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Immune Activation and Lineage Fate Decisions: Inducing a Shift of Program

Sten F. W. M. Libregts

S. libregts@gmail.com
donderdag
26 september 2013
om 12.00 uur
in de Agnietenkapel
Oudezijds Voorburgwal 231
Amsterdam
met na afloop een receptie
ter plaatse
Paranimfen
Alex de Bruin
&
Claudia Brandão Silva
Immune Activation and Lineage Fate Decisions: Inducing a Shift of Program

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Immune Activation and Lineage Fate Decisions: Inducing a Shift of Program

ACADEMISCH PROEFSCHRIFT

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aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. D.C. van den Boom
ten overstaan van een door het college voor promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
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Faculteit der Geneeskunde
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Chapter 1

General Introduction
General Introduction

Hematopoiesis

Blood can be considered as the elixir of life. Besides that blood plasma transports nutrients to cells and exchanges that for waste product from the same cells, it also contains red blood cells that deliver oxygen to cells, white blood cells that form the bodies’ natural defense mechanism by targeting invading pathogens, and platelets which are involved in hemostasis. Hematopoiesis is the process involving the development, production and specialization of blood cells. Because of its importance in the fetomaternal transport of nutrients, waste elimination and the exchange of gasses, the hematopoietic system is one of the first complex tissues to develop during embryogenesis. The yolk sac is the first site where cells with hematopoietic potential are found, however, it was shown that these cells do not develop in the yolk sac itself but are generated as mesodermal precursors in the primitive streak. These hemangioblasts migrate to the yolk sac and give rise to both endothelial and hematopoietic precursors, of which the first give rise to the vasculature of the yolk sac and the latter contribute to the formation of blood islands and primitive erythrocytes. Nonetheless, using transplantation and labeling assays it was revealed that none of these precursor cells are capable of contributing to the definitive hematopoietic system and that the origin of the adult hematopoietic system lies within the intraembryonic mesoderm that develops later during embryogenesis.

Definitive hematopoietic stem cells (HSCs) are defined as cells that are capable of giving complete long-term multi-lineage hematopoietic reconstitution in irradiated adult mice, meaning that these cells have the capacity to give rise to new HSCs in a process termed self-renewal, and generate all lineages of blood cells via multiple proliferation and differentiation steps. In mouse, the first definitive HSCs appear at embryonic day 10.5 (E10.5) within the aorta-gonad-mesonephros (AGM) region and in the vitelline and umbilical arteries. Immunohistological analysis revealed the presence of hematopoietic cell clusters sprouting from the aortic endothelium and it was hypothesized that from these structures the first HSCs arise. More recently, using de novo imaging techniques, it was indeed shown that HSCs emerge directly from hemogenic endothelial cells within the dorsal aorta. Because shortly after their development HSCs are also found in the placenta, yolk sac and fetal liver, it is believed that HSCs also develop independently in these organs, or, alternatively, recirculate through the fetus and then repopulate these hematopoietic sites. The fetal liver, although not generating hematopoietic stem cells de novo, serves as the primary hematopoietic organ in the fetus. Between E9.5-10.5 myeloerythroid progenitors seed the fetal liver and start generating definitive erythrocytes. With the arrival of definitive HSCs at E11.5, hematopoiesis takes a leap as HSCs expand and differentiate into multi-potent progenitors, single-lineage progenitors and more differentiated precursors and mature blood cells.

During adult life hematopoiesis primarily takes place in bone marrow. Development of the mouse skeletal system starts at E12.5 when mesenchymal condensations develop. Within these condensations mesenchymal cells give rise to chondrocytes that built a cartilaginous framework. Through endochondral ossification by osteoblasts this framework is then calcified to bone. With the invasion of the vasculature within the bone, blood circulation is induced and the opportunity for hematopoietic precursors and HSCs to seed into the bone is facilitated. From E17.5 onwards, definitive HSCs and hematopoiesis can then be found in the long bones of the fetus, while hematopoiesis in mouse fetal...
liver is slowly declining until after birth. In humans however, hematopoiesis in the liver is absent after birth and fully takes place in the bone marrow. Furthermore, in mice all bones keep supporting hematopoiesis during life, while in aging humans hematopoiesis is slowly shifted from the long bones to the pelvis, sternum, cranium and vertebrae as red marrow gets replaced by yellow marrow. However HSCs have multi-lineage potential and are very well capable of differentiating into fully mature hematopoietic cells in the bone marrow, further development, maturation and activation of certain hematopoietic cells takes place in specialized secondary organs like the thymus, spleen and lymph nodes. The process where hematopoiesis occurs outside of the bone marrow medulla is termed extramedullary hematopoiesis. Although being physiological in fetal development and for the final maturation steps in the generation of certain hematopoietic cells, during adult life extramedullary hematopoiesis is often seen as a compensatory mechanism associated with hematopoietic stress or pathological conditions like myelofibrosis, leukemia and red blood cell disorders.

**Lineage Differentiation**

HSCs are at the top of the hierarchy of the hematopoietic system and are multipotent cells that via self-renewal and differentiation can give long-term hematopoietic reconstitution of irradiated adult recipients by self-renewal and the capacity to give rise to all lineages of blood cells (Figure 1). The hematopoietic lineages can be divided into the myeloid lineage (monocytes/macrophages, neutrophilic granulocytes, eosinophilic granulocytes, basophilic granulocytes, mast cells and myeloid dendritic cells (mDCs)), the erythroid lineage (erythrocytes and megakaryocytes) and the lymphoid lineage (T-cells, B-cells, natural killer (NK)-cells and plasmacytoid dendritic cells (pDCs)); the development and differentiation into these lineages are called myelopoiesis, erythropoiesis and lymphopoiesis respectively. The identification of hematopoietic stem and progenitor cells (HSPCs) from which all these terminally differentiated cells arise, required the development of specific assays to test the clonality and multipotency for all lineages and the generation of monoclonal antibodies directed against cell surface antigens. With the finding in mouse that most of the clonogenic precursors do not or show only very low expression of markers for lineage commitment, but do express Sca-1 (Ly6A/E) and the stem cell factor receptor c-Kit, an important step was made in identifying HSCs and hematopoietic progenitor cells. Within this so-called Lin- c-Kit+ Sca-1+ (LKS) population further distinction of HSCs can be made based on the expression of CD34, FMS-like tyrosine kinase 3 (Flt-3) and by the degree of self-renewal. Long term reconstituting HSCs (LT-HSCs), which are defined as Lin- c-Kit+ Sca-1+ CD34- Flt-3-; are HSCs that have life-long self-renewal capacity and can generate and sustain multi-lineage reconstitution after serial transplantation into recipient mice. LT-HSCs give rise to short-term reconstituting HSCs (ST-HSC, Lin- c-Kit+ Sca-1+ CD34+ Flt-3+), which have limited self-renewal potential, but are capable of quickly reconstituting myeloablated recipients. ST-HSCs in turn give rise to multipotent progenitors (MPP, Lin- c-Kit+ Sca-1+ CD34+ Flt-3+). Although MPPs are still multipotent and can generate blood cells of all lineages, they bare no or very limited self-renewal potency. More recently an alternative method for the identification of more pure populations of HSCs is achieved by the expression pattern of the signaling lymphocyte activation molecules (SLAM) members CD150, CD48 and CD244. This panel of markers can either be used by itself or by incorporating them to the LKS-population...
Figure 1. Schematic overview of the hematopoietic system.

Long term-repopulating hematopoietic stem cells (LT-HSCs) give rise to short term repopulating stem cells (ST-HSCs), that produce multipotent progenitors (MPPs). MPPs can subsequently differentiate into lineage restricted progenitors; common lymphoid progenitors (CLPs), common myeloid progenitor (CMPs), megakaryocyte-erythroid progenitors (MEP) and granulocyte-monocyte progenitors (GMPs). These lineage restricted progenitors ultimately give rise to all cell lineages from the hematopoietic system. Recent literature showed that downregulation of Flt3 allows the generation of MPPs that can give rise to myeloid and erythroid progeny, but lost lymphoid progeny, while MPPs with high expression of Flt3 have lost erythroid potential, but have myeloid and lymphoid potential\cite{49,61}. In addition, it was found that ST-HSC can directly give rise to MEPs, thereby skipping differentiation into a MPP- and CMP-stage\cite{64}. 
Furthermore, HSCs can be enriched by exploiting the physiologically capability of HSCs to quickly efflux the fluorescent vital dye Hoechst 33342\textsuperscript{53}. Based on the strength of this capacity lineage-biased HSCs can be purified\textsuperscript{54}.

Differentiation of MPPs results in the formation of two oligopotent progenitor cells; the common lymphoid progenitor (CLP, Lin\textsuperscript{-} C-kit\textsuperscript{low} Sca-1\textsuperscript{low} IL-7Rα\textsuperscript{+}) which can give rise to B-cells, T-cells, NK-cells and pDCs and the common myeloid progenitor (CMP, Lin\textsuperscript{-} C-kit\textsuperscript{+} Sca-1\textsuperscript{-} CD34\textsuperscript{low} CD16/32\textsuperscript{low}), which give rise to all cells of the myeloid and erythroid lineages\textsuperscript{55-57}. CMPs further differentiate into the granulocyte-monocyte progenitor (GMP, Lin\textsuperscript{-} C-kit\textsuperscript{+} Sca-1\textsuperscript{-} CD34\textsuperscript{+} CD16/32\textsuperscript{+}) and the megakaryocyte-erythroid progenitor (MEP, Lin\textsuperscript{-} C-kit\textsuperscript{+} Sca-1\textsuperscript{-} CD34\textsuperscript{-} CD16/32\textsuperscript{-})\textsuperscript{57}. From GMPs and MEPs unipotent progenitor and precursor cells arise which proliferate and eventually mature into terminally differentiated blood cells from the myeloid and erythroid lineages respectively. GMPs thus differentiate into neutrophilic, basophilic and eosinophilic granulocytes, monocytes, macrophages, mDCs and mast cells. MEPs give via multiple maturation steps rise to erythrocytes and megakaryocytes, of which the latter generate platelets. Complexity is added to the hematopoietic differentiation tree as a macrophage-DC progenitor (MDP) has been described that downstream of the CMP gives rise to pDCs, mDCs, monocytes and macrophages, but not to granulocytes\textsuperscript{58} and a common dendritic cell progenitor that only has mDC and PDC potential\textsuperscript{59,60}. More recently, it was described that all hematopoietic cells develop from Flt-3\textsuperscript{+} progenitor cells\textsuperscript{50} and it has been suggested that the MPP population can be divided in two populations based on the differential expression of Flt-3\textsuperscript{61}. MPPs expressing high levels of Flt3 are termed lymphoid-primed MPPs (LMPPs) as they have lost erythroid potential, but are still capable of generating lymphoid and myeloid progeny via CLPs and GMPs, whereas MPPs expressing low levels of Flt3 can directly give rise to MEPs, next to the CMPs and CLPs\textsuperscript{62}. In addition it was suggested that cells with erythroid and megakaryocytic potential can directly be generated from HSCs or MPPs\textsuperscript{63,64}.

The HSC Niche

To maintain hematopoietic homeostasis during adult life, human HSPCs generate approximately \(1 \times 10^9\) red blood cells and \(1 \times 10^8\) white blood cells every hour. Lineage differentiation therefore is tightly regulated, but the exact processes involved are not fully understood yet. More and more evidence is however emerging that the input for this regulation is generated by the microenvironment in which HSPCs reside; the hematopoietic niche\textsuperscript{65,66}. Initially the niche was described as the interaction between the stem cell and cells in its proximity that determined its behavior and prevented differentiation\textsuperscript{67}, but it remained sort of a black box until more recently new life was breathed into the concept with the discovery of new cell types, molecules, anatomical locations and signaling cascades that control HSPC behaviour. As the HSCs are on top of the hematopoietic hierarchy, significant effort is put into revealing how cell extrinsic cues from the niche specifically regulate HSC quiescence, survival and self-renewal, but also how they affect their differentiation behavior\textsuperscript{68}. Although under homeostatic conditions a small number of HSCs can be found in circulation\textsuperscript{69}, the majority of HSCs are located in close proximity to the cellular lining that divides bone from bone marrow or in close relation to the sinusoidal blood vessels found within the bone marrow\textsuperscript{51,70-72}. These anatomical locations are called the endosteal and the vascular HSC niche respectively and contain several different types of cells. Whereas
osteoclasts are dispensable for HSC maintenance within the endosteal niche\textsuperscript{73}, osteoblast numbers correlate with the number of HSC and have been shown to play an important role in the maintenance and proliferation of HSCs by the production of a large number of factors that regulate HSC function\textsuperscript{70,71,74-75}. These factors include angiopoietin-1 (Ang-1), stem cell factor (SCF), thrombopoietin (TPO) and CXC-chemokine ligand 12 (CXCL12) and have all been implicated in the maintenance of long-term repopulation capacity and quiescence of HSCs\textsuperscript{75-81}. Although debatable because of discrepancies found between in vitro and in vivo data, also Wnt, Notch and Sonic hedgehog (Shh) signaling have been implicated in affecting the hematopoietic lineage fate of HSC\textsuperscript{82-88}. In addition to osteoblasts, depletion of osteoblast supportive macrophages (osteomacs) and CD169\textsuperscript{+} macrophages results in the disruption of the endosteal niche and the concomitant mobilization of HSCs\textsuperscript{89,90}. Depletion of nestin\textsuperscript{+} mesenchymal stem cells (MSCs) and stromal cells results in the same observation\textsuperscript{91}. Nestin\textsuperscript{+} MSCs are not only found in the endosteal niche, but also scattered in the bone marrow and surrounding vascular endothelial cells. They therefore also belong to the vascular niche and are considered as one of the key constituents of the HSC niche. Compared to other niche cells, nestin\textsuperscript{+} MSCs produce the highest levels of HSC maintenance factors like CXCL12, SCF and Ang-1\textsuperscript{91}. Interestingly, nestin\textsuperscript{+} MSCs are tightly associated with neurons from the sympatetic nervous system and alterations in innervation via sympatetic nerve fibres results in HSC mobilization\textsuperscript{92}. Recently, glial fibrillary acidic protein (GFAP)-positive Schwann cells that surround sympatetic nerve fibers were found to promote transforming growth factor-\textbeta (TGF-\textbeta) activation\textsuperscript{93}. TGF-\textbeta promotes HSC quiescence by inhibiting lipid raft clustering, which is important for cytokine-induced cell cycle entry\textsuperscript{94}. In addition, adrenergic signals from the sympatetic nervous system allow cyclical circulation of HSCs in the bloodstream based on circadian oscillations\textsuperscript{95}. Just like nestin\textsuperscript{+} MSCs, also CXCL12-abundant reticular (CAR) cells are ascribed to belong to both the vascular and osteoblastic niche. Disrupting the interaction of CXCL12 on CAR-cells with CXCR4 on HSCs severely reduces the number of HSCs as this interaction keeps HSCs quiescent\textsuperscript{81}. Whereas deletion of SCF from nestin-expressing cells does not affect HSC frequency and function, deleting SCF from endothelial cells and leptin receptor-expressing perivascular cells depletes the niche of HSCs, making them essential vascular niche constituents\textsuperscript{96,97}. Besides expressing SCF, bone marrow sinusoidal endothelial cells promote HSC self-renewal and expansion via Notch-signaling and by maintaining the sinusoidal blood vessel network via vascular endothelial growth factor (VEGF)-signaling\textsuperscript{97,98}. To what extent the endosteal and perivascular niche are functionally different remains to be elucidated, however it has been proposed that the most quiescent HSCs reside in the endosteal niche, while the more frequently dividing HSCs occupy the perivascular niche\textsuperscript{96,99}. Whether HSCs travel between niches is unknown, but HSCs are not trapped within their niche as they have been shown to be mobile, independently of cell-cycle\textsuperscript{100}.

