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WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences

Developmental Biology

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Hematopoietic Stem Cells and Progenitors in Murine Autoimmune Arthritis

by

Kwadwo Asare Oduro, Jr.

A dissertation presented to the
Graduate School of Arts and Sciences
of Washington University in
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LIST OF ABBREVIATIONS

B6	C57BL/6 mice
B6xG7	Progeny of crossing B6 and G7 mice
B6xKRN	Progeny of crossing B6 and KRN mice
BM	Bone Marrow
BrdU	Bromodeoxyuridine
CLP	Common Lymphoid Progenitor
c-Mpl	Myeloproliferative Leukemia Virus Oncogene
EMH	Extramedullary Hematopoiesis
FACS	Fluorescence Activated Cell Sorting
G7	B6 congenic mice with MHC II I-Ag7 instead of I-Ab
G-CSF	Granulocyte Colony Stimulating Factor
GFP	Green Fluorescent Protein
GMP	Granulocyte Monocyte Progenitor
HSC	Hematpoietic Stem Cell
IFN	Interferon
Ig	Immunoglobulin
IgH	Immunoglobulin Heavy Chain
IL	Interleukin
K/BxN	Progeny of crossing KRN and NOD mice
KRN	A T cell receptor transgene or mice with this transgene
KRNxG7	Progeny of crossing KRN and G7 mice
KSL	Kit+Sca1+Lin-
Lin	Lineage (Mature Cell) Markers
LPS	Lipopolysaccharide
LT-HSC	Long Term Reconstituting Hematopoietic Stem Cell

MEP	Megakaryocyte Erythroid Progenitor
MPP	Multi Potential Progenitor
PB	Peripheral Blood
PTX	Pertussis Toxin
QRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RBC	Red Blood Cell
SDF-1	Stromal Derived Factor - 1
TCR	T cell Receptor
TPO	Thrombopoietin

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ABSTRACT OF THE DISSERTATION

Hematopoietic Stem Cells and Progenitors in Murine Autoimmune Arthritis

by

Kwadwo Asare Oduro, Jr.

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Professor Kyunghee Choi, Chairperson

Although the physiological role of stem cells in generating and maintaining a functional biological system is widely appreciated, very little is known about the behavior of these cells in pathological conditions. If and how these primitive cells are affected by a disease process or may contribute to the disease process has not been adequately explored. We have studied the Hematopoietic System, with particular emphasis on Hematopoietic Stem Cells (HSCs) and Progenitors, in a spontaneous murine autoimmune arthritis model. We found that these mice have a systemic increase in myeloid cells, which correlated with an increased frequency of granulocyte-monocyte progenitors relative to other committed progenitors in arthritic marrow. Gene expression analysis of uncommitted Kit+Sca1+Lin- (KSL) cells from arthritic bone marrow revealed increased expression of myeloid cell related transcripts at the expense of megakaryocyte and erythroid transcripts. *In vitro*, KSL cells from arthritic mice were markedly superior in generating myeloid cells, including osteoclasts. When KSL cells from arthritic and control mice were competitively transplanted arthritic KSL cells performed better in reconstituting the myeloid compartment. Sustained myeloid output *in vivo* was environment dependent since it was abolished in young recipients but sustained in old recipients. In these old recipients, enhanced myeloid output from arthritic KSL cells could be detected 6 months post transplantation suggesting that the myeloid primed state exists in the most primitive long term reconstituting HSC (LT-HSC) fraction of KSL cells. Consistent with this, we identify a “myeloid inflammatory signature” – defined by

upregulation of S100a8, S100a9, Chi3l3 and SOCS3 transcripts – that is triggered not only in KSL cells but also to an even greater degree in prospectively sorted arthritic CD150+CD48-CD34-KSL LT-HSCs. Our results indicate that primitive *uncommitted progenitors* of arthritic mice are molecularly altered to have increased myeloid potential while preserving multipotentiality and self-renewal. Since myeloid cells are critical in the inflammation and destruction that accompany arthritis, our results further suggest that *uncommitted progenitors* adopt a pathologic state that favors disease persistence. Therefore we identify the apparent existence of “inflammatory stem cells”. This has implications for our understanding of mechanisms driving chronicity of chronic diseases and the potential involvement of stem cells in the process.

CHAPTER 1

INTRODUCTION

Physiological Maintenance of the Hematopoietic System by Hematopoietic Stem Cells

The hematopoietic system consists of a number of different mature cells with distinct functions including oxygen transport (mediated by erythrocytes), blood clotting (mediated by platelets) and immune defence (mediated by granulocytes, monocyte lineage cells and lymphocytes). All these mature hematopoietic cells have a finite life span ranging from a few hours in the case of neutrophils to months in the case of erythrocytes. Regeneration of the hematopoietic system as mature cells die is possible because of the existence of hematopoietic stem cells (HSCs). HSCs are cells that have the potential to give rise to all cells of the hematopoietic system (hence “multipotential” or “uncommitted”) but also have the ability to self-renew. Functionally, HSCs, upon transplantation, can reconstitute all hematopoietic lineages of a lethally irradiated mice over the long term [1].

As HSCs differentiate (Figure 1), they progressively lose their self-renewal ability while still retaining multipotentiality, giving rise to multipotential progenitors (MPPs) which lack self-renewal ability [2]. HSCs and MPPs form a pool of uncommitted progenitors (also referred to as “primitive progenitors” in this dissertation). As differentiatiation ensues, particular lineage potentials are lost resulting in the formation of committed progenitors. These committed progenitors include Common Lymphoid Progenitors (CLP), Megakaryocyte-Erythroid progenitors (MEP) and Granulocyte-Monocyte Progenitors (GMPs). As their names suggest, CLPs have the potential to generate only lymphoid lineages including B cells, T cells and Natural Killer cells [3-5], MEPs generate only megakaryocytes and erythrocytes while GMPs generate only granulocytes and monocyte lineage cells [6, 7]. Other committed progenitors like Common Myeloid Progenitors (CMP; putative precursors of GMPs and MEPs) and lymphoid primed multipotential progenitors (LMPPs; putative precursors of GMPs and CLPs) have been reported but also disputed [6-10]. Downstream of committed progenitors, mature cells develop via further lineage restricted precursors For example, eosinophils develop from GMPs via eosinophil lineage committed progenitors [11] and erythrocytes develop from MEPs via erythroid restricted precursors [7].

The differentiation of HSCs into mature cells, as described above, occurs primarily in the bone marrow. Here it is thought that various cells including mesenchymal stem cells, macrophages and possibly endothelial cells provide a niche for the HSCs [12-17]. Molecular signals from the niche retain HSC in the bone marrow, regulate HSC quiescence, self-renewal and differentiation and preserves HSC function over the long term. Molecular niche signals include stromal cell derived factor-1 (SDF-1), Kit Ligand and Thrombopoietin (TPO) which interact with CXCR4, Kit and c-Mpl respectively on HSC and primitive progenitors [18-24].

Lineage Priming and Phenotypic Identification of Hematopoietic Progenitors

Low level expression of transcripts that are abundant and/or functionally important in mature cells is thought to underlie multipotentiality [6, 25-29]. The pattern of expression of these transcripts is reflective of differentiation potential such that uncommitted progenitors (i.e. HSCs and MPPs), express transcripts of all hematopoietic lineages while committed progenitors express a more restricted lineage transcript repertoire corresponding to their downstream progeny. This phenomenon, termed “lineage priming”, has been shown at a clonal level to be compatible with functional properties of hematopoietic reconstitution and *in vitro* colony generation [26, 30]. Similar to other stem/progenitor populations [31], HSCs also have chromatin epigenetic modifications consistent with being poised to generate multiple lineages. Specifically, there is the co-existence of activating histone modifications like H3K4me3 and deactivating H3K27me3 at mature cell genes leading to low expression of these genes in the primitive cells [32, 33]. Differentiation is coincident with the resolution of these “bivalent marks” into predominantly activating or deactivating modifications with promotion or complete suppression of gene expression in different progeny [31].

Although hematopoietic progenitors express mature cell mRNA to a low extent, they are largely devoid of cell surface protein expression of a number of mature cell markers. Therefore, all committed and uncommitted hematopoietic progenitors are phenotypically designated lineage marker negative (or abbreviated “Lin-”) (Figure 1). To identify specific hematopoietic progenitors,

additional markers are used. For example, primitive progenitors (HSCs and MPPs) are identified using the marker combination Kit+Sca1+Lin- (abbreviated KSL or KLS+ to distinguish these from committed progenitors many of which are Kit+Sca1-Lin- hence KLS-). KSL (KLS+) cells enrich 1000 fold for *in vivo* long term reconstituting (LT-HSC) activity compared with whole bone marrow. HSCs within the KSL fraction can be enriched further by staining for additional markers such as CD150, CD48, CD41 and CD34 [1, 17, 34, 35]. Since the cell numbers recoverable from such extensive enrichment (about 0.01% of bone marrow cells) are prohibitively more difficult to work with, KSL cells are still widely used in the study of primitive progenitors.

Primitive Progenitors in Non-physiological states – Rationale for this study

While much is known about the physiological function and properties of primitive progenitors, knowledge of their behavior in non steady-state, including pathological conditions, remains very limited. Hematopoietic progenitors express a number of receptors for cytokines that are upregulated during inflammation and other non-steady states [36, 37]. These include receptors for Interleukin 1 (IL-1), IL-3, IL-6, Granulocyte Colony Stimulating Factor (G-CSF) and Erythropoietin among others. However, it is not clear if these cytokines exert their effects by acting directly on primitive progenitors or on more committed progenitors. It has been shown for instance that although 70-80% of KSL cells express the G-CSF receptor [37] the expression of this receptor on these cells is irrelevant for their G-CSF mediated mobilization [38]. Hirai et al, has also shown that C/EBPβ dependent emergency granulopoiesis driven by G-CSF, which is important in combating certain infections, leads to upregulation of C/EBPβ in GMPs but not in primitive progenitors (KSL cells) [39]. In some cases where cytokines have been shown to act on primitive progenitors, they exert completely different effects as in the case of the platelet producing cytokine, TPO, which acts through c-mpl to regulate HSC quiescence [23, 24].

Primitive progenitors have to strike the right balance between meeting the physiological demand of homeostatically replenishing the hematopoietic system over the short term, while still having enough reserve to sustain hematopoiesis over the long term. Therefore, it makes sense

that they would be relatively protected from inflammatory signals. The relative quiescence of primitive progenitors especially HSCs makes them relatively resistant to cell-cycle dependent cytotoxic drugs like 5-FU [40]. Furthermore, the HSC niche contains regulatory T cells that helps to dampen local inflammation [41]. In spite of this, recent studies have shown that various inflammatory stimuli including those of microbial origin may lead to primitive progenitor proliferation, expansion and functional exhaustion either directly or indirectly [42]. For example G-CSF treatment of mice increases KSL frequency [43]. Alum based immunization also leads to increased proliferation and expansion of the entire KSL compartment in an IL-1RI dependent manner [44]. Type I and II interferon (IFN) increase proliferation of HSCs leading to expansion of primitive hematopoietic compartments [45-48]. Polymicrobial sepsis, infection with specific microbes such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Mycobacterium avium* as well as administration of the bacterial antigen, lipopolysaccharide (LPS) change the cycling and/or pool size of primitive progenitors [43, 47, 49-53].

The disease relevance of these changes are however unclear for a couple of reasons. First, a number of these studies have utilized individual cytokines or microbial agents while disease phenomena are usually more complex involving multiple cytokines that individually may have opposing effects. Second, while it is easy to understand how an increase in frequency or proliferation of *committed* progenitors such as GMPs will consequently lead to increase in disease relevant innate immune cells like neutrophils, it is not immediately clear how an increase in *uncommitted* progenitors would lead to a specific mature cell outcome. Proliferation/Expansion of primitive progenitors is not specific to inflammatory states as it is also seen in other situations like increased red blood cell demand [54, 55] indicating that it may be a non-specific “knee jerk” response to stress rather than a specific immune response. Therefore, it was important to study primitive progenitors in a disease model and in addition to examining their cycling to also examine their differentiation potential in the disease context.

KRNxG7 arthritis model

We have studied alterations in primitive progenitor homeostasis in inflammatory conditions using a mouse autoimmune arthritis model [56, 57] (Figure 2). This model was made by Kouskoff et al who discovered fortuitously that when transgenic mice expressing the Vα4β6 (KRN) T Cell Receptor (TCR) were crossed with NOD mice, F1 progeny (KRNxNOD or K/BxN) spontaneously developed inflammatory arthritis with 100% penetrance [56]. The MHC II molecule I-A^{g7} in NOD mice is the key molecule for disease occurrence. Consequently while KRN on a C57BL/6 (B6) background (I-A^{b} MHC II) is disease free, all offspring (hereafter referred to as KRNxG7) derived from crossing KRN mice with B6 congenic mice bearing I-A^{g7} MHC II (G7) developed arthritis. We used KRNxG7 mice predominantly because they are more appropriate for transplantation experiments, which are indispensable for analysis of stem cell function, since it is free from some of the histocompatibility concerns associated with NOD mice. In our colony, joint swelling in most KRNxG7 mice begins around 3.5 to 4 weeks of age, a little earlier than what is reported for K/BxN arthritis which initiates around 4-5 weeks of age [58]. A few mice have delayed onset but by 6 weeks, all mice show some degree of joint swelling. This earlier disease onset in KRNxG7 mice may be reflective of more severe disease than in K/BxN disease because of deficiency of complement C5 in NOD mice, a key component of disease pathogenesis [59, 60].

The mechanism of disease *initiation* involves the recognition, by KRN TCR on T cells, of an epitope of the endogenous glucose phosphate isomerase (GPI) enzyme presented by the I-A^{g7} MHC II molecule on antigen presenting cells [61]. During T cell development, most KRN expressing T cells are negatively selected since they are autoreactive. However, thymic negative selection is incomplete allowing release of autoreactive T cells and their subsequent activation [56]. Activated autoreactive T cells stimulate anti-GPI antibody production from MHC II I-A^{g7} expressing B cells in draining lymph nodes of the joint [58]. K/BxN mice with complete deletion of Recombination Activating Gene (Rag-/-; these mice lack B and T cells), T cell receptor α chain (Cα; these mice lack T cells) or Immunoglobulin μ heavy chain (these mice lack B cells) are

completely resistant to disease. Furthermore, arthritis can be induced by transfer of KRN expressing T cells into non-arthritic Cα-/-B6xG7 mice or transfer of KRN T cells plus B cells from B6xNOD into B6xNOD Rag-/- mice [62, 63]. These results indicate that B and T cells are required for disease initiation [62].

It is thought that the anti-GPI antibodies produced by autoreactive B cells complex with circulating GPI and are then deposited at the joint in an FcγR dependent manner [64]. This subsequently leads to recruitment of complement factors and cells of the innate immune system, direct effectors of mediate joint pathology [64]. Various models have been put forward to explain the joint specificity of this disease such as presence of unique GPI-binding molecules [65], particularities of the vasculature facilitating inflammatory cell accumulation [66] and lack of cellular inhibitors [67] at the joint.

Arthritis can be induced in naïve B6 mice by injecting K/BxN serum (which contains anti-GPI autoantibodies) or purified anti-GPI autoantibodies [62, 68]. The serum transfer model is widely used in studies of the mechanisms of disease since arthritis can be induced in multiple mouse strains without the need for complicated and time consuming genetic crosses [59]. However, it is worth noting that unlike K/BxN, KRNxG7 or arthritis induced by T cell transfer, which result sustained chronic disease, arthritis induced by serum transfer is transient with disease peaking about 7 days after serum transfer and subsequent return to baseline. Furthermore, the initiation stages prior to autoantibody production is not recapitulated in the serum transfer model. Nevertheless, the ability to transfer disease to genetically unmanipulated mice allows one to isolate effects of disease independent genetic effects. Therefore, this serum transfer model, in addition to the KRNxG7 model, is extensively utilized in this thesis work.

Importance of Myeloid Cells in Arthritis

Although arthritis initiated by auto-antibodies [61] as described in the previous section, myeloid cells are the direct effectors mediating joint and systemic pathologies of the disease. Using an anti-Gr1 depleting antibody, Wipke and Allen have shown that Gr1+ cells are required

for the joint inflammation (swelling) in arthritis [69]. Subsequent studies have corroborated the indispensable role of neutrophils and monocyte lineages in the arthritic joint inflammation [70-74]. Osteoclasts, which are also of myeloid origin, are indispensable for the bone erosions since they are the sole cells capable of bone resorption. In the absence of osteoclasts, joint swelling can proceed but joint bone erosions are spared in various arthritis models [75-77].

Relevance of K/BxN or KRNxG7 arthritis model to human disease

K/BxN or KRNxG7 arthritis shares several clinical, histological and molecular features with human rheumatoid arthritis (RA), the most prevalent autoimmune disease [78]. Clinically, bilaterally symmetrical joint swelling with a decreasing distal to proximal involvement is reminiscent of human RA. Histological features such as joint pannus formation, osteoclast mediated joint erosions and consequent ankylosing and deformation of joints due to aberrant repair are also reminiscent of human RA. This murine model also has extraarticular manifestations/complications such as splenomegaly, lymphadenopathy and osteopenia which are also seen in some human RA patients. Elevated levels of circulating antibodies to ubiquitous antigens [79] are found in human RA and anti-GPI antibodies in particular is elevated in RA patients with extra-articular complications [80]. Human RA in general and anti-GPI positive human RA in particular are also strongly associated with MHC polymorphisms. Therefore, this arthritis model is highly relevant to human disease and has been extensively used in teasing out basic mechanisms in inflammatory disease.

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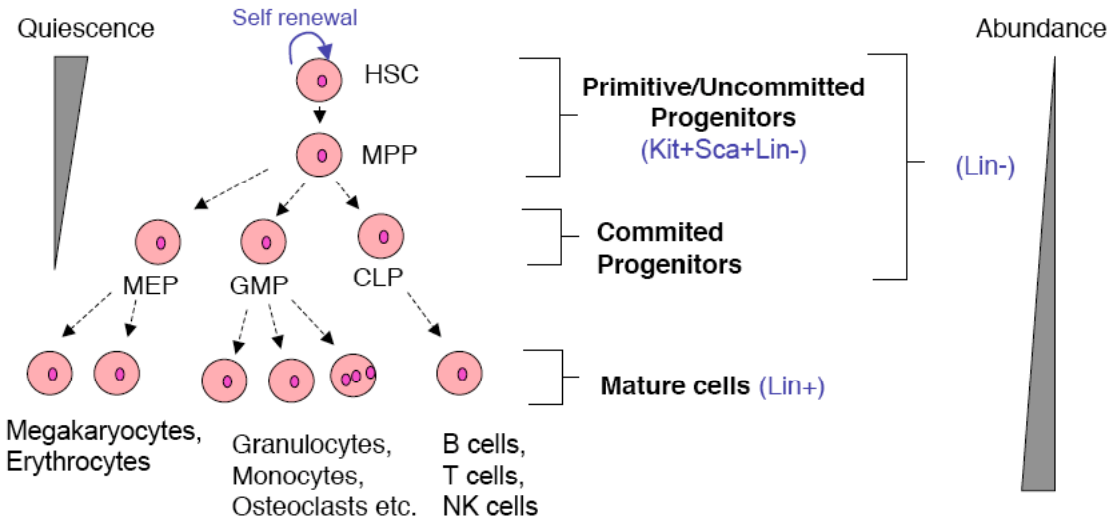

Figure 1

Figure 1. Physiological Generation of the Hematopoietic System from Primitive Progenitors

Primitive/Uncommitted Progenitors consists of Hematopoietic Stem Cells (HSCs), which have self-renewal ability, and Multipotential Progenitors (MPPs), which have lost self-renewal ability. They generate the committed progenitor pool which consists of Megakaryocyte Erythroid Progenitors (MEP), Granulocyte Monocyte Progenitors (GMP) and Common Lymphoid Progenitors (CLP). Cell cycling is directly related to maturity of progenitors so that HSCs are most quiescent while commited progenitors are most cycling. Committed progenitors generate mature cells. Mature cells express mature lineage markers and are thus designated Lineage Marker + (or Lin+). Progenitors, both committed and uncommitted, lack expression of these markers and are thus Lin-. Primitive progenitors are identified as Kit+Sca1+Lin-.

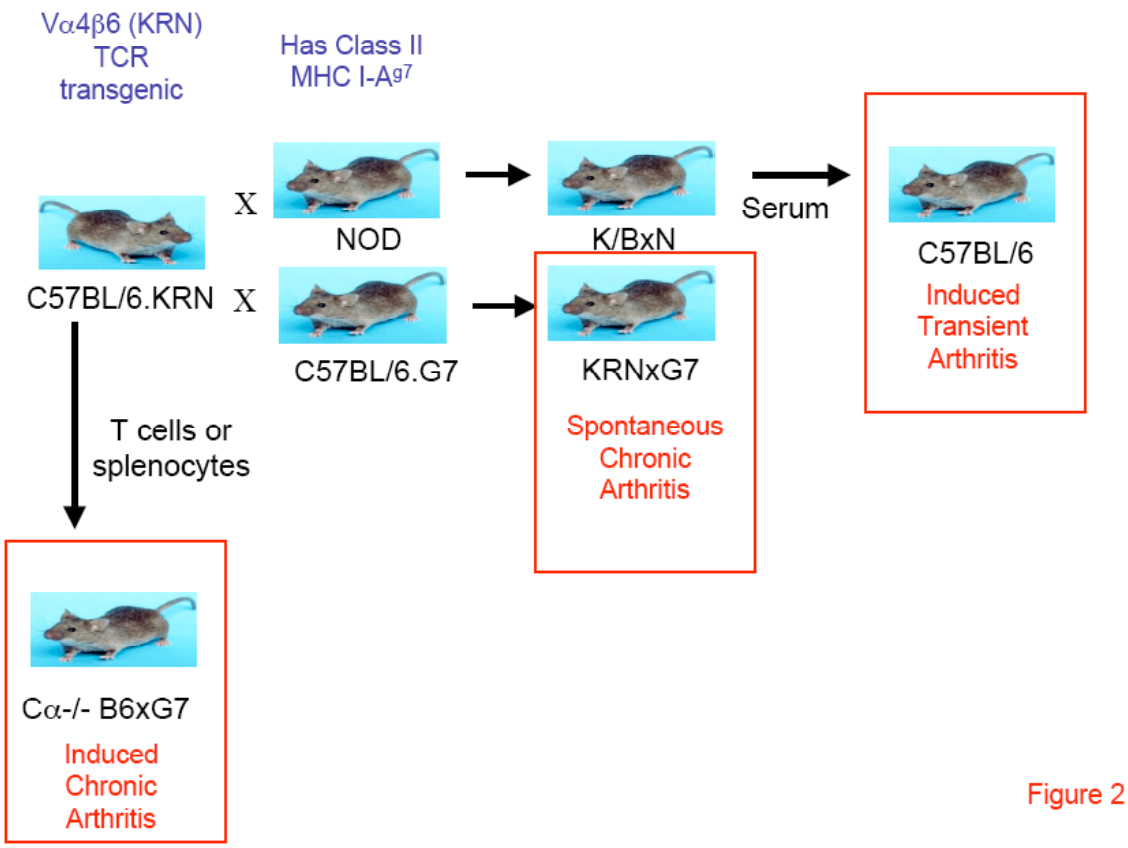

Figure 2. Arthritis Models used in this study

Arthritis models utilized in this study are boxed in red.

Spontaneous Chronic Arthritis: KRN TCR transgenic mice crossed with C57BL/6.G7 (G7) mice which have the class II MHC molecule I-A^{g7} generates KRNxG7 progeny which all develop arthritis spontaneously. Joint swelling usually begins around 3.5-4 weeks of age and peaks around 6 weeks of age. The disease remains chronic.

Induced Transient Arthrits (Serum Transfer Arthritis): Serum from the chronic model can be transferred to naïve mice to induce a transient arthritis that peaks about 1 week after injection followed by complete resolution.

Induced Chronic Arthritis: T cells transferred to T cell deficient (Cα-/-) B6xG7 mice induces arthritis which peaks about 2 weeks after transfer and remains chronic.

CHAPTER 2

GENERAL CHARACTERIZATION OF HEMATOPOIESIS IN ARTHRITIC MICE

Part of the contents of this chapter has been published in a co-authored manuscript (Ma Y et al, 2009. *Blood* 114:4402). To differentiate my work from that of others, I only cite this publication in this chapter when it refers to work done by others

OVERVIEW

Very little is known about primitive progenitors in non-steady state conditions especially in a disease model. To study primitive progenitors in a disease model we have chosen the KRNxG7 model of autoimmune arthritis. In this chapter we present analyses of frequencies of progenitors and mature cells in arthritic and control mice that form the basis for subsequent analyses presented in chapters 3 and 4. The significant findings from this chapter are that although BM primitive progenitor frequency is similar in arthritic bone marrow, frequencies of committed progenitors and mature cells are markedly changed in favor of myeloid lineages. We also demonstrate an increase in splenic primitive progenitors in arthritic mice that are unlikely due to immigration from the bone marrow, challenging the prevailing dogma for initiation of extramedullary hematopoiesis in adult spleen.

PRIMITIVE PROGENITOR FREQUENCY ANALYSIS

Similar Bone Marrow Primitive Progenitor Frequency and Turnover

Various genetic mutations and inflammatory stimuli cause an expansion or decline in primitive progenitor frequency often accompanied by changes in proliferation status. To address the question of whether primitive progenitors are perturbed in arthritic mice, we examined the BM KSL frequency before (1-3 week old) and during (6 week and older) signs of visible disease (Figure 1A). BM KSL frequency was unchanged in young arthritic mice (1 week, 3 weeks, 6-9 week old). The frequency of CD150+CD48-CD41 (hereafter “SLAM HSC”) cells, which analyzes a more purified HSC population [1], was also similar between arthritic and control mice. Slight increase in KSL frequency of arthritic mice as they got older (15-20 weeks old) was not associated with a change in SLAM HSC frequency indicating that HSC frequency is unchanged even at these ages.

To determine if arthritis leads to changes in proliferation of primitive progenitors, we assessed the fraction of KSL cells that had incorporated the administered nucleotide analog bromodeoxyuridine (BrdU) in replicating DNA (Figure 1B). BrdU injected 2-3 hours before

sacrificing mice is taken up by cells and incorporated into new DNA synthesized during this period. Therefore, BrdU+ fraction gives the fraction of cells traversing S-phase during the period of exposure to the BrdU reagent. Consistent with previous reports [2], we found that KSL cells from young mice (4 week old) had a higher BrdU+ fraction than KSL cells from 6-8 week or 16 week old mice (Figure 1B and Data Not Shown). KSL (or KLS+) cells also cycled less (lower BrdU+ fraction) than Kit+Sca1-Lin- (KLS-) cells, which contain committed progenitors and hence are less primitive and more proliferative [3]. Within this KLS- population, MEPs cycle more frequently than GMPs [3] which may account for the lower BrdU+ fraction of arthritic KLS- cells; arthritic mice have increased GMP to MEP ratio in the BM (see later – Figure 7). However at no time point did we see a significant difference between BrdU+ fraction of the primitive progenitor enriched KSL (KLS+) cells from KRNxG7 versus age matched control mice.

Administered BrdU could itself induce cell cycling [4] although it was unlikely based on the dose we used. We were also concerned about the bioavailability of injected BrdU in arthritic mice versus controls. Therefore, we also assessed cell cycling by a different approach that does not depend on administration of exogenous agents. Ki67 is an intracellular molecule expressed by all cycling cells regardless of the stage, while non-cycling, quiescent, G0 cells are Ki67 negative [5]. As an internal control, we verified that KLS- committed progenitors were less quiescent than KSL cells and that KSL cells from young (3 week old) mice were more proliferative than KSL cells from older (6-8 week old) mice (Data Not Shown). KSL cells are heterogeneous with only about 5% being long term self renewing HSCs. It was possible that cycling in arthritic HSCs was changed even if bulk KSL cell cycling was not changed. Therefore, we examined Ki67 expression in CD150+CD48-KSL cells, which are highly enriched in HSC activity (at least 1 out of 2 cells [1]) in addition to Ki67 expression of total KSL cells. Consistent with their primitive status in both arthritic and control mice, about 70% of CD150+CD48-KSL cells were $Ki67^{neg}$ compared with about 15-20% of KSL cells (Figure 1C-F). We however detected no difference in $Ki67^{neg}$ fraction when comparing the same population from arthritic and control mice consistent with our BrdU results.

Therefore unlike in a number of inflammatory states, primitive progenitor frequency and cycling is unchanged in arthritic mice. This is consistent with the similar ability of KRNxG7 arthritic and B6xG7 control bone marrow to rescue lethally irradiated mice in serial transplantation experiments (Yunglin Ma, [6]). It is however worth noting that total BM cellularity is increased about 50%, implying that absolute primitive progenitor counts are also increased in arthritic mice. We checked for differences in apoptosis by annexin V mediated recognition of cell surface phosphatidylserine staining. However, only a very small fraction (about 2%) were annexin V+ in both arthritic and control KSL cells with no difference between the two strains (Data Not Shown). Highly efficient clearance of apoptosing cells *in vivo* [7] may however obscure accurate evaluation of KSL cell apoptosis in arthritic and control mice.

Increased Primitive Progenitors in Arthritic Spleen

HSC frequency was similar in arthritic marrow despite the fact that arthritic bones had an osteoblast deficiency resulting in low bone formation rate and low-turnover osteoporosis [6]. This was unexpected because osteoblasts have been proposed as putative niche cells for HSCs [8, 9]. Previous studies had indicated that suppression of osteoblast activity with G-CSF or conditional ablation of osteoblasts by ganciclovir treatment of Collagen 2.3 promoter driven thymidylate kinase transgenic mice (Col2.3-TK) resulted in splenic extramedullary hematopoiesis (EMH) [10-12]. Therefore, we examined the spleen and found that although HSC frequency was similar in arthritic and control bone marrow, KSL and SLAM HSC frequencies were markedly higher in 6 week old arthritic spleens compared with age matched control spleens (Figure 2A & 2B). This was corroborated by transplantation experiments showing superior rescue of lethally irradiated recipients by Lin- cells from arthritic spleen than Lin- cells from control spleens (Yunglin Ma, [6]).

To determine how early increased primitive progenitors occur in the spleen, we examined the spleens of young KRNxG7 mice prior to disease (joint swelling) onset (i.e.1-3 wk old) and age matched controls. KRNxG7 KSL and SLAM HSC frequencies were similar to control mice at 1-3 weeks of age supporting the fact that increased splenic primitive progenitors occur after initiation

of joint swelling or is coincident with it (Figure 2A & 2B). However, both KSL and SLAM analyses yielded different results. KSL frequency in 6 week old KRNxG7 arthritic spleen was similar to KSL frequency in 2 week old KRNxG7 and age matched controls (0.15-0.3%); what was different was the drastic *reduction* of KSL cells in 6 week old control spleen by an order of magnitude (Figure 2A). SLAM HSC frequency on the other hand was the same in 8 week old control mice as it was in 3 week old KRNxG7 and control mice (0.001-0.004%); what was different was a drastic increase in SLAM HSC in 8 week old arthritic spleens (about 0.04%) (Figure 2B).

