF8-EGFP (Supplemental Fig. 1B). We believe that the existence of IRF8-EGFPPhi subset in the Fr. A compartment could be a true nature of this population. A strong bias of IRF8-EGFPPhi Fr. A cells to develop into DC–myeloid lineages as assessed by adoptive transfer assays (Fig. 5D) is consistent with the notion that some DCs bear rearranged Ig D_{HJH} sequences (45, 46). This is consistent with the idea that DCs might be generated from different developmental pathways, including at least the MDP–CDP pathway and the pre–pro-B pathway. Therefore, the IRF8-EGFP reporter mouse could be a valuable tool for investigating the nature of the pre–pro-B cell population.

Another intriguing finding of this study is that differentiation to the DC lineage, and pDC in particular, is associated with very high expression of IRF8. Both MDPs and CDPs are uniformly IRF8-EGFPPhi. This suggests that commitment to the DC lineage is highly dependent on IRF8-regulated gene programs. Indeed, IRF8-deficient mice exhibit a complete blockade in the development of pDCs and CD8α+cDCs (21, 22). Although this study was in preparation for submission, Rosenbauer and colleagues reported a very similar finding in DC progenitors by using an IRF8-Venus reporter mouse (47).

As with any other EGFP reporter models, there is a concern of reporter efficiency during a dynamic differentiation process. On the one hand, a prolonged requirement for IRF8-EGFP to build up to a sufficient level for detection would underreport true IRF8 pro-
Finally, the IRF8-EGFP reporter mouse recently has been demonstrated to faithfully reveal dynamic changes of IRF8 expression during progression of an experimental autoimmune encephalomyelitis disease (52) and in differentiating Langerhans cells (53). Altogether, the IRF8-EGFP reporter appeared to faithfully reflect true IRF8 expression in both physiological and pathological conditions.

In view of the data indicating that IRF8-EGFP could identify subsets of GMPs and pre–pro-B cells with distinct lineage potentials, we propose that there exists an IRF8 concentration-dependent mechanism for myeloid and lymphoid lineage specification and commitment. In this model, the development of macrophages and DCs requires very high levels of IRF8, whereas the generation of B lineage cells depends on intermediate levels. Minimal or no requirement of IRF8 is found for the development of megakaryocytic and erythroid cells as well as the T cell lineage in general. The phenotypes of IRF8-EGFP reporter mice described in this study and IRF8-deficient mice reported previously support this conclusion.

In addition to its function in regulating immune cell differentiation under the steady-state conditions, IRF8 also plays important roles in the effector stages of innate and adaptive immune responses against a variety of microbial pathogens (54–59). This is at least partially achieved by IRF8-mediated transcriptional control of cytokine expression, including IL-12p40 and type I IFNs (60). Because both B and T cells significantly upregulate IRF8-EGFP expression following in vitro stimulation with LPS, LPS plus IFN-γ, anti-IL-4 (which stimulates B cells), or anti-CD3/CD28 (which stimulates T cells) (Fig. 8), the IRF8-EGFP reporter mouse could be valuable for studying the functions of IRF8 in the settings of inflammatory responses such as the EAE (52). Furthermore, this IRF8-EGFP reporter should be useful for studying the functions of IRF8 in other cell types such as microglia and smooth muscle where IRF8 has been recently found to be present and function (13, 14, 61–63).

Acknowledgments

We thank Mehrnoosh Abhari for assistance in cell sorting and Alfonso Macias for mouse colony maintenance.

Disclosures

The authors have no financial conflicts of interest.

References


Corrections


The institutional affiliations of the second and eighth authors were published incorrectly. The corrected author line is shown below along with the corrected affiliations. Also, the funding footnote is incorrect. The corrected footnote is shown below.