Besides cellular interactions or secreted protein factors that contribute to HSC behaviour, also physical and mechanical input is incorporated into the HSC niche. LT-HSC are mostly found in hypoxic environments and the levels of hypoxia-inducible factor 1\alpha (HIF-1\alpha) have been shown to affect the behavior of HSCs\textsuperscript{101,102}. Stimulation of the calcium ion-sensing receptor on HSCs results in enhanced homing capacity upon transplantation, while deletion of this receptor decreases this capacity, indicating that calcium fluxes regulate HSC function\textsuperscript{103,104}. Furthermore, shear stress in the AGM-region upregulates Runt-related transcription factor (RUNX)1, which is essential for regulation of the hematopoietic program,
and initiates the development of definitive HSCs in the embryo\textsuperscript{105}. The bone marrow has a different extracellular organization though, lacking the shear stress of blood flow that is found in the dorsal aorta, but the mechanical support given by the extracellular matrix in the bone marrow has been found to affect HSC differentiation\textsuperscript{106}.

**Lineage Commitment**

Lineage commitment requires the expression of lineage-specific transcription factors at early stages of hematopoiesis. Forced expression or gradual loss of these transcription factors directs differentiation of HSPCs into a specific lineage. Maintaining HSC quiescence is required to ascertain life-long generation of blood cells. GATA-3 is the gatekeeper of this process as it is required for the cell-cycle entry and maintenance of LT-HSCs\textsuperscript{107}. PU.1 is the main transcription factor that drives myelopoiesis, however, it also plays an important role in lymphopoiesis. PU.1-deficient mice die shortly after birth as they have impaired myelopoiesis and have a severely impaired T cell, NK cell and B cell development\textsuperscript{108,109}. Conditional disruption of PU.1 in bone marrow HSCs of adult mice resulted in impaired HSC function and the loss of CMPs and CLPs, indicating that early PU.1 expression in HSPCs is essential for differentiation of cells into myeloid and lymphoid lineages\textsuperscript{110}. Interestingly, overexpression of PU.1 blocks erythropoiesis by binding to GATA-1 and blocking GATA1 DNA binding\textsuperscript{111,112}. Both conditional and full GATA1 ablation results in developmental arrest of immature erythroid cells in bone marrow and fetal liver respectively, thereby displaying GATA1’s essential role in erythroid differentiation\textsuperscript{113,114}. More recently it was shown that reporter mice for PU.1 and GATA1 develop two distinct MPP populations, one PU.1\textsuperscript{+}, the other GATA1\textsuperscript{+}. Whereas the PU.1\textsuperscript{+} MPP displays myeloid and lymphoid potential, but no erythroid and megakaryocytic potential, the GATA1\textsuperscript{+} MPP bears myeloid and erythroid potential, but no lymphoid capacities, indicating that regulation of transcription factor expression already early during hematopoiesis plays an important role in lineage commitment\textsuperscript{115}. Further downstream, in the CMP, it has been shown that the level of PU.1 expression determines the transition from CMP to either GMP or MEP\textsuperscript{116}. Besides lowered PU.1 expression, upregulation of GATA-1 is essential for the transition of CMP to MEP. Whereas expression of FOG-1, Gfi-1b and EKLF becomes increasingly important for further differentiation into erythroid cells\textsuperscript{117-119}, GATA-2, FOG-1, Fli-1 and Nf-E2 expression enhance megakaryocytic differentiation from MEPs\textsuperscript{117,120,122}. High levels of PU.1 in combination with high expression of CCAAT/enhancer-binding protein α (C/EBPα) drives differentiation from CMPs to GMPs\textsuperscript{123}. Within the GMP population high levels of PU.1 and interferon- consensus sequence-binding protein (ICSBP) induce monocyte differentiation, while timed expression of C/EBPα, C/EBPβ, C/EBPε, GATA-2 and Gfi-1 induce granulocytic and mast cell differentiation\textsuperscript{124,125}. Gfi-1 is also described to play an important role in the lymphoid lineage as it represses the PU.1 gene expression that is needed for the initial lymphoid priming during differentiation of CLPs to mature lymphocytes. By displacing PU.1 from positive autoregulatory elements, Gfi-1 thereby enhances B-cell development\textsuperscript{126}. Gfi-1 is induced by the expression of Ikaros, another transcription factor that is required for induction of lymphopoiesis\textsuperscript{127,128}. Ikaros mutated mice lack all T-cells, B cells, NK cells and their progenitors, while Ikaros deficient mice lack B cells and the initial development of T cells, indicating Ikaros’s essential role in lymphocyte development. Upstream of Ikaros, E2A is required for the development of LMPPs by inducing lymphocyte-associated genes, while preventing expression of HSC-related and non-lymphoid genes\textsuperscript{129}. During further B-cell
differentiation, EBF-1$^{130,131}$, Pax5$^{132,133}$, Sox4$^{134}$ and Bcl1$^{135,136}$ play essential roles, while GATA-3$^{137}$ and Notch-signaling$^{138}$ are involved in the differentiation into T-cells and Ets-1$^{139}$ and E4BP4$^{140}$ in the differentiation into NK cells.

Besides that transcription factors often repress the function of other instructive transcription factors, they also largely control the expression of lineage specific cytokine receptors that support survival, proliferation, maturation and differentiation of progenitor cells to the fully mature cells of the hematopoietic system. Depending on the progenitor cell, cytokine signaling can be permissive as it supports the progenitor cell, instructive as it induces commitment into a certain lineage, or restrictive as it inhibits differentiation into a certain lineage$^{141-146}$. Whereas IL-3, IL-6, SCF, TPO, Flt3-ligand (Flt3L) and granulocyte macrophage-colony stimulating factor (GM-CSF) support a broad array of progenitor cells, cytokines like IL-7, Flt3L, granulocyte colony-stimulating factor (G-CSF), macrophage colony-stimulating factor (M-CSF), the hormone erythropoietin (EPO) and TPO, trigger specific progenitor populations and can direct lineage differentiation of lymphocytes, granulocytes, monocytes, erythrocytes and megakaryocytes, respectively. More recently, it was shown that forced expression of cytokine receptors or their downstream transcription factors can convert or instruct differentiation of progenitors into certain lineages$^{143-145}$, indicating that extrinsic signals, next to intrinsic signals, can drive lineage differentiation.

The Immune System

The defense of the human body against viruses, bacteria, fungi, parasites, malignantly transformed cells and harmful substances is orchestrated by the cooperative actions of the innate and adaptive immune system. Cells from the innate immune system form the first line of defense against pathogens, however, their response is limited as they rely on invariant pattern recognition receptors (PRRs) which can only recognize common features and molecules of pathogens, the so called pathogen-associated molecular patterns (PAMPs)$^{147-149}$. The innate immune system is largely made up out of cells from the myeloid lineage. By a process called phagocytosis these cells are capable of engulfing pathogens and destroy them by either merging phagocytic vesicles with vesicles containing degradative enzymes and toxic molecules or by the extracellular release of vesicles containing a plethora of pro-inflammatory and anti-microbial molecules$^{150,151}$. Most abundantly present are the short-lived granulocytes, which based on the make up of their granules, can be distinguished as neutrophilic, eosinophilic or basophilic granulocytes. Very similar to basophilic granulocytes are mast cells, which are found as residents in tissues and release granules to induce allergic inflammation$^{152}$. Monocytes circulate in the blood and migrate into tissues where they mature into relatively long-lived macrophages or DC’s. Beside their major role in the clearance of apoptotic cells, cell debris and cells targeted for degradation by the adaptive immune system, macrophages help inducing inflammation by the release of pro-inflammatory molecules and initiate the development of an adaptive immune response by presenting antigenic peptides to T cells$^{153,154}$. Whereas granulocytes and macrophages are primarily involved in pathogen degradation, DCs specifically bridge the gap between innate and adaptive immunity as they serve as specialized antigen-presenting cells (APCs) that allow activation, proliferation and differentiation of naïve T cells$^{155,156}$. Upon engulfing foreign molecules in peripheral tissues, DC’s mature and migrate to secondary lymphoid organs like lymph nodes and the white pulp of the spleen, where they present antigens via
an immunological synapse to T cells that each bear a single type of receptor with a unique specificity\textsuperscript{157,158}. NK cells take up a special place within the innate immune system as they are lymphocytes that play a role in the early defense of infection by intracellular pathogens and tumor cells. Activation of NK cells is achieved by altering the balance between activating and inhibitory invariant receptors. Upon activation targeted cells get killed by the release of cytotoxic granules\textsuperscript{159}. Interestingly, although NK cells belong to the innate immune system, they are closely related to T and B cells, which comprise the adaptive immune system.

Whereas cells of the innate immune system only have limited recognition capacity for foreign antigens, T and B lymphocytes are capable of detecting an infinite amount of antigens as they bear receptors that are generated by somatic recombination of variable, diverse and joining gene segments encoding for these receptors\textsuperscript{160-163}. T and B lymphocyte precursors are both generated in the bone marrow, but where B lymphocyte precursors mature in the bone marrow, T lymphocyte restricted precursors migrate to the thymus for positive and negative selection and further maturation\textsuperscript{164}. After maturation both T and B lymphocytes recirculate through the body and enter secondary lymphoid organs where they encounter activated APCs presenting antigens. Upon recognition of their specific antigen via their specific T cell receptor (TCR) or B cell receptor (BCR), activation, proliferation and differentiation of these lymphocytes is induced, resulting in the formation of effector and memory cells\textsuperscript{165,166}.

B lymphocytes are involved in the humoral immune response to infection as upon activation naïve B lymphocytes differentiate into antibody producing plasma cells\textsuperscript{167,168}. Antibodies are soluble BCRs that recognize, opsonize and neutralize specific pathogens and target them for destruction by the innate immune system\textsuperscript{169,170}. For induction of a humoral immune response help from T lymphocytes is needed as they instigate proliferation, BCR-mutations and immunoglobulin class switching to generate high-affinity antibodies\textsuperscript{171,172}. T lymphocytes come in two flavors; CD8\textsuperscript{+} cytotoxic T cells that recognize and kill virus-infected cells that express antigens via major histocompatibility complex (MHC) class I molecules on their cell surface and CD4\textsuperscript{+} helper T cells (Th cells), that, based on their cytokine profile, can exhibit different effector functions\textsuperscript{173,174}. For full activation of T cells three signals are required that are provided by the APCs they encounter and which are given via the formation of an immunological synapse\textsuperscript{156,175}. Signal one is the antigen specific signal that is derived from the interaction between the antigen presented by MHC complexes on the APC and its recognition by the TCR on the T cell. A second signal is provided by co-stimulatory molecules expressed on both APC and T cells. These co-stimulatory molecules generally belong to the B7 or tumor necrosis factor (TNF) superfamily and promote or inhibit survival and proliferation of T cells\textsuperscript{176,177}. A third signal is commonly, but not exclusively, delivered by the cytokine milieu in which the T cells reside upon activation. These cytokines are produced by the APC, but also by the local environment, and are particularly important in the differentiation of naïve CD4\textsuperscript{+} T cells into distinct T helper subsets\textsuperscript{178,179}. IL-12\textsuperscript{180,181} and IFN-γ\textsuperscript{182} induce the expression of the transcription factor T-bet, which drives the development of naïve CD4\textsuperscript{+} T cells into IL-2 and IFN-γ producing Th1 cells\textsuperscript{183,184}. Th1 cells stimulate the development of a cell-mediated and phagocyte dependent immune response, but also activate B cells to produce antibodies of the strongly opsonizing IgG subclasses\textsuperscript{185}. IL-4 induces the transcription factor GATA-3 that drives the development of Th2 cells\textsuperscript{186,187}. Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, which evoke a strong humoral immune response against extracellular pathogens. In the presence of TGF-β naïve CD4\textsuperscript{+} T cells upregulate expression of FoxP3, which differentiates them into
T regulatory cells (Tregs)\textsuperscript{188,189}. Tregs control and dampen adaptive immune responses by the production of IL-10 and TGF-β. Other regulatory T cell subsets like Tr1 and Th3 cells are induced when signal three is IL-10\textsuperscript{190,191}. If the APC produces TGF-β together with IL-6 or IL-21 and IL-23, then RORγT expression is induced in T cells. RORγT drives the development of Th17 cells that produce IL-17, IL-21 and IL-22\textsuperscript{192,193}. Th17 cells play an important role in the immunity of epithelial and mucosal barriers as they help protecting against bacteria and fungi by the recruitment and activation of neutrophils. They however are also implied in the induction of the development of severe autoimmune diseases\textsuperscript{194-196}. More recently, also a Th9 subset has been described. Upon activation by an APC the presence of IL-4 and TGF-β allows the differentiation of naïve CD4\textsuperscript{+} T in to Th9 cells, which produce high levels of IL-9 and IL-10. Although still largely unknown what their role is during an immune response, they are considered an autonomous Th cell subset as they lack the expression of transcription factors and the production of cytokines related to other Th cell subsets\textsuperscript{197}.

\textbf{Immune Activation and Lineage Instruction}  
As hematopoietic cells have only a certain lifespan, homeostatic blood production generates new accretion at a constant rate. Extra output of the hematopoietic system is required when immune cells are consumed and need to be replenished as they get recruited to sites of infection and die because of apoptosis while fighting pathogens\textsuperscript{146,198}. For a swift response to this cellular stress the output of the hematopoietic system therefore needs to be quickly enhanced or shifted to cope with the body's needs\textsuperscript{146,199}. Different pathogens require the differentiation of progenitors to specific immune cells that are capable of combatting the invading pathogen. As a consequence the output of other immune cells is then often diminished\textsuperscript{199-203}. Classically it is believed that activation of PRRs on hematopoietic and non-hematopoietic cells at the entry site of the pathogen induces the secretion of a cocktail of chemokines and cytokines\textsuperscript{204}. Whereas chemokines attract immune cells to fight the pathogen\textsuperscript{205,206}, cytokines induce survival and enhance effector functions of local and attracted immune cells\textsuperscript{207}. As immune cells get depleted at the site of infection, a systemic cytokine and chemokine response is induced which promotes granulopoiesis or monopoiesis\textsuperscript{146}. Furthermore, DCs travel to secondary lymphoid organs where they initiate an adaptive response to help clearing the invading pathogen\textsuperscript{208}. While poorly understood, more and more data imply that feedback to the hematopoietic system and its niche is not only provided by systemic responses, but also by immune cells that actively migrate to the bone marrow\textsuperscript{75}.