During normal ontogeny of the hematopoietic system, the main site for hematopoiesis shifts from the fetal liver to the bone marrow starting from late gestation. In the early days of life, hematopoietic activity is pronounced not just in the bone marrow but also in the liver and spleen [13, 14]. As mice grow, the primitive progenitors in extramedullary sites including the spleen disappear and the bone marrow becomes the major site for hematopoiesis. The decreased splenic KSL frequency in 6 week old non arthritic mice compared with 1wk or 2wk old mice (Figure 2A) reflects this normal ontogenic process. In contrast, the maintenance of splenic KSL frequency in 6 week old arthritic KRNxG7 mice at levels similar to KSL frequency in the neonatal stage suggested that there was disruption of normal ontogenic decline of splenic progenitors in arthritic mice. In other words, do primitive progenitors increase in spleen by a passive mechanism of *persistence* of primitive progenitors or by a more active process of accumulation as the SLAM HSC frequency analysis (Figure 2B) would suggest?

Increased splenic primitive progenitors can be induced by T cell transfer arthritis but not by serum transfer arthritis

To address the mechanism of increased primitive progenitor frequency in spleens of arthritic mice, we induced arthritis in wild type mice at 2.5-3 months of age. Wild type mice at this age have negligible splenic progenitors. We reasoned that splenic progenitors will increase upon arthritis induction if this occurred by an “active” process such as immigration of HSCs from the bone marrow, decreased emigration of constitutively circulating HSCs from spleen and/or

proliferation of resident HSCs in the spleen. However, if spleen progenitors are high in arthritic mice due to disrupted ontogeny, as explained in the previous section, then arthritis induction in 2.5-3 month old wild type mice, which have already undergone ontogenic decline of splenic progenitors, would fail to recapitulate this phenotype.

We injected 2.5-3 month old B6 mice with repeated doses of arthritogenic serum to trigger a sustained arthritis as done previously [15] (Figure 2C). We confirmed that these mice developed arthritis based on joint swelling. However, at no time point during the course of the experiment did we detect a significant increase in splenic KSL cells, even 6 weeks post the initial injection (Figure 2D). In contrast, we found that arthritis induction by KRN T cell transfer into Cα-/- B6xG7 mice [16] elicited a close to 10-fold increase in splenic KSL frequency (Figure 2D).

Therefore, splenic primitive progenitors are actively induced during arthritis development and not just passively retained from the neonatal period. Arthritis per se is insufficient for the splenic progenitor increase since splenic KSL frequency remains at a very low level in serum transferred arthritic mice. Rather, increased KSL cells are associated with early events in arthritis initiation prior to anti-GPI secretion since these events are recapitulated in the T cell transfer model but not in the serum transfer model. These early events include activation of autoreactive T cells and B cells. Activated T cells have been reported to regulate the frequency of primitive hematopoietic progenitors in the spleen [17, 18] and future work can address how T cell activation participates in the augmentation primitive progenitors in arthritic spleens.

Splenic primitive progenitors likely increase by expansion of in situ progenitors rather than mobilization from the bone marrow

Inflammatory stimuli like G-CSF, LPS or Pertussis Toxin (PTX) and conditional ablation of osteoblasts can mobilize hematopoietic progenitors from the bone marrow [10, 19-22]. In the cases of G-CSF and LPS treatment, these lead to EMH that has been shown to result from the increased emigration of BM progenitors [19, 20]. Recently it has also been suggested that EMH could also result from inflammation driven impaired egress of constitutively circulating progenitors

transiting extramedullary organs [23]. Therefore, it was possible that increased primitive progenitors in arthritic spleen were derived from the bone marrow either by *increased mobilization* or *impaired egress* from the spleen. We found that peripheral blood (PB) KSL frequency was increased in PB of KRNxG7 arthritic mice (Figure 3A) rendering *impaired egress* an unlikely mechanism for increased splenic progenitors; impaired egress should result in decreased or similar PB KSL frequency.

Increased splenic progenitors could also result from *expansion* of the few progenitors that normally reside in the spleen at steady state. The spleen is the most prominent extramedullary localization of progenitors in adult mice. At steady state, there are about 700 colony forming units of progenitors (CFU) in the spleen; compared with 10000-20000 in the bone marrow of a single femur or about 100 in the liver [23]. Monette et al showed that the increase in splenic CFU activity that occurred following immunization of mice with killed *Bordetella pertussis* consisted of a hydroxyurea sensitive period of proliferating and expanding splenic progenitors prior to a hydroxyurea insensitive immigration of progenitors [24]. The early part of the expansion phase was associated with a rise in PB CFU activity and a dip in BM and spleen CFU activity indicating that the spleen is not simply a reservoir that only receives progenitors from the bone marrow in response to inflammatory stimuli.

Therefore either *mobilization* or *expansion* or both were plausible mechanisms for expansion of splenic progenitors in arthritic mice. To test whether mobilization of primitive progenitors contributes to the increased primitive progenitors in arthritic spleens we generated asplenic mice. We reasoned that if arthritic spleens siphoned off mobilized primitive progenitors then PB primitive progenitors would increase in the absence of a spleen. We generated genetically asplenic KRNxG7 arthritic mice (KRNxG7 Tlx1-/-). Tlx1 (Hox11) is a transcription factor that specifies splenic development during embryogenesis. Tlx1-/- mice are viable with the only prominent defect being the absence of spleen unlike other asplenic mice that have a multitude of defects [25, 26]. We also surgically splenectomized KRNxG7 mice at 3 weeks old and compared their PB KSL frequency to that of sham operated mice at 8 weeks of age.

Genetically (Tlx1-/-) and surgically splenectomized KRNxG7 mice developed arthritis similar to KRNxG7 mice with intact spleen (Figure 3B and Data Not Shown).

Like B6xG7 non arthritic control mice, asplenic B6xG7 mice had very low PB KSL frequency (Figure 3C). More importantly we found that PB KSL frequency in asplenic KRNxG7 mice (Tlx1-/- and surgically splenectomized mice were pooled) was not changed compared with KRNxG7 mice having an intact spleen (Figure 3C). BM KSL frequency was not changed either so cells destined for the spleen were not "backed up" in the BM (Figure 3D). In the absence of compensating mechanisms like translocation of progenitors to other extramedullary sites [27] or increased apoptosis, these data (Figure 3C & 3D) suggest that mobilization from the bone marrow likely plays a minimal role in the increased splenic primitive progenitors in arthritis. This result contrasts with G-CSF or LPS treatment of splenectomized mice which both result in augmentation of PB hematopoietic progenitors following splenectomy confirming BM to spleen mobilization of progenitors by these factors [19, 20].

Increased splenic progenitors in arthritic spleen is independent of TNFα signaling

Physiological retention of primitive progenitors in the bone marrow is mediated by SDF-1/CXCR4 interaction and a number of molecules that mobilize BM primitive progenitors do so by downregulating SDF-1, CXCR4 or by blocking their interaction [11, 22, 28-30]. TNFα which is upregulated in human arthritis and in our arthritic mice (Changwon Park, [6]) is capable of downregulating BM SDF-1 leading to mobilization of osteoclast precursors in arthritis [31-33]. TNFα also mobilizes B cell precursors to the spleen as occurs in alum immunization [34]. Furthermore, TNFα inhibits osteoblastogenesis [35] and promotes osteoclast activation [36] both conditions that have been reported to promote mobilizaton of BM progenitors [10, 37]. Therefore we hypothesized that blocking TNFα signaling in arthritic mice would block mobilization of primitive progenitors; the sensitivity of splenic primitive progenitors would indicate whether this pathway is involved in accumulation of splenic primitive progenitors.

To test this, we blocked TNFα signaling in KRNxG7 arthritic mice by 2 approaches. Firstly we treated a cohort of KRNxG7 mice with anti-TNFα antibody [38] and a control cohort with anti-GST antibody continuously for 3 weeks starting from 3 weeks of age (Figure 3E). In the second approach, we generated KRNxG7 mice lacking the proinflammatory TNF receptor TNFRI (p55) [39]. The p55 receptor mediates the pro-osteoclastogenic [36, 40] as well as anti-osteoblastogenic effects of TNFα [35]. Consistent with previous reports [41, 42], these TNFα deficient mice still developed arthritis. However, we failed to see any significant differences in splenic KSL frequency nor colony formation (Figure 3F & 3G) indicating that absence of TNFRI does not impair accumulation of splenic progenitors.

Summary of Primitive Progenitor analysis

Therefore in summary, primitive progenitor frequency and cell cycling is unchanged in arthritic bone marrow while it is increased in the peripheral blood and spleen. The increase in primitive progenitors in the spleen is unrelated to the peripheral blood changes and likely results from expansion of the few resident progenitors that remain in the spleen during development. The mechanism of expansion is associated with early events in arthritis initiation prior to antibody generation and activated T cells may be involved.

MATURE CELL AND COMMITTED PROGENITOR FREQUENCY ANALYSIS

To determine the mature cells output in arthritic mice we examined bone marrow, spleen and peripheral blood for frequency of myeloid cells (Gr1+), B lymphoid cells (B220+) and T lymphoid cells (CD3+). We found a systemic increase in Gr1+ cells and a decrease in the lymphoid populations (Figure 4A). We also noticed that long bones of arthritic mice and bone marrow suspensions retrieved from these mice were pale (Figure 4B). Since the red color of normal bone marrow is due to erythrocytes this observation suggested that red blood cells (RBC) were depleted in arthritic marrow and that arthritic mice were consequently anemic. Peripheral

blood complete blood counts (CBC) confirmed that arthritic mice were mildly anemic compared with controls (Figure 4C). RBC counts, RBC volume and consequently hemoglobin amount were all decreased in arthritic peripheral blood indicating a microcytic anemia. RBC Distribution Width, a measure of anisocytosis is increased. Therefore, arthritic mice have microcytic anemia with increased anisocytosis, which are clinical signs of "anemia of chronic disease". We next examined the changes to the leukocyte populations further.

Defective Bone Marrow B lymphopoiesis in arthritic mice

In the bone marrow B220+ cells can be divided into more mature/recirculating $B220^{hi}$ cells which are also IgM+ and immature $B220^{lo}$ cells which are also AA4.1+ [43, 44]. We found that the residual B220+ cells in arthritic bone marrow were almost all $B220^{hi}$IgM+AA4.1- with almost complete depletion of $B220^{lo}$IgM-AA4.1+ (Figure 5A). PreB, ProB and PreproB cells, which represent increasingly more immature B cell precursors contained within the B220+IgM- population, were all depleted in arthritic marrow (Figure 5B). The fact that frequency of mature and immature subsets of B220+ cells were differentially affected in arthritic mice implied that the diminished B220+ frequency was not simply an artifact of increased Gr1+ frequency.

This B lymphopoiesis defect occurred in the context of osteoblast deficiency in arthritic mice [6]. Previous studies have also reported that manipulation of osteoblasts lead to B lymphopoietic defects implicating osteoblasts in normal development of B lymphocytes [10, 45, 46]. For example, Wu et al, showed that deleting Gsα in osteoblast lineage cells using Osterix Cre caused reduction in the B lymphopoietic cytokine, IL-7, and a B cell defect at the level of ProB cells [46]. Zhu et al, also showed that conditional ablation of mature osteoblasts by ganciclovir treatment of Col2.3 TK transgenic mice led to a more severe depletion of B cells at the pre-pro B level with concomitant reduction SDF-1 and Flt3L in addition to IL-7 [45]. Therefore, we examined production of B lymphopoietic cytokines in the marrow and found reductions in SDF-1 (Changwon Park, [6]), Flt3L and IL-7 as well (Figure 5C). A recent study has however suggested that the main SDF-1, Flt3L and IL-7 producing cells in the bone marrow are not bone-

building osteoblasts but stromal cells dispersed in the marrow [47]. Consistent with this we found that expression of these cytokines were not changed in 3 week old KRNxG7 mice (Figure 5D) even though osteoblast deficiency had already begun with reduction in osteoblast and osteoclast markers (Figure 5E & 5F).

Arthritic marrow is myeloid skewed

We also examined the increased Gr1+ cells in the bone marrow of arthritic mice further. As expected most of these Gr1+ cells co-stained with Mac1 thus confirming they are indeed myeloid cells rather than other cells like T cells and plasmactyoid dendritic cells that have also been reported to be Gr1+ [48, 49]. Two populations of Mac1+Gr1+ cells could be distinguished based on level of Gr1 expression. $Gr1^{hi}$ cells, which are mature neutrophils, were of similar frequency in arthritic and control marrow (Figure 6A). Mean frequencies differed by less than 20% and rarely reached significance. On the other hand the increased overall Gr1+ frequency was most pronounced in the $Gr1^{lo}$ subset. Mac1+Gr1lo frequency was 100-200% increased in arthritic marrow (Figure 6A).

This population has been reported to contain monocytes [50] as well as immature neutrophils – promyelocytes and myelocytes [51-53]. Consistently, whereas Mac1+$Gr1^{hi}$ cells expressed high levels of the neutrophil marker Ly6G (detected with antibody clone 1A8), Mac1+Gr1lo cells expressed lower and heterogeneous levels of Ly6G (Figure 6B). On the other hand, Mac1+$Gr1^{lo}$ uniquely contained cells expressing the monocyte specific c-Fms.molecule and expressed higher levels of FcγRIII/IIb, Ly6C and the chemokine receptors Ccr1 and Ccr2 important in monocyte trafficking and in osteoclastogenesis [54-56] (Figure 6B, 6C and data not shown). Both monocytes (defined as Mac1+Gr1lo cFms+$Ly6C^{hi}$) and immature neutrophils (defined as Mac1+Gr1lo cFms-$Ly6C^{int}$) are increased in arthritic marrow although the magnitude of the increase is slightly greater for the latter (Figure 6C and Data not shown). Macrophages (defined as F480+Gr1loMac1+ cells) were also elevated in arthritic marrow (Figure 6D).

Therefore the increased myeloid output in arthritic mice is not restricted to a particular type of myeloid cell.

Myeloid skewing in arthritic marrow is evident at the committed progenitor level

Myeloid cells, lymphoid cells and RBCs are derived from different committed progenitors – GMP, CLP and MEP respectively. The fact that increased BM Gr1+ and decreased BM B220+ were most pronounced in more immature subsets (Mac1+Gr1lo and B220+AA4.1+ respectively) suggested that the increased myeloid cells and reciprocally decreased lymphoid and erythroid cells in arthritic marrow was developmentally regulated. Therefore we examined committed progenitors to determine if myeloid skewing existed at this level. GMPs and MEPs are both contained within the Kit+Sca1-Lin-IL7Rα- fraction of the bone marrow. We found a skewing towards increased FcγRIII/IIb^{hi}CD34+ GMPs and decreased FcγRIII/IIb-CD34- MEPs (Figure 7A). The degree of skewing seemed to correlate with clinical severity of disease since it was more pronounced in mice that were more emaciated, less mobile and had more brittle bones (data not shown). Furthermore, we found that Flk2+IL7Rα+Lin- cells which contain CLPs and B cell committed progenitors that are more immature than any B220+ expressing cell fraction [57, 58] was completely depleted in arthritic marrow (Figure 7B). Frequency of Lymphoid primed multipotential progenitors (LMPPs), a subset of KSL cells with myeloid and lymphoid potential but no erythroid potential [59, 60] and hence presumptive precursors of CLPs and GMPs, was unchanged (Figure 7C). Therefore, arthritic marrow have increased myeloid cells and committed myeloid progenitors at the expense of lymphoid and erythroid cells and their committed progenitors.

Erythroid and B lymphoid lineage cells are expanded in distinct extramedullary sites

The peripheral anemia in arthritic mice is not as severe as would be predicted based on bone marrow analysis presumably due to partial compensation by splenic erythropoiesis. We

found enhanced splenic erythropoiesis in arthritic mice with increased CD71+ erythroid precursors (Figure 8A). This is reminiscent of “stress erythropoiesis” that is triggered by a variety of anemia inducing conditions such as irradiation or phenylhydrazine treatment [61, 62]. Megakaryocytes (CD41+CD61+ cells), which are also derived from MEPs were also increased in arthritic spleen (Figure 8B). Therefore it appears while the bone marrow of arthritic mice is inhospitable to the differentiation of MEP and downstream progeny, differentiation into these cells in the spleen is not impaired. Alternatively, the primitive progenitors in arthritic spleen have a different lineage potential from the primitive progenitors in arthritic bone marrow.

The diminished B220+ cells in KRNxG7 mice was paradoxical because this arthritis model is antibody mediated and it has been reported that K/BxN mice have high amounts of circulating immunoglobulins [15, 63]. Previous studies have suggested that immunization, infection, and inflammatory cytokines can mobilize B-cell precursors into the periphery [34, 52, 64]. Therefore, we conducted further analysis on spleen and lymph node to determine whether B lymphopoiesis is relocated to other sites, which might account for the increased IgG in serum of arthritic mice. We found a 10-fold increase in B cell derived plasma cell frequency in the spleen despite the fact that splenic B220+ frequency was decreased (Figure 8C). We also found that unlike in other organs examined, frequency of B cells and B cell precursors were increased in arthritic lymph nodes (Figure 8D & 8E).

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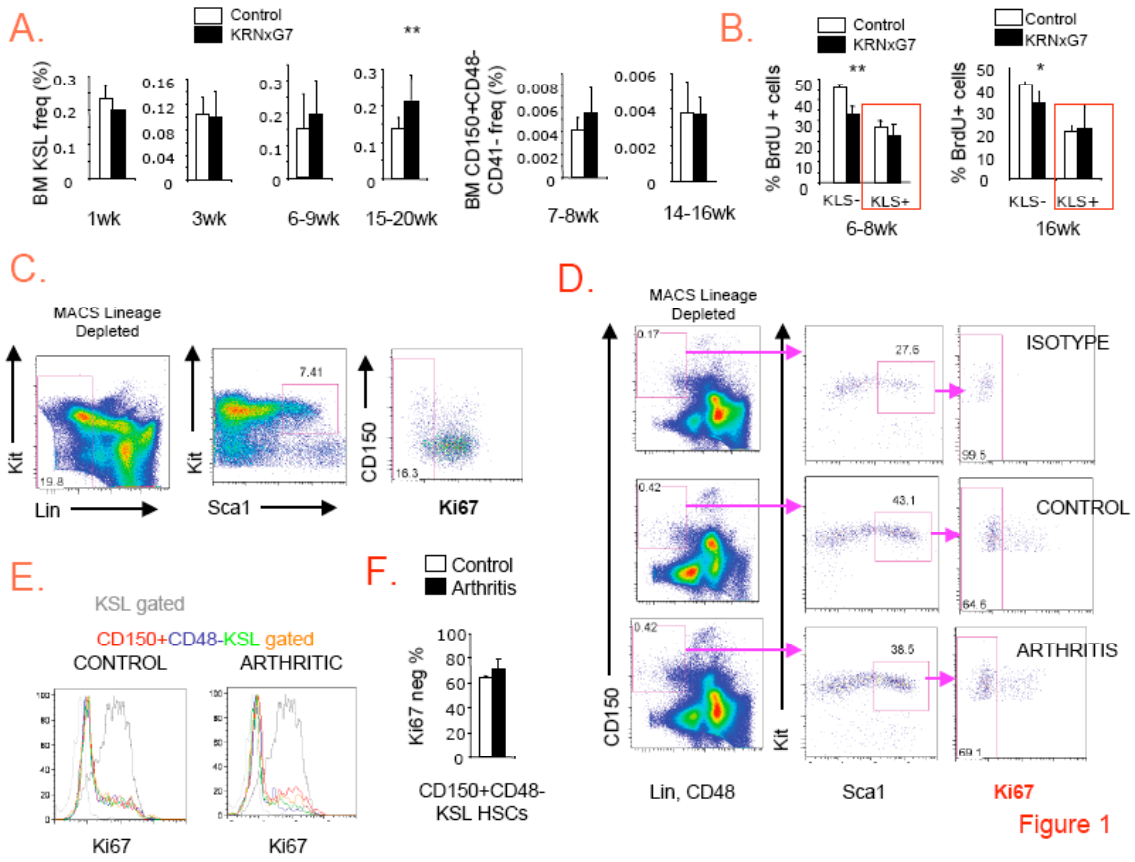

Figure 1. Frequency and Proliferation of primitive progenitors in arthritic (KRNxG7) bone marrow is unchanged

A. Flow cytometry determined Bone marrow (BM) primitive progenitor frequency of KRNxG7 and control mice of different ages.

1 week (wk) KSL: 2 KRNxG7 mice and 4 control (B6xG7) mice were used.

3 wk KSL: Data is pooled from 2 experiments with total of 9 KRNxG7 and 9 control (G7 and KRN) mice.

6-9 wk KSL: Data is pooled from 5 experiments with total of 26 KRNxG7 and 24 control (G7, B6xG7, KRN, B6xKRN) mice.

15-20 wk KSL: Data is pooled from 3 experiments with total of 13 KRNxG7 and 13 control (B6xG7, KRN) mice.

7-8 wk CD150+CD48-CD41-: 4 KRNxG7 and 7 control (B6xG7, B6xKRN) were used.

14-16 wk CD150+CD48-CD41-: 4 KRNxG7 and 3 control (B6xG7) were used.

B. Flow cytometry determined BrdU+ fraction of KLS+ cells (primitive progenitors; same as KSL cells, highlighted with red box) and of KLS- cells (committed progenitors) in arthritic and age matched control bone marrow. 3-5 mice in each group were analyzed at each time point. 6-8 week analysis is pooled from 2 independent experiments. B6 mice were used as controls for 6-8 week analysis. KRN mice were used as control for 16 week analysis.

C. FACS plots depicting Ki67 analysis of KSL cells.

D. Representative FACS plots depicting gating scheme for analysis of quiescent fraction of CD150+CD48-KSL IL7Rα- cells (HSCs) in bone marrow of arthritic and control mice based on intracellular Ki67 expression. An isotype control antibody (Top panel) aided in setting the Ki67 negative gate.

E. Most KSL cells (dark grey solid histogram) are Ki67+ (non quiescent). However most KSL CD150+CD48- cells (colored histograms) from both arthritic and control mice are Ki67- (quiescent). Light grey/hatched histogram represents isotype control.

F. Quantification of Ki67 negative (quiescent) fraction of HSCs.

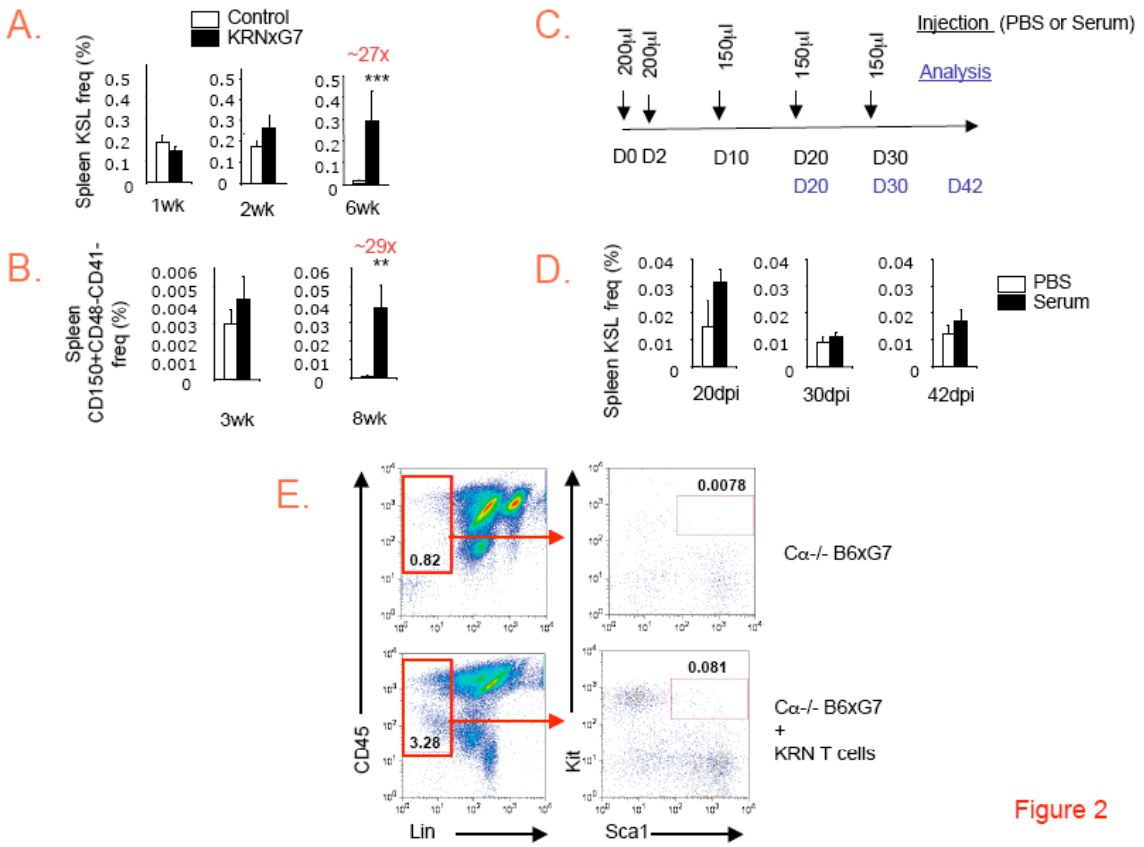

Figure 2. Primitive progenitor frequency is increased in KRNxG7 arthritic spleen and can be induced by T cell transfer arthritis

A. Flow cytometry determined splenic KSL frequency in KRNxG7 mice and age matched controls.

1 week (wk): 2 KRNxG7 mice and 4 control (B6xG7) mice were used.

2 wk: 6 KRNxG7 mice and 3 control (B6xG7) mice were used. KRN control mice gave similar results as B6xG7 controls (not shown).

6 wk: Data is pooled from 2 experiments. 11 KRNxG7 mice and 7 control (B6xG7, G7) mice were used. At 6 weeks, Arthritic KSL cells are also increased relative to KRN controls (N=8,8 pooled from 2 experiments) although the fold change is less (Not Shown). KRN controls

seem to have higher basal splenic KSL cells and Gr1+ cells than non-TCR transgenic control mice (Data Not Shown).

B. Flow cytometry determined splenic CD150+CD48-CD41 ("SLAM HSC" frequency in KRNxG7 and control mice. 3wk: 5 KRNxG7 and 5 control (B6xG7) mice were used. 8wk: 4 KRNxG7 and 3 control mice were used. As for KSL analysis SLAM HSC frequency is also increased relative to KRN control at 8 weeks (N=4 KRN, 3 KRNxG7) but the fold difference is less as KRN spleens have more SLAM HSCs than non TCR transgenic controls (Not shown).

C. Experimental scheme for testing induction of splenic progenitor increase by arthritogenic serum transfer into B6 mice. The first serum injection is administered on Day 0 (D0). Dose of serum (or PBS) administered on different days post the initial injection is indicated in black. Days post initial injection when cohorts of mice are sacrificed for analysis are indicated in blue.

D. Flow cytometry determined Splenic KSL frequency at different days post initial serum or PBS injection (dpi). None of the differences are statistically significant

E. FACS plots illustrating Induction of splenic KSL frequency by KRN T cell transfer into T cell deficient Cα-/- B6xG7 mice to cause arthritis. Control mice are Cα-/- B6xG7 mice that were not given exogenous T cells.

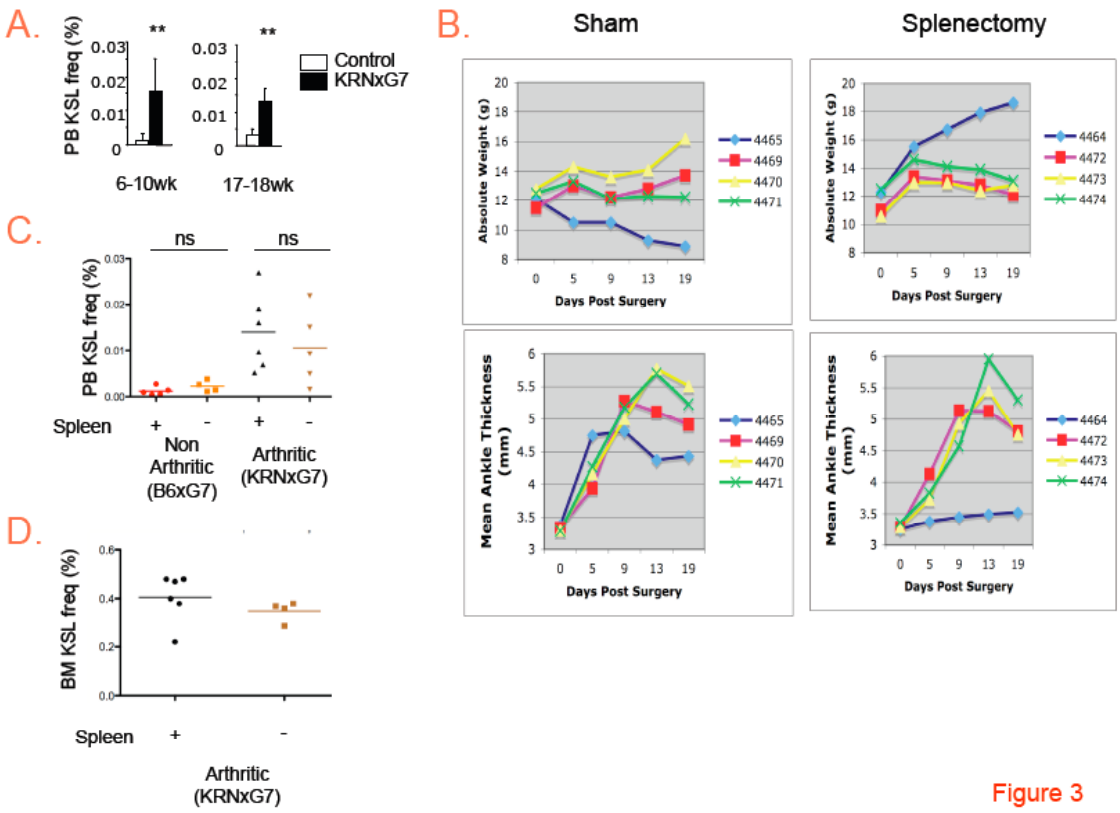

Figure 3

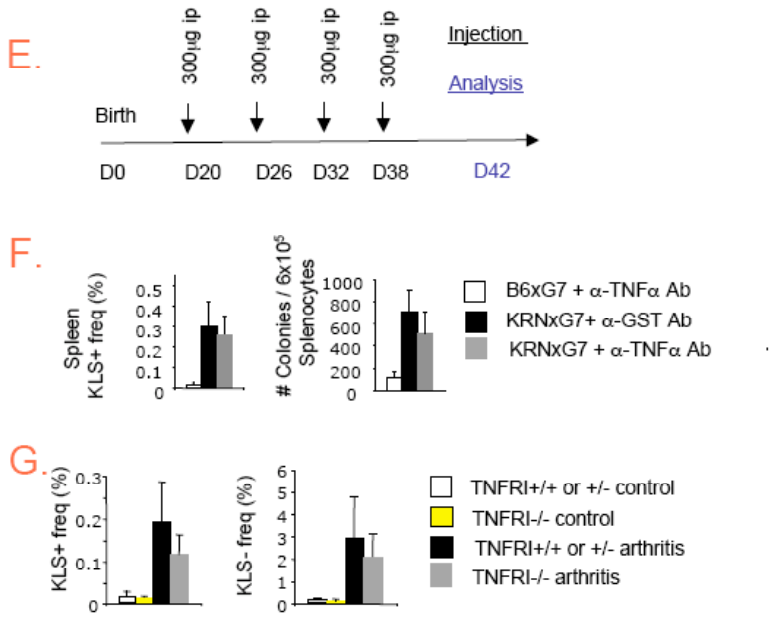

Figure 3

Figure 3. Increased primitive progenitors in arthritic spleen likely occurs by expansion of resident splenic progenitors rather than by mobilization

A. Flow cytometry determined PB KSL frequency in KRNxG7 and age matched controls. 6-10wk: Data is pooled from 2 experiments. 7 KRNxG7 and 7 control (G7, B6xG7) mice were used. 17-18wk: 4 KRNxG7 and 4 control (B6xG7) mice were used.