Hongsheng Wang,* Ming Yan,†,‡ Jiafang Sun,* Shweta Jain,* Ryusuke Yoshimi,§ Sanaz Momben Abolfath,* Keiko Ozato,¶ William G. Coleman, Jr.,†,‡ Ashley P. Ng,§,¶ Donald Metcalf,§,¶ Ladina DiRago,§ Stephen L. Nutt,§,¶ and Herbert C. Morse, III*

*Virology and Cellular Immunology Section, Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD 20852; †Division of Intramural Research, National Institute on Minority Health and Health Disparities, National Institutes of Health, Bethesda, MD 20892; ‡Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892; §Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892; ¶Department of Internal Medicine and Clinical Immunology, Yokohama City University Graduate School of Medicine, Yokohama 236-0004, Japan; *Program in Genomics of Differentiation, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20892; ‡Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria 3052, Australia; and §Department of Medical Biology, University of Melbourne, Parkville, Victoria 3010, Australia

This work was supported by the Intramural Research Program of the National Institutes of Health’s National Institute of Allergy and Infectious Diseases (to H.W., J.S., S.J., S.M.A., and H.C.M.), the National Institute of Child Health and Human Development (to R.Y. and K.O.), the National Institute of Diabetes and Digestive and Kidney Diseases (to M.Y. and W.G.C.), and the National Institute on Minority Health and Health Disparities (to M.Y. and W.G.C.). A.P.N., L.D., and D.M. were supported by Program Grant 1016647 and Independent Research Institutes Infrastructure Support Scheme Grant 361646 from the Australian National Health and Medical Research Council. This work was also supported by the Carden fellowship (to D.M.) from the Cancer Council of Victoria, a Cure Cancer Australia/Leukaemia Foundation Australia postdoctoral fellowship, a Lions fellowship from the Cancer Council of Victoria (to A.P.N.), and the Victorian State Government Operational Infrastructure. S.L.N. was supported by an Australian Research Council Future fellowship.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1490036
Supplemental Fig. 1. Analysis of early B cell populations using the Weissman nomenclature (A) or the modified Hardy nomenclature (B). The BM cells were stained and analyzed by FACS. The numbers are percentages of cells falling in each gate. The lineage panel (Lin) contains anti-CD3, CD11b, Gr-1, Ly6C, DX5, Ter119 Abs. The overlays are expression of IRF8-EGFP over WT controls. Data are representative of three independent experiments.
Supplemental Fig. 2. IRF8-EGFP expression in splenic B cell subsets, GCs, PCs, NK and neutrophils/macrophages. Splenocytes of WT and IRF8-EGFP mice were stained and analyzed by flow cytometry. The dot plot showed gating schemes used to define specific populations. The overlays (right panels) are expression of IRF8-EGFP over WT controls. Data are representative of more than five independent experiments. Note that GC and PC analysis was done with mice immunized with NP-KLH/alum for 8 days.
Supplemental Fig. 3. Evaluation of IRF8-EGFP (A and B) or IRF8 (C) expression in early B cells. (A) IRF8-EGFPlo/- pre-pro-B cells become IRF8-EGFP+ cells in vitro. BM pre-pro-B cells were sort-purified for EGFPlo/-, EGFPint and EGFPbri as shown in Fig. 5A and were cultured with IL-7, Flt3L, SCF, and IL-15 for 5 days. The cells were then analyzed by flow cytometry. The numbers are percentages of cells falling in each gate. Data are representative of three independent experiments with similar results. (B) BM B cell subsets were defined as depicted in Fig. 2 and Fig. 4. The HSC included both LT- and ST-HSCs. The MP included GMPs, CMPs and MEPs. MFI are absolute values excluding background values obtained from a wild-type control mouse. (C) FACS analysis of intracellular IRF8 protein expression in B6 BM B cells. BM cells were pre-purified with biotin-labeled antibodies against Gr-1, CD11b, Ter119, CD3, Ly6C and Dyna beads. The cells were then stained with antibodies as indicated, followed by fixation, permeabilization and intracellular staining with an anti-IRF8 Ab (C-19x, Santa Cruz) and an anti-goat secondary antibody. Left panel, gating schemes used for defining each B cell subsets. Right panel, MFI are absolute values excluding background which was determined from an IRF8-/- sample. Error bars are of 3 mice. Data represent four independent experiments.
### Supplemental Table 1. Antibodies for FACS analysis