Besides the fact that bone marrow is the primary site for hematopoiesis and serves as a reservoir of myeloid immune cells, it has been found to actively control immune responses. Bone marrow is an important organ in T cell trafficking in both human and mouse as approximately 3-8% of nucleated cells in the bone marrow are mature T cells, with the majority of T cells having a memory phenotype\textsuperscript{209-212}. Memory T cells preferentially accumulate in the bone marrow\textsuperscript{213,214} and have been found to specifically migrate to the perivascular regions of the bone marrow upon adoptive transfer\textsuperscript{215}. In the perivascular region, where also HSCs reside, memory T cells are then maintained by keeping close contact with IL-7 producing stromal cells\textsuperscript{216,217}. Upon antigen stimulation by either resident or migrated DC’s, bone marrow can serve as a site for recruitment and proliferation of naïve and memory T cells\textsuperscript{212,218,219}. Interestingly, clusters of DCs in the perivascular space of the bone marrow have
been found to interact with recirculating T cells and B cells\textsuperscript{220}, indicating that activation of the adaptive response can take place in close proximity of HSCs. Upon activation, also B cells can migrate back to the bone marrow where they differentiate into long-lived antibody-secreting plasma cells\textsuperscript{221}. Retention and survival-signals for plasma cells in the bone marrow are provided by CXCL12, a proliferation-inducing ligand (APRIL) and B cell activating factor of the TNFR family (BAFF)-signaling\textsuperscript{222,223}. Plasma cells are therefore found in close proximity to CXCL12 expressing stromal cells\textsuperscript{224}. As these CAR-cells also harbour hematopoietic progenitors, this gives more reason to believe that direct communication between immune cells and hematopoietic progenitors is feasible and that hematopoiesis can be directly influenced by immune cells.

Besides the release of the cytokines with known hemato-modulatory functions like G-CSF and M-CSF, a plethora of other pro-inflammatory cytokines with yet poorly understood hemato-modulatory functions are produced by activated immune cells that are present in or migrate to the bone marrow upon infection. Although these cytokines are primarily known for their involvement in enhancing and guiding immune responses, receptors for IL-1, tumor necrosis factor α (TNF-α), transforming growth factor-β (TGF-β) and type I (IFN-α and IFN-β) and II interferons (IFN-γ) are also found on HSPCs\textsuperscript{146}. Of the IFNs, the inhibitory effects of IFN-γ on hematopoiesis have been most extensively studied. IFN-γ has been proposed to activate quiescent HSCs and promote long-term repopulating HSC proliferation upon bacterial infection\textsuperscript{225}. However, recently published data from our lab suggest that IFN-γ rather impairs the self-renewal capacity of HSC by blocking TPO-mediated STAT-5 phosphorylation\textsuperscript{226}. Studies using IFN-γ\textsuperscript{−∥} and IFN-γR\textsuperscript{−∥} mice further demonstrate that IFN-γ plays an essential role in mounting a myeloid immune response upon bacterial infection\textsuperscript{202,227}. Simultaneous to the induction of myelopoiesis, IFN-γ skews the outgrowth of monocytes at the expense of neutrophilic and eosinophilic granulocytes\textsuperscript{228,229}. In addition, IFN-γ is well known for its inhibitory effects on erythropoiesis and detrimental effect on B cell lymphopoiesis\textsuperscript{230-232}, but the exact mechanisms involved in their suppression remain to be explored.

To fully activate lymphocytes, both antigen-specific and antigen non-specific triggering is needed. An antigen specific signal is provided to the cell by the interaction of the TCR with peptides presented on MHC class II molecules. Non-specific triggering is provided by co-stimulatory molecules expressed on the cell surface of APCs and provides the cell with signals for survival, proliferation and differentiation\textsuperscript{156}. The tumor necrosis factor (TNF) receptor superfamily member CD27 is an important co-stimulatory molecule that is expressed on T cell, B cell, and NK cell subsets\textsuperscript{233-235}. The type II TNF-related transmembrane glycoprotein CD70 is the unique ligand of CD27 and is only transiently expressed during immune activation on activated DCs, B cells and T cells\textsuperscript{236-239}, indicating the unique roles of these molecules during immune activation. CD27-triggering has been found to have the most profound effects on the T cell pool as it enhances the proliferation and survival of activated T cells\textsuperscript{240,241}. Furthermore, CD27-signaling induces the expansion of effector T cells and is required for the formation and maintenance of T cell immunity\textsuperscript{232,242}. More recently, it was also shown that CD27-triggering allows the formation of T cell responses against low-affinity antigens by lowering the threshold for TCR-triggering, thereby allowing the formation of broader immune responses and the generation of T cells against virus variants\textsuperscript{243}. CD70-binding to CD27 however does not only affect T cells. CD27-triggering on B-cells promotes the differentiation to plasma cells and stimulates immunoglobulin
production\textsuperscript{244,245}. In addition, it enhances the formation of germinal centers when triggered on somatically hypermutated B cells\textsuperscript{246}. The outgrowth of B-cells from the bone marrow on the other hand is diminished during constitutive triggering of CD27\textsuperscript{232,247}. CD27-triggering furthermore induces proliferation of NK-cells and enhances the production of IFN-γ and their cytotoxic capacity\textsuperscript{248,249}.

Strikingly, also HSPCs have been found to constitutively express CD27 on their cell surface\textsuperscript{247,250}. As interaction between CD70 and CD27 is dependent on immune activation, it is thus suggested that activated immune cells can actively give feedback to the hematopoietic system\textsuperscript{247}. Constitutive CD27-triggering on HSCs leads to enhanced HSC self-renewal and accumulation of HSCs. Functionally, CD27-triggered HSCs display a myeloid biased gene signature and when adoptively transferred into recipient mice CD27-triggered HSC display reduced lymphoid potential, but increased myeloid potential (De Bruin, unpublished). In line with these results CD27-triggered HSPCs have diminished colony-forming potential and inhibited lymphocyte outgrowth \textit{in vitro}\textsuperscript{247}. To what extent CD27 can further alter lineage differentiation when triggered on hematopoietic progenitors is largely unknown and remains to be elucidated.

**Scope of This Thesis**

Given the fact that activated immune cells migrate back to the bone marrow or can get activated in close proximity of HSPCs, direct feedback mechanisms by activated immune cells are proposed to play an important role in altering hematopoiesis. Gaining insight in how hematopoiesis and immune activation can alter each other’s function therefore could lead to the knowledge needed to develop therapies that interfere or prevent the development of adverse conditions associated with chronic immune activation, like i.e. anemia or neutropenia. In this thesis we describe how triggering of the TNF-receptor superfamily member CD27 by CD70 and the pro-inflammatory cytokine IFN-γ influence lineage commitment during immune activation. In chapter two we investigated the expression of the TNF-receptor superfamily member CD27 on definitive HSC during ontogeny of the mouse fetus and whether the absence or triggering of this co-stimulatory molecule influences the development of the hematopoietic system. In chapter three we sought to determine whether CD27-singaling instructs for the development of specific T helper subsets by using mice with either a Th1- or Th2-prone background and by using \textit{in vitro} skewing assays. Chapter four deals with the effects of the pro-inflammatory cytokine IFN-γ on the development of new erythrocytes and the breakdown of effete erythrocytes in a mouse model of chronic immune activation. As mice can respond to hypoxia by induction of extramedullary stress-erythropoiesis, chapter five discusses the influence of IFN-γ on the stress-erythropoietic response. By using \textit{in vitro} culture systems for erythroid precursor proliferation and erythroid differentiation, chapter six describes how IFN-γ affects the cellular fate of expanding and differentiating erythroid precursors. In chapter seven we discuss the findings described in this thesis regarding the influence of immune activation and hematopoiesis. Moreover, we provide an interesting parallel of the 3-signal model for T cell differentiation with the processes that shape and regulate hematopoietic differentiation.
Reference List


2. Sabin FR. Studies on the origin of blood-vessels and of red blood-corpuscles as seen in the living blastoderm of chicks during the second day of incubation. Contributions to Embryology 1920;9:215-262.


14. de Bruijn MF, Speck NA, Peeters MC, Dzierzak E. Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. EMBO J. 2000;19:2465-2474.


52. Yilmaz OH, Kiel MJ, Morrison SJ. SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. Blood 2006;107:924-930.


Chapter 2

Function of CD27 in T cell differentiation

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Abstract
Differentiation of naïve CD4+ T cells to functional effector T-helper (T_H) cells is driven by both costimulatory molecules and cytokines. Although polarizing cytokines can induce the differentiation into a particular T_H-subset, certain costimulatory molecules also seem to affect this polarization process. We have previously found that CD70-transgenic (CD70TG) mice develop large numbers of IFN-γ-producing CD4+ T cells and we therefore questioned whether CD27 triggering provides an instructive signal for T_H1 differentiation or rather supports T_H cell formation in general. Although CD70TG mice on a T_H1-prone C57Bl/6J background develop more T_H1 cells, we found that this phenotype is lost when CD70TG mice are fully backcrossed on a T_H2-prone Balb/c background, but is not replaced with more T_H2 cells. Furthermore, CD70-overexpression is not sufficient to drive T_H17 cell formation, nor does it affect the generation of FoxP3+ regulatory T cells. Using an in vitro setting, we found that CD27-triggering does not provide instructive signals for a specific T_H cell subset, but, depending on the cytokine milieu and genetic background, supports T_H1 cell formation, while it inhibits the formation of T_H17 but not T_H2 cells. Induction of allergic airway inflammation in CD70TG Balb/c mice further illustrates that CD27 plays a supportive role in T_H1 differentiation in vivo, without modulating the classical T_H2 response. This supportive role of CD27 in T_H cell polarization could not be attributed to a specific change of transcription factor expression levels. In summary, this study indicates that CD27 signaling does influence T_H cell differentiation, but that it is highly dependent on the conditions and genetic background.
**Introduction**

Recognition of a MHC-peptide complex via the TCR is the first signal required for effector T cell formation, as it initiates T cell activation and clonal expansion. Subsequent to MHC-peptide binding, T cells depend on a second signal for their survival and proliferation, which is provided in the form of costimulatory molecules. For the final differentiation and polarization to effector cells, activated T cells require a third signal, which is provided by polarizing cytokines\(^1\);\(^2\). Thus, naive T cells rely on a triad of signals for their activation and differentiation into an effector population. The large number of molecules that have been implicated in this process either instruct, support or permit the formation of a specific effector T cell population.

Within the CD4 T cell population, a large variety of helper T (T\(_H\)) cell subsets has been identified, such as T\(_H_1\), T\(_H_2\), T\(_H_17\), T\(_H_3\), T\(_R\) and T\(_{Reg}\), which have been attributed a specific function in the immune system. Classically, these T\(_H\) subsets can be distinguished by their cytokine production and/or transcription factor expression. As such, T\(_H_1\) cells are characterized by the ability to produce high levels of IFN-γ and TNF-α, thereby supporting cell-mediated immunity. On the other hand, humoral immunity is linked to T\(_H_2\) formation and increased secretion of IL-4, IL-5, IL-6, IL-10 and IL-13\(^3\). Protective anti-bacterial immunity as well as development of autoimmunity is generally linked to an increase in IL-17 producing T\(_H_17\) cells\(^4\);\(^5\). Next to these effector T cell subsets, two inducible regulatory T cell subsets can be identified by their production of IL-10 and TGF-β, which are respectively referred to as T\(_R_1\) and T\(_R_3\) cells\(^6\);\(^8\). Finally, naturally occurring regulatory T cells (T\(_{Reg}\)) are not characterized by their cytokine production, but are generally distinguished by their expression of the transcription factor FoxP3\(^9\);\(^10\). The function of these T\(_{Reg}\) subsets is to regulate inflammatory responses and to prevent the induction of autoimmunity.

The differentiation of naïve CD4\(^+\) T cells towards these different helper T cell lineages is classically driven by polarizing cytokines, which affect the expression and/or function of instructive transcription factors. T\(_H_1\) polarization occurs subsequent to the production of IL-12 by antigen presenting cells (APCs), which results in the upregulation of the transcription factor TBet in T cells\(^11\). In contrast, T\(_H_2\) formation is enhanced following IL-4 signalling and through the upregulation of the transcription factor GATA-3\(^12\). Commitment of a T cell to the T\(_H_17\) lineage is induced by the transcription factor RORγt. In mice, this transcription factor is upregulated by the combination of TGF-β and IL-6, whereas in humans the combination of TGF-β and IL-1β is necessary\(^5\);\(^13\)-\(^16\). Regulatory T cells are induced by increased levels of TGF-β and retinoic acid and result in the upregulation of the transcription factor FoxP3\(^17\);\(^20\). Although polarizing cytokines clearly fulfill a key function in T\(_H\) cell formation, costimulatory molecules may also play an important role in T cell differentiation and polarization. Several lines of evidence suggest that engagement of the TNFR superfamily member CD27 by its ligand CD70 enhances T\(_H_1\) cell development. Whereas CD70 is only transiently expressed on APCs and lymphocytes during immune activation\(^21\)-\(^25\), we have previously shown that constitutive expression of CD70 on B cells induces a strong increase in the numbers of IFN-γ producing CD4\(^+\) and CD8\(^+\) T cells, thereby enhancing T cell mediated immunity\(^25\);\(^28\). In addition, microarray analysis of activated effector-type CD4\(^+\) T cells from WT vs CD27-deficient mice revealed that CD27-triggering can induce a Th1-like gene expression profile\(^29\). In human T cells, CD27 ligation drives proliferation of CD4\(^+\) T cells, but also T\(_R_1\) polarization via upregulation of IL-12Rβ2 and T\(_{Bet}\)\(^30\). Moreover, human TNF-α-induced CD70\(^+\) DCs can
evoke \(T_h^1\), but also \(T_h^1\)7 responses, although it was not shown whether these responses are indeed dependent on CD27-engagement31. Finally, CD27 ligation in mice can under certain conditions promote \(T_h^1\) cell formation independently of IL-1232. Overall, these data suggest that CD27, as a typical "signal 2", can directly induce \(T_h^1\) cell differentiation without the need for the classical "signal 3".

Based on these observations, we questioned whether triggering through CD27 provides instructive signals for \(T_h^1\) differentiation, or that it rather supports the formation of \(T_h^1\) cells. As the genetic background of mice has been associated with a predisposition to \(T_h^1\) cell polarization and disease development33-36, we decided to approach our question by backcrossing CD70TG mice from a \(T_h^1\)-prone C57Bl/6J to a \(T_h^2\)-prone Balb/c background. Our data indicate that the strong \(T_h^1\) skewing observed in CD70TG mice is highly dependent on the genetic background, as it does not induce \(T_h^1\), nor \(T_h^2\) skewing on a Balb/c background. Importantly, CD27 ligation during the induction of allergic airway inflammation (AAI), a typical \(T_h^2\) response, enhanced the generation of \(T_h^1\) cells without affecting the formation of \(T_h^2\) cells. Together with \textit{in vitro} polarization studies, our data indicate that CD27 does not instruct, but rather supports the formation of \(T_h^1\) cells, both \textit{in vitro} and \textit{in vivo}.