B. Absolute Weight and Mean ankle thickness trajectories after surgical splenectomy or sham surgery of 3 week old KRNxG7 mice. Each line (and number) corresponds to an individual mouse. 8 mice were splenectomized but only 4 are shown. #4464 never developed arthritis as evinced by continuous weight gain and ankle thickness that remains relatively constant. The trajectory of this mouse is similar to the trajectory of wild type control mice (Not shown).

C. Flow cytometry determined PB KSL frequency in peripheral blood of 7-10 week old KRNxG7 and B6xG7 mice with or without spleens. B6xG7 mice without spleens are all genetically asplenic (Tlx1-/-). 2 KRNxG7 mice without spleens are genetically asplenic (Tlx1-/-) while the remaining 3 were surgically splenectomized. 1 KRNxG7 mouse with a spleen underwent a sham surgical operation; the rest are un-operated. Each dot represents an individual mouse. Data is pooled from 3 experiments. PB KSL

D. Flow cytometry determined BM KSL frequency of 7-10 week old KRNxG7 mice from Figure 3C above.

E. Experimental scheme for administration of α-TNFα neutralizing antibody or anti-GST control antibody to arthritic mice.

F. Left: Flow cytometry determined splenic KSL frequency
Right: Methylcellulose colony formation from total spleen
Of KRNxG7 arthritic and control mice following treatment with α-TNF-α neutralizing antibody or an α-GST control

G. Flow cytometry determined splenic KSL frequency in KRNxG7 arthritic and B6, G7 or B6xG7 control mice with or without the TNFα Receptor I (TNFRI)

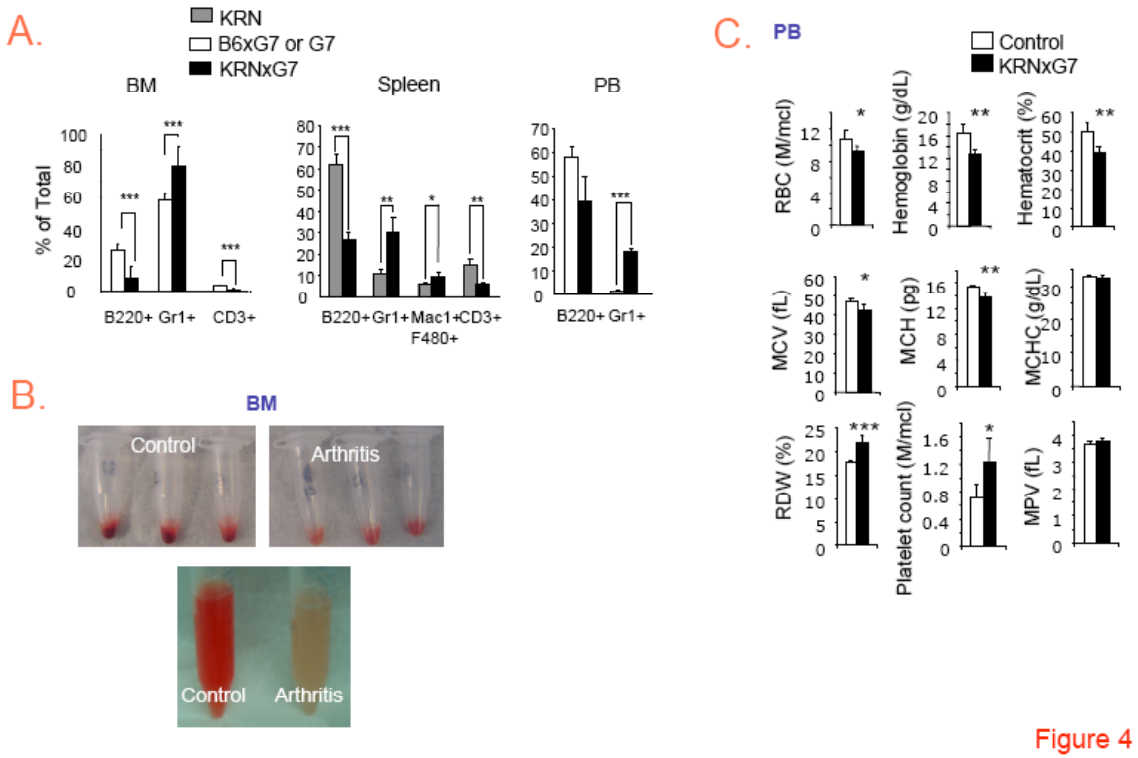

Figure 4

Figure 4. Reciprocal expanded myeloid cells and diminished lymphoid cells in arthritic mice

A. Flow cytometry determined frequency of myeloid (Gr1+), B lymphoid (B220+) and T lymphoid (CD3+) in bone marrow, spleen and PB of 6-8 week old arthritic (KRNxG7) and control mice. Control mice used were KRN for spleen and G7 or B6xG7 for BM and PB mice. Gr1+ cells in spleen is higher in KRN controls than in TCR non transgenic controls. Mac1+F480+ macrophage frequency in the spleen is also shown. Shown is the mean for 3 to 11 mice analyzed per strain for each tissue.

B. Top Panels: Bone marrow pellet from arthritic and control mice.

Bottom Panel: Marrow suspensions from tibiae and femur of control and arthritic mice. Red color is indicative of red blood cell content in bone marrow. Arthritic bone marrow pellet is paler indicating a relative deficiency of red blood cells in the marrow.

C. Erythrocyte parameters in Peripheral blood CBC confirming mild anemia in arthritic mice. RBC = RBC count, MCV=Mean Corpuscular Volume, MCH=Mean Corpuscular Hemoglobin Amount, MCHC=Mean Corpuscular Hemoglobin Concentration, RDW=Red Blood Cell Distribution Width. 2 male and 2 female of control and arthritic mice were used. This experiment was repeated with identical results with N=5 mice in each group.

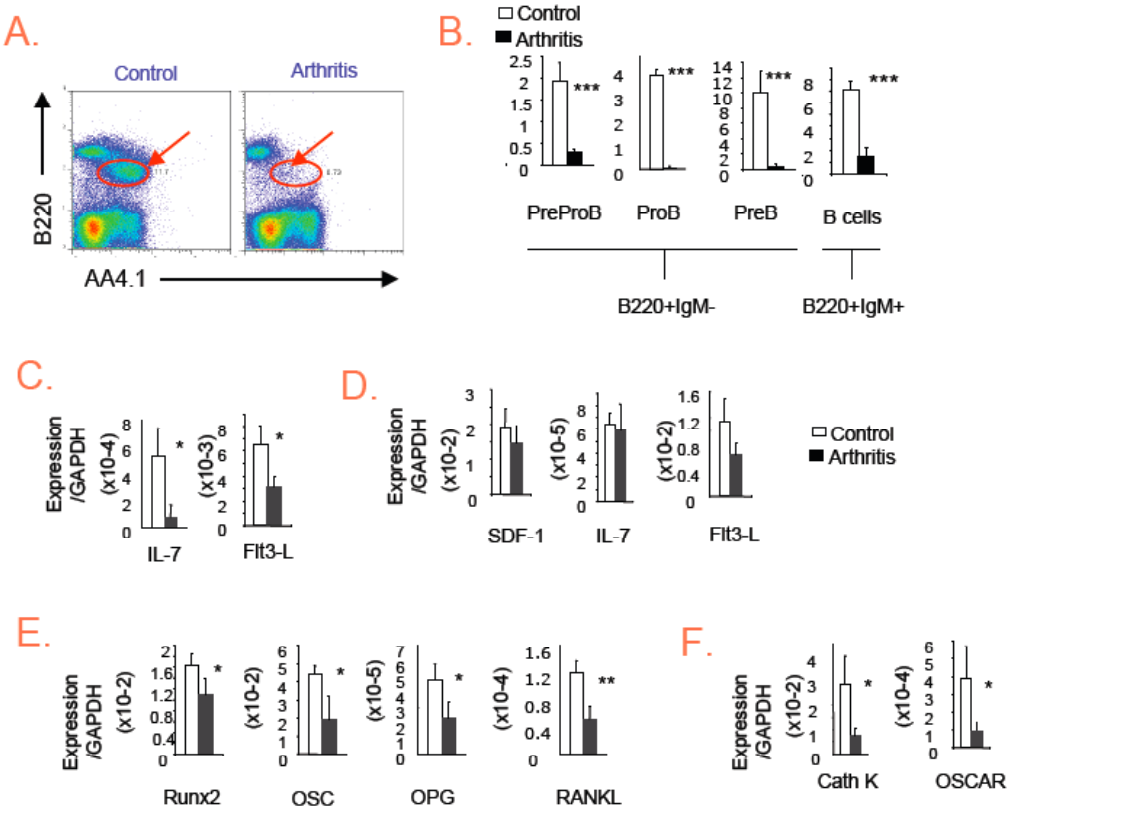

Figure 5. Defective marrow B lymphopoiesis and associated bone microenvironment changes

A. Representative FACS plot for B220+ cells in BM of KRNxG7 arthritic or G7 control mice. Whole bone marrow is stained for B220 and AA4.1 (a marker for B-cell precursors). B-cell

precursors (B220loAA4.1+; arrow) are virtually depleted in KRNxG7 BM, whereas most of the residual cells are B220hi and IgM+ (IgM staining not shown).

B. Flow cytometry determined frequencies of B220+IgM- B cell precursors in the BM. From least mature to most mature: PreproB (B220+IgM-CD19-CD43+NK1.1-CD11c-), ProB (B220+IgM-CD19+CD43+), PreB (B220+IgM-CD19+CD43-), and B cells (B220+IgM+).

C. Expression of various B lymphopoiesis promoting cytokines in 6 week old arthritic (KRNxG7) and control (G7) marrow. Expression was determined by quantitative real-time PCR (qRT-PCR), followed by normalization to *GAPDH*. IL-7 (Interleukin 7), Flt3-L (ligand for Flk2).

D. QRT-PCR of B lymphopoiesis promoting cytokines in 3 week old KRNxG7 and B6xG7 control marrow. SDF-1 (Stromal Cell Derived Factor -1)

E. QRT-PCR of osteoblast related genes in 3 week old KRNxG7 and B6xG7 control marrow. Runx2 is a transcription factor required for early steps in osteoblastogenesis. OSC (osteocalcin), OPG (osteoprotegerin) and RANKL are all produced by osteoblasts.

F. QRT-PCR of osteoclast specific genes in 3 week old KRNxG7 and B6xG7 control marrow. Cath K (Cathepsin K).

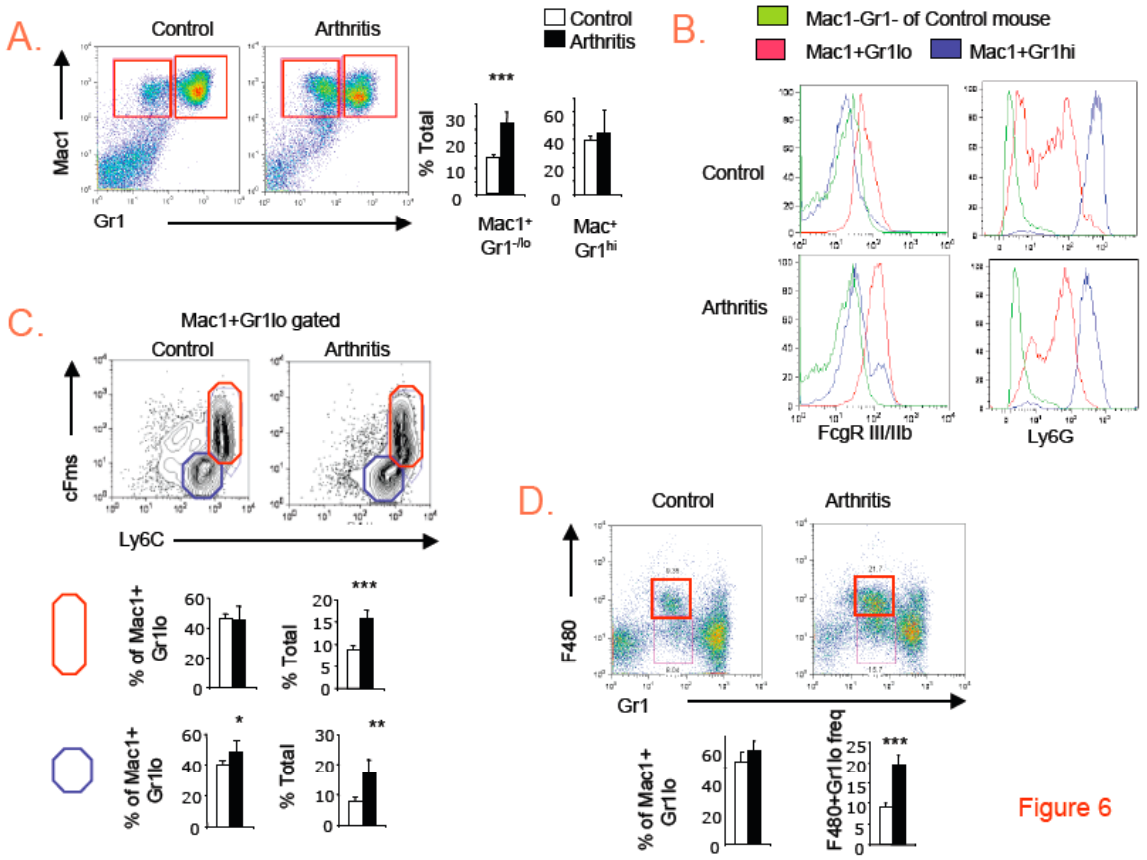

Figure 6. Myeloid skewing in arthritic bone marrow

A. Left Panel: Representative FACS plots of Mac1 and Gr1 stained bone marrow (BM) with gated Mac1+Gr1lo and Mac1+Gr1hi populations (red gates).

Right Panel: Mean frequency and standard deviation of BM Mac1+Gr1lo and Mac1+Gr1hi populations.

B. Flow cytometry histograms depicting expression of FcγRIII/IIb and Ly6G (detected with antibody clone 1A8) on Mac1+Gr1lo and Mac1+Gr1hi cells of KRNxG7 arthritic and control mice. The expression of these markers on Mac1-Gr1- cells of a non-arthritic control mouse is also shown (green histogram) to serve as a basis for comparison

C. Top Panel: Representative FACS plots showing Mac1+Gr1lo cells (See Figure 1a) subfractionated based on expression of cFms and Ly6C. cFms+Ly6C+ cells (red gate) are monocyte lineage cells. The remaining cFms-Ly6Clo (blue gate) are therefore enriched in

immature neutrophils (promyelocytes, myelocytes) which are also Mac1+Gr1lo. Bottom Panel: Quantification of subpopulations of Mac1+Gr1^{lo}.

D. Top Panel: FACS plot of F480 versus Gr1 of bone marrow cells. Bottom Panel: Quantification of F480+Gr1lo cells as a fraction of Mac1+Gr1^{lo} cells and as a fraction of total bone marrow live cells. 4 arthritic mice and 4 control mice were used.

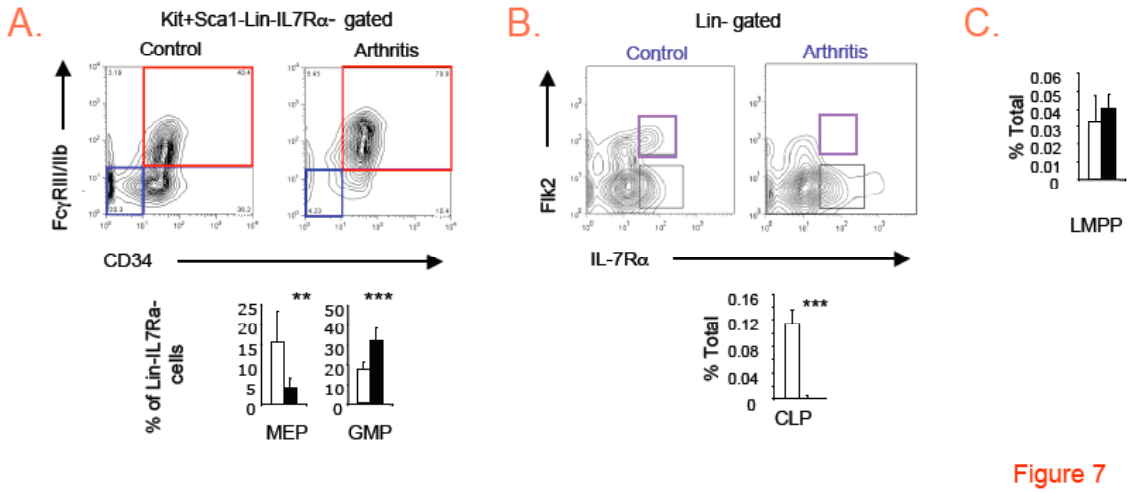

Figure 7

Figure 7. Myeloid skewing in arthritic marrow is evident at the committed progenitor level

E. Top Panel: Representative FACS plot for analysis of BM committed progenitors. GMPs and MEPs are FcγRIII/II+CD34+ (red gate) and FcγRIII/II-CD34- (blue gate) respectively within Kit+Sca1-Lin-IL7Rα- cells.

Bottom Panel: GMP and MEP frequencies normalized to total Lin-IL7Rα- myeloerythroid progenitors. GMP analysis is pooled from 3 independent experimental sets total of 10 mice per each group. MEP analysis is pooled from 2 independent experimental sets with 7 mice per each group.

G. Top Panel: Representative FACS plot depicting depleted CLP (purple gate) in KRNxG7 bone marrow. CLPs are identified as Flk2^{+}IL-7Rα^{+}Lin^{-}.

Bottom Panel: CLP frequency analysis.

H. Frequency of Lymphoid primed multipotential progenitor (LMPP; Kit+Sca1+Lin-Flk2hiCD34+) in the bone marrow

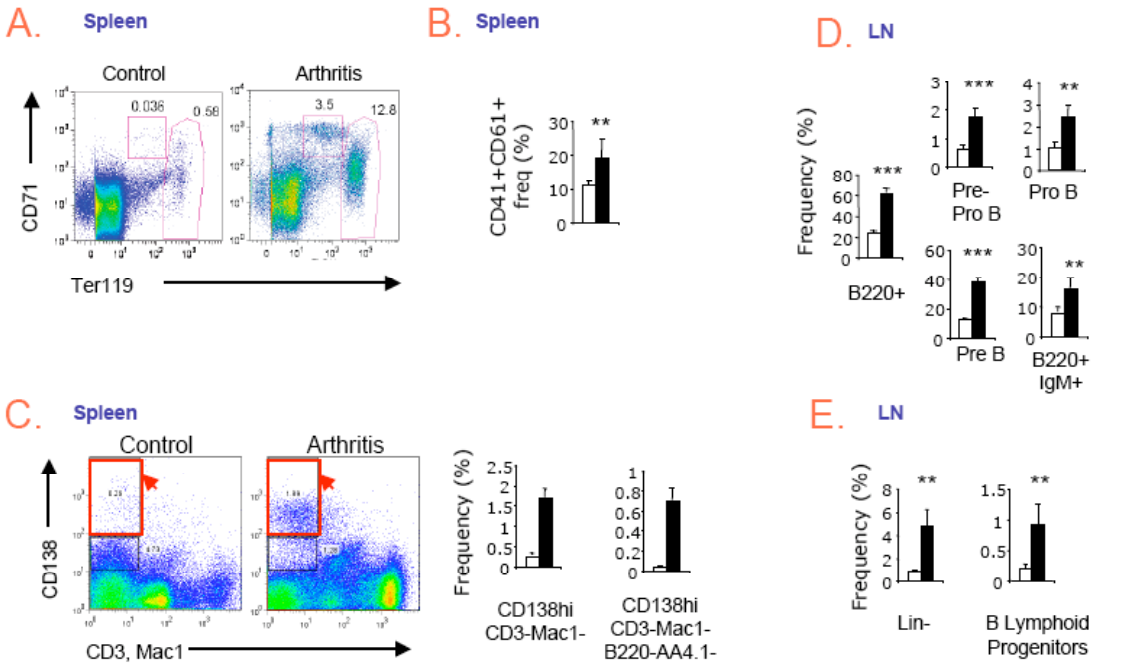

Figure 8

Figure 8. Erythroid and B lineage cells are increased in distinct extramedullary sites

A. FACS plots indicating CD71 and Ter119 expression of cells of RBC lysed spleen from arthritic and control mice. In addition to RBC lysis this plot is pre-gated using forward scatter and side scatter parameters that exclude the most mature red blood cells. Increased frequency of immature RBC lineage cells including Ter119^{hi} cells and Ter119^{lo}CD71^{hi} (both gated) indicates robust erythropoiesis in arthritic spleens. Arthritic spleens also had increased MEP frequency (not shown).

B. Mean splenic CD41+CD61+ frequency and standard deviation. CD41+CD61+ phenotypically identify megakaryocytes. Together with Figure 5C above supports increased activity of MEP in arthritic spleens unlike their depletion in arthritic marrow.

C. Representative FACS plots and bar graphs of plasma cells in spleens of control and arthritic mice. Splenocytes that were not myeloid cells nor T cells (CD3-Mac1-) but expressed the plasma cell marker CD138 (syndecan-1) at high levels were identified as plasma cells.

D. Mean frequencies of various B lineage populations in lymph node (LN). Frequencies shown are total B220+ cells and subsets of these cells from least mature to most mature – preproB, proB, preB (all B220+IgM-) and B220+IgM+ cells. Frequencies of all subsets of B220+ cells are increased in arthritic lymph nodes.

E. Mean frequencies of total progenitors (Lin-) and B lymphoid progenitors ($Kit^{lo}Sca1^{-/lo}$Lin-IL7Rα+) in arthritic and control lymph node (LN). Both total Lin- and B lymphoid progenitor frequencies are increased in arthritic LN. B220 is included in the "Lin" cocktail and hence these cells are B220-, even more immature than the B cell precursors examined in Figure 8D. To exclude T cells from the Lin- gate, CD3 was used.

CHAPTER 3

CELL INTRINSIC MOLECULAR DIFFERENCES BETWEEN ARTHRITIC AND CONTROL PRIMITIVE PROGENITORS

Most of the contents of this chapter has been published in Oduro KAJ et al, 2012. *Blood* 120:2203

OVERVIEW

Our preliminary analysis of primitive progenitors in arthritic mice (Chapter 2) revealed that the frequency and cycling of these cells in the bone marrow are unchanged compared with age matched non-arthritic controls. In contrast we demonstrated marked differences in the frequency of various mature cells and the committed progenitors that give rise to them in the bone marrow. Specifically, we found that myeloid cells and GMPs were increased while lymphoid cells, erythrocytes and their respective committed progenitors – CLPs and MEPs – were decreased in arthritic marrow.

In this chapter, we take this analysis a step further by asking the question - Is the increased GMPs and myeloid cells in arthritic mice at the expense of other lineages due to changes at primitive progenitor level not revealed by frequency or cell cycle analyses? As our analysis in Chapter 2 illustrates, it is possible that the skewed myelopoiesis in arthritic marrow is simply a consequence of relocation of erythroid and lymphoid progenitors to other organs. Ueda et al has suggested that marrow myeloid and lymphoid development share a common developmental niche such that mobilization of B lymphoid precursors out of the bone marrow by alum immunization for example reallocates spatial resources for myeloid development [1]. Altered turnover rates of the different committed progenitors in arthritic mice could also affect abundance of the committed progenitors and consequently their mature progeny.

All these potential explanations invoke possible mechanisms for myeloid skewing that do not depend on any cell intrinsic contributions from primitive progenitors. Therefore to determine whether uncommitted progenitors are involved in the myeloid skewing we decided to sort these cells and analyze them prospectively. We demonstrate in this Chapter, cell intrinsic molecular differences between primitive progenitors obtained from arthritic and non-arthritic control mice. These differences suggest that primitive progenitors are myeloid primed by the arthritic environment.

MYELOID PRIMING IN ARTHRITIC PRIMITIVE PROGENITORS

KSL cells from KRNxG7 arthritic mice upregulate myeloid genes and downregulate erythroid genes

The KSL phenotype contains all primitive progenitors and enriches about 1000 fold for the HSC activity compared with whole bone marrow. The similar cell cycling of KSL cells from KRNxG7 arthritic and control mice (Chapter 1) indicated that this phenotype identified a similar population in arthritic and control mice that is different from the faster cycling committed progenitors [2]. We sorted Kit+Sca1+Lin- (KSL) cells from arthritic and control mice to high purity (Figure 1A). To verify the immaturity of KSL cells from both arthritic and control mice, relative to committed progenitors i.e. GMPs, we cultured sorted KSL cells and GMPs in methylcellulose media containing various hematopoietic cytokines. Although both KSL cells and GMPs formed colonies in the presence of myelopoietic cytokines, KSL cells had superior plating efficiency than GMPs as well as bigger colonies indicating greater per cell output (Figure 1B & 1C). In the presence of erythropoietic cytokines, only KSL cells formed colonies (Figure 1B). KSL cells but not GMPs also survived in B lymphopoietic culture media (See Chapter 4). Therefore, KSL cells from KRNxG7 arthritic mice just like control KSL cells are *uncommitted* – unlike GMPs, which are *committed* since they lack the *capacity* to thrive in erythroid or lymphoid restricted media.

To determine cell intrinsic molecular differences that might exist between arthritic and control KSL populations, we compared their transcriptomes using mouse Affymetrix 430 2.0 microarray. This array uses about 45000 probesets to assess the abundance of over 39000 transcripts. We have previously shown that stem cell enriched populations, transient amplifying progenitor populations and mature cell populations are distinguishable based on gene ontology (GO) analysis of genes enriched in the population [3, 4]. Using a similar analysis we confirmed that KSL cells from arthritic and control mice cluster together and with other stem cell enriched populations from previous studies (Figure 1D). In fact, out of over 45000 probesets assayed, only about 700 (1-2%) detected transcripts that were differentially regulated in arthritic versus control

KSL cells. We reasoned that the identity of these differentially regulated genes and more importantly their associated functions will indicate how arthritic KSL cells differ from control KSL cells.

Therefore we performed *in silico* functional annotation of the differentially regulated genes using DAVID Bioinformatics – an online genome anaylsis resource that integrates several *in silico* analysis tools [5]. We found that functional annotation categories such as "cell surface" (7X enriched; $p=1.5x10^{-7}$), "transmembrane protein" (5X enriched; $p=1.7x10^{-6}$) were significantly enriched in genes downregulated in arthritic KSL cells but not in the upregulated genes (Figure 2A & not shown). Interestingly the few cell surface/ transmembrane protein genes that were upregulated in arthritic KSL cells included several receptors for inflammatory cytokines such as IL6st (gp130) and IL17Rα (Figure 2B).

We specifically inquired whether lineage specific genes were differentially regulated in arthritic KSL cells. We found increased expression of the C/EBP family of myeloid transcription factors - C/EBP α, β and δ - and other myeloid associated genes (Figure 2C & 2D). On the other hand a number of erythroid and megakaryocyte genes including key transcription factors like GATA1, EKLF (Klf1), Fog1 (Zfpm1) and Gfi1b were downregulated in arthritic KSL cells (Figure 2E). To validate these microarray results, we analyzed expression of 24 genes, including some upregulated and downregulated genes from the microarray, using quantitative real time PCR on KSL cells from arthritic and control mice that were independent from the pools used for the microarray. As an internal control we verified that the sorted KSL cells expressed much lower levels of the GMP markers FcγRIII and FcγRIIb than sorted GMPs (Figure 3A). We also verified that arthritic KSL and control KSL cells expressed similar levels of the cell cycle inhibitor and stemness gene p21 [6] (Figure 3B). Out of 24 genes assessed, expression of 18 was changed in a manner consistent with the microarray experiment (Figure 3C & data not shown). Notably we were able to validate the increased expression of highly myeloid specific genes like S100a8 and

Chi3l3 as well as decreased expression of key erythropoietic transcription factors like Gata1 and EKLF.

We also detected increased cell surface protein expression of FcγR III/IIb on arthritic KSL cells (Figure 3D) consistent with upregulation of FcγRIII and FcγRIIb transcripts from the microarray analysis (Figure 2D). During normal myelopoiesis FcγR III/IIb cell surface expression is upregulated at the GMP level and is sustained in mature cells. In fact, FcγR III/IIb is used as a marker for myeloid committed progenitors, GMPs [7, 8]. The level of FcγRIII/IIb cell surface protein and mRNA expression in arthritic KSL cells were much less than on GMPs (Figure 3A & 3D) thus implying that arthritic KSL cells are not misidentified GMPs but rather bonafide KSL cells on a path towards myeloid differentiation.

Induction of the "myeloid Inflammatory signature" by arthritogenic serum transfer

Since mice having *both* KRN transgene and MHC II IA^{g7} always develop arthritis, our non-arthritic control mice are always genetically different from the KRNxG7 arthritic mice in terms of lacking either the KRN transgene or the arthritogenic MHC II IA^{g7} allele. It is theoretically possible although unlikely that the myeloid biased gene expression pattern we observed in KRNxG7 arthritic KSL cells was a consequence of genetic differences with control mice. To determine if this myeloid bias was indeed arthritis environment dependent, we utilized the serum transfer model of arthritis [9]. Naïve C57BL/6 wild type mice when injected with serum from arthritic mice, develop arthritis that peaks around day 7. These serum induced arthritic mice also have relatively increased GMPs and Mac1+Gr1lo cells in the bone marrow compared to PBS injected controls (Figure 4A & 4B). We sorted KSL cells from serum transfer and PBS injected mice and performed qRT-PCR analyses. Again, KSL cells from serum transfer arthritic mice expressed higher levels of the myeloid specific genes Chi3l3, S100a8, S100a9, SOCS3 and lower levels of key erythropoietic transcription factors GATA1 and Fog1 than PBS injected controls (Figure 4C) recapitulating what we saw in KRNxG7 arthritic KSL cells.