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>Clone</th>
<th>Fluorochrome</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>145-2C11</td>
<td>PE.Cy7, biotin</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD4</td>
<td>GK1.5</td>
<td>FITC, PE, Pacific blue (PB), Percp.Cy5.5</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD5</td>
<td>53-7.3</td>
<td>PE</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD8</td>
<td>53-6.7</td>
<td>FITC, PE</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD11b</td>
<td>M1/70</td>
<td>Alexa Fluor 700, 647</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD11c</td>
<td>HL3</td>
<td>PE, PE.Cy7</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD19</td>
<td>ID3</td>
<td>FITC, PE, APC, Percp.Cy5.5</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD21</td>
<td>4E3</td>
<td>FITC, PE</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD23</td>
<td>B3B4</td>
<td>PE, biotin</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD24</td>
<td>M1/69</td>
<td>FITC, PE</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD25</td>
<td>PC61.5</td>
<td>PE</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD27</td>
<td>LG.3A10</td>
<td>PE, APC</td>
<td>BD Biosciences, Biolegend</td>
</tr>
<tr>
<td>CD16/CD32</td>
<td>2.4G2</td>
<td>PE</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD34</td>
<td>RAM34</td>
<td>PB</td>
<td>eBioscience</td>
</tr>
<tr>
<td>B220</td>
<td>RA3-6B2</td>
<td>FITC, PE, APC, PB, APC.Cy7, Alexa Fluor 700, biotin, Qdot655</td>
<td>BD Biosciences or invitrogen</td>
</tr>
<tr>
<td>CD43</td>
<td>S7</td>
<td>PE</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD44</td>
<td>IM7</td>
<td>FITC, Alexa Fluor 700</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD71</td>
<td>R17217</td>
<td>PE</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD115</td>
<td>AF598</td>
<td>APC</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD117 (cKit)</td>
<td>2B8</td>
<td>APC, APC.Cy7</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD138</td>
<td>281-2</td>
<td>PE</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Gr-1</td>
<td>RB6-8C5</td>
<td>PE.Cy7, PB, APC.Cy7, biotin</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>MHC II</td>
<td>AF6-120.1</td>
<td>Biotin</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>Siglec H</td>
<td>551</td>
<td>PB</td>
<td>Biolegend</td>
</tr>
<tr>
<td>pDCA</td>
<td>eBio927</td>
<td>FITC, Percp-eFluor 710</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Ter119</td>
<td>Ter-119</td>
<td>Biotin</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>IL-7R</td>
<td>A7R34</td>
<td>PE</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Ly6D</td>
<td>49-H4</td>
<td>APC</td>
<td>BD Biosciences (self-labeled)</td>
</tr>
<tr>
<td>CD135(Flt3)</td>
<td>A2F10</td>
<td>PE.Cy5, BV421</td>
<td>eBioscience, Biolegend</td>
</tr>
<tr>
<td>DX5</td>
<td>DX5</td>
<td>PE.Cy7</td>
<td>eBioscience</td>
</tr>
<tr>
<td>GL7</td>
<td>GL-7</td>
<td>FITC</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>PNA</td>
<td>-</td>
<td>Biotin</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sca-1</td>
<td>E13-161.7 or D7</td>
<td>FITC, Alexa Fluor 700</td>
<td>BD Biosciences or eBioscience</td>
</tr>
<tr>
<td>AA4.1</td>
<td>AA4.1</td>
<td>PE</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>BP-1</td>
<td>6C3</td>
<td>Biotin</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>IgM</td>
<td>II/41</td>
<td>APC</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>NK1.1</td>
<td>PK136</td>
<td>Percp.Cy5.5, biotin</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>FAS (CD95)</td>
<td>Jo2</td>
<td>PE</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD4</td>
<td>RM4-5</td>
<td>Percp.Cy5.5</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Ly6C</td>
<td>AL-21</td>
<td>Biotin</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD40</td>
<td>3/23</td>
<td>biotin</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD86</td>
<td>GL1</td>
<td>PE</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD150</td>
<td>TC15-12F12.2</td>
<td>APC</td>
<td>Biolegend</td>
</tr>
</tbody>
</table>