**Materials and Methods**

**Mice**

CD70TG mice were generated on a C57Bl/6J background, maintained heterozygously and bred in the animal department of the Academic Medical Center (Amsterdam, The Netherlands) under specific-pathogen-free conditions26. To generate CD70TG Balb/c mice, mice were backcrossed 10x with wild type (WT) Balb/c mice (Harlan). WT mice were obtained from CD70TG C57Bl/6J or Balb/c littermates. Mice were used at 6-12 weeks of age, age- and sex-matched within experiments and were handled in accordance with institutional and national guidelines.

**Cell staining and flow cytometry**

Single-cell suspensions were obtained by mincing the specified organs through 40 µm cell strainers (Becton Dickinson). Erythrocytes were lysed with an ammonium chloride solution and cells were subsequently counted using an automated cell counter (Casy, Schärfe system). Cells (5 x 10^5- 5 x 10^6) were collected in staining buffer (PBS with 0.5% bovine serum albumin (Sigma)) and stained for 30 min at 4°C with antibodies in the presence of anti-CD16-CD32 (FcBlock, clone 2.4G2; kind gift from Dr. Louis Boon, Bioceros, The Netherlands). The following monoclonal antibodies were obtained from Pharmingen: allophycocyanin-conjugated anti-B220 (clone RA3-6B2); peridinin chlorophyll protein-conjugated (PerCP) anti-CD3ε (clone 145-2C11); Fluorescein isothiocyanate-conjugated (FITC) anti-CD3ε (clone 17A2); PE- or PerCp-conjugated anti-CD4 (clone L3T4); PerCp-, FITC or allophycocyanin-conjugated anti-CD8 (clone Ly-2); PE- or allophycocyanin-conjugated anti-CD62L (clone MEL-14). Antibodies used from eBioscience: PE-conjugated anti-FoxP3 (clone NRRF-30); FITC-conjugated anti-CD44 (clone IM7); FITC-conjugated anti-CD27 (clone LG.7F9). Intracellular stainings for FoxP3 were performed using Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBioscience), according to the manufactures protocol. Data were collected on
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a FACSCalibur or FACSCanto (Becton Dickinson) and were analyzed with FlowJo software (Treestar, Inc.).

**T cell stimulation assays**

*Direct ex vivo cytokine production*

Splenocytes were plated at 1 x 10^6 cells/well in a 96-well round-bottom plate and stimulated for 6 hours with 1 ng/ml PMA and 1 mM ionomycin, of which the last 4 hours was in the presence of 1 ng/ml Brefeldin A (Sigma). Hereafter, cells were stained for CD4 and CD8 followed by fixation and permeabilization. Cells were then incubated for 30 min with fluorescent labelled antibodies against either IFN-γ, IL-17, IL-4, IL-5, IL-13 (eBioscience/BD).

*TH cell polarization*

Naïve (CD44-CD62L^+) CD4^+ T cells and B (B220^+) cells were electronically gated and sorted using a FACSaria cell sorter (Becton Dickinson). The purity of cells sorted using this method was consistently > 96%. Sort purified naïve CD4^+ T cells from WT mice were then stimulated for 3 days under TH0, TH1 or TH17 polarizing conditions, or 7 days under TH2 polarizing conditions, in the presence of WT or CD70TG derived B cells in a 1:1 ratio. All T cell polarization conditions included plate-bound αCD3 (clone 145-2C11, 5 μg/ml), soluble αCD28 (clone PV-1, 1 μg/ml) (both a kind gift from Dr. Louis Boon, Bioceros, The Netherlands) and soluble IL-2 (25 ng/ml) (Invitrogen). For TH1 polarization 10 ng/ml IL-12 (R&D Systems) and 5 μg/ml aIFN-γ (clone 11B11, a kind gift from Louis Boon, Bioceros) was added. For TH2 polarization 50 ng/ml IL-4 (R&D Systems), 5 μg/ml αIL-12 (clone c17.8) and 20 μg/ml αIFN-γ (clone XMG 1.2, both mAbs were a kind gift from Louis Boon, Bioceros) was added. For TH17 polarization, 3 ng/ml TGF-β (R&D Systems) and 20 ng/ml IL-6 (Peprotech) was included. At the end of the culture period, cells were washed and stimulated with 1 μM ionomycin, 1 ng/ml PMA and 1 μg/ml Brefeldin A for 5 hours. Cells were then stained for CD4 and CD8 followed by fixation and permeabilization and stained for IL-4, IL-5, IFN-γ, IL-17, IL-10 and IL-13 as described above.

**Allergic Airway inflammation**

Wild type and CD70TG Balb/c mice were sensitized to OVA by i.p injection of 20 μg OVA (Fluka, Switzerland) in a 200 μl aluminum potassium sulfate suspension (Sigma-Aldrich, Germany) on day 0 and 14. Mice were then challenged on day 28, 29 and 30 by i.n. administration of 100 μg OVA in 50 μl of PBS. Control sensitization was performed with aluminum potassium sulfate in PBS and control challenge was performed with PBS alone. All mice were sacrificed on day 32 and serum, spleen and lung was collected.

**Quantitative real-time PCR (qPCR) analysis**

RNA was isolated from naïve T cells and polarized cells (as described above) using TRIzol (Invitrogen), and cDNA was prepared by reverse transcription of 0.5 μg RNA. The resulting cDNA was subjected to qPCR analysis with the LightCycler System (Roche Diagnostics) in microcappilary tubes with a QuantiTect SYBR Green PCR kit solution (Qiagen). HPRT was used as a reference. Relative changes were calculated by the 2^(-ΔΔCT) method.38
The primers used to detect mRNA transcripts are as follows:

\[
\begin{align*}
\text{mT}_{\text{Bet}}: & \quad 5'-\text{CAACAACCCCTTTGCCAAAG}-3' \text{ (forward)} \\
& \quad 5'-\text{TCCCCCAAGCAGTTGACAGT}-3' \text{ (reverse)}; \\
\text{mGATA3}: & \quad 5'-\text{AGAACCGGCCCCTTATCAA}-3' \text{ (forward)} \\
& \quad 5'-\text{AGTTCGCGCAGGATGTCC}-3' \text{ (reverse)}; \\
\text{mRORγt}: & \quad 5'-\text{TGTCCTGGGCTACCCTACTG}-3' \text{ (forward)} \\
& \quad 5'-\text{GTGCAGGAGTAGGCCACATT}-3' \text{ (reverse)}; \\
\text{mTWIST}: & \quad 5'-\text{CGCACGCAGTCGCTGAACG}-3' \text{ (forward)} \\
& \quad 5'-\text{GACGCGGACATGGACCAGG}-3' \text{ (reverse)}; \\
\text{mFOG}: & \quad 5'-\text{TCCCCTGAGAGAGAAGAACCG}-3' \text{ (forward)} \\
& \quad 5'-\text{GCAGCATCCTAGCCAGCA}-3' \text{ (reverse)}; \\
\text{mHPRT}: & \quad 5'-\text{TGAAGAGCTACTGTAATGATCAGTCAAC}-3' \text{ (forward)} \\
& \quad 5'-\text{AGCAAGCTTGCAACCTTAACCA}-3' \text{ (reverse)}. 
\end{align*}
\]

Statistical analysis
Statistical analysis of the data was performed using the unpaired Student’s *t*-test. Asterisks denote significant differences (* \( p<0.05 \), ** \( p<0.005 \)).

Results

Transgenic CD70 expression on B cells on a C57Bl/6J and Balb/c background
Ensuing T cell activation, the genetic background predisposes polarization to a specific helper T cell subset\(^{33,39}\). To determine whether CD27 ligation differentially affects helper T cell polarization depending on the genetic background, we backcrossed the CD70TG C57Bl/6J mice on a Balb/c background to study the \textit{in vivo} effects of CD70-driven costimulation. Flow cytometric analysis confirmed high CD70 expression on transgenic B cells in both backgrounds, though expression was slightly lower in Balb/c mice (Fig. 1a). CD70 overexpression induced a significantly downregulation of CD27 in all T cells from CD70TG mice in both strains (Fig. 1b), which is indicative of a productive interaction between CD27 and CD70. The functional consequence of this enhanced CD27-mediated costimulation is an increase in effector-memory T cell (CD44\(^{hi}\) CD62L\(^{lo}\)) formation in CD4 and CD8 T cells\(^{26}\), though this was more pronounced in C57Bl/6J mice than in BALB/c mice (Fig. 1c-d). Although the phenotype of CD70TG mice seemed less severe on a BALB/c background, the degree of B cell depletion\(^{26}\) was comparable to C57Bl/6 mice (Fig. 1e). Thus, enhanced CD27-driven costimulation induces strong effector T cell formation and a concomitant loss of B cells, both on a C57Bl/6J and Balb/c background.

Strain specific enhancement of T\(_{h,1}\) polarization via CD27 ligation under homeostatic conditions
To investigate the impact of CD27-triggering on T\(_{h,1}\)-cell polarization, we analyzed the cytokine profile of CD4 T cells by direct stimulation of WT and CD70TG splenocytes with PMA/ionomycin and brefeldin A. Based on these experiments, we conclude that CD70TG C57Bl/6J mice have increased percentages and absolute numbers of IFN-γ production by
CD4+ T cells and decreased production of the Th2 cytokines IL-5 and IL-13 by CD4+ T cells (Fig. 2a-b). No significant changes were identified for IL-17 and a small increase in the absolute, but not relative number of IL-4 producing T cells (Fig. 2a-b). In contrast with C57Bl/6 mice, we found no significant difference in IFN-γ-producing CD4+ T cells in CD70TG mice on a Balb/c background (Fig. 2c-d). Importantly, constitutive CD27 triggering in Balb/c mice did not enhance Th2 differentiation either, but even reduced the levels of IL-5 and IL-13 producing CD4+ T cells (Fig. 2c-d). These data demonstrate that in the steady state situation CD27 triggering reduces Th2 differentiation and enhances Th1 differentiation, but the latter only on a Th1-prone background.

**CD27 ligation does not affect the regulatory T cell compartment**

Since regulatory T cells also express CD2740,41, we assessed whether enhanced CD27 triggering by CD70 could affect the formation and/or activation of this distinct T cell subset.

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**Figure 1. Impact of CD70-overexpression in C57Bl/6J and Balb/c mice.**

CD70 expression on B220+ B cells (a) and CD27 expression on CD3+ T cells (b) in WT and CD70TG spleens from C57Bl/6J or Balb/c mice. Control in b represents background staining on T cells without the CD27 antibody. Quantification of T_em cells (CD44hi CD62Llo) in CD4 (c) or CD8 (d) T cells in WT and CD70TG spleens from C57Bl/6J or Balb/c mice. (e) Quantification of the relative (left) and absolute (right) number of B220+ B cells in WT and CD70TG spleens from C57Bl/6J or Balb/c mice.
Figure 2. TH profile of CD70TG mice on different genetic backgrounds.
WT and CD70TG C57Bl/6J and Balb/c mice were analyzed for their intrinsic cytokine production capacity directly ex vivo. A representative staining on a (a) C57Bl/6J and (c) Balb/c background for IFN-γ, IL-4, IL-5, IL-13 and IL-17 production by CD4+ T cells in WT and CD70TG mice after stimulation with PMA/ionomycin is shown. The percentage of cytokine producing cells by WT or CD70TG derived CD4+ T cells on a (b) C57Bl/6J (average of 4 mice ± SD) or (d) Balb/c (average of 3 mice ± SD) background. Asterisks denote significant differences (* p<0.05; ** p<0.005).
This is particularly important because T_{reg} might also influence T_{H} cell formation. Regulatory T cells are characterized by their expression of the transcription factor FoxP3, high levels of CD25 and can be distinguished into two subsets based on CD103 and CD62L expression. CD70TG mice showed no significant differences in the percentage and absolute numbers of CD4^{+} regulatory T cells on a C57Bl/6J background (Fig. 3a-b), though numbers were reduced in Balb/c mice (Fig. 3e-f). The expression of CD27 on regulatory T cells was significantly downregulated in CD70TG mice (Fig. 3c,g). However, this did not correlate with an increase in the activation state of these regulatory T cells. CD70TG C57Bl/6J mice showed a small increase in CD103 expression, but no difference was observed for CD62L and CD25 expression (Fig. 3c-d). In addition, CD70TG Balb/c mice did not show relevant changes in expression of CD25, CD103 and CD62L (Fig. 3g-h). These data indicate that although CD27 is expressed on T_{reg}, enhanced ligation through CD70 does not affect the activation status of these cells. Overall, the observed changes in T_{reg} homeostasis in CD70TG mice on a C57Bl/6 vs. Balb/c background do not explain the respective changes in effector CD4^{+} T cell profile.

Strain-specific enhancement of IFN-γ producing cells via CD27 ligation under non-polarizing conditions

The cytokine profile of WT and CD70TG splenocytes indicated that CD27-costimulation has a stimulating effect on the formation of T_{H}^{1} cells, at least in C57Bl/6 mice, and an inhibitive effect on T_{H}^{2} cells on both backgrounds (Fig. 2). However, since the conditions and timing under which these T cells were initially activated is unknown, these data do not allow us to conclude whether CD27 triggering provides instructive or supportive signals for T_{H} cell polarization. Therefore we performed T cell stimulation assays under specific polarizing conditions using naive WT T cells and providing CD27 triggering by the addition of either WT or CD70TG B cells. Based on two independent experiments we conclude that that CD27 ligation enhanced the formation of IFN-γ producing T cells under non-polarizing (T_{H}^{0}) conditions for C57Bl/6J derived cells, but not for Balb/c derived cells (Fig. 4). IL-4 production was not affected under these conditions. Importantly, CD27 ligation did neither enhance nor inhibit the formation of IFN-γ or IL-4 producing cells under T_{H}^{1} and T_{H}^{2} polarizing conditions in both genetic backgrounds (Fig. 4). Interestingly, CD27 triggering did reproducibly inhibit the formation of IL-17 producing T cells derived from C57Bl/6 mice, but this was not consistently found with Balb/c-derived T cells (Fig. 4). These data indicate that CD27 ligation provides supportive signals for IFN-γ production under non-polarizing conditions, but repressive signals for IL-17 production; however, this could only be concluded for C57Bl/6-derived cells. Because of this strain-specific effect and because CD27-triggering did not affect T cell differentiation under T_{H}^{1} or T_{H}^{2} conditions, we conclude that CD27-mediated costimulation does not provide instructive signals for T_{H} cell polarization.