The gene expression pattern of serum transfer KSL cells and KRNxG7 KSL cells were not identical though. For instance C/EBPβ and Ccr2, which were increased and EKLF, which was decreased in KRNxG7 KSL cells (Figure 3C) were not changed in the serum transfer KSL cells (Figure 4C). These differences may represent changes that require prolonged duration of arthritis since the serum transfer model only induces a transient arthritis. In fact the mild (30-40%) increase in bone marrow Mac1+Gr1^{lo} frequency (Figure 4A) in the serum transfer model is similar to KRNxG7 mice at the very early stages of disease prior to joint swelling (i.e. 3-week old; Figure 4D); it is unlike the 100-200% increase in the frequency of this population in KRNxG7 mice with full blown disease (Chapter 2; Figure 6). The gene expression differences may also be due to differences in disease severity. Unlike the KRNxG7 mice where all distal limb joints are affected, disease development in the serum transfer model is more heterogeneous with some distal limb joints spared [9]. Not all joints were affected in the cohort of serum transferred mice used for the gene expression analysis. Furthermore, in our experience, mice with serum transfer arthritis do not appear as sick clinically (i.e. they are not emaciated and their bones are not brittle unlike KRNxG7 arthritic mice). They also lack the marked increase in peripheral blood Mac1+Gr1+ cells seen in KRNxG7 arthritis mice (Chapter 2: Figure 4 and Data Not Shown).

Nevertheless our data shows that the myeloid primed state of KRNxG7 KSL cells is arthritis dependent since we can induce key molecular expression changes in KSL cells of mice after just 7 days of arthritis exposure. We refer to upregulation these genes – SOCS3, S100a8, S100a9 and Chi3l3 – as a "myeloid inflammatory signature" based on consistent induction in both KRNxG7 and serum transferred arthritic mice.

"Myeloid Inflammatory Signature" Genes are upregulated in Primitive HSCs

Only about 5% of KSL cells are true HSCs. It was of interest to know if the myeloid priming we observed in KSL cells also existed at this level. These cells have self-renewal capability and for this reason can replenish the hematopoietic system over the long term. Therefore myeloid priming at this level might be of significance in terms of understanding chronic

inflammation. Previous studies have shown that all long term reconstituting HSC (LT-HSC) activity is contained within the CD150+CD48-CD34- fraction of KSL cells [10, 11]. Specifically 40-50% of CD150+CD48-KSL [11] and 40% of CD34-KSL cells [12] have LT-HSC activity in single cell transplantation experiments implying that CD150+CD48-CD34-KSL represents a highly enriched LT-HSC population.

We prospectively sorted CD150+CD48-CD34-KSL cells from arthritic and control mice and performed a gene expression analysis. We found that although the expression of a number of transcription factors (TF) was unchanged (Figure 5A), all the myeloid genes constituting the myeloid inflammatory signature identified earlier – SOCS3, S100a8, S100a9 and Chi3l3 – were increased in arthritic HSCs compared with control HSCs (Figure 5B). In fact the magnitude of the increase at the HSC level was greater than the fold increase at the bulk KSL level (Figure 5C). . It is worth noting that myeloid committed progenitors (i.e. GMPs) are CD150- and CD34+ [7, 8, 13] making it even more unlikely that the myeloid inflammatory signature we have identified is due to contamination by GMPs.

TF activity can be increased by mechanisms other than increased expression such as post-translational modification or subcellular localization. Future work can address if these other mechanisms rather than increased expression of myeloid TFs or decreased expression of erythroid TFs explain the markedly enhanced transcriptional activity at myeloid genes in HSCs.

In summary, activation of a myeloid program by the arthritic environment is initiated at the most primitive HSC level.

FEATURES OF NORMAL AGING IN ARTHRITIC KSL CELLS

How might such lineage-biased transcription be compatible with a primitive progenitor status? We mentioned earlier that only 2% of probe sets were differentially regulated in KSL cells from KRNxG7 arthritic KSL cells versus control KSL cells and only a fraction of these could be considered lineage specific genes. Furthermore for most differentially regulated genes the changes were modest – less than 3 fold. To determine if the changes observed at the mRNA

level are generally also reflected at the protein level we performed FACS analysis of a number of differentially regulated genes using KSL cells. Proteins which are typically expressed on the cell surface were chosen due to relative ease of assessment compared with cell permeabilization to access intracellular proteins. In contrast with what we showed for FcγR III/IIb earlier (Figure 3D), 13 out of 17 genes changed at the mRNA level including all the cytokine receptors assayed were not changed at the cell surface protein level (Figure 6 and Data not shown). Although changes to intracellular protein levels for these and other genes is possible, these data support the idea that primitive progenitors are thrust in a state of "readiness" (i.e. "primed") that is measured presumably to avoid compromising their primitive progenitor status.

Two of the genes whose protein expression we were able to confirm were explored further because of their previously association with normal aging – another epigenetic state where myeloid potential is increased in primitive progenitors.

Elevation of IgG and P selectin proteins in arthritic KSL cells

Three probe sets corresponding to P selectin and 4 probe sets corresponding immunoglobulin heavy chain (IgH) were increased in KRNxG7 arthritic KSL cells relative to both KRN and B6xG7 non-arthrtic control KSL cells (Figure 7A). In fact IgH transcripts, together with transcripts of the myeloid gene Chi3l3, were the most upregulated in arthritic KSL (about 10 fold upregulated); all 4 IgH probe sets were in the top 5 upregulated "microarray hits". Previous studies have found that P selectin mRNA and protein are upregulated in primitive progenitors in aged (21-28 month old) mice relative to younger mice [14, 15]. Chambers et al found that while only about 3% of KSL side population (SP) cells in 2 month old mice are P selectin positive, about 81% of these cells were P selectin positive in 28 month old mice [14]. We checked and confirmed that consistent with our microarray result, P selectin protein was increased in arthritic KSL cells even though these cells were obtained from mice that were only 6-8 weeks old (Figure 7B). Therefore, similar to normal aging, KSL cells in KRNxG7 arthritis upregulate P selectin.

Presence of IgH in KSL cells is unusual because, physiologically, immunoglobulins are associated with B lineage cells. Furthermore at steady state, the earliest progenitors to display VDJ recombinase activity, which is a pre-requisite for immunoglobulin production, are CLPs [16]. Although IgH transcripts have previously been reported in KSL cells using the same microarray platform (Mouse 430 2.0) that we utilized in our study [17], and are upregulated by normal aging, [14] these transcripts have been considered “sterile”, resulting from aberrant transcprition of non recombined IgH loci that consequently do not code for any protein [14, 17].

To gain further insight into the upregulated IgH transcript in arthritic KSL cells, we first determined which specific IgH transcripts were upregulated in arthritic KSL cells. Examination of the target sequences recognized by all 4 affymetrix IgH probe sets (Figure 7A) revealed that they were partially overlapping and all mapped to the 4^{th} exon of the IgG1 constant region gene ($C\gamma1$) (Figure 7C & 7D). To determine if full-length $C\gamma1$ transcript was upregulated in uncommitted progenitors in arthritis, we performed qRT-PCR experiments on independently sorted cells using primer sets designed to recognize exons 1, 2 and 3 of $C\gamma1$ (Figure 7E). As expected, $C\gamma1$ transcripts were virtually undetectable in KSL cells or highly purified HSCs (CD150+CD34-CD48-KSL) cells sorted from control animals. However, levels of $C\gamma1$ was markedly upregulated in arthritic KSL cells and even more so in purified HSCs regardless of the primer sets used (Figure 7F). These results suggested that there is upregulation of full length $C\gamma1$ transcript, not just fragments, in primitive progenitors, including HSCs, in arthritic mice.

To determine if these transcripts were sterile or were translated, we examined cell surface immunoglobulin protein expression on KSL cells by flow cytometry. Consistent with previous reports, we were unable to detect any immunoglobulin on the surface of KSL cells from young wild type mice (Figure 7G). KSL cells from KRNxG7 arthritic mice expressed cell surface IgG, but not IgM, the first immunoglobulin synthesized during physiologic B cell development. (Figure 7G and Data Not Shown). Furthermore, we found using monoclonal to different IgG isotypes that the surface IgG detected on arthritic KSL cells was specifically of the IgG1 isotype

(Figure 7G), consistent with our transcript analysis. This phenomenon was arthritis dependent because a similar pattern of cell surface IgG1 protein expression was detected on KSL cells from wild type B6xG7 mice induced to develop arthritis by serum transfer (Figure 7H). Interestingly, KSL cells from aged wild type mice, which also upregulate IgH transcripts [14], previously regarded sterile, also upregulate cell surface IgG protein (Figure 7I). However in contrast to arthritic KSL cells, IgG2b, not IgG1, is the isotype upregulated (Figure 7I). Therefore, arthritic uncommitted progenitors specifically upregulate IgG1 at the mRNA and protein levels.

These result raise a number of questions. What is the mechanism of IgG1 synthesis in arthritic primitive progenitors? Is there still a germline configuration at the IgG locus in these cells or does VDJ recombination and class switching occur? Physiologically IgG1 protein cannot be synthesized until after class switch recombination in activated B cells. This results in reduced $C\mu$ and $C\gamma$ germline transcripts (GLTs) and increased $C\gamma$ post switch transcripts (PST). However we found that $C\mu$ GLT were still very prominent in arthritic KSL cells as in controls while $C\gamma 1$ PSTs was still very low (Figure 7J). Therefore it is possible that some other mechanism, such as alternative processing of IgH transcripts [18] occurs in arthritic primitive progenitors.

Another question based on these findings is the significance of IgG upregulation of primitive progenitors in arthritis and aging. It is interesting to note that the predominant IgG isotype produced in KRNxG7 arthritis is also IgG1 [17-20]. It is not clear though if this increased surface IgG on arthritic KSL cells is capable of recognizing arthritogenic epitopes of GPI, but GPI transcripts are curiously also upregulated in arthritic KSL cells as well (Figure 2D).

In summary KSL cells from arthritic mice have upregulated P selectin and IgG reminiscent of primitive progenitors in normal aging, another epigenetic state characterized by myeloid skewing.

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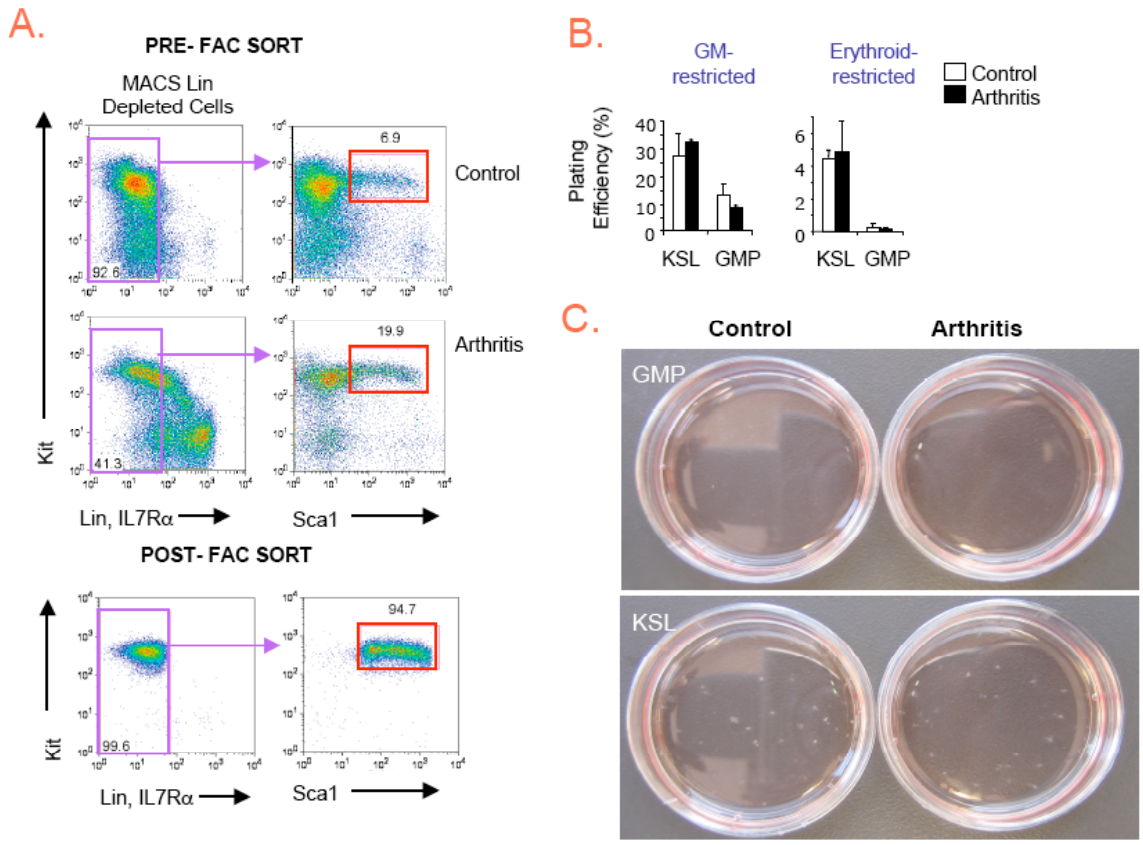


Figure 1. KSL sorting and validation

A. Scheme for sorting KSL cells and checking purity of sort. Residual Lin+ cells after lineage depletion (more prominent in arthritic samples) were efficiently excluded during FAC sorting. Besides reduced Kit^{hi}Sca1-Lin- MEPs (Figure 1C and unpublished), Kit versus Sca FACS plot of arthritic mice is similar to that of control mice, and is devoid of the overt Sca1 shift present in some models of inflammation (See Figure S2B).

B. Methylcellulose colony replating of sorted BM GMP and KSL cells. “GM restricted” medium contains recombinant KitL, Interleukin (IL)-3 and -6 without erythropoietin (EPO). “Erythroid restricted” medium is serum free and contains EPO. (See Methods for source of media).

C. GM restricted methylcellulose plates (day 7-8) containing recombinant SCF, IL-3 and IL-6 depicting macroscopic KSL derived colonies from arthritic or control mice, visible as specks. GMP derived colonies are only visible under a microscope.

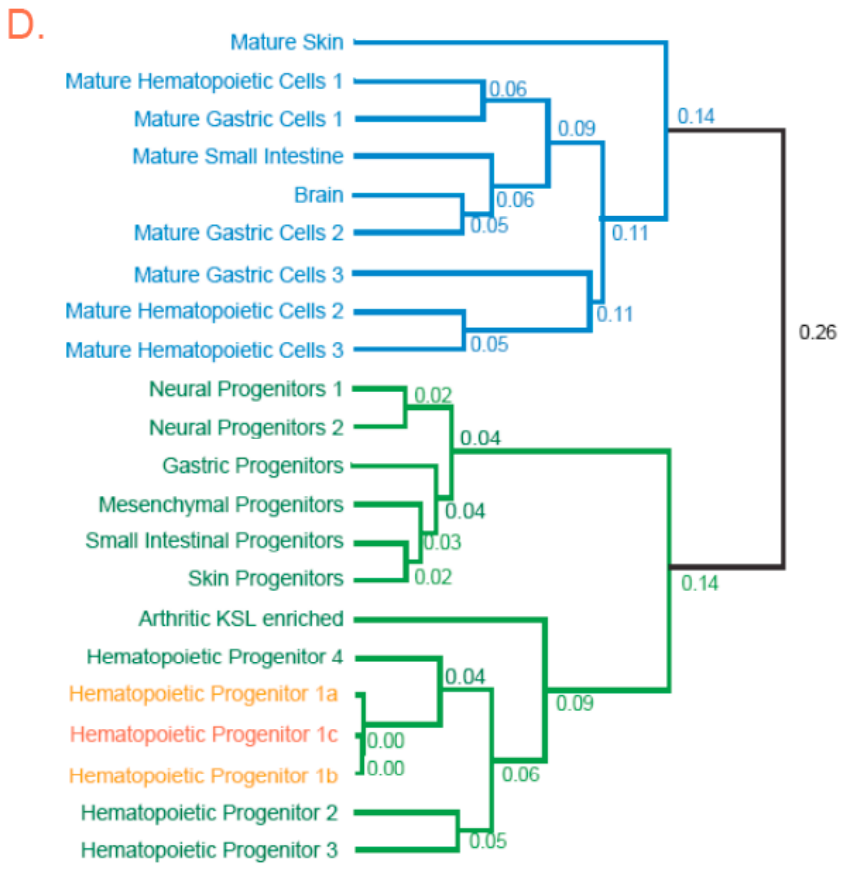


Figure 1

D. Dendrogram comparing arthritic KSL enriched expression profile ("hematopoietic progenitor 1c; red color) and control KSL enriched expression profile ("hematopoietic progenitor 1a & 1b") to each other and to profiles of stem cells (green) and mature cells (blue) from previous studies (See Table S2). Mature Lin+ expression was used as a baseline for determining KSL enriched genes. Profile of genes enriched in arthritic KSL cells relative to control KSL cells is represented by "arthritic KSL enriched".

A.

Category	Term	RT	Genes	Count	%	P-Value	Fold Enrichment
GOTERM_CC_FAT	external side of plasma membrane	RT		13	9.4	1.5E-8	9.1
GOTERM_CC_FAT	cell surface	RT		14	10.1	1.5E-7	6.6
KEGG_PATHWAY	Allograft rejection	RT		8	5.8	5.3E-7	15.5
KEGG_PATHWAY	Graft-versus-host disease	RT		8	5.8	5.3E-7	15.5
KEGG_PATHWAY	Type 1 diabetes mellitus	RT		8	5.8	9.4E-7	14.3
SP_PIR_KEYWORDS	transmembrane protein	RT		14	10.1	1.7E-6	5.4
GOTERM_BP_FAT	immune response	RT		15	10.9	3.2E-6	4.7
GOTERM_BP_FAT	negative thymic T cell selection	RT		4	2.9	2.4E-5	64.9
GOTERM_BP_FAT	antigen processing and presentation	RT		7	5.1	2.7E-5	11.8
KEGG_PATHWAY	Autoimmune thyroid disease	RT		7	5.1	3.2E-5	10.9

B.

probe set	NAME
1452843_at	Il6st: interleukin 6 signal transducer
1443115_at	Tgfbr2: transforming growth factor, beta receptor II
1421034_a_at	Il4ra: interleukin 4 receptor, alpha
1429959_at	Il10rb: interleukin 10 receptor, beta
1448950_at	Il1r1: interleukin 1 receptor, type I
1450456_at	Il21r: interleukin 21 receptor
1420904_at	Il17ra: interleukin 17 receptor A

C.

Category	Term	RT	Genes	Count	%	P-Value	Fold Enrichment
PIR_SUPERFAMILY	PIRSF005879:CCAAT/enhancer-binding protein	RT		3	0.7	1.8E-3	41.8
PIR_SUPERFAMILY	PIRSF005879:CCAAT/enhancer-binding	RT		3	0.7	1.8E-3	41.8
INTERPRO	CCAAT/enhancer-binding	RT		3	0.7	2.4E-3	36.7
PIR_SUPERFAMILY	PIRSF037286:Smad protein	RT		3	0.7	4.5E-3	27.9
GOTERM_BP_FAT	embryonic hemopoiesis	RT		5	1.1	3.8E-5	23.4
INTERPRO	Dwarfin	RT		3	0.7	1.1E-2	18.4
INTERPRO	SMAD domain, Dwarfin-type	RT		3	0.7	1.1E-2	18.4
INTERPRO	MAD homology, MH1	RT		3	0.7	1.1E-2	18.4
GOTERM_BP_FAT	inactivation of MAPK activity	RT		3	0.7	1.2E-2	17.6
UP_SEQ_FEATURE	domain:MH2	RT		3	0.7	1.3E-2	16.5

Figure 2

Figure 2. Upregulated myeloid genes and cytokine receptors in arthritic KSL cells based on microarray analysis

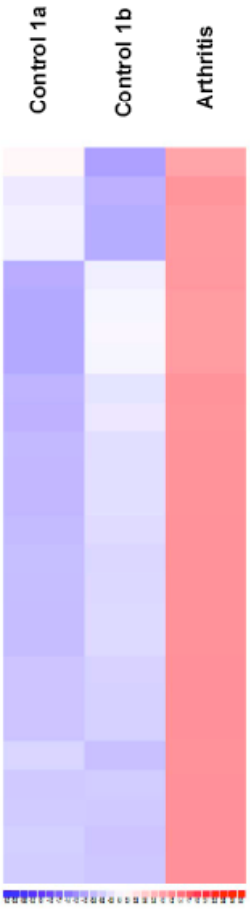
A. Functional annotation terms enriched in genes downregulated in arthritic KSL cells based on DAVID Bioinformatics analysis (Reference #5, Huang da et al, 2009). Only terms with at least

3 genes represented in the downregulated gene list with p<0.05 were used in the analysis. Top 10 most statistically significant terms (based on “p value”). Orange arrows highlight GO terms “External Side of Plasma Membrane”, “Cell Surface” and “Transmembrane protein”.

B. Microarray based heat map depicting cytokine receptors upregulated in arthritic KSL relative to both B6xG7 (control 1a) and KRN (control 1b) KSL populations.

C. Functional annotation terms enriched in genes upregulated in arthritic KSL cells based on DAVID Bioinformatics analysis (Reference #5, Huang da et al, 2009). Only terms with at least 3 genes represented in the upregulated gene list with p<0.05 were used in the analysis. Top 10 most enriched terms (based on “fold enrichment”). Orange arrows highlight GO terms “PIRSF005879:CCAAT/enhancer-binding protein”, PIRSF005879:CCAAT/enhancer-binding and CCAAT/enhancer-binding.

D.



probe set	NAME
1427126_at	Hspa1b: heat shock protein 1B
1449924_at	Prg3: proteoglycan 3
1452388_at	Hspa1a: heat shock protein 1A
1422873_at	Prg2: proteoglycan 2, bone marrow
1455899_x_at	Socs3: suppressor of cytokine signaling 3
1435477_s_at	Fcgr2b: Fc receptor, IgG, low affinity IIb
1423233_at	Cebpd: CCAAT/enhancer binding protein (C/EBP), delta
1421186_at	Ccr2: chemokine (C-C motif) receptor 2
1419691_at	Camp: cathelicidin antimicrobial peptide
1419394_s_at	S100a8: S100 calcium binding protein A8 (calgranulin A)
1418982_at	Cebpa: CCAAT/enhancer binding protein (C/EBP), alpha
1458279_at	Foxo3: Forkhead Box O 3
1448756_at	S100a9: S100 calcium binding protein A9 (calgranulin B)
1425451_s_at	Chi3l3 /// Chi3l4: chitinase 3-like 3 /// chitinase 3-like 4
1448377_at	Slpi: secretory leukocyte peptidase inhibitor
1419764_at	Chi3l3: chitinase 3-like 3
1448898_at	Ccl9: chemokine (C-C motif) ligand 9
1456909_at	LOC676974: similar to Glucose-6-phosphate isomerase (GPI)
1436778_at	Cybb: cytochrome b-245, beta polypeptide
1418901_at	Cebpb: CCAAT/enhancer binding protein (C/EBP), beta
1455486_at	Pias1: protein inhibitor of activated STAT 1
1421802_at	Ear1: eosinophil-associated, ribonuclease A family, member 1
1448620_at	Fcgr3: Fc receptor, IgG, low affinity III
1448328_at	Sh3bp2: SH3-domain binding protein 2
1422412_x_at	Ear3: eosinophil-associated, ribonuclease A family, member 3
1426587_a_at	Stat3: signal transducer and activator of transcription 3

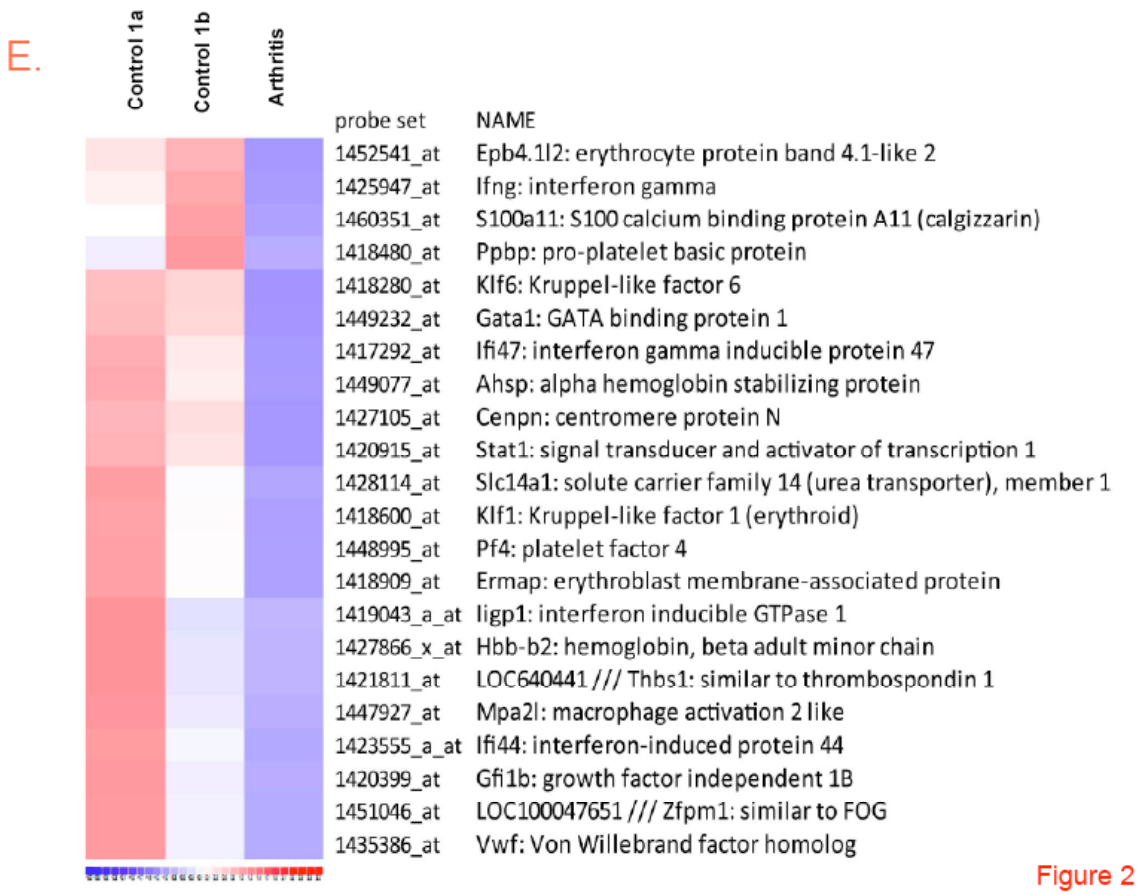


Figure 2

D. Microarray based heat map depicting selected genes upregulated in arthritic (KRNxG7) KSL cells relative to both B6xG7 (control 1a) and KRN (control 1b) KSL populations.

E. Microarray based heat map depicting selected genes downregulated in arthritic (KRNxG7) KSL cells relative to both B6xG7 (control 1a) and KRN (control 1b) KSL populations.

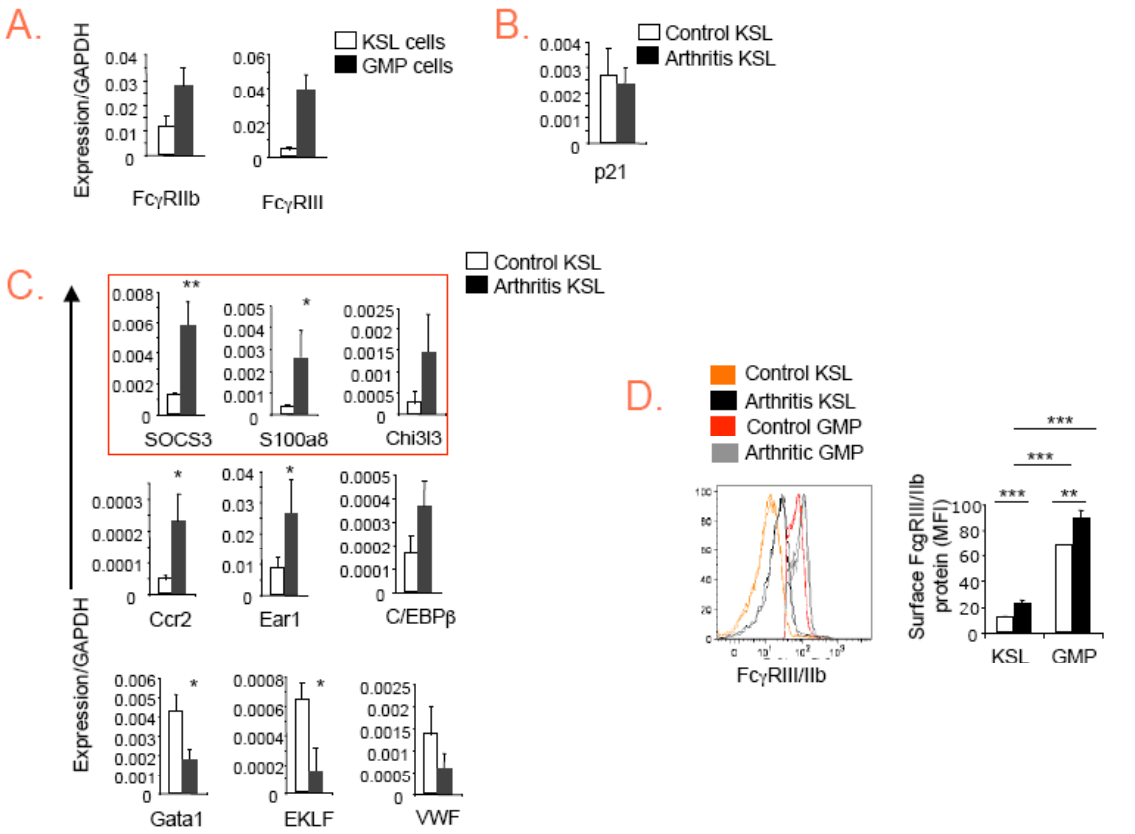


Figure 3

Figure 3. Validation of upregulated myeloid genes and downregulated erythroid genes in KRNxG7 arthritic KSL cells obtained from microarray analysis

A. QRT-PCR analysis of FcγRIIb and FcγRIII transcripts in sorted KSL and GMPs. Internal control for subsequent qRT-PCR analysis

B. QRT-PCR analysis of cyclin dependent kinase inhibitor, p21 in sorted arthritic (KRNxG7) and control (B6xG7) KSL cells. Internal control for subsequent qRT-PCR analysis

C. QRT-PCR validation of selected genes based on microarray. GAPDH normalized expression using 3 independently sorted KSL RNA different from pools used for microarray. Genes in red box are part of a “myeloid inflammatory signature” based on consistent upregulation in KSL cells from KRNxG7 arthritis and serum transfer arthritis models (See Figure 4 & 5)

D. Left: FcγRIII/IIb protein expression on KSL cells and GMP determined by FACS. Histograms from 2 arthritic mice (KRNxG7) and 2 control mice (KRN) are shown.

Right: Median fluorescence intensity (MFI) of Anti-FcγRIII/IIb-PE signal on gated KSL cells and GMP as a surrogate quantification of FcγRIII/IIb surface protein level. Representative of at least 2 experiments with at least 3 arthritic and control mice per experimental set up.

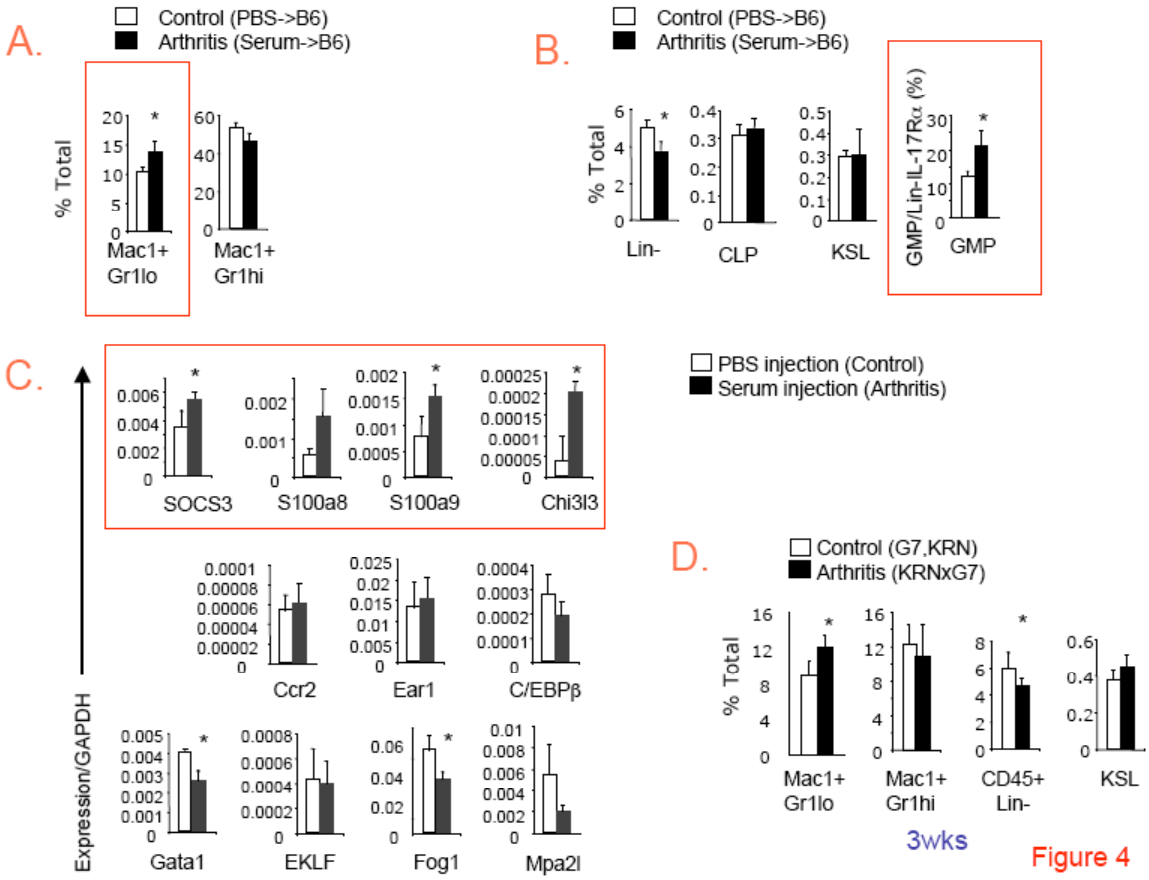


Figure 4

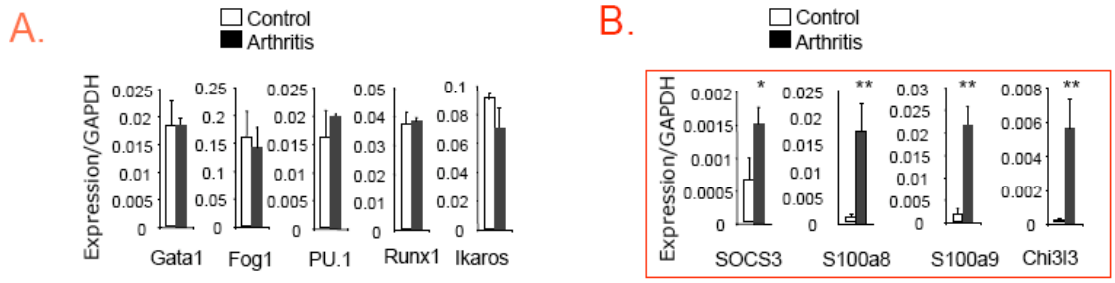
Figure 4. Myeloid Priming can be induced in naïve KSL cells by arthritogenic serum transfer

A. Analysis of bone marrow Mac1+Gr1+ frequencies in serum injected (arthritis) and PBS injected (control) B6 mice 7 days post injection. Mean and standard deviation for N=3 mice each is shown.

B. Analysis of bone marrow progenitor frequencies in serum injected (arthritis) and PBS injected (control) B6 mice 7 days post injection. Mean and standard deviation for N=3 mice each is shown.

C. Quantitative RT-PCR of selected genes in KSL cells from serum transfer arthritic mice and control PBS injected mice. “Myeloid inflammatory signature genes” are highlighted with a red box (See Figure 3 & 5)

D. Analysis of hematopoietic frequencies in 3 wk old KRNxG7 (prior to detectable joint swelling) and control mice. Modest Mac+Gr1lo increase is similar to serum transfer arthritis.



C.

CELL TYPE (CONDITION)

GENES	KSL (KRNxG7/Control)	KSL (Serum/PBS)	CD150+CD48-CD34-KSL (KRNxG7/Control)
SOCS3	4.7	1.6	2.3
S100a8	7.5	2.8	18.1
S100a9	ND	2.0	14.0
Chi3l3	5.8	5.6	41.9

Figure 5

Figure 5. Upregulation of myeloid inflammatory signature genes exists at the most primitive HSC subset of KSL cells

A. Quantitative RT-PCR of transcription factors in arthritic and control HSCs (CD150+CD48-CD34-KSL IL7Rα-). Mean and standard deviation of GAPDH normalized expression is shown.

B. Quantitative RT-PCR of "myeloid inflammatory signature genes" (See Figure 3 & 4) in arthritic and control HSCs (CD150+CD48-CD34-KSL IL7Rα-).

C. Table comparing fold increase in expression of "myeloid inflammatory signature genes" in arthritic KSL cells and HSCs relative to controls (based on Figures 3C, 4C & 5B).

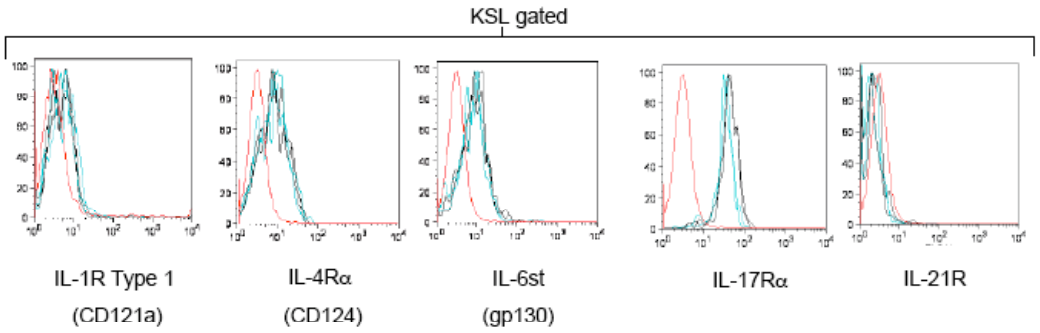
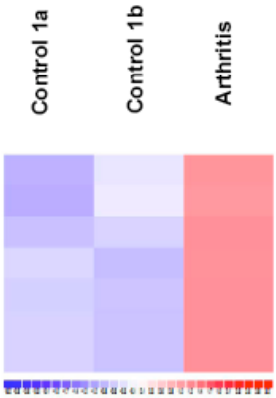


Figure 6

Figure 6. Protein expression of cytokine receptors on KSL cells

Protein expression of cytokine receptors on KSL cells from KRNxG7 arthritic mice (black histograms) and KRN control mice (blue histograms) were determined by flow cytometry. Red histogram represents isotype control staining. Besides IL-21R, the remaining cytokine receptors are detected on KSL cells. However no difference can be delineated between KRNxG7 and KRN KSL cells. Similar results were obtained using G7 as control.

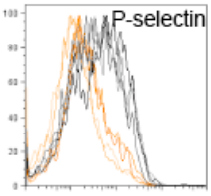
A.



(dChip Annotation)

probe set	NAME
1449906_at	Selp: selectin, platelet
1420558_at	Selp: selectin, platelet
1440173_x_at	Selp: selectin, platelet
1427870_x_at	Igh-6: Immunoglobulin heavy chain 6 (heavy chain of IgM)
1427756_x_at	Igh-6: Immunoglobulin heavy chain 6 (heavy chain of IgM)
1425247_a_at	Ighg1: immunoglobulin heavy constant gamma 1 (G1m marker)
1425324_x_at	Igh-6: Immunoglobulin heavy chain 6 (heavy chain of IgM)

B.



Control

Arthritis (KRNxG7)

C.

Target Sequence	Affymetrix Probe ID
ccactctcctggtaaatgatccccagtgtccttggagccctctggtcctacaggactctgacacctacctcca	1427870_x_at
gacaagaaaattgtgcccagggattgtggttgtaagccttgcatatgtacaggcagaccgaaggctccacaggtgtacaccattccacctcccaaggataaagtcagtctgacctgcatgataacagacttcttccctgaagacattactgtggagtggcagtggaatgggcagccagcggagaactacaagaacactcagcccatcatggacacagatggctcttacttcgtctacagcaagctcaatgtgcagaagagcaactgggaggcaggaaatactttcacctgctctgtgttacatgagggcctgcacaaccaccatactgagaagagcctctccccactctcctggtaaatgatctcagtgtccttggagccctctggtcctacaggac	1427756_x_at
tctacagcaagctcaatgtgcagaagagcaactgggaggcaggaaatactttcacctgctctgtgttacatgagggcctgcacaaccacca	1425247_a_at
aggagcagttcaacagcactttccgctcagtcagtgaacttcccatcatgcaccaggactggctcaatggcaaggagttcaaatgcagggtcaacagtgcagctttccctgcccccatcgagaaaaccatctccaaaaccaaaggcagaccgaaggctccacaggtgtacaccattccaccctcccaaggagcagatggccaaggataaagtcagtctgacctgcatgataacagacttcttccctgaagacattactgtggagtggcagtggaatgggcagccagcggagaactacaagaacactcagcccatcatggacacagatggctcttacttcgtctacagcaagctcaatgtgcagaagagcaactgggaggcaggaaatactttcacctgctctgtgttacatgagggcctgcacaaccaccatactgagaagagcctctccccactctcctggtaaatnatcccagtgtccttggagccctctggtcctacaggactctgacaccta	1425324_x_at

Figure 7

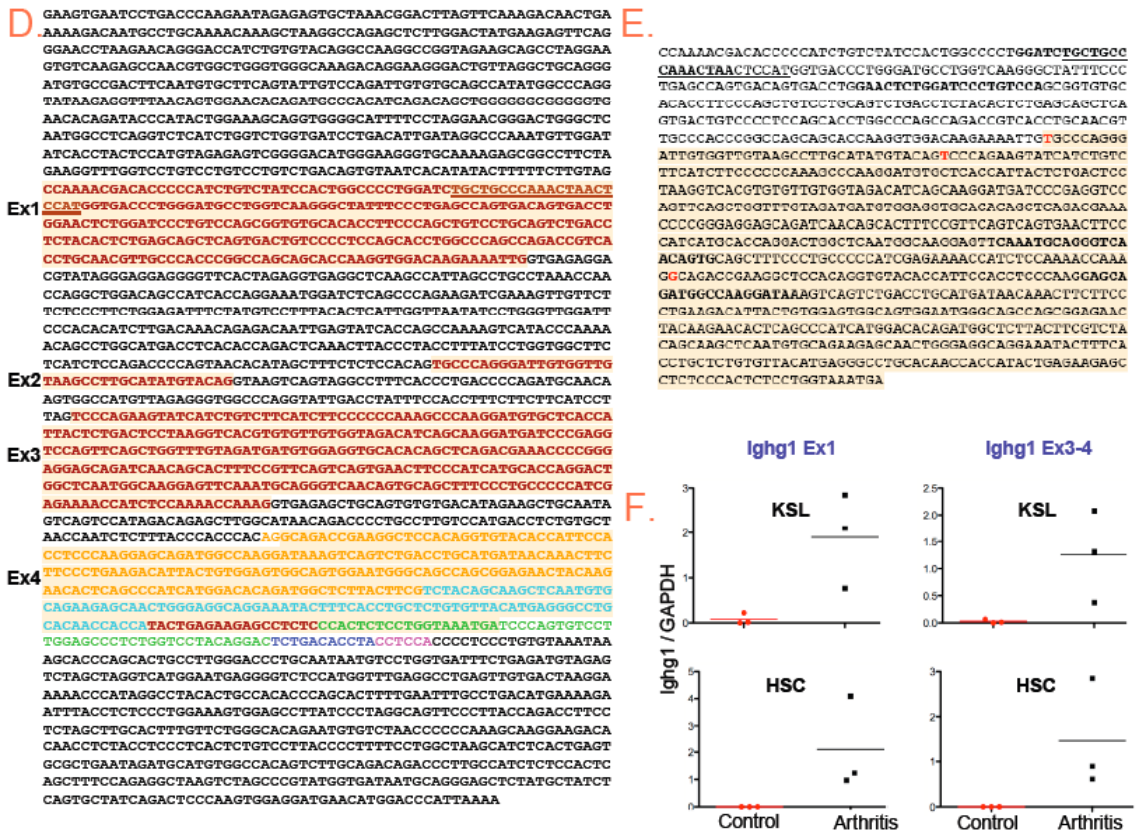


Figure 7. Upregulatin of P selectin and IgG1 protein in arthritic KSL cells

A. Heat map illustrating upregulated P selectin and immunoglobulin heavy chain transcripts in arthritic (KRNxG7) KSL cells relative to B6xG7 (Control 1a) and KRN (Control 1b) controls. The dChip annotations given does not accurately reflect the gene identified by these probesets. See Figures 7C & 7D.

B. Flow cytometry showing P-selectin protein expression on arthritic and control KSL cells. Each histogram is from a single mouse. Histograms from 4 arthritic mice (black) and 4 control mice (orange) are shown. Rightward shift of arthritic KSL cells indicate increased cell surface P-selectin protein expression and corroborates upregulation of P selectin transcript in arthrtic KSL cells based on microarray experiment.

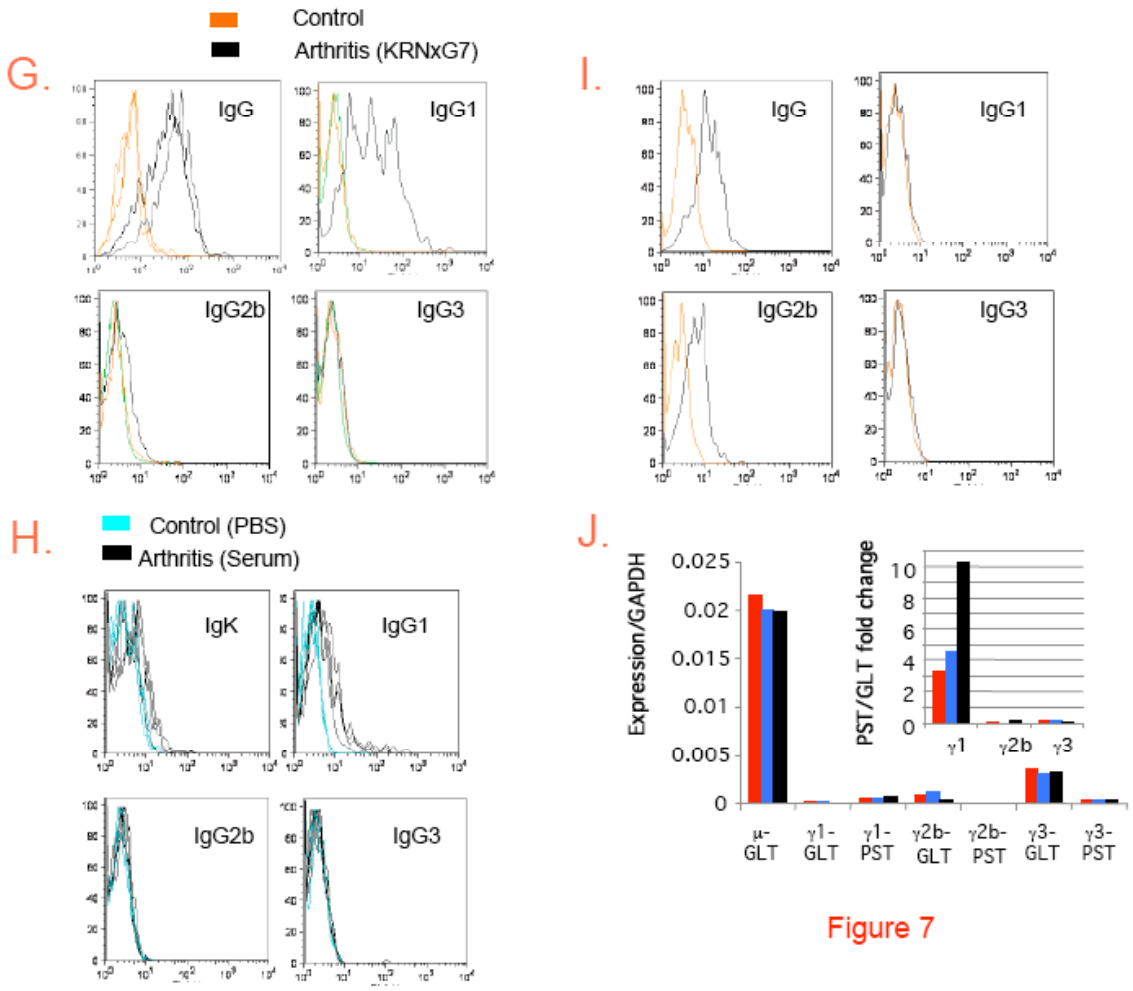


Figure 7

C. Target sequences for all immunoglobulin heavy chain affymetrix microarray probes upregulated in KRNxG7 KSL cells from Figure 7A.

D. Genomic sequence of the mouse IgG1 constant region (Cγ1) locus extracted from Ensemble. There are 4 exons (Ex) highlighted by light brown background shading and Ex1,2,3,4 annotations. Target sequences for all immunoglobulin heavy chain affymetrix microarray probes upregulated in KRNxG7 KSL cells (See Figure 7C) are located in exon 4. Sequence letters are colored to match the colors of target sequences shown in Figure 7C. Other sequence letters within exons are in dark brown font. Non coding regions (e.g. introns) are in black font Reverse primer for GLT/PST analysis used in Figure 7J below is underlined and lies within the 1st exon.

E. IgG1 constant region cDNA showing sequences corresponding to primers used for qRT-PCR validation. The start of exons 2,3,4 are shown in red. QRT-PCR primer sequences are shown in bold. In primer set 1, forward and reverse primers are in exon 1. In primer set 2, forward primer is in exon 3, reverse primer is in exon 4.

F. QRT-PCR of IgG1 constant region (Cγ1) expression by KSL cells and HSCs (CD150+CD48-CD34-KSL). KSL cells and HSCs were sorted from pools of mice independent from those used for microarray experiment. Each dot represents expression from an individual mouse or pool of mice.

G. Flow cytometry of cell surface immunoglobulin (IgG) protein on KSL cells. IgG – Total IgG using rat polyclonal antibody. A goat polyclonal antibody to mouse IgG also gave the same result (Not shown). IgG1, IgG2b and IgG3 isotypes are detected using specific rat monoclonal antibodies. All staining was performed in the presence of FcγR blocking antibody to prevent non-specific interaction between cell surface IgG and fluorophore conjugated anti-IgG antibodies. Isotype control is shown in green.

H. Surface immunoglobulin expression on KSL cells from young B6xG7 mice induced to develop arthritis by serum transfer and control B6xG7 mice injected with PBS. Ig heavy chain molecules γ1, γ2b, γ3 and Ig light chain molecule κ were assayed.

I. Surface IgG expression of KSL cells from old (22 months) B6xG7 mice (Black Histograms) compared with young B6xG7 mice (Orange Histograms). Same antibodies used in Figure 7G were used.

J. QRT-PCR analysis of germline and post switch transcripts (GLT and PST respectively) of immunoglobulin heavy chain genes (μ, γ1, γ2b, γ3) in sorted arthritic KSL cells (black) and KSL cells from two control strains (B6xG7 – red, KRN – blue).

Insert: PST abundance normalized to GLT abundance of the γ heavy chain isotypes.

CHAPTER 4

CELL INTRINSIC FUNCTIONAL DIFFERENCES BETWEEN ARTHRITIC AND CONTROL PRIMITIVE PROGENITORS

Most of the contents of this chapter has been published in Oduro KAJ et al, 2012. *Blood* 120:2203

OVERVIEW

Our gene expression analysis (Chapter 3) suggested that primitive progenitors including HSCs were primed towards myeloid development by virtue of upregulated myeloid specific transcripts. KSL cells from arthritic mice also shared certain unique similarities with primitive progenitors from aged wild type mice specifically the upregulation cell surface P selectin and IgG. These KSL changes correlated with increased myeloid output in arthritic mice (Chapter 2). However, the magnitude of gene expression changes in arthritic KSL cells for most genes was modest. Increased cell cycling, which is typically associated with activated progenitors, does not occur in arthritic primitive progenitors either (Chapter 2). Therefore the question still remains whether the gene expression changes in arthritic KSL cells have any functional significance with respect to KSL properties.

Therefore in this chapter, we sorted KSL cells from arthritic and control mice and prospectively tested their lineage potential *in vitro* and *in vivo*. With the help of competitive and non-competitive, instructive and permissive assay conditions, we define concepts of *capacity* and *propensity* in understanding progenitors that on the one hand are still “primitive” but on the other hand are “lineage primed”. We demonstrate functionally that arthritic primitive progenitors indeed have superior myeloid generation potential and are thus myeloid primed.

IN VITRO ASSESSMENT OF MYELOID PRIMING

Arthritic KSL cells have increased myeloid potential *in vitro*

As mentioned earlier, *in vitro* colony formation in semi-solid medium replete with differentiation promoting cytokines was not significantly different between arthritic and control KSL cells while both populations had superior activity to GMPs (Chapter 3; Figure 1b). However, recent studies have shown that myelopoietic cytokines in culture act *instructively* rather than providing a *permissive* environment for hematopoietic differentiation [1-3]. Therefore while they allow evaluation of differentiation *capacity*, as utilized earlier for clarifying KSL versus GMP

properties (Chapter 3), they are inadequate in evaluating the relative *propensities* of arthritic and control KSL cells in generating particular lineages.

We therefore established a "competitive in vitro culture system" in which equal numbers of arthritic and control KSL cells were mixed in liquid culture without differentiation cytokines but with KitL and Flt3L to promote viability [4-6] (Figure 1A). Arthritic and control KSL cells were obtained from mice with different CD45 (Ly5) alleles to allow tracking of their relative cell output. Under this permissive condition there was about up to a 13-fold expansion of cells after 3-4 days of culture although this was variable. Twenty percent of these cells still remained Kit+Lin- indicating they were still immature (Data Not Shown). About 40-60% of all cells generated were Mac1/Gr1+ but negative for other lineage markers (B220 for B cells, CD3 and TCRβ for T cells, and Ter119 for erythrocytes) and therefore were myeloid cells (Figure 1B). Thirty percent of these myeloid cells expressed Kit indicating that the myeloid population consisted of a mixture of cells with different levels of maturity (Data Not Shown). Consistent with a greater myeloid propensity of arthritic KSL cells, myeloid cells (Mac1/Gr1+B220-CD3-TCRβ-Ter119-) derived from arthritic KSL cells was about 3-fold more than those derived from control KSL cells (Figure 1B & 1C).

While the inflammatory phenomenon in arthritic mice is characterized by increased neutrophils, the bone destruction that occurs at the joint is mediated by osteoclasts, which are also of myeloid lineage. The bone marrow Mac1+Gr1lo population that was increased in arthritic mice (Chapter 2: Figure 6A) has previously been reported to also contain osteoclast precursors [7]. Furthermore our KSL gene expression analysis revealed upregulation in arthritic KSL cells of several genes previously implicated in osteoclastogenesis including Chi3l3 [8, 9], S100a8 [10], Ccr2 [11], Ccl9 [12-14] and Sh3bp2 [15]. On the other hand IFNγ, which suppresses osteoclastogenesis [16], and several IFNγ regulated genes were reduced in arthritic KSL cells while Adar1, which is a negative regulator of interferon signaling [17] was increased (Chapter 3: Figure 2E and Not Shown). Therefore, we also tested if arthritic KSL cells had a specific increase in osteoclastogenic potential as a part of the overall increase in myeloid generation propensity.

Osteoclasts are multinucleate cells resulting from cell fusion that are identified by a histochemical stain. This precludes the use of competitive assays to compare osteoclastogenic potential. However, previous attempts to generate osteoclast *in vitro* from early progenitors found that osteoclast generation occurred only after prior activation of the progenitors with IL-3 [18]. Therefore we surmised that an increased osteoclastogenic potential of arthritic KSL cells would be detectable even in non-competitive assays.

We prospectively sorted KSL cells from arthritic and control mice and cultured them *in vitro* in osteoclastogenic conditions – that is in the presence of M-CSF and RANKL – without any prior activation. Consistent with previous attempts, KSL cells from control mice largely failed to develop osteoclasts in vitro while KSL cells from arthritic mice were able to generate osteoclasts (TRAP+ multinucleate cells) (Figure 1D). Arthritic KSL containing wells displayed osteoclast potential ~80% (15/19) of the time. On the rare occasion that control KSL cells formed osteoclasts, these cells were invariably smaller and/or fewer than those formed from arthritic KSL cells (Figure 1D and not shown). This property was arthritis dependent because KSL cells sorted from 2 week old KRNxG7 mice, which is prior to onset of visible disease (joint swelling) also failed to develop osteoclasts (data not shown). Only 2000 KSL cells from arthritic mice was sufficient to form *in vitro* osteoclasts although the osteoclasts were not generated as robustly as could be generated from GMP committed progenitors (Figure 1D). Taken together, arthritic KSL cells have increased *in vitro* myeloid generation propensity that is also reflected in an increased *in vitro* osteoclastogenic potential.

Arthritic KSL cells have diminished lymphopoietic *propensity* in vitro

It still remained possible that arthritic KSL cells performed better in these assays because of some non-specific reason such as better viability in vitro. For example from microarray analysis (Chapter 3: Figure 2D) arthritic KSL cells had marked upregulation of the stress combative inducible heat shock proteins Hspa1a and Hspa1b, which may protect arthritic KSL cells during *in vitro* culture. Therefore we examined lymphopoiesis of KSL cells *in vitro*. KSL cells from either

KRNxG7 arthritic or control mice cultured on OP9 stromal cells with B lymphoid promoting cytokines KitL, Flt3L and IL-7 thrived and expanded 100-1000 fold relative to input KSL cell number after about a week of culture (Figure 2A). About 50% of these cells produced were B220+ cells, which were also Gr1- (Figure 2B and Data Not Shown). These B220+ cells segregated into 2 main populations: B220+CD11c-Mac1- B cell precursors and B220+CD11c+NK1.1- putative lymphoid dendritic cell precursors [4, 19] (Figure 2C). Under these *non-competitive* culture conditions, KSL cells from arthritic or control mice generated similar cell output, similar frequency of total B220+ cells and similar frequency of the B220+ subsets indicating they had similar lymphopoietic *capacity*. By comparison, GMP cells, which lack lymphopoietic *capacity* failed to appreciably expand under these conditions (Figure 2A); GMP seeded wells accumulated debris, and most cells were dead as determined by eosin viability dye staining (not shown).

Next, we compared lymphoid generation *propensity* of arthritic and control KSL cells in *competitive in vitro* cultures under the same conditions – on OP9 cells in the presence of KitL, Flt3L and IL7. B220+Gr1- cells were preferentially (about 2 fold) derived from control KSL cells (Figure 2D). B220+CD11c- B cell precursors and especially B220+CD11c+ putative lymphoid dendritic cells precursors were both preferentially derived from control KSL cells (Figure 2D). Therefore arthritic KSL cells have diminished lymphopoietic *propensity* compared with control KSL cells. This suggests that the increased myeloid output of arthritic KSL cells *in vitro* that we previously demonstrated (Figure 1) is specific and unlikely due to non-specific factors like increased survival. While arthritic KSL cells still have the *capacity* for differentiation into other lineages such as lymphoid lineages their *propensity* is compromised presumably resulting from their myeloid primed state.

Increased in vitro myeloid potential and decreased lymphoid potential is inducible in naïve mice by arthritogenic serum transfer

We showed earlier (Chapter 3) that upregulation of "myeloid inflammatory signature" genes defined in KRNxG7 arthritic KSL could be triggered in KSL cells in naïve mice induced to develop arthritis by arthritogenic serum transfer. This demonstrated the disease dependence of the gene expression changes. To determine if the increased myeloid propensity and decreased lymphoid propensity of KSL cells from KRNxG7 mice was indeed arthritis dependent, we induced serum transfer arthritis in B6xG7 and injected control mice with PBS. KSL cells from serum injected and PBS injected mice were then sorted and cultured competitively in both stromal cell free and OP9 culture media to test myeloid and lymphoid propensity respectively (Figure 3A). In all cases, KSL cells from serum transferred mice contributed more to Mac1/Gr1+ myeloid output (Figure 3B) while the very same KSL cells contributed less to B220+CD11c-Mac1- lymphoid output (Figure 3C). This corroborates the results with KRNxG7 arthritic KSL cells and shows that the increased myeloid and decreased lymphoid potential in vitro are arthritis dependent.

IN VIVO ASSESSMENT OF MYELOID PRIMING

Arthritic KSL cells have increased myeloid potential *in vivo*

To determine whether myeloid priming of arthritic KSL cells could also be manifested in an *in vivo* setting we performed competitive transplantation experiments. We initially generated mice that were histocompatible to both KRNxG7 arthritic mice and B6xG7 control mice but were Ly5 congenic with respect to both prospective donors to serve as transplantation recipients. Equal numbers of sorted KSL cells from arthritic and control mice with different Ly5 alleles were then transplanted into lethally irradiated young (2-3 months old) recipients (Figure 4A). This experimental set up allowed us to differentiate cells derived from arthritic KSL donor, control KSL donor and residual cells from the recipient based on Ly5 staining. Myeloid and B lymphoid cell output were tracked using Mac1 and B220 respectively. Co-staining with Gr1 and CD19 respectively confirmed the myeloid and B lymphoid status of cells analyzed (Not shown).