CD70-driven costimulation does not inhibit T_{H}^{2} polarization during allergic airway inflammation

Although CD27-costimulation in vitro does not instruct T_{H} cell development, this does not explain why CD70TG mice had significantly fewer T_{H}^{2} cells (Fig. 2). To test whether CD27 triggering inhibits T_{H}^{2} formation in vivo, we exposed WT and CD70TG BALB/c mice to the allergic airway inflammation (AAI) model. This model induces T_{H}^{2} cells that amplify allergic inflammation via the production of cytokines, chemokines and enhancing IgE production.
Figure 3. Impact of CD70-driven costimulation on regulatory T cells.
The regulatory T cell compartment was analysed for WT and CD70TG C57Bl6J (a-d) and Balb/c (e-h) mice. (a,e) Representative staining for FoxP3 within the splenic CD4+ T cell population. (b, f) The percentages and absolute numbers of splenic derived regulatory T cells in mice. (c, g) Representation and (d, h) quantification of CD4+FoxP3+ T cells that express CD27, CD25, CD103 and CD62L, based on the gate in the respective histograms. Asterisks denote significant differences (* p<0.05; ** p<0.005).
Figure 4. Impact of CD70 triggering on T cell polarization in vitro.

T cell polarization assays were performed using WT derived naive CD4+ T cells and WT or CD70TG derived B cells. T cells were cultured under TH0 (non-polarizing), TH1 or TH17 polarizing conditions for a period of 3 days or under TH2 polarizing conditions for 7 days. C57Bl/6J or Balb/c polarized cells were studied for their capacity to produce TH1, TH2 or TH17 associated cytokines upon PMA-ionomycin stimulation after the specified polarization. This experiment was performed twice with similar outcome.

Although the pathogenesis of AAI also includes the recruitment of other T cell subsets into the lung, TH2 cells play an essential role in the inflammatory response. In addition, blockade of the TH2 cytokines IL-4 and IL-13 by antibodies or neutralizing fusion proteins, respectively, resulted in reversing and/or preventing allergen-induced airway hyperresponsiveness during sensitization and challenge phases. We found that both WT and CD70TG Balb/c mice had a significant infiltration of CD4+ T cells in the lung, which allowed us to investigate...
the effect of CD27 ligation on T<sub>H</sub> cell polarization in vivo (Fig. 5a). WT mice showed increased numbers of IL-4, IL-13 and IFN-γ producing CD4<sup>+</sup> T cells in the lung of OVA challenged mice compared to PBS-treated mice (Fig. 5b). Importantly, CD70TG mice showed normal numbers of IL-4 and IL-13 producing CD4<sup>+</sup> T cells and rather unexpectedly even showed a significant increase of IFN-γ producing CD4<sup>+</sup> T cells compared to challenged WT mice (Fig. 5b). Thus, we conclude that CD27-mediated costimulation does not inhibit T<sub>H2</sub> formation, neither in vitro nor in vivo, though it can under certain circumstances promote T<sub>H1</sub> differentiation.

CD70-driven transcription factor regulation

To investigate how CD27 ligation affects CD4 T cell differentiation on a molecular level, we examined the impact of CD70-driven costimulation on transcription factor expression, as changes on this level would be expected if CD27 ligation would provide instructive signals for T<sub>H</sub> cell formation. The transcription factor T<sub>Bet</sub> is the main transcription factor associated with T<sub>H1</sub> polarization. T<sub>Bet</sub> induces the expression of IL-12Rβ2, thereby allowing cells to differentiate to T<sub>H1</sub> cells following IL-12 signalling<sup>11,49,50</sup>. We found that T<sub>Bet</sub> mRNA is induced under T<sub>H0</sub> polarizing conditions compared to naïve T cells, maintained under T<sub>H1</sub> polarizing conditions, but downregulated under T<sub>H2</sub> or T<sub>H17</sub> conditions. Importantly, CD27 triggering did not affect mRNA expression of T<sub>Bet</sub> under these conditions (Fig. 6a). GATA3 is the central transcription factor responsible for T<sub>H2</sub> polarization<sup>12</sup>, and is essential for the cytokine profile associated with T<sub>H2</sub> polarized cells<sup>12,51,52</sup>. We found that GATA3 transcript levels remained similar to levels found in naïve T cells under T<sub>H0</sub> conditions, were downregulated under T<sub>H1</sub> and T<sub>H17</sub> conditions, and were upregulated under T<sub>H2</sub> conditions. However, CD27 triggering did not affect the GATA3 transcription expression levels under any conditions (Fig. 6b). The transcription factor RORγt, which is important for T<sub>H17</sub> lineage commitment<sup>16</sup>, was only found under T<sub>H17</sub> conditions and was not regulated by CD27 stimulation (Fig. 6c). The transcription factors TWIST and FOG have been implicated in a negative feedbackloop for
Function of CD27 in T helper cell differentiation

T<sub>H</sub>1 and T<sub>H</sub>2 polarization, respectively<sup>53-55</sup>. We found that both factors were downmodulated with respect to naïve T cells under all polarizing conditions and that CD27 ligation did not affect the respective transcription factor expression levels (Fig. 6d-e). Thus, we conclude that CD27 triggering does not influence the expression of instructive transcription factors, which is in line with the notion that CD27 supports, but does not instruct the formation of IFN-γ producing CD4<sup>+</sup> T cell following T cell activation.

![Figure 6. Enhanced CD70-driven IFN-γ production is not transcriptionally regulated.](image)

Naive CD4<sup>+</sup> T cells were stimulated under specific TH0, TH1, TH2 and TH17 polarizing conditions in the presence of WT or CD70TG C57Bl/6J derived B cells. Transcript levels encoding (a) TBet, (b) GATA3, (c) RORγt, (d) TWIST, and (e) FOG were analysed by qPCR, normalized with HPRT for each condition and depicted as the number of copies per HPRT transcript. This experiment was performed twice with similar outcome.
Discussion

In the present study, we show that although CD70-overexpression strongly promotes the formation of IFN-γ producing CD4+ T cells, costimulation through CD27 does not induce differentiation of T_H1 cells per se. Instead, CD27 seems to support T_H1 cell formation, but this is dependent on the culture conditions and genetic background of the mice. The latter has been associated with a predisposition towards T_H1 or T_H2 polarization, i.e. C57Bl/6J mice are more prone towards T_H1 cell development, whereas Balb/c mice are more T_H2 prone. This variance in polarization can be attributed to a difference in a dominant genetic locus between the different genetic backgrounds, but could also be related to differences in chromatin remodelling subsequent to receptor signalling. In this respect, it has been suggested that Balb/c mice are less capable of sustaining responsiveness to IL-12 compared to C57Bl/6J mice, thereby decreasing their T_H1 polarizing capacity. Although it is unknown which genetic differences are responsible for the distinct phenotypes of C57Bl/6J and Balb/c CD70TG mice, it could be that epigenetic regulation of the Th1 locus plays an important role in this process. It has been reported that CD27-triggering on CD4 T cells downregulates expression of Prmt1, an enzyme that methylates histones. If CD27-costimulation influences the Th1 locus, this could explain why the effects at steady state are more clear in C57Bl/6 mice (Fig. 2), because this locus might be less accessible in BALB/c mice. The fact that there is IFN-γ production during AAI even in BALB/c mice implies that the Th1 locus does become accessible during these conditions, which could be the reason why CD27 ligation is capable of enhancing the formation of IFN-γ producing CD4+ T cells also in this model (Fig. 5). It could also be that the CD27-costimulatory pathway acts in concert with transcription factors involved in Th1 formation and is therefore more effective on the C57Bl/6 background. Moreover, it has been shown that CD27 exerts its costimulatory effect by enhancing T cell proliferation and/or survival, though it is not clear if these effects are relevant for our current study, as it is difficult to comprehend why such effects would be dependent on a particular T cell subset and the genetic background of the mice. In comparison, ligation of OX40, another member of the TNFR superfamily, can also induce proliferation and survival of CD4 T cells and thereby enhance the pool of T_H1, but also of T_H2 cells, depending on the model. More molecular studies are required to understand the genetic differences between C57Bl/6J and Balb/c mice regarding Th1 cell formation and how CD27-mediated costimulation influences this process.

Whereas CD70 clearly stimulates T_H1 cell development, it has also been shown that TNF-α-induced CD70+ DCs can induce Th17 responses in humans. Our data clearly indicate that CD27 triggering in T cells, at least on the C57Bl/6J background, inhibits the formation of T_H17 cells in vitro (Fig. 4). It could well be that this is due to the enhanced IFN-γ secretion, since loss of IFN-γ secretion promotes T_H17 formation, whereas IFN-γ negatively affects formation of the T_H17 lineage. This also correlates with the strain-specific effect, since the strong increase on IFN-γ production was more profound on cells from C57Bl/6J than Balb/c mice. Interestingly, it has recently been shown in γδ T cells that CD27-positive cells produce IFN-γ and that IL-17 production is restricted to CD27-negative cells, also indicating that CD27-signaling and IL-17 production are not compatible. IL-17 producing T cells were not significantly altered in CD70TG mice, but numbers were very low and it would therefore be important to induce T_H17 formation in these mice, for instance by bacterial infection, to examine the impact of CD27-triggering on IL-17 production in vivo. We have previously
shown that absence of CD27 does at least not affect the outcome of a bacterial infection with the intracellular pathogen Mycobacterium tuberculosis\(^{67}\), but IL-17 production by CD4 T cells was unfortunately not investigated in this study.

As T cell immunity can be seen as a balance between activation and regulation, we postulated that CD27 signalling could influence T\(_{\text{Reg}}\) numbers and/or their activation state. Importantly, other TNFR superfamily members have been shown to influence T\(_{\text{Reg}}\) numbers and/or function. GITR is capable of promoting T\(_{\text{Reg}}\) proliferation without impairing its regulatory function\(^{68,69}\), whereas OX40 inhibits the induction of regulatory T cells from effector T cells\(^{70}\). We found that T\(_{\text{Reg}}\) express CD27, and that transgenic overexpression of CD70 resulted in a significant reduction of membrane bound CD27, indicative for an interaction with CD70. However, both the activation state and absolute numbers of regulatory T cells were not affected in CD70TG mice in C57Bl/6J mice, though T\(_{\text{Reg}}\) numbers were modestly reduced on the Balb/c background. Nevertheless, these changes do not correlate with the observed changes in CD4 effector T cells, suggesting that regulatory T cells are not accountable for the differences observed in helper T cell formation between C57Bl/6J and Balb/c mice.

Classically, the generation of IFN-γ producing T\(_{1}\) polarized cells occurs following the upregulation and activation of the transcription factor T\(_{\text{Bet}}\). T\(_{\text{Bet}}\) activation results in an upregulation of IL-12R\(_{\beta2}\), and the subsequent formation of active IL-12R. In addition, T\(_{\text{Bet}}\) plays an important role in chromatin remodeling\(^{71}\), thus allowing transcription of T\(_{1}\) dependent genes and instructing T\(_{1}\) cell formation. Steinman et al.\(^{32}\) showed that CD27 ligation could enhance formation of IFN-γ producing CD4\(^+\) T cells in an IL-12 independent manner, suggesting that CD27 signalling can promote helper T cell polarization independently of the classical polarization via the cytokine environment. Although the underlying mechanism was not investigated in that study, the findings are congruent with the hypothesis that CD27 triggering does not instruct, but rather supports T\(_{1}\) cell formation. This supporting effect is substantiated by the finding that CD27-triggering does not affect T\(_{\text{Bet}}\) expression levels (Fig. 6). It has been reported that IFN-γ can enhance T\(_{\text{Bet}}\) expression\(^{72}\), but this feedback system might not be relevant in this system, as T\(_{\text{Bet}}\) levels were also comparable between T\(_{0}\) and T\(_{1}\) cells (Fig 6), despite a much higher number of IFN-γ producing cells under T\(_{1}\) compared to T\(_{0}\) conditions (Fig. 4).

In conclusion, these data indicate that CD27 signalling specifically enhances the pool of IFN-γ producing CD4\(^+\) T cells, not by providing instructive polarizing signals, but most likely by the combination of sensitizing these cells for IL-12 mediated signaling and by acting on the proliferation and/or survival of these cells.

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Reference List


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64. Ohshima Y, Yang LP, Uchiyama T et al. OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human CD4(+) T cells into high IL-4-producing effectors. Blood 1998;92:3338-3345.


Chapter 3

CD27 expression and function during ontogeny of the hematopoietic system

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Submitted
Abstract

The observation that tumor necrosis factor receptor (TNFR) superfamily member CD27 is expressed on adult hematopoietic stem and progenitor cells (HSPCs) strongly suggests that direct cell-cell contact between immune cells and HSPCs plays a role in regulating hematopoiesis and raised the question whether CD27-signaling could also be involved in the development of the hematopoietic system during embryogenesis. We here report that CD27 is expressed on the majority of definitive hematopoietic stem and progenitor cells in mice from E11.5 onwards. While absence of CD27 on adult HSPCs results in enhanced myeloid-colony forming potential, hematopoiesis is unaffected in CD27-/- fetuses when compared to wildtype controls. By overexpressing CD70, the unique ligand of CD27, we previously showed that constitutive triggering of CD27 on HSPCs has pronounced effects on hematopoiesis in adult life. In contrast, in this study we found that CD27-triggering on fetal liver HSPCs via CD70 transgenic (CD70TG) B-cells does not affect hematopoiesis during embryogenesis. Furthermore, our findings reveal that in CD70TG mothers, which have a chronically activated immune system, neither CD70-expressing B cells, nor activated T cells and the pro-inflammatory cytokine interferon-γ (IFN-γ) affect the ontogeny of the hematopoietic system of the fetus. In contrast to the expression of CD27 on fetal HSPCs from the mouse, CD27 expression is lacking on human fetal HSPCs. Taken together, these data show that the fetus is well protected against the activated immune system of the mother and that CD27-expression on fetal HSPCs does not play a pivotal role during embryogenesis.
Introduction

In the mouse embryo the first definitive hematopoietic stem cells (HSCs) arise from haemogenic endothelium in the dorsal aorta at embryonic day 10.5 (E10.5)\(^1\)-\(^4\). After emergence, HSCs are thought to enter the circulation and colonize the fetal liver (FL) from E11 onwards. Thereafter, HSCs eventually colonize other secondary hematopoietic niches like the bone marrow from where the hematopoietic system is further developed\(^5\). Via a delicate balance between self-renewal and differentiation, HSCs are capable of generating and maintaining the blood and immune system throughout the entire life-span of an organism\(^6\);\(^7\). Although being dormant most of the time, the generation and maintenance of HSCs during periods of cellular stress imposes a dynamic and tight regulation of these cells\(^8\). An important role in regulation of HSC function lies within the regulatory microenvironment in which these cells reside, the HSC niche\(^9\). Within the bone marrow (BM), where most of the adult HSCs reside, two niches have been described. One HSC niche was found near the border of bone marrow and endosteum\(^10\);\(^11\), the other is located in the perivascular region of blood vessels\(^12\);\(^13\). During embryogenesis, FL-HSCs are found within and adjacent to sinusoidal networks\(^14\). Dependent on cell-cell interaction and signals received within the niche, the fate of HSCs can be altered. HSCs then proliferate and differentiate into more committed progenitor at the expense of maintaining their quiescence and self-renewal capacity. The more differentiated progenitors cells will subsequently give rise to the fully differentiated blood cells of all lineages of the hematopoietic system\(^15\). While HSC emergence and differentiation to mature blood cells in the fetus is well described, the actual signals and cell-cell interactions affecting this process still remain to be explored in detail.

Although first described to be solely expressed on lymphoid cells\(^16\)-\(^19\), high expression of the tumor necrosis factor (TNF) receptor superfamily member CD27 was also found on adult hematopoietic stem and progenitor cells (HSPCs) in adult mouse BM\(^20\);\(^21\). CD27 acts as a co-stimulatory molecule during immune responses and induces activation, expansion and differentiation of lymphocyte populations\(^22\);\(^29\). More recently it is shown that CD27 co-stimulation also lowers the threshold of T cell receptor triggering. This mechanism enables the immune system to generate T-cell responses against low-affinity antigens, thereby generating a broader response to pathogens\(^26\). In the adult mouse, expression of the unique ligand of CD27, the type II TNF-related transmembrane glycoprotein CD70, is restricted and is only transiently expressed on T cells, B cells and dendritic cells upon immune activation\(^19\);\(^27\)-\(^29\). The observation that CD27 is expressed on adult HSPCs therefore suggested that the activated immune system could affect hematopoiesis. CD27 triggering on hematopoietic stem and progenitor cells (HSPCs) by overexpression of CD70 on B cells indeed inhibits HSPC colony forming potential \textit{in vitro} and especially B cell differentiation in the BM is abrogated \textit{in vivo} in adult mice\(^21\). In addition, CD27-triggering enhances self-renewal of adult HSCs, thereby inducing accelerated aging of the HSC compartment (De Bruin, unpublished data). Moreover, enhanced CD70 expression also has secondary hematopoietic effects in adult mice due to enhanced interferon-γ (IFN-γ) production by an extended effector T cell pool\(^23\). Enhanced production of IFN-γ due to CD27-triggering further depletes B cells\(^23\), hampers erythropoiesis\(^30\) and the formation of eosinophilic and neutrophilic granulocytes\(^31\);\(^32\), while myelopoiesis is enhanced\(^32\).

Given the fact that the absence of CD27 and CD70-mediated triggering of CD27 on adult mouse HSPCs have profound effects on hematopoiesis\(^21\), and other immune-system related
signals, like interleukin-1 and tumor growth factor (TGF)-β, have been found to affect early hematopoiesis, we sought to determine whether CD27 is already expressed on fetal HSCs and whether its expression has a function in shaping the developing hematopoietic system. We here describe the emergence of CD27-expressing HSPCs within the aorta-gonad-mesonephros-region (AGM) and FL of mice as early as E11.5 of gestation. At E12.5 the majority of HSPCs within the FL express high levels of CD27. Absence of CD27 on fetal HSPCs had no effect on hematopoiesis in vivo, nor are CD27 HSPCs impaired in their functional capacity in vitro. Furthermore, by using CD70 transgenic (CD70TG) mice we show that CD27-triggering on HSPCs during ontogeny does not affect hematopoiesis in vivo or affects the hematopoietic output in vitro.

In humans maternal immune activation during pregnancy can have serious consequences for the conceptus. Not only can it affect development and cause per-term birth in diseases like inflammatory bowel disease, diseases like rubella, hepatitis and cytomegalovirus can cause malformations of the fetus and induce abortion. By using CD70TG mice as a model for sterile immune activation, we show that the chronic immune activation that is present in CD70TG mothers has no impact on hematopoiesis in the fetus. In contrast to mouse, where CD27-expression on HSPCs is eminent, we found that HSCs from human FL, fetal BM and cord blood do not express CD27.

Taken together, we conclude that the presence, absence or triggering of CD27 on HSPCs does not influence the development of the hematopoietic system in the fetus, but rather has a role in affecting hematopoietic output during immune activation after birth. In addition, we show that the fetus is well protected from maternal immune activation.

Materials and Methods

Mice
WT, CD27 and CD70TG mice on a C57BL/6 background were used for experiments. Mice were bred and had access to water and food ad libitum in specific pathogen free conditions in the animal facility of the Academic Medical Center (University of Amsterdam, Amsterdam). All animal experiments were approved by the Experimental Animal Committee of the Academic Medical Center in Amsterdam, The Netherlands, according to institutional and national guidelines.

Preparation of the AGM Region and Fetal Liver
For the generation of embryos, adult mice were submitted to timed pregnancies in the late afternoon. Appearance of a vaginal plug the next morning was considered E0.5. At time-points between E10.5 and E17.5 pregnant females were sacrificed and embryos were isolated. The AGM-region and FL were then dissected using a dissection microscope. AGM regions were first dissociated by immersion in IMDM supplemented with collagenase (0.12% w/v type I, a kind gift from Prof. Dr. E. Dzierzak) for 45 minutes at 37°C. Subsequently, the AGM cell suspensions and FLs were strained through a 70 μm cell strainer to obtain single cell suspensions. For all washing and additional handling steps, cells were taken up in PBS supplemented with 10% fetal calf serum.
Flow Cytometry and Cell Sorting
Monoclonal antibodies used (all from eBioscience, unless stated otherwise) were CD19-Fluorescein isothiocyanate (FITC; 1D3), B220-Phycoerythrin(PE)-Cy7 (RA3-6B2), CD4-PE (GK1.5), CD8a-allophycocyanin (APC; 53-6.7), GR-1-FITC (RB6-8C5), CD11b-APC-Alexa780 (M1/70), Ter119-PE-Cy5.5 (Ly-76) and CD70-PE (FR70). For identification of HSPCs by flow cytometry, cells were first incubated with a lineage cocktail of biotin-conjugated antibodies directed against CD4, CD8a, B220, Gr1 and Ter119. As CD11b / MAC-1 is described to be expressed on fetal hematopoietic progenitor cells, this marker was not used in our lineage cocktail. Subsequently, cells were washed and incubated with streptavidin-PE, Sca-1-PE-Cy7 (D7), c-Kit-APC-Alexa780 (2B8) and CD27-APC (LG.7F9). All stainings were performed in the presence of anti CD16/32block (2.4G2, a kind gift of Louis Boon, Bioceros, Utrecht, The Netherlands). HSPCs were defined as c-Kit+ Sca-1+ in the AGM-region at all time-points and E11.5 from FL. From E12.5 onwards FL-HSPCs were defined as Lin- C-Kit+ Sca-1+ (LKS). Data acquisition was done using a FACS Calibur or FACS Canto II (BD Biosciences). Cells were sorted using a FACS Aria (BD Biosciences). Data was analyzed using FlowJo Software (Treestar).

Semi Solid Colony Assays
FACS-sorted FL-HSPCs or total FL cells were cultured in complete methylcellulose medium (Methocult m3434, Stem Cell Technologies) at 250 or 100,000 nucleated cells, respectively, in 6-wells plates. For FACS-sorted LKS cells, colonies were typed and scored at day 12. For total FL cells analysis on the number and type and colonies generated was done at day 8.

Human Fetal Liver, Fetal Bone Marrow and Cord Blood Analysis
With informed consent and approval of the Medical Ethical Committee of the Academic Medical Center / University of Amsterdam, 14 to 20 week old human FL and fetal BM samples were obtained from elective abortions. Cord blood was obtained with informed consent and approval of the Medical Ethic Committees of local hospitals. To obtain single cells suspensions from fetal BM and FL, tissues were first mechanically disrupted and subsequently filtered over a mesh. FL, fetal BM and cord blood CD34+ cells were then isolated from single cell suspensions using a Ficoll-Paque Plus (GE-Healthcare) density gradient and subsequent magnetic enrichment using the CD34 Progenitor Cell Isolation Kit (Miltenyi Biotech). Obtained cell fractions from FL and fetal BM were labelled with CD3-FITC (SK7), BDCA2-FITC, CD56-FITC, CD10-FITC, CD34-PE-Cy7, CD38-PerCP (HIT2), CD133-PE, CD45-Alexa700 (2D1 and HI30), and CD27-APC (LG.7F9) for FACS analysis. Cord blood samples were labelled with CD34-PE-Cy7 (8G12), CD38-PerCP-Cy5.5 (HIT2), CD45-PacificBlue (T29/33) and CD27-APC (LG.7F9). Data acquisition was done using a LSR II cytometer (BD Biosciences) and analyzed using FlowJo Software (Treestar).

Statistical analysis.
Results are expressed as mean ± SD. Statistical analysis of groups was performed using Graphpad Prism 5, using either a non-paired 2-tailed Student t-test when comparing two groups or a one-way ANOVA with Bonferroni correction when more then 2 groups. P<0.05 was considered statistically significant.
Figure 1. CD27-expressing HSPCs in mouse embryonic tissues.

CD27 expression analysis on definitive HSPCs from AGM and fetal liver. (a) Representative dot plots and histograms from flow cytometric analysis of CD27 expression on HSPCs from E12.5 fetal liver. The % of positive cells is based on stainings on HSPCs from CD27-/- fetal livers. Data is representative for 2 independently performed experiments containing at least 5 mice per group. (b) Flow cytometric analysis of CD27 expression on HSPCs from E10.5-E12.5 AGM regions and E11.5-E12.5 fetal livers. E10.5 and E11.5 data are representative for 1 experiment containing at least 4 mice per group, E12.5 data are representative for 2 experiments containing at least 5 mice per group. (c) Colony forming capacity of sorted CD27+ and CD27- WT fetal liver HSPCs (Lin- C-kit+ Sca-1+) at E12.5 from 2 different experiments. Data is expressed as bar graphs +/- SD and represents counts in duplo for each sample.
Results

**CD27-expressing HSPCs in mouse embryonic tissues.**

While CD27-expressing HSPCs can be found in adult mouse BM\(^{20,21}\), it is unknown when CD27 expression is initiated during development and whether it is functionally involved on emerging HSPCs in early life. Therefore, CD27 expression was analyzed by FACS on HSPCs from E10.5-E12.5 mouse AGM-regions and FLs (Fig. 1a-b). No CD27-expression was found on HSPCs from the AGM-region at E10.5, using CD27\(^{-/-}\) embryos as negative controls. However, profound expression of CD27 was found on HSPCs in both the AGM-region and FL from E11.5 onwards (Fig. 1a-b). Although the majority of FL-HSPCs at E12.5 express CD27, there is a small population where CD27-expression is absent (Fig. 1a-b). To examine functional differences between CD27\(^{+}\) and CD27\(^{-}\) HSPCs these subsets were sorted and cultured in

![Graphs showing data](image)

**Figure 2. Absence of CD27 on fetal HSPC does not impair HSC function during ontogeny.**

Analysis of the hematopoietic precursor compartment of WT and CD27\(^{-/-}\) fetal livers at E12.5. (a) Absolute number of cells in fetal liver as determined with flow cytometric analysis. Data are expressed as bar graph +/- SD from mice analysed in two independent experiments, with each group containing 4 mice. * indicates a significant difference (P<0.05) between using a 2-tailed Student \(t\)-test. (b) Absolute number of LKS cells in fetal liver at E12.5 as determined with flow cytometric analysis. Data are expressed as bar graph +/- SD from mice analysed in two independent experiments, with each group containing 4 mice. (c) Colony forming capacity of sorted WT and CD27\(^{-/-}\) sorted fetal liver HSPCs (LKS-cells) from 2 different experiments. Data is expressed as bar graphs +/- SD and represents counts in duplo for each sample.
semi-solid colony forming assays. The presence or absence of CD27 on HSPCs had no effect on the functional capacity of these cells as both CD27⁺ and CD27⁻ HSPCs gave rise to the same amount and type of colonies in two independently performed experiments (Fig. 1c). CD27 is thus expressed on fetal HSPCs from E11.5 onwards, but does not discriminate between functionally different subsets of HSPCs in the embryo.

Absence of CD27 on fetal HSPC does not impair HSC function during ontogeny. Next we checked whether the absence of CD27 affected the development of the hematopoietic system. CD27⁻/⁻ mice are viable and show no apparent phenotype (data not shown). We observed that in the E12.5 wildtype embryos total FL cell numbers are slightly higher than in FLs of CD27⁺ embryos (Fig. 2a). However, both the percentage and absolute number of LKS cells of E12.5 CD27⁻/⁻ FLs was comparable to wildtype FL (Fig. 2b). In addition, E12.5 CD27⁻/⁻ FL LKS cells gave rise to the same amount and types of hematopoietic colonies when compared to WT FL LKS cells, suggesting that E12.5 CD27⁻/⁻ FL cells are similarly competent as WT FL cells and indicating that CD27 is not required for hematopoiesis during ontogeny (Fig. 2c).

Triggering of CD27 by CD70 does not impair HSC function during ontogeny. Expression of CD27 on HSPCs during ontogeny suggests that triggering of CD27 by CD70 affects HSPC function. To test this hypothesis we made use of CD70 transgenic (CD70TG) mice, which express CD70 under control of the B-cell specific human CD19 promotor. At E17.5, when a substantial amount of CD19⁺ cells can be found in the FL, we observed in CD70TG embryos a clear population of B220⁺ CD19⁺ B cells that expressed high levels of CD70 (Fig. 3a). Total FL B cell numbers were comparable between CD70TG and control mice (Fig. 3b), this in contrast to adult CD70TG mice, where B cell differentiation is impaired and B cells are gradually depleted. Compared to WT embryos, expression of CD27 is downregulated on LKS cells in CD70TG embryos, indicating that CD27 on HSPCs has been triggered (Fig. 3c). This is in line with earlier results that show downregulation of CD27 expression upon interaction with CD70. Surprisingly, triggering of CD27 induced a modest, but significant, increase in the number of LKS cells compared to WT (Fig. 3d), which was also seen in CD27⁻/⁻ embryos. The presence of a higher number of LKS cells however had no consequences for hematopoietic lineage differentiation as the absolute cell number of FLs was comparable between groups (Fig. 3e). In addition, the functional hematopoietic differentiation capacity of CD27-triggered LKS cells was unaltered as both the number and types of colonies formed were comparable to WT and CD27⁻/⁻ embryos (Fig. 3f). These data thus show that CD27 indeed can be triggered on HSPCs during ontogeny, but it does not have clear consequences for the number of HSPCs and hematopoietic cells in vivo or the functional capacity of these cells in vitro.

Chronic immune activation in the mother does not affect ontogeny of her progeny. Although the maternal and fetal blood supply are separated by the placenta, it is unclear whether immune activation in the mother, or activated immune cells from the mother that cross the placenta, affect the unborn fetus. As described earlier, CD27-triggering itself, and the consequential high number of effector T cells and elevated IFN-γ levels in CD70TG mice have profound effects on hematopoiesis in adult mice. This chronic state of immune
Figure 3. Triggering of CD27 by CD70 does not impair HSPC function during ontogeny.