Six days after transplantation, we found that myeloid cells were preferentially derived from arthritic KSL cells (about 5 fold) (Figure 4B & 4C). Sixteen days post transplantation, myeloid cells were still preferentially derived from arthritic KSL cells although less so (about 2 fold) (Figure 4C). However at 5 weeks and beyond, myeloid cells were equally derived from arthritic and control KSL cells. On the contrary, B lymphoid contribution was greater from control mice with the difference disappearing with increased time post transplantation. Therefore arthritic KSL cells generate myeloid cells with relatively faster kinetics and lymphoid cells with slower kinetics compared with control KSL cells. In some experiments, both myeloid and lymphoid engraftment from arthritic KSL cells were lower than from control KSL cells (Data Not Shown). However even in this situation, myeloid engraftment from arthritic KSL cells was superior to lymphoid engraftment.

The similar contribution to myeloid and lymphoid cells from arthritic and control KSL cells beyond the very early weeks post transplantation was consistent with our previous transplantation experiments using whole bone marrow instead of sorted KSL cells [20]. Furthermore, the restoration of a balanced myeloid and lymphoid output from arthritic KSL cells corroborated our *in vitro* findings that arthritic KSL and control KSL cells have similar differentiation *capacity* even though arthritic KSL cells have a skewed differentiation *propensity* towards myeloid development.

Myeloid priming occurs in the most immature HSC fraction of arthritic KSL cells

Although our data so far suggests myeloid priming in the arthritic KSL population, it is unclear if the most immature self-renewing HSCs are also affected. Unequivocal detection of HSC contribution in *in vivo* transplantation requires assessment several weeks (at least 16 weeks and more convincingly 6 months) post transplantation [21]. At earlier time points, contribution by this so called long term reconstituting HSCs (LT-HSCs) is minimal and is confounded by contribution by less primitive HSPPCs with limited or absent self-renewal. Therefore the detection of preferential myeloid contribution of arthritic KSL cells only at early time points (<5 weeks; Figure 4C) suggested that myeloid priming or arthritic KSL cells might not exist in the self-

renewing LT-HSC fraction. Alternatively it was possible that LT-HSCs are indeed affected but in an environment dependent manner. Functional outcomes due to cell autonomous *genetic* modifications are easily detected long after transplantation because genetic modifications are stable. On the other hand, since *epigenetic* changes including environmentally induced perturbations are reversible, their functional outcome would only be detectable in the long term if the change is inherently stable or if the recipient environment maintains the change. We surmised that the environment of young B6xG7 recipients (Figure 4) could erase/reverse myeloid priming of arthritic LT-HSCs and was hence unsuitable to test myeloid priming of arthritic LT-HSCs.

Therefore we considered using old mice as recipients. Similar to arthritic mice, aged wild type mice have an increase in myeloid cells and an increased myeloid potential of HSCs [22]. This phenomenon is at least in part environmental driven because transplanted HSC enriched population from young mice generate significantly more myeloid cells and less B lymphoid cells in the old recipient relative to young recipient [22]. We reasoned that since aged environment supports myeloid development it would be less likely to reverse myeloid priming of transplanted cells. We competitively transplanted equal numbers of arthritic (KRNxG7) and control (B6xG7) KSL cells from young mice (2-3 months old) into old (20 months old) B6xG7 recipients (Figure 5A). Donors and recipients were congenic with respect to Ly5 allowing us to distinguish KRNxG7 donor, B6xG7 donor and B6xG7 recipient derived cells.

Eight weeks after transplantation, myeloid cell frequency in peripheral blood was around 40%, higher than myeloid cell frequency of young recipients, and indicating that environmental effects of the aged recipient is preserved despite lethal irradiation and transplantation (Figure 5B). At this time point (8 weeks) myeloid (Mac1+Gr1hi) cells in the old recipient mice were preferentially derived from arthritic KSL cells (1.7 fold) while in contrast B lymphoid (B220+) cells were equally derived from donor arthritic and control KSL cells (Figure 5C & 5D). In fact relative contribution to Mac1+Gr1hi myeloid cells by arthritic KSL cells was still greater than relative contribution to B220+ cells at 16 weeks and 6 months post transplantation (Figure 5D), time points reconstituted solely by LT-HSCs [21]. Therefore, LT-HSCs within arthritic KSL cell

population have greater *in vivo* myeloid potential than control LT-HSCs but is undetectable when young recipients are used.

Primitive progenitors from arthritic mice also engraft old recipients better than control cells as evinced by bone marrow KSL chimerism analysis 6 months post transplant (Figure 5E). This might also explain the increase in arthritic KSL derived lymphoid and myeloid cells at 16wks and 6 months post transplantation (Figure 5D). In fact, similar results are obtained when young recipients which showed a balanced lineage output 9 weeks post transplantation (Figure 4C) are analyzed several (7-10) months after transplantation when these recipients are no longer chronologically "young" (Figure 5F & 5G). 10 months after transplantation contribution of arthritic KSL cells to both lymphoid and myeloid lineages (Figure 5F) as well as to bone marrow KSL cells is increased (Figure 5G).

Lastly, we transplanted KSL cells from old donors into young recipients using KSL cells from young B6xG7 donors as competitors (Figure 6A). 20 month old KRNxG7 arthritic KSL cells or B6xG7 KSL cells reconstituted 50-70% of myeloid cells but only 15-30% of lymphoid cells consistent with the known lineage skewing of primitive progenitors with age [22]. At 16 weeks post transplantation when HSC output can be assessed, we found that KSL cells from old (~20 month old) KRNxG7 arthritic mice performed better in myeloid reconstitution than in lymphoid reconstitution (Figure 6B). Myeloid reconstitution from old KRNxG7 arthritic mice was not significantly different from old B6xG7 myeloid reconstitution (Figure 6B). However, lymphoid reconstitution from old KRNxG7 arthritic mice was about half the lymphoid reconstitution from old B6xG7 mice (Figure 6B). We did not see these effects when we used 5 month old donors instead of 20 month old donors (Figure 6C). Therefore increased duration of arthritis – 20 month instead of 2 months (Figure 4C) or 5 months (Figure 6C) – increases the epigenetic stability of the myeloid primed state in arthritic KSL cells permitting its detection in young recipient mice.

MOLECULAR MECHANISM OF MYELOID PRIMING

Possible Roles for TLR4 and S100 proteins

The molecular mechanism by which myeloid priming occurs in arthritic primitive progenitors is unclear at the moment. Transcription factors Stat3 and C/EBPβ and the IL6 co-receptor IL-6st (gp130), which play crucial roles in non-steady state granulopoiesis [23-25], are all upregulated in arthritic KSL cells. However it remains to be established whether they act at the KSL level or in downstream GMPs or myeloid precursors. On the other hand, TLR4 stimulation using LPS drives proliferation and differentiation of KSL cells into myeloid cells [4, 26]. A number of studies have highlighted the importance of TLR4 in various arthritis models [27-34]. In the K/BxN arthritis model in particular, it has been shown that while TLR4 mutant mice developed arthritis upon K/BxN arthritogenic serum transfer, the arthritis was short lived compared with arthritis induced in TLR4 sufficient mice [27]. Furthermore, the TLR4 ligand, LPS exacerbates arthritis in TLR4 sufficient animals while permitting arthritis development in IL-1R knockout mice, which are otherwise resistant to serum transfer arthritis [27, 35].

Therefore we first examined expression of TLR4 on the surface of KSL cells. TLR4 is detectable on KSL cells as previously reported [4] but is very low (Figure 7A). Interestingly we found that the TLR4 signal was diminished on the surface of arthritic KSL cells while intracellular TLR4 was abundant and similar between arthritic and control KSL cells (Figure 7B). Even though the KRNxG7 model is free of LPS, this result was reminiscent of the effect of LPS stimulation, which also decreases cell surface TLR4 signal by receptor internalization or altered conformation [36, 37]. We therefore predicted that arthritic KSL cells might exhibit diminished responsiveness to *de novo* LPS stimulation. We sorted KSL cells from KRNxG7 arthritic mice and CD45 congenic B6xG7 mice and set up *in vitro* stromal cell free competitive culture in the presence of different concentrations of LPS (Figure 7C). We found that while arthritic KSL cells generated more myeloid cells than control KSL cells did in the absence of LPS, consistent with the myeloid primed state of arthritic KSL, the myeloid contribution from arthritic KSL cells decreased with increasing LPS dose (Figure 7D). This effect was recapitulated in *in vitro* competitive stromal cell free culture

using KSL cells from B6xG7 serum transferred arthritic mice versus B6xG7 PBS transferred control mice (Figure 7E). Therefore arthritis renders KSL cells more refractory to LPS stimulation by reducing responsive TLR4 on the cell surface.

This raises two questions. First, is signaling through TLR4 required for the myeloid primed state of arthritic primitive progenitors such that signaling by some endogenous molecule drives the myeloid primed state and downregulates surface TLR4 in the process? A candidate molecule in this respect is S100a8 which we found to be consistently and markedly increased in arthritic KSL cells and more primitive HSCs (CD150+CD48-CD34-KSL; See Chapter 3). Recently, it was reported that S100a8 is capable of acting as an endogenous TLR4 ligand [38]. S100a8 protein typically heterodimerizes with the related protein S100a9, which probably stabilizes the S100a8 protein since S100a8 protein is undetectable in S100a9-/- mice despite the presence of S100a8 mRNA [39]. S100a9 was also increased in arthritic KSL cells and HSCs. S100a8 and S100a9 are in fact the most abundant protein in the cytoplasm of mature neutrophils and have been implicated in arthritis and other inflammatory states [40, 41]. It has also been reported that transgenic overexpression of S100a9 skews development of myeloid derived suppressor cells over dendritic cells and macrophages in cancer [42] indicating that S100 proteins can affect differentiation outcome. We are currently conducting experiments to test the hypothesis that endogenous S100a8/S100a9 produced by arthritic HSCs promote the myeloid propensity of these cells by autocrine stimulation of TLR4 pathways or other means.

The second possibility is that cell surface TLR4 signaling is a negative regulator of the myeloid primed state such that its downregulation on the cell surface promotes myeloid priming of primitive progenitors in arthritic mice? Endogenous TLR4 ligands like S100a8 could still be involved in this mechanism but in this case, TLR4 downregulation not necessarily TLR4 signaling is what drives myeloid priming.

To determine the role of TLR4 in myeloid priming of primitive progenitors in arthritis we would use TLR4 -/- mice. The most obvious experiment will be serum arthritis induction in TLR4-/- versus TLR4+/+ mice followed by KSL sorting and functional assessment. However since the

course of arthritis disease is different in these two strains we would be unable to distinguishably attribute a reduced myeloid potential in KSL cells from TLR4-/- mice to disease severity (KSL TLR4 independent) or to a direct TLR4 dependent effect on KSL cells.

Therefore we have established an experimental scheme which would be widely applicable in teasing out the molecular mechanisms of KSL myeloid priming *in vivo* (Figure 8). This involves generating bone marrow chimeric mice from the mutant mice (e.g. TLR4-/-) and CD45 congenic wild type mice. This is followed by serum arthritis induction of the chimeric mice after engraftment and steady state is achieved (typically 6-8 weeks). In most cases mice will still be susceptible to the disease because of the presence of wild type cells in the chimeric mice. More importantly, both wild type and mutant KSL cells would be exposed to the same disease environment. Mutant and wild type KSL cells can then be sorted based on different CD45 allele expression and myeloid potential assessed prospectively.

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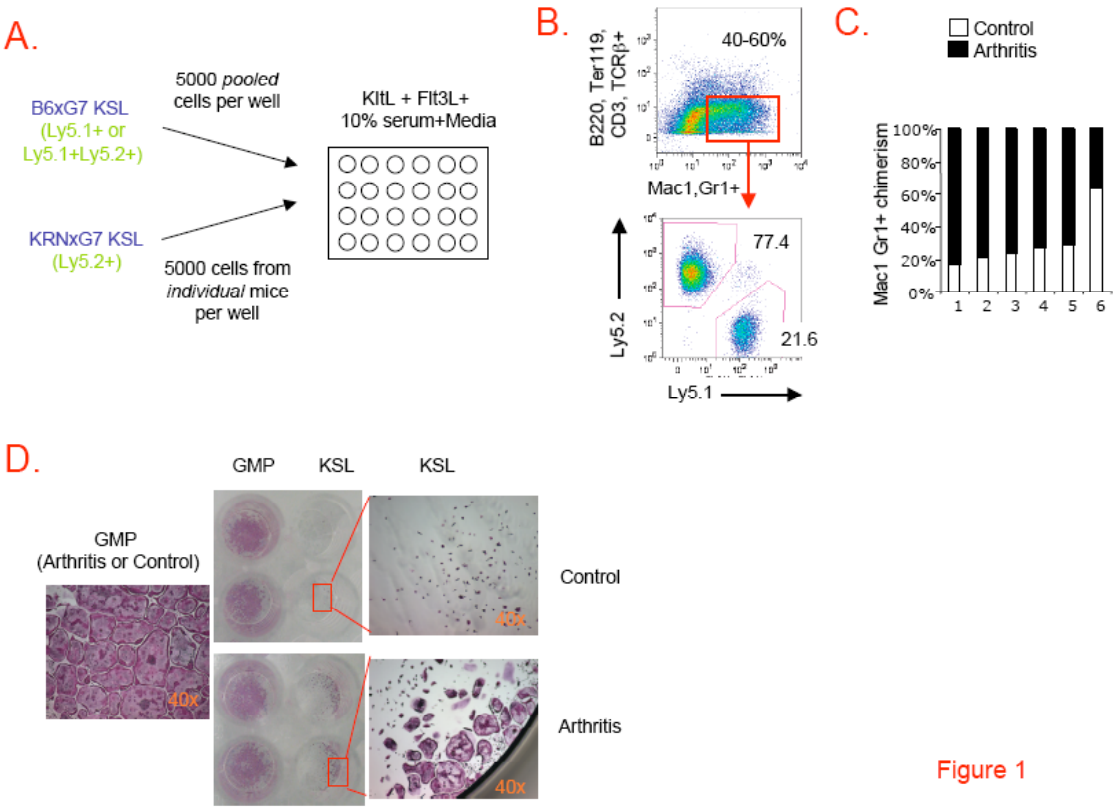

Figure 1. Arthritic KSL cells have increased *in vitro* myeloid potential

A. Experimental scheme for *in vitro* competitive culture of arthritic and control KSL cells. Arthritic and control mice have different CD45 (Ly5) alleles. 5000 each of arthritic and control KSL cells were mixed in the same well of a 24 well plate with survival cytokines, KitL+Flt3L and media containing 10% serum.

B. *Top*: FACS plot depicting myeloid differentiation of KSL cells *in vitro* after 3.5 days of culture. Myeloid cells (red gate) are Mac1+ and/or Gr1+ and negative for B cell (B220), T cell (CD3, TCRβ) and Erythrocyte (Ter119) markers. About 40-60% of cells were present in this gate. *Bottom*: FACS plot depicting chimerism analysis to determine arthritic KSL derived (Ly5.2+) and control KSL derived (Ly5.1+) myeloid cells.

C. Summary of *in vitro* competitive culture results. Each bar represents outcome of KSL cells from 1 independent arthritic mouse competed with control KSL cells. In 5/6 cases arthritic KSL cells (black) contributed greater to myeloid cell output.

D. TRAP stained wells at least 6 days after culturing sorted KSL and GMPs with M-CSF and RANKL to promote osteoclastogenesis. Low magnification camera images and high magnification microscopy images of wells are shown. TRAP+ cells are pink/purple. The few TRAP+ cells in wells seeded with control KSL cells are not multinucleate unlike arthritic KSL and GMP derived cells.

***p<0.001

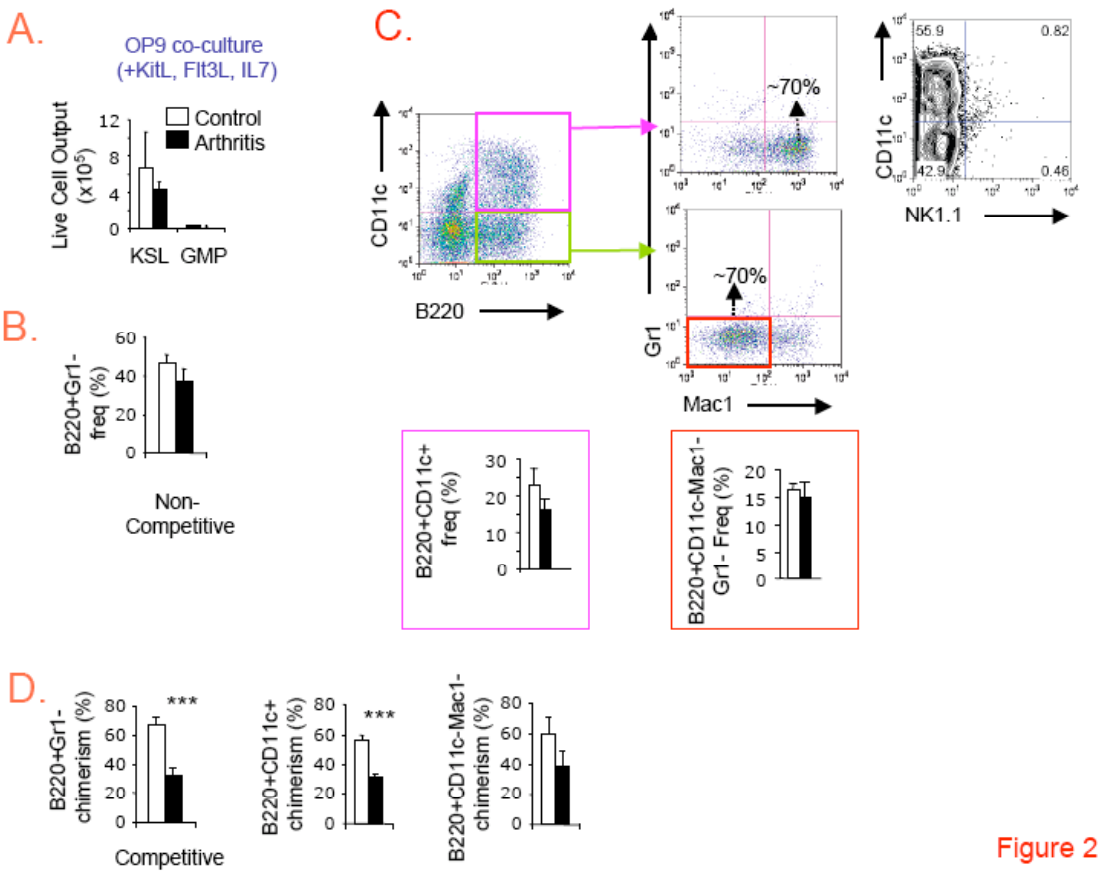

Figure 2

Figure 2. Arthritic KSL cells have reduced *in vitro* lymphoid potential

A. Cell output 6-7 days after growing 3000 sorted BM KSL cells or GMPs from arthritic (KRNxG7) and control (G7) mice on irradiated OP9 feeder cells in the presence of KitL, Flt3L and IL-7 to promote B cell differentiation.

B. Frequency of B220+Gr1- after culturing arthritic (KRNxG7) and control (G7) KSL cells on irradiated OP9 cells and media containing 10% serum, KitL, Flt3L, IL-7 to promote lymphoid cell formation. “Non competitive” culture means KSL cells from arthritic and control mice were seeded into different wells and were not mixed. Although G7 control mice are used here, similar results are obtained when other control strains are used (Data not shown).

C. Schematic of detailed FACS analysis of B220+ cells from D5-7 OP9 culture of KSL cells and frequency of B220+ subsets. B220+ consist of a CD11c+ subset that are predominantly Mac1+ but Gr1- that are also NK1.1- (putative dendritic cell precursors) and CD11c- subset that are predominantly Mac1-Gr1- (putative early B cell precursors). NK1.1+ cells (NK lineage cells), subsets of which express B220, are not generated.

D. Relative contribution of arthritic (KRNxG7) and control (KRN) KSL cells to total B220+Gr1- cells, B220+CD11c+ and B220+CD11c-Mac1- cells when KSL cells are seeded in the same wells and grown competitively (1:1) on irradiated OP9 cells with KItL, Flt3L and IL-7. Control and arthritic KSL cells were distinguished by CD45 (Ly5) allele expression. A different cohort of mice (N=3 each time) was used for the total B220+ analysis and the B220+ subset analysis.

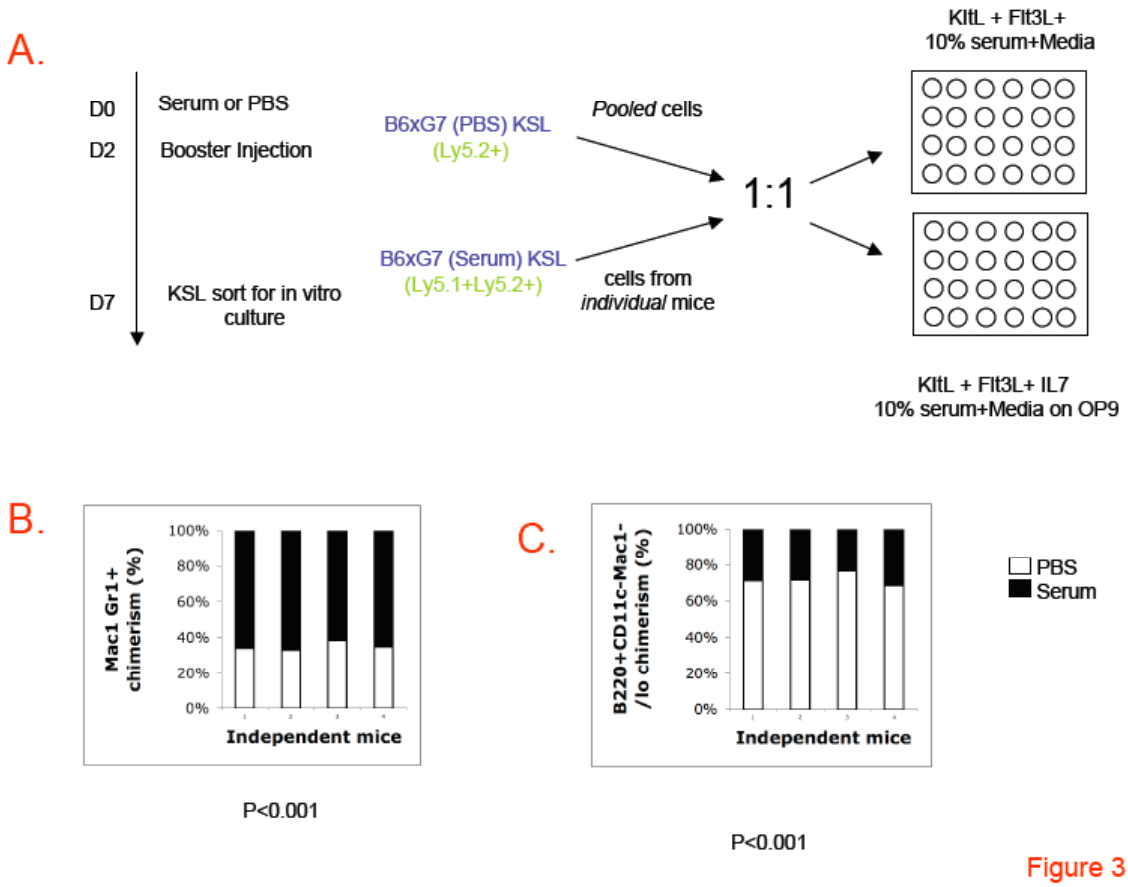

Figure 3. Increased myeloid and decreased lymphoid potential can be induced in KSL cells by arthritogenic serum transfer into naïve mice

A. Experimental scheme arthritis induction in naïve mice (B6xG7) by injection of serum. Control mice were injected with PBS. Serum injection was administered on Day 0 (D0) and D2 and KSL cells sorted for competitive *in vitro* stromal cell free and OP9 culture with indicated cytokines on D7.

B. Relative contribution of arthritic (serum injected) and control (PBS injected) KSL cells to Mac1/Gr1+ cells generated in *in vitro* stromal cell free culture. Each bar represents outcome of KSL cells from 1 independent arthritic mouse competed with control KSL cells.

C. Relative contribution of arthritic (serum injected) and control (PBS injected) KSL cells to B220+CD11c-Mac1- B cells generated in *in vitro* culture on OP9 cells with lymphopoietic

cytokines. Each bar represents outcome of KSL cells from 1 independent arthritic mouse competed with control KSL cells.

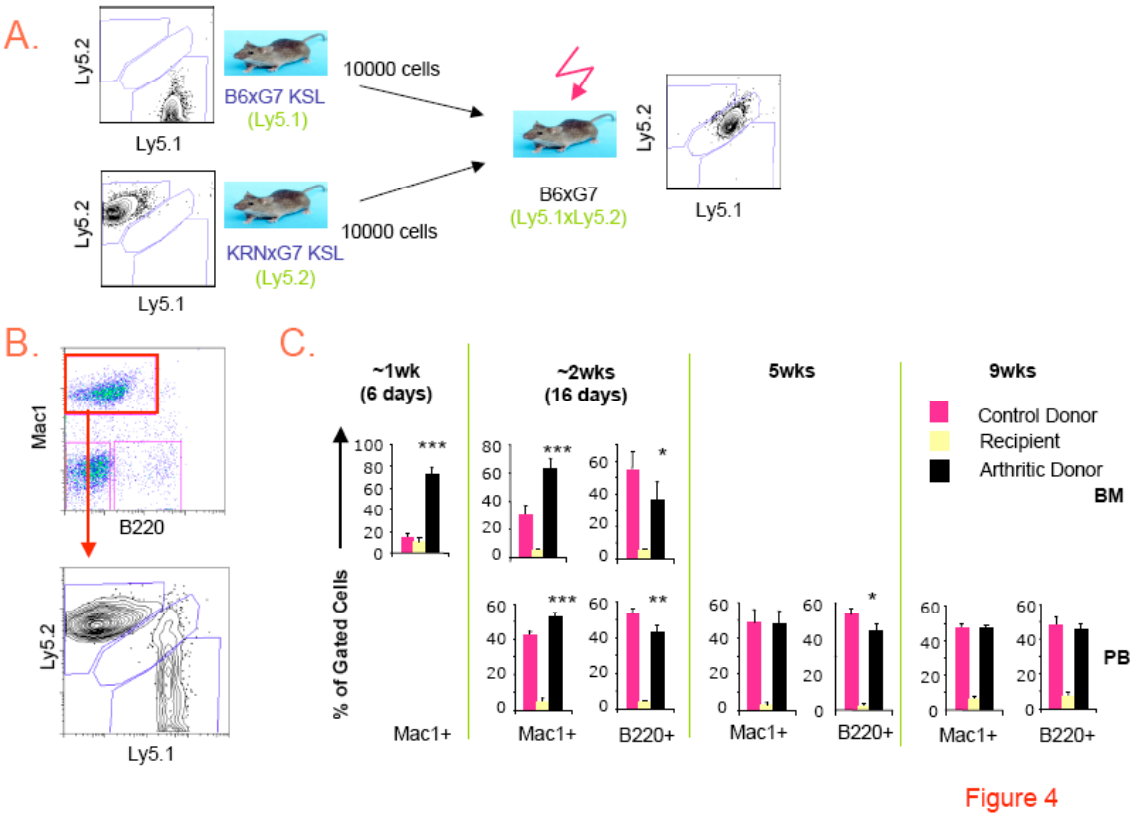

Figure 4

Figure 4. Arthritic KSL cells have increased *in vivo* myeloid potential although it is not sustained in young recipients

A. Experimental scheme for competitive *in vivo* transplantation. Donors and recipients have distinct Ly5 alleles.

B. FACS plot depicting analysis of Ly5 chimerism of bone marrow Mac1+ cells in recipient mice 6 days after transplantation.

C. Quantification of arthritic and control KSL contribution to Mac1+ and B220+ cells in bone marrow (BM) and peripheral blood (PB) based on Ly5 chimerism.