Analysis of the effects of constitutive CD27-triggering on E17.5 fetal liver HSPCs. (a) Representative histogram from CD70 expression on B-cells using flow cytometric analysis. Data are representative for at least 3 mice per group. (b) Absolute B cell numbers in fetal liver. Data are expressed as bar graph +/- SD. Each group contains at least 3 mice per group. (c) Representative histogram from CD27 expression on E17.5 HSPCs (LKS-cells) using flow cytometric analysis. Data are representative for at least 3 mice per group. (d) Absolute number of HSPCs (LKS-cells) in fetal liver as determined with flow cytometric analysis. Data are expressed as bar graph +/- SD. Each group contains at least 3 mice. * indicates a significant difference (P<0.05) between WT and the analyzed groups using a one-way ANOVA with Bonferroni correction (e) Absolute number of cells in fetal liver as determined with flow cytometric analysis. Data are expressed as bar graph +/- SD. Each group contains at least 3 mice. (f) Colony forming capacity of sorted fetal liver HSPCs from WT, CD27-/- and CD70TG embryos. Data is expressed as bar graphs +/- SD and represents counts in duplo for three samples per group.
Figure 4. Chronic immune activation in the mother does not affect ontogeny of her progeny.
Comparison of the hematopoietic compartment of E12.5 embryos from a WT and CD70TG mother. (a) Representative dot plots from cytometric analysis of Sca-1 expression on HSPCs from fetal livers from embryos isolated from a WT or CD70TG mother and bone marrow from a WT or CD70TG mother. (b) Representative histograms from flow cytometric analysis of CD27 expression on fetal HSPCs from a WT or CD70TG mother. (c) Absolute HSPC cell number in fetal liver. Data are expressed as bar graph +/- SD. Each group contains at least 5 mice per group. (d) Colony forming capacity of sorted fetal liver HSPCs from embryos from a WT and CD70TG mother. Data is expressed as bar graphs +/- SD and represents counts in duplo for three samples per group. (e) Absolute number of cells in fetal liver as determined with flow cytometric analysis. Data are expressed as bar graph +/- SD. Each group contains at least 5 mice per group. (f) Representative dot plots from cytometric analysis of hematopoietic lineages present in fetal livers from embryos isolated from a WT or CD70TG mother.
activation of the mother could potentially also affect the development of the hematopoietic system of the fetus. To investigate this, we analysed the hematopoietic compartment in fetuses of CD70TG mothers at E14.5, when the adaptive immune system is undeveloped. As IFN-γ is known to induce Sca-1 expression on HSPCs\textsuperscript{21,30-32}, Sca-1 expression was analysed on Lin\(^{-}\) c-Kit\(^{+}\) cells of E14.5 FLs. In contrast to CD70TG mothers themselves, where Sca-1 was highly expressed on BM-HSPCs, embryos carried by these mice did not display upregulation of Sca-1 (Fig. 4a), strongly suggesting that IFN-γ, produced by the mother, does not pass the placenta. Although still being controversial, it has been proposed that both maternal and fetal cells in general, and lymphocytes in particular, might cross the placenta and induce tolerance\textsuperscript{41-45}. Therefore, we next checked whether B cells or activated T cells from a CD70TG mother could cross the placenta and alter hematopoiesis in the fetus. In contrast to the mother itself, where CD27 is downregulated on BM HSPC, we did not observe downregulation of CD27 on FL LKS cells in any of her fetuses (Fig. 4b), indicating that CD70TG B cells do not or only in very small numbers cross the placenta. In addition, we did not find alterations in the absolute number of LKS cells present in the FLs of embryos from CD70TG mothers when compared to their WT littermates (Fig. 4c), nor was their functional capacity affected or was the FL cellularity altered (Fig. 4d-e). Furthermore, we did not observe any differences in lineage development in the FL as the percentages of granulocytic (GR-1\(^{+}\)), monocytic (CD11b\(^{+}\)) and erythroid (Ter119\(^{+}\)) cells are similar between fetuses from WT and CD70TG mothers (Fig. 4f). The absence of B220\(^{+}\) B cells and CD4\(^{+}\) and CD8\(^{+}\) T cells at this time-point (Fig. 5f) further indicate that during immune activation lymphocytes do not detectably cross the placental barrier and do not affect hematopoiesis. Taken together, within this model the placenta thus protects the developing hematopoietic system of the fetus from chronic immune activation within the mother.

**Human fetal HSPCs lack CD27-expression.**

Although CD27 expression was not found on the vast majority of CD34\(^{+}\) cells from BM of adult humans\textsuperscript{20,46}, it was recently reported that CD27 is expressed to a certain extent on normal HSCs in human and that it is particularly high on human leukemic HSCs\textsuperscript{47}. To further investigate whether CD27 is expressed on human fetal HSPCs as in mouse fetal tissues, we also examined human FL, fetal BM and cord blood. Whereas we could find clear expression of CD27 on Lin\(^{-}\) CD34\(^{+}\) cells from FL and fetal BM, CD27 is largely absent from the HSCs (Lin\(^{-}\) CD34\(^{+}\) CD38\(^{+}\)) in these organs (Fig. 5a-b) as well as in cord blood (Fig. 5c). Also the hematopoietic progenitor populations (Lin\(^{-}\) CD34\(^{+}\) CD38\(^{+}\)) of FL, fetal BM and cord blood are mostly negative for CD27 expression. These data demonstrate that, in contrast to mouse, the vast majority of human fetal tissue HSPCs lack the expression of CD27, suggesting that CD27 also does not play a major role in shaping the human hematopoietic system during ontogeny.

**Discussion**

Here we describe the expression of the TNF-receptor superfamily member CD27 on definitive HSPCs in mouse fetuses from E11.5 onwards (Fig. 1a-b), suggesting that CD27 could play a role in development of the hematopoietic system. These data correlate well with a recently described comprehensive transcriptomic analysis of developing HSCs in murine embryos\textsuperscript{48},...
in which CD27 mRNA could be first detected in HSCs from the AGM-region at E11.5 and in FL HSCs from E12.5 onwards (see also http://hsc.hms.harvard.edu). In contrast to the adult situation however, neither the absence, nor the triggering of CD27 has an effect on hematopoiesis in the fetus as in both cases the number of LKS-cells and the functional capacity of these cells is comparable to control cells (Fig. 1c, Fig. 2a-c, Fig. 3d/f). The absence of hematopoietic effects in the fetus due to constitutive CD27-triggering could be due to the duration of CD70-binding. As it takes up until E17.5 for a substantial amount of CD70TG B cells to arise, constitutive CD27-triggering on fetal HSPCs is up until birth relatively short, giving little time to develop hematopoietic differences. This in contrast to adult CD70TG mice, where from E17.5 onwards CD27-triggering on HSPC occurs and which in time could induce more pronounced hematopoietic effects. Although we found some significant differences between WT, CD27-/- and CD70TG embryos regarding cell numbers (Fig. 2a, Fig. 3d), these differences might reflect small discrepancies between gestation time within an experiment.
Another explanation for the lack of effects of CD27-triggering could be lying in differences in the downstream signalling upon CD27-triggering between adult and fetal HSCs. Upon binding to CD70, the intracellular tail of CD27 is engaged to TNFR-associated factor 2 (TRAF2) and TRAF5, ultimately activating the canonical and non-canonical nuclear-factor-κB (NFκB) pathways, as well as the c-Jun-N-terminal kinase (JNK) pathway. Activation of these pathways upon CD27 triggering in activated CD4+ T cells leads to the transcription of genes related to induction of Th1 responses and the upregulation of the anti-apoptotic B-cell chronic lymphocytic leukemia / lymphoma 2-like 1 (Bcl-XL) in adult mice. In CD8 T cells it mainly induces survival by induction of IL-2, Bcl-XL and Pim expression. The effects on gene expression by CD27 triggering on HSPCs is largely unknown, but recent data from our lab show that constitutive CD27-triggering on adult long term repopulating HSCs results in upregulation of genes related to cell cycle progression, DNA replication, DNA repair responses, myeloid differentiation and stress responses, while genes related to regulation of adaptive immune responses, induction of cell death and cell cycle arrest are downregulated, ultimately leading to enhanced HSC self-renewal and accelerated ageing of HSCs (De Bruin; unpublished data). Although CD27 expression on fetal HSPCs is downregulated in CD70TG embryos, which is indicative for CD27-triggering, we show that this has no effect on the number of HSPCs in vivo and the functional capacity of HSPCs in vitro (Fig. 3d,f). Although we did not specifically analyse cell cycle status, these data suggest that cycling of HSPCs is not induced. In addition we did not find an enhanced myeloid output based on the total cell numbers and functional capacity of fetal HSPCs (Fig 3e-f), as seen in adult CD70TG mice. Although we cannot exclude any effect of CD27-triggering on HSPCs based on gene expression, our data suggests that CD27-signaling on fetal hematopoietic progenitors might be blocked or overruled by other developmental signalling cues. From these data we can thus conclude that although CD27 is expressed on fetal hematopoietic progenitors of the fetus, this co-stimulatory molecule is not involved in shaping the hematopoietic system of the fetus. We therefore state that it isn’t until immune activation is induced after birth that CD27-triggering on HSPCs becomes important in affecting the hematopoietic output.

Whereas CD27-expression is evident on human leukemia stem cells (LSCs) and contributes to disease progression by induction of Wnt target genes, CD27 expression on healthy human HSPCs is absent or low. In addition to these data, we show that we did not find convincing CD27 expression on fetal HSCs from FL, fetal BM and cord blood (Fig. 5). As expression was very low and only found on a minority of HSCs, the impact of CD27-signaling on hematopoiesis therefore would not be of significant influence. Given the fact that in adult mice CD27 stimulation on HSPCs extensively influences the hematopoietic system, it is tempting to explore whether other co-stimulatory proteins on both adult and fetal HSPCs are expressed, and whether their triggering is involved in the shaping of the hematopoietic system during fetal life and subsequent alterations of it during immune activation in adult life. This could give more insight in how the immune system can regulate the hematopoietic system and give feedback at the progenitor level.

Within a mother’s womb, the fetus is considered an allograft. Besides that the placenta allows nutrient and gas exchange from the mothers blood to the fetus and waste exchange from fetus to mother it also serves as a physical immunological barrier that prevents transmission of pathogens from mother to child and avoiding the immune system from the mother attacking the fetus. A variety of immune cells have however been proposed to play
a role in the maternal tolerance to the fetus. Early during pregnancy decidua natural killer cells (dNKs) are present in copious amounts in the decidua, accompanied by macrophages, T cells and DCs. To date it is generally accepted that cell-free fetal DNA can be found in the maternal circulation and maternal IgG can cross the placental barrier to induce passive immunisation in the fetus. However still controversial, maternal lymphocytes and other hematopoietic cells have been described to pass the placenta as well. Using CD70TG mice we however did not find any evidence for the transfer of maternal activated lymphocytes or inflammatory cytokines, such as IFN-γ, across the placental barrier, as neither CD27 down-regulation nor Sca-1 upregulation was found on FL-HSPCs (Fig. 4a-b). Although we cannot exclude that lymphocytes did pass the placental barrier, the numbers would have been very low and certainly did not severely affect the hematopoietic output from fetal HSPCs (Fig. 4c-f). Interestingly, IFN-γ is produced in relatively high levels by dNKs and plays an important role in vasculature remodelling and angiogenesis upon implantation of the placenta. IFN-γ is nevertheless not required for the implantation of the conceptus and further pregnancy, as IFN-γ−/− mice breed normally and generate normal litters (data not shown). Although IFN-γ might be beneficial for the conceptus, elevated levels of IFN-γ in the placenta have also been linked to pregnancy loss, as IFN-γ inhibits T regulatory cell (Treg) formation. Trophoblasts within the placenta induce systemic proliferation of Tregs and attracts them to the fetal-maternal interface of the placenta by production of chorionic gonadotropin. Here Tregs contribute to the maternal tolerance to the fetus by suppressing autoimmune responses and allogeneic responses against the fetus. Absence of Tregs in the placenta leads to a failure of gestation. Although CD70TG mice have elevated numbers of IFN-γ producing effector T cells, we have no indications that the litter number of CD70TG mice is smaller compared to control mice (data not shown) and that Treg function is abrogated in the placenta of CD70TG mice.

With respect to the influence of maternal immune activation on fetal development we did not observe any effects on the conceptus regarding hematopoiesis in mice (Fig. 5). To what extent other parameters are influenced is unknown, but has been described that immune activation during pregnancy can induce spontaneous abortion, cause pre-term birth, affect birth weight, affect fetal brain development, affect the fetal adaptive immune system and can lead to cognitive impairment and dopaminergic hyperfunction in humans. In utero infection with pathogens like rubella, hepatitis, congenital cytomegalovirus (CMV), herpes simplex virus (HSV) and human immunodeficiency virus (HIV) can result in severe congenital disorders, malformations and pregnancy loss, especially during the first trimester of gestation when the fetus is still incapable of mounting immune responses. Here we show by using a model of sterile chronic maternal immune activation that excludes vertical transmission of pathogens, that a high degree of chronic maternal immune activation is not sufficient to influence hematopoietic development in the fetus. To what extent fetal hematopoiesis is affected by maternal immunity in general and CD27 triggering on HSPCs in particular in case of pathogen spreading over the placenta largely unknown and requires further research using disease models. Gaining further insight in how maternal immune responses develop while maintaining tolerance to the conceptus could give valuable leads to prevent congenital defects and pregnancy loss due to infection.
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Chapter 3

Reference List

1. de Bruijn MF, Speck NA, Peeters MC, Dzierzak E. Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. EMBO J. 2000;19:2465-2474.