*p<0.05**p<0.01***p<0.001 relative to control KSL derived cells

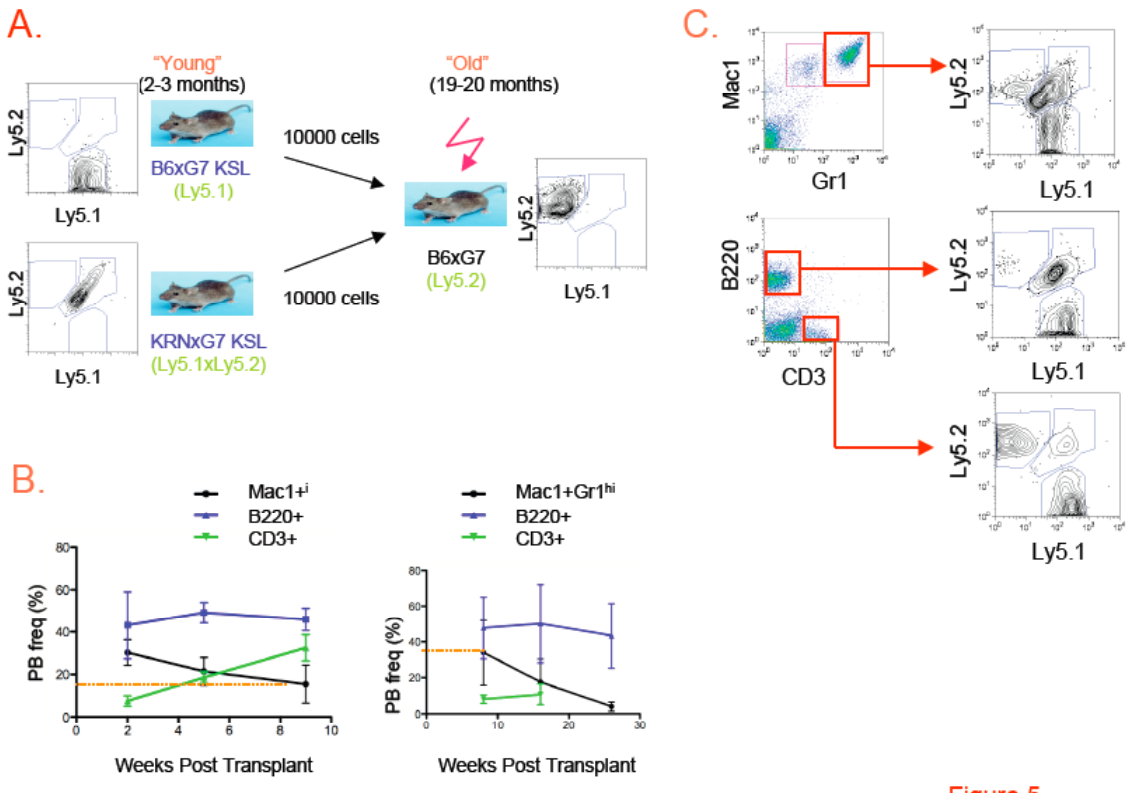

Figure 5

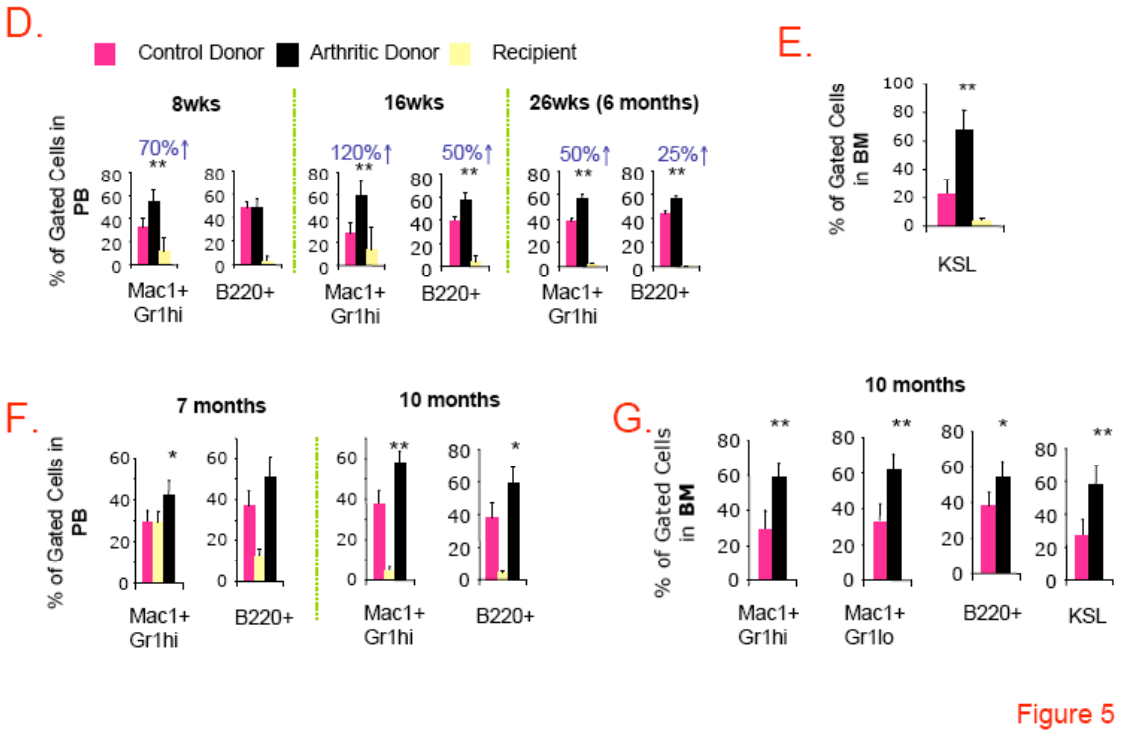

Figure 5

Figure 5. Long term *In vivo* myeloid potential of most primitive HSC subset of arthritic KSL cells is revealed by old recipient transplantation

A. Experimental scheme for transplantation into old (~20 month old) B6xG7 recipients. Note that CD45 alleles of B6xG7 donor, KRNxG7 donor and B6xG7 recipient mice are slightly different from what was used for the young recipient experiment.

B. Peripheral blood (PB) frequency of myeloid (Mac1+), B cells (B220+) and T cells (CD3+) after competitive transplantation of arthritic and control KSL cells. Extrapolation (orange hatched line) indicates myeloid frequency about 2 months post transplantation.

   Left: Young (2-3 month old) recipients (See Figure 4). At early time points Mac1+ cells express Gr1 homogenously and $Gr1^{hi}$ versus $Gr1^{lo}$ cells cannot be resolved (Not Depicted). PB Myeloid frequency 2 months post transplantation is <20%.

   Right: Old (20 month old) recipients (See Figure 5A). PB Mac1+ frequency 2 months post transplantation is about 40%. CD3 frequency was not determined at the 26 wk time point.

C. Representative FACS plots showing gating scheme for analysis of myeloid cells (Mac1+Gr1hi), B cells (B220+; also CD3-) and T cells (CD3+; also B220-) in peripheral blood of recipient mice 8 weeks after transplantation. Gated cells are further analyzed for CD45.1 (Ly5.1) versus CD45.2 (Ly5.2) to determine arthritic donor derived cells (Ly5.1+) versus control donor derived cells (Ly5.1+) versus recipient derived cells (Ly5.1+Ly5.2+).

D. PB chimerism 8 weeks (wks), 16wks and 26wks (6 months) post transplantation using old recipients gated on Mac1+Gr1hi myeloid cells or B220+ cells. Percentage change of arthritic KSL derived Mac1+Grhi or B220+ cells relative to control KSL derived cells is indicated in blue.

E. BM KSL chimerism 26wks post transplantation using old recipients

F. PB chimerism 7 months and 10 months post transplantation using young (2-3 month old) recipients gated on Mac1+Gr1hi myeloid cells or B220+ cells. These are the same recipients used for 5 week- and 9 week- analyses in Figure 4c.

G. BM chimierism 10 months post transplantation into young recipients. These are the same recipients used for 5 week- and 9 week- analyses in Figure 4c and 7 month analysis in Figure 5F.

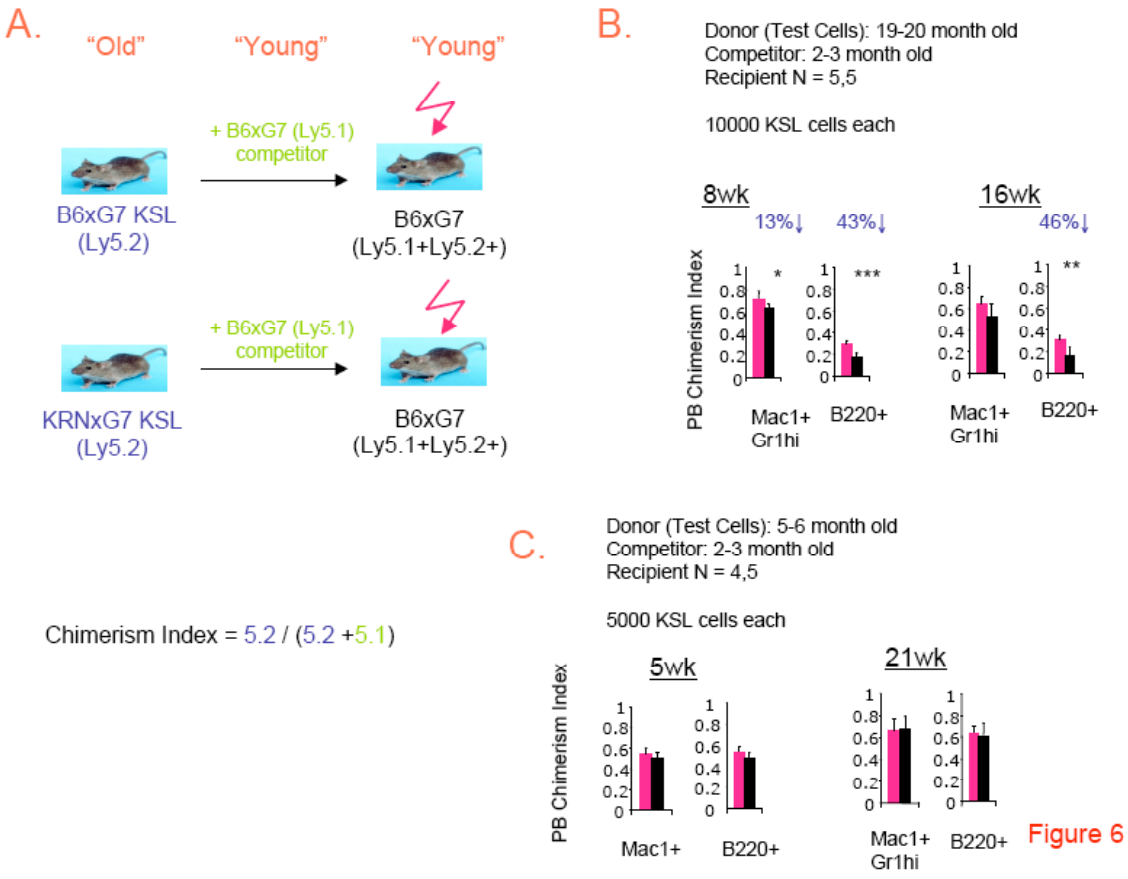

Figure 6. *In vivo* transplantion of old donors in young recipients

A. Experimental scheme for transplantation of KSL cells from "old" donors into young recipients. Old donors were either 5 months or ~20 months old; KRNxG7 and B6xG7 test cells were always age matched in 2 different experiments. "Old" B6xG7 or KRNxG7 with a CD45 allele other than CD45.2 was not available. Therefore rather than compete the old B6xG7 and KRNxG7 KSL cells head-to-head in the same recipient as done in Figure 4 and 5, they were each competed with an equal number of young congenic B6xG7 (Ly5.1) KSL cells.

Chimerism index, calculated as shown, gives the contribution of B6xG7 or KRNxG7 KSL test cells normalized to total cell output in the recipient.

B. PB chimerism index for 20 month old KRNxG7 and B6xG7 donors

C. PB chimerism index for 5 month old KRNxG7 and B6xG7 donors

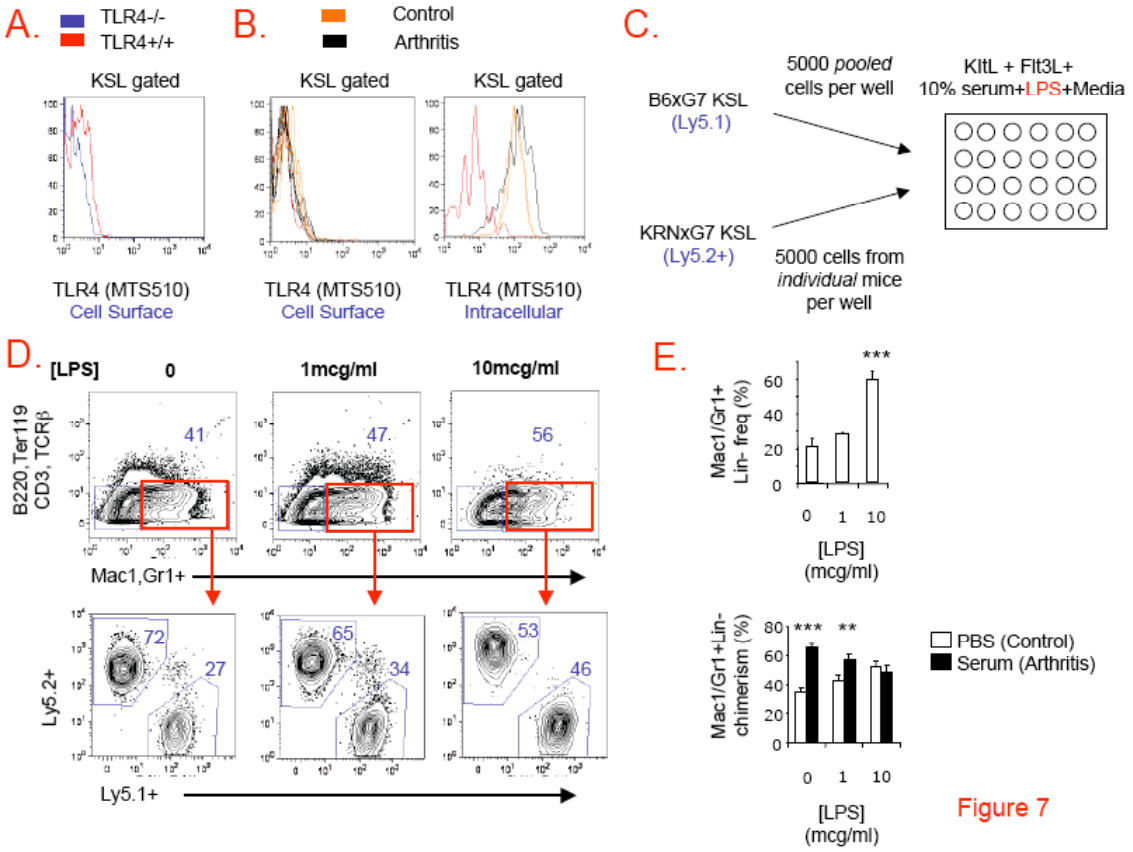

Figure 7. Arthritic KSL cells downregulate surface TLR4 protein and are refractory to LPS stimulation

A. Validation of α TLR4 antibody (clone MTS510). BM from TLR4-/- and B6 (Ly5.1) mice, which are TLR4+/+ were stained with KSL markers as well as PE conjugated αTLR4 antibody. Histogram indicates cell surface TLR4 protein expression by KSL cells

B. Cell surface and intracellular TLR4 protein expression in arthritic (KRNxG7) and control KSL cells. Isotype control staining is shown in red.

C. Experimental scheme for *in vitro* competitive culture of arthritic and control KSL cells. Arthritic and control mice have different CD45 (Ly5) alleles. 5000 each of arthritic and control KSL cells were mixed in the same well of a 24 well plate with survival cytokines, KitL+Flt3L and media containing 10% serum with different concentrations of LPS.

D. *Top*: FACS plot depicting myeloid differentiation of KSL cells *in vitro* after 3.5 days of culture with different doses of LPS. KRNxG7 KSL cells and B6xG7 KSL cells were used. Myeloid cells (red gate) are Mac1+ and/or Gr1+ and negative for B cell (B220), T cell (CD3, TCRβ) and Erythrocyte (Ter119) markers.

   Bottom: FACS plot depicting chimerism analysis to determine arthritic KSL derived (Ly5.2+) and control KSL derived (Ly5.1+) myeloid cells.

E. *Top*: Mac1/Gr1+B220-CD3-TCRβ-Ter119- frequency after in vitro competitive culture of arthritic KSL cells (serum injected B6xG7 mice) and control KSL cells (PBS injected B6xG7 mice).

   Bottom: Relative contribution of KSL cells to Mac1/Gr1+B220-CD3-TCRβ-Ter119- cells.

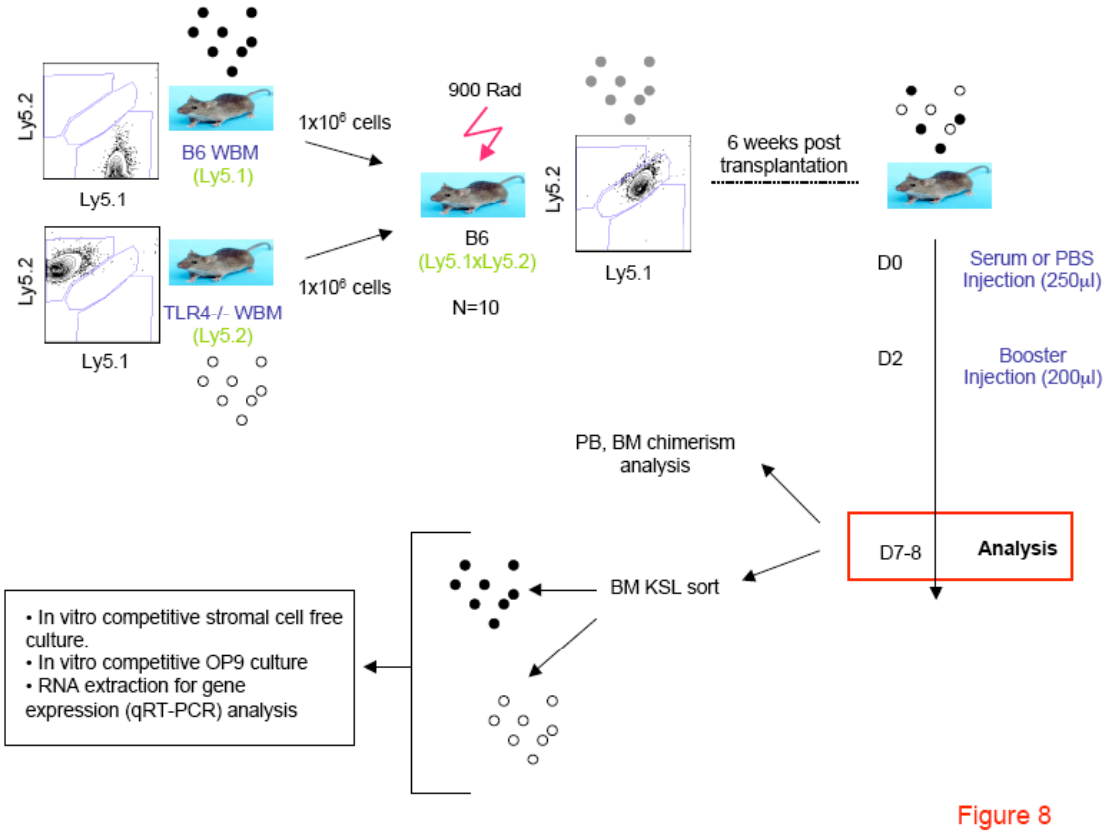

Figure 8. Experimental scheme for determining the role of TLR4 in the myeloid priming induced in KSL cells by arthritis

Bone marrow chimeric mice are generated from TLR4-/- and CD45 congenic wild type mice. This is followed by serum arthritis induction of the chimeric mice after engraftment and steady state is achieved (typically 6-8 weeks). TLR4-/- and TLR4+/+ KSL cells can then be sorted based on different CD45 allele expression. Gene expression or Prospective differentiation assays can then be performed with these cells.

CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

SUMMARY OF MAJOR FINDINGS

While a lot is known about the behavior of primitive progenitors in physiological states, the question of if and how these cells are affected by disease and how they in turn contribute to the disease process has been minimally explored. The immune response to infectious and inflammatory states has almost invariably been studied at the level of mature cells. In this study, we have shown that bone marrow primitive progenitor frequency and cycling is unchanged in arthritic mice although it is markedly increased in the spleen. Using *in vivo* transplantation, *in vitro* differentiation and gene expression analyses of sorted cells, we have shown that primitive progenitors including HSCs from arthritic mice are myeloid primed although they retain the *capacity* to differentiate into other lineages. These cell intrinsic changes are environment dependent because firstly, it is reversed when arthritic KSL cells are transplanted into young wild type (B6xG7) environment and secondly, it can be triggered in KSL cells from normal B6 mice induced to develop arthritis by arthritogenic serum transfer. This myeloid priming of arthritic HSCs correlates with skewed myeloid frequencies at the committed progenitor and mature cell levels in the arthritic mice.

DISEASE RELEVANCE

Role of primitive progenitor myeloid priming in disease

Myeloid cells including neutrophils, monocyte lineage cells and osteoclasts are crucial for the joint specific inflammation and destruction as well as the systemic inflammation that occur in arthritis [1-9]. Our findings that primitive progenitors in arthritic mice are myeloid primed suggests that primitive progenitors are co-opted to facilitate disease. Even though mechanisms occurring at the mature cell level such as increased survival [4, 10] undoubtedly contribute in prolonging chronic arthritis, these cells have a finite life span and need to be eventually replenished. Primitive progenitors including HSCs, which have self renewal ability, are myeloid primed in order to meet this pathologic demand thus fueling disease. In fact we found that the transcription factor Foxo3a which has been shown to promote arthritis by improving the survival of neutrophils is

already increased in arthritic KSL cells. We propose that there is a positive feedback loop that drives chronic disease; the inflammatory environment triggers myeloid priming and increased myeloid output from primitive progenitors which in turn perpetuates the inflammation in a vicious cycle.

The potential powerful role of HSCs and primitive progenitors in disease cannot be underscored since a single HSC can reconstitute an entire hematopoietic system. However, how do we show that HSCs or primitive progenitors indeed have a role to play in arthritis since these cells do not transfer the disease when transplanted into naïve recipients? The most obvious experiment to propose would be to selectively block myeloid priming in primitive progenitors, such as by a Cre mediated conditional knockout or knockdown of cell intrinsic molecules required for myeloid priming, and then assess the effect on disease outcome. There are a number of reasons why this experiment is not trivial. First, no Cre mouse has been generated that is specific to primitive progenitors. Mx-Cre and Vav-Cre mice that are currently used in HSC research will also be functional in other hematopoietic and/or non hematopoietic lineages. Second, the target molecule chosen should be one that does not also play a role in mature cells since these cells derive from primitive progenitors. This can be ruled out by parallel experiments where the target molecule is conditionally cleaved out using Cre molecules specific to mature cells such as Mac1-Cre or LysozymeM-Cre. The cell intrinsic molecules driving myeloid priming are currently unknown and it remains to be determined if molecules that facilitate disease at the primitive progenitor level but not at the mature cell level exists. Selective manipulation of primitive progenitors is important to rule out the possibility that amelioration of disease is due to changes at the mature cell level.

We are currently establishing a system that will allow us to evaluate the role of primitive progenitors in arthritis. We found that lethally irradiated mice were resistant to serum transfer arthritis most likely because of the ablation of their hematopoietic system especially short-lived neutrophils, which are required for arthritis initiation [1-4]. Lethally irradiated mice succumb to irradiation after 10-14 days allowing a time window for short-term experiments. In these lethally

irradiated mice we can transplant different cell types including primitive progenitors from wild-type and mutant mice and evaluate their ability to restore arthritis susceptibility following serum injection. Based on cell dose injected, volume of arthritogenic serum injected and disease incidence and severity elicited we would be able to score the contribution of a cell to disease induction.

We have found that a single injection of 20000 KSL cells (the only dose we have tried) restored disease susceptibility in all mice injected with 150μl (the only dose we tried) of serum. This in itself is significant because in previous studies, repeated injections of as much as $5x10^6$ to $1x10^7$ wild type BM or PB neutrophils per injection was needed to restore arthritis susceptibility in mutant mice with defective neutrophils [2-4]; the ratio of KSL cells to neutrophils in the bone marrow is about 1:100. Instead of irradiation, these neutrophil defective mice coud also be used as recipients. Irradiation or neutrophil defective mice are better options than using an anti-Gr1 antibody whose lingering effects days after treatment [1] would block the action of de novo generated neutrophils.

***In situ* involvement of primitive progenitors in arthritis**

In addition to increased *magnitude* of myeloid cell output, there is also the need for *directionality* with respect to joint disease. Rapid neutrophil infiltration into the joint following arthritogenic serum transfer is well documented [1, 11]. However, it is not clear for instance what the origin of osteoclasts in diseased joints are or whether like osteoclasts, some of the myeloid cells including neutrophils are generated *in situ* in diseased joints. HSCs and progenitors are known to circulate at low levels in peripheral blood and lymph transiting in and out of different organs and tissues but the biological relevance of this phenomenon has remained obscure [12-14]. Recently, Massberg et al showed that inflammatory signals mediated by Pertussis toxin or LPS can interfere with Sphingosine-1-phosphate mediated egress of progenitors from extramedullary sites and hence retain these cells at sites of inflammation [14]. Si et al have consequently shown that intraperitoneal aseptic inflammation induced by thioglychollate injection

recruits primitive progenitors including HSCs to the peritoneum [15]. In this study, we showed that KSL cells are increased in the peripheral blood of arthritic mice and that these KSL cells are not spleen derived because PB KSL frequency in asplenic arthritic mice is the same as in arthritic mice with intact spleen. Furthermore, unpublished work from the lab (Yang et al) has shown that CD45+Lin- cells are increased in arthritic joints; KSL cells could not be adequately evaluated because tissue preparation involved prolonged incubation with collagenase and dispase which has been shown to downmodulate cell surface Kit [16]. GFP labeled CD45+Lin- cells (from β-actin::GFP mice) injected into arthritic mice are also selectively recruited to the joint (Junjie Yang). These previous findings and ours raise the possibility that myeloid primed primitive progenitors are recruited to arthritic joints where they may contribute directly to myeloid effector cells including osteoclast by differentiating *in situ*.

In preliminary experiments, we have demonstrated that KSL cells from arthritic mice injected into the ankle/foot of RANK-/- mice undergoing serum transfer arthritis were able to develop into osteoclasts *in situ* without any added factors. RANK-/- mice were used because they are completely devoid of endogenous osteoclasts due to a cell autonomous defect; RANKL is still present to promote osteoclastogenesis of the transplanted progenitors. The contralateral ankle/foot, which did not receive any KSL cells, were completely devoid of osteoclasts. This system needs to be optimized. For example it is possible that there is an optimum time after injection of serum when the joint environment is most likely to promote osteoclastogenesis. This might be related to when RANKL is produced at the joint as well as when the relevant endogenous osteoclast precursors traffic to the joint. An optimized system will for instance allow comparison of *in situ* osteoclastogenic potential of circulating monocytes and bone marrow KSL cells. It will also allow molecular manipulation of sorted KSL cells prior to joint injection to determine which genes are mechanistically involved in *in situ* disease relevant joint osteoclastogenesis.

We have also initiated 2-photon microscopy experiments to allow us to monitor the trafficking of primitive progenitor to the joint based on imaging the toe of arthritic mice [17]. In this

system, arthritis is induced by subcutaneous footpad injection of arthritogenic serum (Baomei Wang). Arthritis that develops is initially confined to this foot although it eventually spreads to the contralateral foot. During the period of unilateral arthritis, we can image the diseased foot with the contralateral foot as a control. We were able to detect intravenously injected GFP+ Lin- cells (from β actin::GFP mice) in the diseased joint, although this needs to be repeated. We found that most injected cells were trapped in the spleen. Therefore, using asplenic Tlx1-/- mice in the future might improve sensitivity of detecting injected cells at the joint. Improved sensitivity would allow us to inject fewer cells allowing trafficking experiments of KSL and more enriched HSC populations to be more feasible.

With this system, we can address the question of whether the degree of recruitment of primitive progenitors versus mature cells at different times during the course of the disease is different. Are mature cells like neutrophils initially recruited with a later arrival of primitive progenitors? Also, based on the upregulation of molecules like Ccr2, MMP9 and P-selectin in KSL cells from KRNxG7 arthritic mice, we can also address the question of whether primitive progenitors from arthritic mice are recruited more robustly than primitive progenitors from non-arthritic mice. Test cells can be compared within the same recipients by obtaining cells from transgenic mice with different fluorescent proteins (e.g. GFP and RFP) or by labeling cells with fluorescent dyes like PKH-67 or CFSE and their different color variants. We can also determine if primitive progenitors recruited to the joint generate mature effector cells *in situ*. One way of doing this is to *in vitro* label KSL cells from Lysozyme M::EGFP with a red dye like PKH-26. Since the lysozyme M promoter is very minimally active in KSL cells [18] these cells will be mainly red while still in the primitive progenitor state. As they differentiate, the red dye becomes diluted out and if they differentiate into neutrophils this would correspond to the emergence of green cells as a result of the activation of the Lysozyme M promoter.

MYELOID PRIMED PRIMITIVE PROGENITORS IN ARTHRITIS AND NORMAL AGING

KRNxG7 arthritic mice have a number of features that are reminiscent of normal aging. These include weight loss, thymic atresia and low turnover osteoporosis, that is osteoporosis due to diminished bone formation rather than overactive bone resorption [19]. Within the hematopoietic system, we also find that KRNxG7 mice are mildly anemic, are lymphopenic and have preferential myeloid output all of which occur in normal aging. KSL gene expression analysis also revealed that upregulation of P selectin protein and IgH that occurs in aging primitive progenitors also occurs in KRNxG7 arthritic primitive progenitors.

There are however some important dissimilarities between aged primitive progenitors and arthritic primitive progenitors. Most prominently, some of the genes that were strikingly upregulated in arthritic KSL cells including S100a8, S100a9 and Chi3l3 were all previously reported to be downregulated in the normal aging of stem cells [20]. Furthermore, although increased myeloid potential of arthritic and aged primitive progenitors are both epigenetically induced, it appears the changes induced in the latter are more stable. In our own experiments (Chapter 4: Figure 6), we found that old wild type (B6xG7) KSL cells in competition with young wild type KSL cells still reconstituted the myeloid lineage better than lymphoid lineage (60% versus 30%) 16 weeks post transplantation. On the other hand, when young arthritic KSL cells were competed with young wild type KSL cells (Chapter 4: Figure 4), relative contributions to lymphoid and myeloid lineages were the same as early as 9 weeks post transplantation. Recent studies have suggested that normal aging is associated with the expansion of HSC clones identified by high expression of CD150 [21]. However, we do not see clear differences in the CD150 expression in the HSC enriched CD48- fraction of arthritic KSL cells compared with control KSL cells. This may in part underlie the differences between arthritic primitive progenitors and physiologically aged primitive progenitors. Interestingly, a recent study has also found that even though mice with accumulating mitochondrial DNA mutations share several hematopoietic features with physiological aging, there are still distinct differences [22, 23].

As we gain a better understanding of the cell intrinsic molecular underpinnings of myeloid priming in arthritic primitive progenitors, it will be interesting to discover how it converges and diverges from the mechanisms of physiological aging. It will also be interesting to study what aspects of the aged environment allow arthritic KSL cells to engraft better than control KSL cells and maintain their myeloid primed output. It has been found for instance that HSCs with the same phenotype located at different sites within the bone marrow have different lineage potentials [24]. Furthermore, primitive progenitors migration between niches within the bone marrow influences myeloid versus lymphoid fate *in vivo* [25]. Therefore, it is possible that myeloid supportive niches abound in aged mice and that these niches are preferentially occupied by arthritic KSL cells after transplantation leading to the results we obtained.

TOWARDS THE STUDY OF PRIMITIVE PROGENITORS IN OTHER DISEASED STATES

Primitive Progenitor Identification in Non-Steady State – Altered markers or Altered properties?

The use of fluorophore-conjugated antibodies to molecules expressed by cells, as is extensively used in this study, has greatly facilitated the identification and sub-fractionation of cells. This has proven indispensable in the prospective isolation and study of primitive progenitor at steady state. However, since much of these molecular markers have been validated based on cells at steady state, it cannot be guaranteed that the same cells in non-steady states will express the same markers. As different cytokines, antigens or diseases may have different unpredictable effects on primitive progenitors, it is important to confirm the applicability of the immunophenotype used for isolation of primitive progenitors in the diseased/treated mouse.

A very relevant molecule in this regard is Sca1, one of the molecules used for identifying "KSL cells". Various molecules particularly Type I and II interferons are potent inducers of Sca1 [26-38]. Since hematopoietic progenitors express cell surface interferon receptors [39] microbial

products or diseased states that induce interferon production upregulate Sca1 in hematopoietic progenitors. This can lead to Kit+Sca1-Lin- committed progenitors masquerading as HSC containing Kit+Sca1+Lin- (KSL) cells with concomitant depletion of phenotypic Kit+Sca1-Lin- cells. Very conspicuous Sca1 upregulation in hematopoietic progenitors, evident as a shift in a Kit versus Sca1 FACS plot of Lin- gated cells, is observed in mice following infection with *Mycobacterium avium*, *Escherichia coli*, *Ehrlichia muris, Plasmodium chabaudi* and Vaccinia virus [39-43]. Even though these phenotypic changes are reported, the significance of this is not acknowledged nor tested in the interpretation of the results of functional assays. Consequently, expansion and increased cycling of phenotypic “KSL cells” (or subsets) is interpreted as increased proliferation of HSC and primitive progenitors rather than due to the more numerous and proliferative committed progenitors with altered phenoytpe. Defects in long term multilineage reconstitution of irradiated recipients by these cells is interpreted as an HSC functional defect as is seen in genetic knockouts that result in loss of HSC quiescence [44] rather than due to dilution of true primitive progenitors by contaminating committed progenitors. Abolishment of these properties in interferon receptor knockout mice is interpreted as a role for interferon in modulating these properties rather than modulating Sca1 expression which is more likely. The inflammatory stimulus, for example LPS, sometimes induce emigration of HSCs and/or increase in cellularity of bone marrow in addition to Sca1 upregulation rendering whole bone marrow analysis inadequate in evaluating functional changes due to the inflammatory stimulus. Therefore, Sca1 upregulation may lead to internally consistent yet erroneous conclusions about primitive progenitor cell cycle state, function and molecular mechanism of these changes in some diseased state.

The arthritis model we used in this study is free of these issues that have plagued previous studies of primitive progenitors in non steady state. Arthritic KSL cells do not upregulate Sca1 at the mRNA level and neither do we notice any Sca1 shift in FACS analysis reminiscent of an immunophenotypic aberration. Cell cycling which is closely associated with the primitive status of progenitors is unchanged in arthritic KSL cells and CD150+CD48-KSL cells relative to controls. We show that although the arthritic KSL cells are myeloid primed they still retain a similar

multilineage *capacity* as control KSL cells by virtue of their similar methylcellulose colony output, mature cell output in non-competitive lymphogenic OP9 co-culture and restoration of a balanced lineage output in long term transplantation experiments. In this regard we implicitly confirmed that the KSL immunophenotype represents a bona fide primitive progenitor population as in control mice free from Sca1 or other cryptic identification artifacts. Beyond examining KSL cells, we also conducted gene expression analysis on CD150+CD48-CD34-KSL cells. At steady state, GMPs are actually CD150-CD34+. This makes it even more unlikely that the myeloid priming we see is due to GMPs masquerading as HSCs. This study shows that increased proliferation can be dissociated from other inflammation driven effects on HSCs, such as myeloid priming, in contrast with previous studies. Absence of HSC proliferation in inflammation does not imply HSC homeostasis is not affected just as increased proliferation does not only occur in a setting of inflammation.

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CHAPTER 6

EXPERIMENTAL PROCEDURES

ANIMALS AND ANIMAL TREATMENT

Animals

C57BL/6 (B6; Ly5.2) and B6.SJL-Ptprc^{a}Pep3^{b}/BoyJ (B6; Ly5.1) were purchased from Jackson Laboratory and maintained in our suite. KRN TCR transgenic mice on a B6 background (KRN; Ly5.2) and C57BL/6 congenic mice bearing arthritogenic I-A$^{g7/g7}$ MHC-II molecule (G7; Ly5.2) [1] were both obtained from Dr. Paul Allen (Washington University) and maintained in our suite. The G7;Ly5.1 strain was generated by crossing G7;Ly5.2 and B6;Ly5.1 mice first to generate B6xG7 (Ly5.1xLy5.2), and then intercrossing F1s to generate G7;Ly5.1. B6xG7 mice were generated from B6;Ly5.2 or B6;Ly5.1 crossed with G7;Ly5.2 or G7;Ly5.1 parents to get mice with different Ly5 allleles - Ly5.1+Ly5.2-, Ly5.1+Ly5.2+, Ly5.1-Ly5.2+. G7 genotyping was performed using a biotin conjugated anti – G7 antibody kindly provided by Dr. Emil Unanue (Washington University). KRN, G7 and or B6xG7 mice, which are all non-arthritic were used as controls in this study to arthritic KRNxG7 mice.

KRNxG7 were generated by crossing KRN mice with G7;Ly5.2 or G7;Ly5.1 mice to get arthritic mice of different Ly5 isotypes – Ly5.1-Ly5.2+ and Ly5.1+Ly5.2+ respectively. We found that placing food directly on the bedding of mouse cages drastically improved survival of KRNxG7 mice allowing some arthritic mice to attain a full lifespan. For the splenectomy experiment, mice were sedated, followed by shaving and sterilization of the surgical site. The spleen was pulled after making a small incision on the lateral skin. Vessels supplying the spleen were tied off and both the abdominal cavity and skin were sutured. Splenectomy experiments were done with Julie Ritchey a technician in Dr. John Dipersio's lab at Washington University.

Tlx1-/- mice previously generated [2] was obtained from Dr. Qiufu Ma (Harvard University). Genotyping primers for Tlx1 had to be redesigned since we found that published primers [3] contained nucleotide differences with the mouse genome that impaired the accuracy of genotyping results. For wild type Tlx1, forward primer: CTT CGG TAT CGA CCA GAT CC, reverse primer CGG AGC CCC CGC CGC CGT AAG TG. For mutant Tlx1, published primers [3] were used.

TNFRI-/- and TNFRI, II double knockout mice were obtained from Dr. Steven Teitelbaum (Washington University). MMP9-/- mice were obtained from Dr. Robert Senior (Washington University). IL6-/- mice were purchased from Jackson Laboratories. For generation of KRNxG7 mice deficient in specific genes (X), homozygous knockout mice (X-/-) were bred with KRN and G7 to obtain double heterozygotes (X+/-KRN+/- and X+/-G7+/-). Siblings were crossed to generate homozygous knockouts of the gene of interest with G7 homozygotes or with the KRN transgene (X-/-KRN+ and X-/-G7). These were then bred together to generate X-/- KRNxG7 mice. This complicated mating scheme had to be undertaken because KRNxG7 mice are non sterile precluding simply crossing these mice with X-/- mice. With the exception of TNFRI-/- and Tlx-/-, we failed to obtain KRNxG7 with homozygous knockouts of the other genes. For unknown reasons, generation of X-/- mice that were also KRN+ or the fertility of these mice was the major bottle neck in generating KRNxG7 X-/- mice.

Arthritis Transfer Models

C57BL/6 (B6; Ly5.2) or B6.SJL-Ptprc^{a}Pep3^{b}/BoyJ (B6; Ly5.1) were given a single intraperitoneal injection with 250μl of K/BxN arthritogenic serum (obtained from Dr. Paul Allen). Mice 7 days post serum transfer were used for experiments – hematopoietic frequency analysis or KSL sort for qRT-PCR experiments. Development of arthritis was confirmed visually and by measurement of ankle thickness. Arthritis development was variable and not as robust as arthritis in the KRNxG7 spontaneous chronic model. Based on past literature that arthritis severity occurs in a distal to proximal manner and our observation that some mice had toe/finger swelling without wrist swelling, we scored joint inflammation clinically as follows: Toe/Finger swelling = 1 point, Ankle/Wrist swelling = 2 points. Scores for each of 4 limbs are then added together to give a maximum score of 8 (most severe). Such a score is similar to what has been published before. The cohort of mice that were used for the frequency analysis had scores of 2,6,7 out of 8.

We found that a booster injection of 200μl of arthritogenic serum injected on day 2 led to more robust and uniform joint swelling. Therefore, this 2-dose regimen was used in the experiments to assess *in vitro* potential of sorted KSL cells.

For T cell transfer arthritis experiment, splenocytes of Cα B6xG7 mice [4] transplanted with KRN T cells polarized in vitro by Th17 promoting conditions [5] and control non-transplanted Cα B6xG7 were obtained and analyzed for KSL cells. We also found that transplantation of 1-2 x 10^6 splenocytes from KRN mice also induced disease in Cα B6xG7 mice. This would be used in future experiments.

Mouse Antibody Treatment

300μg of Anti Murine TNFα antibody (TN3-1912) or Anti GST control antibody (both kind gifts from Dr. Robert Schreiber, Washington University, St. Louis) was injected every 6 days from 20 days of age. Mice were analyzed at 6 weeks old.

Tissue Preparation

Mice were sacrificed by cervical dislocation or CO_2 asphyxiation. If peripheral blood (PB) analysis was to be performed, PB was collected prior to sacrificing mice by retro-orbital bleeding into buffer containing 4% Bovine Serum and 0.1-0.2% heparin in phosphate buffered saline (PBS) using heparinized capillary tubes.

Bone marrow was collected from tibia(e) and/or femur(s) by flushing using 25 gauge needles as previously described [6] or by brief high speed centrifugation of bones longitudinally aligned in a perforated eppendorf tube. Spleen was crushed between 2 glass slides or by crushing with the plunger of a 5ml syringe onto a 40μm nylon filter (BD). Inguinal, brachial and/or axillary lymph nodes were retrieved from mice and crushed between glass slides.

BM, PB or spleen cells were RBC lysed using RBC lysis buffer (Roche) or antibody stained for magnetic sorting without RBC lysis as described below. Peripheral blood was

subjected to 2 rounds of RBC lysis due to abundant red blood cells. Cellularity (of marrow or spleen) were always determined after RBC lysis and hence do not account for RBCs. Residual RBCs (low forward and side scatter) were also gated out during FACS analysis. Lymph nodes were not subjected to RBC lysis since they contain very few RBCs.

FLOW CYTOMETRY

General Flow Cytometry

For progenitor cell sorting, mature cells were initially depleted from whole bone marrow by magnetic sorting using the MACS lineage depletion kit (Miltenyi) and LS columns (Miltenyi). Bone marrow from 2 tibiae and 2 femurs (about $50x10^6$ cells) were incubated with 40μl of lineage antibody cocktail followed by 80μl of microbead conjugated anti-biotin antibody. For antibody staining, cells were typically incubated with antibodies in phosphate buffered saline (PBS) containing 4% serum. Staining was usually performed in 96-conical well plates. Staining was performed in eppendorfs when analyzing very small cell numbers (e.g. staining cells produced in *in vitro* stromal cell free cultures). Incubation was typically for 15-20 minutes in the dark at 4°C. Antibodies used in this study are listed in Table 1.

Magnetic sorting does not completely deplete Lin+ cells especially for arthritic mice, probably because the Lineage antibody cocktail formulation is tailored to lineage frequencies present in control mice which are different from those in arthritic mice. Therefore, we stained lineage-depleted bone marrow with fluorophore conjugated antibodies to lineage markers to allow gating out of residual Lin+ cells during FACS analysis. Lineage antibodies used were α-Ter119 (for erythrocytes), α-B220 (for B cells), Mac1 and Gr1 (for myeloid cells) and CD3, CD4, CD8 and/or TCRβ (for T cells). We found that a number of T cells in KRNxG7 mice lacked expression of CD4 or CD8 but were still CD3+TCRβ+ identifying them as T cells. This is possibly due to aberrant T cell development caused by the transgenic KRN TCR as previously reported [7, 8].

The utilization of CD3 and/or TCRβ was especially important in progenitor (CD45+Lin-) identification in the PB, spleen and lymph node. KSL analysis was not affected because these T cells lack Kit expression.

α-IL-7Rα was always added to lineage cocktail for GMP sorts and most of the time for KSL sorts to ensure that the KSL or GMP gates excluded committed lymphoid progenitors. "Natural Helper Cells" which are non progenitor lymphoid clusters located in adipose tissue that provide cytokine help for antibody production and self-renewal of B1 cells are also Kit+Sca1+Lin- but express IL7Rα [9]. These cells were also stained with fluorophore conjugated progenitor marker antibodies: α-Kit, α-Sca1, α-FcgR.

For IgG FACS FcγR blocking prior to and during antibody staining was performed to rule out the possibility of non-covalent FcγR-IgG interaction as the source of the signal. Cells were incubated in 1μg of purified unconjugated FcγR blocking antibody (Clone 2.4G2 from BD or Clone 93 from Biolegend) per 50μl of 4% serum/PBS for 5-10 minutes. 50μl of a 2X solution of fluorophore conjugated antibodies also containing 1μg of FcγR blocking antibody was then added to the cells followed by the typical 15 minute incubation at 4°C. Data is reproduced for KSL cells from several young mice and both old (22 months) mice analyzed. Isotype control antibodies was sometimes utilized for weak protein expression signal such as during IgG or TLR4 analyses of KSL cells.

Analysis was performed on a FACScalibur (BD) and modified FACScans (BD) with 2 and 3 lasers capable of 5 and 8 color analyses respectively. For BM KSL analysis, $1x10^5$ live cells are typically analyzed during flow cytometry. When the estimated progenitor frequency is quite low such as PB and spleen KSL frequency or analysis of purified BM HSC populations (such as CD150+CD41-CD48- cells), $2x10^5$ to $1x10^6$ cells were typically analyzed. Data was analyzed using FlowJo software (Treestar). Cell sorting for subsequent functional or gene expression analysis was perfored with a MoFlo (Dako). Purity of KSL sorting was about 95% determined by post sort analysis.

Cell Cycle and Apoptosis Analyses

For 5-Bromodeoxyuridine (BrdU) analysis, KRNxG7 arthritic mice and age matched control mice were injected intraperitoneally with a single dose (1 mg per 6 g of body mass) of sterile-filtered BrdU (Sigma-Aldrich) dissolved in PBS. Mice were killed 2 to 3 hours later and BM cells harvested as described earlier. Harvested BM cells were subjected to MACS lineage-cell depletion (Miltenyi Biotec) described earlier and lineage depleted cells stained with α-Kit-FITC (eBioscience), α-Sca1-PE and Streptavidin-PerCP-Cy5.5 (BD Biosciences PharMingen). Cells were subsequently fixed, permeabilized and intracellularly stained with APC-conjugated α-BrdU antibody using the APC-BrdU flow kit (BD Biosciences PharMingen). Data was collected using the BD FACScalibur and data analyzed using BD CellQuest software.

For Ki67 analysis, MACS lineage depleted cells (described above) were surface stained with antibodies α-Kit APC-eFluor 780, α-Sca1-PE, α-CD150-APC. PerCP-Cy5.5 conjugated IL7Rα, CD48, and lineage antibodies were used to minimize the number of channels for analysis of CD150+CD48-KSL cells. Cells were then fixed and permeabilized using BD cytofix/cytoperm solution. Fixed cells were then incubated with Ki67 FITC antibody (BD) or FITC conjugated isotype control antibody for 30 minutes at 4°C.

For apoptosis, we used Cy3 conjugated Annexin V antibody (Biovision). After KSL staining, cells were incubated with Annexin V buffer containing Annexin V according to manufacturer's instructions.

GENE EXPRESSION ANALYSIS

Microarray and Bioinformatics Analysis

Total RNA was extracted and purified from FACS sorted CD45+KSL cells and RBC lysed Lin+ fraction of MACS sorted bone marrow using Arcturus Picopure RNA kit (Applied Biosystems). 3 strains were used – KRNxG7 arthritic mice, KRN non-arthritic control mice and

B6xG7 non-arthritic control mice. Therefore, there were 6 samples in total. For each strain, KSL cells and Lin+ cells were from 7-9 pooled mice. All mice were age matched (6-7wks). $3.7x10^5$ B6xG7 KSL, $4.7x10^5$ KSL, $7.4x10^5$ KRNxG7 KSL and $1x10^6$ Lin+ cells from each strain were used for RNA extraction. Purified RNA was linearly amplified and RNA from all 6 samples run on an Affymetrix Mouse 430 2.0 gene chip. Linear amplification and microarray were carried out at the Affymetrix core facility at Washington University School of Medicine http://www.pathology.wustl.edu/research/lcglab/lcgexp.php

Microarray raw data was extracted and analyzed using dChip software (http://www.biostat.harvard.edu/complab/dchip/) with the help of Jason Mills (Washington University). "Upregulated genes" are at least 20% increased in arthritic KSL cells relative to *both* control KSL cell populations (from B6xG7 and KRN – both non-arthritic) examined in this study. Similarly "downregulated genes" were at least 20% reduced relative to *both* control KSL expression levels.

Clustering analyses comparing gene chips from this study to each other and other stem cell and mature cell populations from previous studies were carried out using GOurmet software [10, 11] with the help of Jason Mills (Washington University). Briefly, average expression of various genes in mature cells was determined from the 3 Lin+ microarray results using dChip. Lists of genes enriched in each KSL population relative to mature cells were then determined by comparing raw expression in the KSL population with the predetermined average expression of the mature cells. Fractional representation of GO terms associated with each gene list was calculated and then each cell population was then compared with previous gene expression studies at the level of the GO term representation (See Table 2).

DAVID online resource [12-14] was used for other bioinformatics analysis particularly functional annotation of differentially regulated genes.

Quantitative Real Time PCR

For qRT-PCR analysis of total marrow hematopoietic and non-hematopoietic cells, non-adhered marrow cells were initially flushed out with buffer pelleted and saved. Adhered marrow cells were retrieved and simultaneously lysed by infusing marrow cavity with 1ml of trizol reagent (Invitrogen). This same solution was also used to lyse non-adhered marrow cells. Trizol containing whole marrow cell lysates was stored at -80°C.

Primitive progenitors (KSL cells or CD150+CD48-CD34-KSL cells) were sorted directly into trizol. Since HSC expression of CD34 changes during ontogeny (from CD34+ in fetal liver HSCs to CD34- in adult mice) [15], we used mice that were 14-15 weeks old. 10000 to 20000 KSL cells per mouse were collected. 3 independent arthritic mice and 3 independent control mice were used. For CD150+48-CD34-KSL cells, 3 independent pools each from arthritic and control mice were used; each pool contained cells from 2 mice. 4000 to 7000 cells per pool were collected and used for RNA extraction. For each experiment (KSL or CD150+CD48-CD34-KSL) sorting from all mice was performed on the same day ensuring exact same gates were used. Collected cells in trizol were vortexed and stored at -80°C.

RNA extraction and purification was performed according to manufacturers instruction for trizol with the exception that linear acrylamide (Ambion) was used as a carrier for RNA extraction from primitive progenitors. For whole marrow, we used 1μg of RNA in subsequent steps. For primitive progenitors, we estimated RNA yield to be less than 1μg. Therefore all RNA extracted was used in subsequent steps. Purified RNA was subjected to DNAse digestion followed by first Strand cDNA synthesis using Superscript III reverse transcriptase (Invitrogen). cDNA obtained was diluted in autoclaved distilled water and qRT-PCR performed with Sybr Green as done previously [6, 16]. QRT-PCR primers used in this study are listed in Table S3.

IN VITRO ASSAYS

Methylcellulose Colony Formation

KSL cells or GMP enriched cells (Kit+Sca1-Lin-IL-7Rα-FcγRIII/II^{hi}) from 6-9 week old age and sex-matched (male) arthritic and control mice were sorted on a MoFlo as described above. KRN, G7 or B6xG7 mice were used as controls. Granulocyte and Macrophage (GM) restricted (HSC 008; R & D) and Erythroid restricted (Methocult SF M3436; Stem Cell Technologies) methylcellulose based media were used for replating according to product information sheet with minor modifications. Each population (KSL or GMP) from each mouse was plated in duplicate. 200 cells per plate were used for replating on GM-restricted plates. KSL cells formed much fewer colonies in erythroid restricted plates and hence 1000 cells per plate was used. GM colonies were enumerated on day 7-10 while erythroid colonies were enumerated on D14. Colony counts were performed in a blinded manner to determine mean colony counts, based on duplicates, for each mouse which were then used in subsequent statistical analysis. Plating efficiency was calculated as (number of colonies/number of progenitor cell input) x 100. Data for colony formation on complete media is not shown but was similar to colony formation on GM-restricted media.

In vitro stromal cell free culture

Sorted KSL cells from arthritic and control mice were mixed in 1:1 ratio (5000 cells each) and cultured in 24 well tissue culture plates (TPP). Culture media consisted of StemSpan serum free base medium (Stem Cell Technologies), 10% serum (Hyclone), KitL (1% supernatant) and Flt3L (Peprotech or Ebiosciences). Cells were incubated at 37°C with 5% CO_2. 3.5 days later, cells were harvested for analysis. Control mice were Ly5.1+ or Ly5.1+Ly5.2+ while arthritic mice were always Ly5.2+. Experiments were performed in 2 sets (N=3 in each set).

Osteoclast *In vitro* assay

Osteoclast culturing media consisted of α10 media (α-MEM + 10% serum + Penicillin & Streptomycin antibiotics), 10% CMG supernatant, as a source of M-CSF, and RANKL. RANKL was typically used at 100ng/ml although different concentrations were also tried. All reagents were obtained from the Teitelbaum lab (Washington University).

Two thousand sorted KSL cells or GMP cells were cultured in 96 well flat bottom plates containing 200μl of osteoclast culturing media. Half the media was changed with fresh media on Day2 and daily thereafter. Plates were fixed on Day 5-9 with 4% paraformaldehyde and stained with a histochemical TRAP staining kit (Sigma). GMP cells from arthritic or control mice that were at least 6 weeks of age robustly and consistently generated osteoclasts *in vitro* and thus were used as a positive control.

Note that typical protocols for generating osteoclasts *in vitro* involve priming whole bone marrow by culturing with M-CSF for about 3 days to generate so called “bone marrow macrophages”. These are then subsequently cultured with M-CSF and the indispensable cytokine, RANKL, to generate mature osteoclasts in about 5 more days. However to test osteoclast potential of specific cell populations, the sorted cells are cultured with M-CSF and RANKL immediately without the need for initial priming with M-CSF.

Differentiation on OP9 stromal cells

Three thousand sorted KSL or GMP cells were cultured on irradiated OP9 cells in 6 well plates. In some experiments, 1000 cells were cultured in 24 well plates. Cells were grown in αMEM with 10% serum 10 KitL (1% of supernatant), 10ng/ml IL-7 (Peprotech) and 20ng/ml Flt3L (Peprotech). After 5-8 days, cultured cells were recovered by vigorous resuspension, stained with fluorescent conjugated antibodies and FACS analyzed. Vigorous resuspension, as opposed to enzymatic digestion, was used to recover cells from culture, since enzymatic digestion could at

times lead to destruction of epitopes for antibody recognition for FACS analysis. Approximately, 90% or more of the cells were recoverable by vigorous resuspension.

IN VIVO ASSAYS

General Competitive Transplantation

Recipient mice were lethally irradiated (10Gy; single dose) a day before cell transplantation. Lethally irradiated mice were administered antibiotic treated water – changed twice weekly - for 3 weeks following irradiation. Sorted cells were pelleted and resuspended in HBSS and a 200μl volume of suspension was transplanted via the tail vein. For competitive transplantation, cell suspensions from 2 competing strains were mixed at the appropriate concentrations and injected in the same 200μl volume. Effectiveness of irradiation was verified by death of non-transplanted irradiated mice less than 2 weeks after irradiation and markedly low chimerism of recipient derived cells in transplanted mice.

We were unable to track erythroid chimerism because mature erythroid cells do not express CD45 and erythroid precursors have very low to no CD45 expression. Even though T cell chimerism analysis was possible, these results do not reflect T lineage potential because KRNxG7 derived T cells, which express autoreactive KRN TCR, are negatively selected in the recipient mice [1, 6]. The elimination of arthritic KSL derived T cells also makes it impossible to determine overall CD45 chimerism of arthritic versus control KSL cells although contribution to individual lineages can be assessed.

Young Recipient Competitive Transplantation – Supplemental Information

Preliminary analysis showed hardly any leucocytes in peripheral blood at very early time point (10 days or less) post transplantation. Therefore for D6 time point only bone marrow was analyzed. In fact total cell count of RBC lysed bone marrow retrieved from 2 tibiae and 2 femurs of recipient mice 6 days after transplantation was about $2x10^5$ cells, about 200 fold lower than

typical cellularity of unirradiated mice. At this early time point B220+ cells were also hardly existent in the bone marrow. By 16 days post transplantation, bone marrow cellularity had largely recovered with cell count of close to $3x10^7$. Mice used for 6 day- (N=4) and 16 day- (N=4) analyses were sacrificed in order to analyze bone marrow. Peripheral blood from remaining recipient mice from the same transplant cohort (N=4) was sampled non-lethally at subsequent time points (5wks, 9wks).

Old Recipient Competitive Transplantation – Supplemental Information

For transplantation into old recipients, 5 recipient mice were used. One failed to engraft donor cells appreciably and was therefore excluded from mean and standard deviation analyses presented in Figure 6B. Nevertheless, this mouse still displayed the same pattern of higher myeloid cell output from arthritic KSL cells. 8wks post transplant Mac1+Gr1hi cells comprised 70% of PB, higher than the other mice in the cohort. Most (>95%) of these Mac1+Gr1hi cells were recipient derived while 3.15% (99% of total donor derived cells) was arthritic KSL derived and only 0.026% (1% of total donor derived cells) were control KSL cell derived. On the other hand, in this same mouse, B220+ frequency at 8wks (11.4%) was lower than the other mice in the cohort, with the majority of these cells coming from control KSL cells (84.5% of all, 95% of donor derived cells). Only 4.2% of the B220+ cells (5% of donor derived cells) came from arthritic mice. This and another recipient died before the 26wk analysis time point. Therefore, 8wk and 16wk analysis was performed with N=4 recipients while 26wk analysis was performed with N=3 recipients. In general, recipient mice were more variable than young recipient mice with respect to contribution from residual recipient cells probably reflecting reduced sensitivity to irradiation.

In situ Osteoclast Differentiation of KSL cells

3 week old RANK-/- mice generated from RANK+/- heterozygotes were given serum transfer arthritis by 2 injections of arthritogenic K/BxN serum at 10μl/g body mass. Sorted KSL

cells were resuspended to a concentration of 20000-30000 cells/10μl of HBSS. 30μl of suspension was injected subcutaneously on the dorsal side of the foot into the ankle.

STATISTICS

Student t-test was used to determine statistical significance for all quantitative data. Bar graphs and error bars representing mean and standard deviation respectively are used to display quantitative data based on multiple mice. * = p< 0.05; **=p<0.01; ***=p<0.001.

TABLES ASSOCIATED WITH EXPERIMENTAL PROCEDURES

Table 1. Antibodies used in this study

Molecule	Clone	Source
Kit	2B8	Biolegend, Ebioscience
Sca1	D7	BD, Biolegend, Ebioscience
CD3	145-2C11	Biolegend, Ebioscience
CD4	GK1.5	BD, Biolegend, Ebioscience
CD4	RM4-5	Ebioscience
CD8	53-6.7	Biolegend, Ebioscience
TCRb	H57-597	Biolegend
B220	RA3-6B2	Biolegend, Ebioscience
Mac1	M1/70	BD, Biolegend, Ebioscience
Gr1	RB6-8C5	BD, Biolegend, Ebioscience
Ly6G	1A8	Biolegend
cFms	AFS98	Biolegend, Ebioscience
Ly6C	AL-21	BD
Ter119	Ter119	Biolegend, Ebioscience
IL-7Ra	A7R34	Ebioscience, Biolegend
FcgR III/lib	2.4G2	BD
FcgR III/lib	93	Biolegend
CD34	RAM34	Ebioscience, BD
CD150	TC15-12F12.2	Biolegend
CD48	HM48-1	Biolegend, Ebioscience
CD11c	N418	Biolegend
CD45	30-F11	BD, Biolegend, Ebioscience
CD45.1	A20	Ebioscience, Biolegend
CD45.2	104	Ebioscience, Biolegend

CD71	C2	BD
CD71	R17217	Ebioscience
NK1.1	PK136	BD
Streptavidin		BD, Ebioscience
Lineage cocktail		Miltenyi

Table 2. References for gene chips used for dendrogram analysis.

Name	Reference
Mature Skin	[17]
Mature Hematopoietic Cells 1	This Study
Mature Gastric Cells 1	[18]
Mature Small Intestine	[19]
Brain	[20]
Mature Gastric Cells 2	[21]
Mature Gastric Cells 3	[18]
Mature Hematopoietic Cells 2	[20]
Mature Hematopoietic Cells 3	[22]
Neural Progenitors	[20]
Neural Progenitors	[22]
Gastric Progenitors	[23]
Mesenchymal Progenitors	[24]
Small Intestine Progenitors	[25]
Skin Progenitors	[26]
Hematopoietic Progenitor 4	[27]
Hematopoietic Progenitor 1a	This Study
Hematopoietic Progenitor 1c	This Study
Hematopoietic Progenitor 1b	This Study
Hematopoietic Progenitors 2	[20]
Hematopoietic Progenitors 3	[22]

Table 3. Quantitative RT-PCR primers used in this study

Gene	Primer Sequence	Amplicon Size (bp)
C/EBPb	AAGCTGAGCGACGAGTACAAGA	116
C/EBPb	GTCAGCTCCAGCACCTTGTG	
Ccr2	ATCCACGGCATACTATCAACATC	104
Ccr2	CAAGGCTCACCATCATCGTAG	
Chi3l3	AGAAGGGAGTTTCAAACCTGGT	109
Chi3l3	GTCTTGCTCATGTGTGTAAGTGA	
Ear1	CTGTTGGTGTGTGTGGAAATCC	119
Ear1	ATTTGTTGCCCGACTGGTGAT	
EKLF (Klf1)	AGACTGTCTTACCCTCCATCAG	165
EKLF (Klf1)	GGTCCTCCGATTTCAGACTCAC	
FcgrIIb	ATGGGAATCCTGCCGTTCCTA	117
FcgrIIb	CCCAGCAGCAAGATTTAGCAC	
FcgrIII	TCACTGTCCAAGATCCAGCAA	137
FcgrIII	CGGGGTTTGAAGATTTCTCCGTA	
Fog1 (Zfpm1)	CAGAGCCTTATCCCCTGAGAG	107
Fog1 (Zfpm1)	CGGCTTCTTCAGTTAGGACCT	
Gata1	ATGGAATCCAGACGAGGAAC	
Gata1	CTCCCCACAATTCCCACTAC	
Ikaros	AGACAAGTGCCTGTCAGACAT	110
Ikaros	CCAGGTAGTTGATGGCATTGTTG	
Mpa2l	GTTCCAGGAAGTAACAAAGGCT	102
Mpa2l	ATCCCTAGTCTATTCCCAGTGAC	
PU.1	GAACAGATGCACGTCCTCGAT	107
PU.1	GGGGACAAGGTTTGATAAGGGAA	
Runx1	CTTCCTCTGCTCCGTGCTA	
Runx1	CTGCCGAGTAGTTTTCATCG	
S100a8	AAATCACCATGCCCTCTACAAG	165
S100a8	CCCACTTTTATCACCATCGCAA	
S100a9	ATACTCTAGGAAGGAAGGACACC	129
S100a9	TCCATGATGTCATTTATGAGGGC	
SOCS3	CAAGAACCTACGCATCCAGTG	101
SOCS3	CCAGCTTGAGTACACAGTCGAA	
VWF	CTTCTGTACGCCTCAGCTATG	125
VWF	GCCGTTGTAATTCCCACACAAG	

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