Chronic IFN-γ production in mice induces anemia by reducing erythrocyte lifespan and inhibiting erythropoiesis through an IRF-1/PU.1-axis

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Abstract
Anemia of chronic disease (ACD) is a complication accompanying many inflammatory diseases. The pro-inflammatory cytokine IFN-γ has been implicated in this form of anemia, but the underlying mechanism remains unclear. Here we describe a novel mouse model for ACD, in which enhanced CD27-mediated costimulation strongly increases the formation of IFN-γ-producing effector T cells, leading to a progressive anemia. We demonstrate that the anemia in these mice is fully dependent on IFN-γ and that this cytokine reduces both the lifespan and the formation of red blood cells. Molecular analysis revealed that IFN-γ induces expression of the transcription factors IRF-1 and PU.1 in both murine and human erythroid precursors. We found that upon IFN-γ-stimulation, IRF-1 binds to the promoter of PU.1 and induces PU.1 expression, leading to inhibition of erythropoiesis. Notably, downregulation of either IRF-1 or PU.1 expression is sufficient to overcome IFN-γ-induced inhibition of erythropoiesis. These findings reveal a molecular mechanism by which chronic exposure to IFN-γ induces anemia.
Introduction

The maintenance of the number of circulating red blood cells (RBCs) is based on a tight balance between the production of new RBCs by erythroid progenitors and the removal of effete RBCs by cells of the hemophagocytic system. Several processes can negatively affect this erythroid homeostasis and subsequently lead to the development of anemia. Iron deficiency is the primary cause of anemia in the human population, but patients suffering from diseases involving chronic immune activation, such as persistent infections, cancer, and autoimmune diseases, are also commonly found to be anemic. This condition is termed Anemia of Chronic Disease (ACD) or Anemia of Inflammation and it is the second most prevalent form of anemia. Although the mechanisms involved in the development of ACD are still a matter of debate, pro-inflammatory cytokines such as IL-1, IL-6, TNFa and IFN-γ are thought to be important players in its development. Particularly IFN-γ has been extensively studied in this respect and it has been shown to have a direct suppressive effect on the formation of erythroid colonies in vitro. Exposure to IFN-γ in vitro contributes to early erythroblast death due to the induction of pro-apoptotic molecules, such as TRAIL, TWEAK and CD95(L). Whether this mechanism is also the cause of anemia following chronic IFN-γ exposure in vivo is not yet clear. Another possibility is that IFN-γ negatively affects the lifespan of RBCs due to its ability to activate macrophages and thereby the hemophagocytic system that removes RBCs from the circulation. Moreover, IFN-γ can also induce iron retention in macrophages, which negatively affects iron homeostasis and thereby the erythroid balance.

We have investigated the consequences of enhanced IFN-γ production on erythroid homeostasis in vivo using a mouse model for sterile chronic immune activation. In this model, we overexpressed the TNF-superfamily member CD70 on B cells, which induces high numbers of IFN-γ-producing effector CD4 and CD8 T cells due to enhanced costimulation through the receptor CD27 on T cells. Consequently, these CD70-transgenic (CD70TG) mice display improved T cell immunity and efficiently counter a challenge with influenza virus or tumor cells. However, the increased pool of effector T cells also seriously disrupts the hematopoietic system at different levels, as CD70TG mice gradually lose their B cells and eosinophilic granulocytes due to chronic exposure to IFN-γ, lose their NK cells, and eventually even exhaust their naïve T cell pool. Here we describe that CD70TG mice also become anemic, which is fully dependent on the production of IFN-γ. We used this model to examine the cellular and molecular mechanism by which IFN-γ induces anemia.

We found that chronic production of IFN-γ in vivo profoundly shortens the lifespan of mature RBCs and also negatively affects the differentiation capacity of early erythroid progenitors in BM. Molecular analysis of the latter revealed that IFN-γ induces the expression of IRF-1 in erythroid precursor cells, which upregulates the transcription factor PU.1 and thereby inhibits erythroid differentiation. These findings demonstrate the profound impact of this cytokine on the erythroid balance in vivo and reveal the molecular mechanism by which IFN-γ inhibits erythropoiesis.
Materials and Methods

Mice
For experiments WT, CD70TG\textsuperscript{10}, IFN-γ\textsuperscript{-/-} and CD70TG*IFN-γ\textsuperscript{-/-} mice were used. Mice were bred and maintained on a C57BL/6 background in the animal facilities of the Academic Medical Center (University of Amsterdam, Amsterdam, The Netherlands) in specific pathogen-free conditions. Mice were given standard chow and acidified drinking water ad libitum. All animal experiments were approved by the Experimental Animal Committee of the Academic Medical Center in Amsterdam, The Netherlands, according to institutional and national guidelines.

Peripheral Blood Analysis
Full blood cell analysis, measuring RBC counts, Hgb content, hematocrit, RDW, MCH, MCHC and MCV, was performed on heparinized blood with an automated hemocytometer (Vet ABC Counter, SCIL, Viernheim, Germany or Coulter Ac•T Diff2, Beckman Coulter, Woerden, The Netherlands).

Flow Cytometry and Cell Sorting
Single cell suspensions from spleen were obtained by mincing the organ through 40 μm cell strainers. To obtain splenic macrophages, spleens were first digested with Liberase / DNAse (Roche) for 30 minutes at 37°C. Single cell suspensions from BM were obtained by crushing femurs and tibiae and subsequently filtering the suspension through a 40 mm cell strainer. Where possible, cells were stained in the presence of anti-CD16/CD32 block (2.4G2; kind gift from Louis Boon, Bioceros, Utrecht, The Netherlands). Monoclonal antibodies used (all from eBioscience, unless stated otherwise) were: c-Kit-APC (2B8); CD71-PE (R17217), Ter119-Pe-Cy5.5 (Ly-76), F4/80-FITC/APC (BM8), CD11b-APC (M1/70), PU.1-Alexa®488 (9G7, Cell Signaling) and MHCII-biotin\textsuperscript{10}. For identification of Common Myeloid Progenitors (CMPs) and Megakaryocyte-Erythroid Precursors (MEPs) by flow cytometry, cells were incubated with a lineage cocktail of biotin-conjugated antibodies directed against CD4 (GK1.5), CD8α (53-6.7), B220 (RA3-6B2), CD11b (M1/70), Gr1 (RB6-8C5) and Ter119 (Ly-76). After washing, cells were incubated with streptavidin-PE, CD34-FITC (RAM34), CD127-PerCP-Cy5.5 (A7R34), CD16/32-PE-Cy7 (93) and c-Kit-APC (2B8). For sorting, BM cells were first enriched with anti-CD117 microbeads (Miltenyi Biotec), stained with the antibodies described above, and CMPs and MEPs were subsequently sorted on a FACSaria (BD). Due to the upregulation of Sca-1 by IFN-γ on all BM cells in CD70TG mice\textsuperscript{15}, this marker was not included in the analysis or sorting strategy for CMPs and MEPs, which did not compromise our findings (Fig. S1a). For FACS analysis of reticulocytes, 3-5 ml of heparinized blood was washed in FACS buffer and surface stained for CD71. Cells were washed and resuspended in FACS buffer, after which Thiazole Orange (Sigma-Aldrich) was added to a final concentration of 1 ng/ml and immediately analyzed. Data acquisition was done with a FACSCalibur (BD) or FACSCanto II (BD) and data was analyzed using FlowJo software (Tree Star, Inc.).

EPO-Elisa
Serum EPO levels of mice were quantified by using the Quantikine Mouse/Rat EPO Immunoassay (R&D Systems) as described by the manufacturer.
IFN-γ induces anemia by reducing erythrocyte lifespan and inhibiting erythropoiesis.

**Biotinylation of RBCs**

*In vivo* biotinylation was achieved by i.v. injection of 100 μl of 30 mg/ml sulfo-N-hydroxysuccinimide-long chain-biotin (sulfo-NHS-LC-biotin, Pierce). At regular time intervals, a few microliters of blood were isolated via vena saphena puncture, stained with PE-conjugated-streptavidin and erythroid specific antibodies and analyzed using FACS. For adoptive transfer of biotin-labelled-RBCs, blood was drawn from WT donor mice, washed with PBS-G (PBS supplemented with 0.1% glucose) and subsequently incubated with 0.1 mg/ml sulfo-NHS-LC-biotin in PBS-G for 15 minutes at RT. After washing, 200 μl of biotin-labelled RBCs was injected intravenously RBC turnover was analyzed as described above.

**Erythrophagocytosis Assay**

WT whole blood was washed with PBS, resuspended to a concentration of 2 x 10⁸ cells/ml and subsequently labelled with a final concentration of 25 μM CFSE (Invitrogen), according to the manufacturer's protocol. Single cell suspensions from Liberase-digested spleens were cultured for 2 hours in 12-well plates in phenol-red free DMEM (Lonza)/10% FCS for 2 hours to allow macrophage adherence. Unbound cells were removed and CFSE-labelled RBCs were added in a concentration of 2 x 10⁷ RBCs/ml and co-cultured for 2 hours. Cells were subsequently washed, non-phagocytosed RBCs were lysed with ammonium chloride and the macrophages were isolated for FACS analysis.

**Semi Solid Colony Assays**

For burst forming unit-erythroid (BFU-e) and colony forming unit-erythroid (CFU-e) assays, 2 x 10⁵ nucleated total BM cells were plated in methylcellulose medium (MethoCult M3234, StemCell Technologies). Medium was supplemented with 4 U/ml human recombinant EPO (Janssen-Cilag), 100 ng/ml murine recombinant SCF, 20 μg/ml human holo-transferrin (SCIPAC, T101-5), 2 x 10⁻⁴ M hemin (Sigma-Aldrich, H9039) and 1% penicillin/streptomycin/L-glutamine solution (Invitrogen). CFU-e colonies were counted on day 3, BFU-e colonies on day 8. When appropriate, mouse recombinant IFN-γ (PeproTech) was added. FACS-sorted BM CMPs and MEPs were cultured in complete methylcellulose medium (Methocult M3434, Stemcell Technologies) at 250 cells per 35 mm culture dish and colony formation was analyzed on day 8.

**Microarray**

CD71⁺ cells were MACS-enriched by labelling with biotin-conjugated CD71 antibodies (eBioscience) and streptavidin-conjugated microbeads (Miltenyi Biotech). Total RNA was extracted using Trizol (Invitrogen). Initial RNA yield and subsequent quality of the labelled fragmented cRNA was determined using the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). 100-300ng of total RNA was hybridized to Mouse Gene 1.0 ST Array GeneChips, according to the manufacturer’s protocols (Affymetrix). Single Array Expression Analysis was performed using Genespring GX Software (Agilent Technologies). This platform generates a list of differentially expressed genes after filtering absent, marginal or AFFY control probe sets and applying a log2 transformation. A 1.5-fold change threshold and test statistic of p<0.05 were used as cut-off. These data have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE22656. Further analysis was performed using Ingenuity Pathways Analysis (Ingenuity Systems).
Potential PU.1 and Gata1 target genes classification was obtained by comparing our list of IFN-γ-dependent differentially expressed genes with unpublished (LG), published PU.1 (GDS9011)\textsuperscript{16} and Gata1 ChIP-seq data (ERA000161)\textsuperscript{17} using Ingenuity Pathways Analysis.

**Quantitative PCR**
Total RNA was isolated using Trizol (Invitrogen) and reverse transcribed to cDNA using random hexamers and Superscript II reverse transcriptase (Roche). Quantitative PCR was performed using the StepOnePlus RT-PCR system (Applied Biosystems) using Express SYBR GreenER (Invitrogen). Primer sequences are available on request.

**Western Blot**
Total protein extracts were made according to standard methods. Proteins were separated on a 12.5\% SDS-PAGE-gels and subsequently transferred onto polyvinylidene difluoride membranes (Sigma Aldrich) by electroblotting. Membranes were blocked with PBS containing 1\% BSA or 3\% milk and 0.25\% Tween. Blots were incubated overnight at 4° C with primary antibodies, thoroughly washed with PBS containing 0.25\% Tween, incubated with appropriate horseradish-peroxidase-conjugated secondary antibodies and developed in enhanced chemiluminescence reagents (Amersham Pharmacia Biotech). Antibodies used were: PU.1 (T21, sc-352), Gata1 (N6, 265 and H200, sc-13053), IRF-1 (H8, sc-74530), GAPDH (MAB374 Chemicon Millipore), Beta-Actin (ab-6276) and Nucleophosmin (FC8229, ab-10530).

**Cell Culture of Human Erythroid Progenitors and shRNA-mediated Knockdown**
In accordance with institutional guidelines provided by the Erasmus MC Medical Ethical Committee (METC), human erythroid progenitor cells were cultured as described\textsuperscript{18}, in the presence of recombinant human EPO (1 unit/ml, kind gift of Ortho-Biotech, Tilburg, The Netherlands), recombinant human SCF (50 ng/ml, kind gift of Amgen, Breda, The Netherlands) and dexamethasone (5 x 10\textsuperscript{-7} M; Sigma).
Lentivirus was produced by transient transfection of 293T cells\textsuperscript{19}. Supernatant was harvested over three consecutive days after transfection, kept at 4° C and pooled. Pooled supernatant was filtered and concentrated by centrifugation at 20 krpm for 2h at 4° C. Human erythroid progenitor cells were transduced in 6-well plates at 2-3 x 10\textsuperscript{6} cells per well and sufficient amounts of virus to transduce ~80\% of the cells. When appropriate, puromycin (1 µg/ml final concentration) was added after 1 day, and selection was performed overnight.
For knockdown experiments, clones from The RNAi Consortium (TRC 15; Sigma) were used. The non-target SHC002 vector was used as a control. (SHC002: 5’-CAACAAGATGAAGAGCACCAA-3’). We tested 5 shRNA clones directed against PU.1 and IRF-1 mRNA, and we selected the ones with stronger downregulation for further experiments: PU.1 (TRCN0000020538: 5’-GAAGAAGCTCACCTACCAGTT-3’), and IRF-1 (TRCN0000014671: 5’-AGATGCTAAGAGCAGGCAAGGCAAGGCAAC-3’).
At day 1-3 after selection cells were counted and plated in triplicate in methylcellulose medium (MethoCult H4434, StemCell Technologies). We used 2 x 10\textsuperscript{5} cells for CFU-e counts and 2 x 10\textsuperscript{6} cells for BFU-e counts. Medium was supplemented with 1\% penicillin/streptomycin solution (Gibco BRL) and when appropriate with 200 ng/ml of human recombinant IFN-γ (PeproTech). CFU-e colonies were counted on day 8 and BFU-e colonies on day 16.
**Chromatin Immunoprecipitation**

Human erythroid progenitor cells were cultured and grown in sufficient numbers and pulsed or mock pulsed overnight with 200ng/ml human recombinant IFN-γ. Cells were collected and Chromatin Immunoprecipitation (ChIP) was performed as described with IRF-1 (H8, sc-74530) and CD71 antibody (347510, BD Biosciences, San Jose, CA, USA) as a negative control. qPCR was performed on the input and immunoprecipitated samples using primers for the IRF-1 binding site at the TAP121 and PU.1 promoter. The relative fold enrichment was calculated as RFE = 2^{(CT ChIP sample – CT input sample)}.

Primers used are:
- TAP1 promoter, amplicon size 94 bp.
  - 5’-GGCGAGAAGCTCAGCATT-3’ and ‘5’-TAGTCTGGGCAGGCCACTTT-3’.
- PU.1 promoter, amplicon size 121 bp.
  - 5’-CTGGTCTGAAGTGCCTTTCTTTG-3’ and ‘5’-AAGAAGGAGTTGAGGAGCCAC-3’.

**Statistical Analysis**

Results are expressed as mean ± SD. Statistical analysis between groups was performed with Graphpad Prism 5, using either a paired or non-paired 2-tailed Student’s t-test when comparing two groups or a one-way or two-way ANOVA test with Bonferroni correction when comparing more than two groups. Area under the curve analysis was performed using SPSS 15.0.1 software (SPSS Inc.). P-values <0.05 were considered to be statistically significant.

**Results**

**CD70TG mice develop an IFN-γ-dependent anemia**

As CD70-overexpression induces the formation of high numbers of IFN-γ-producing effector T cells, we used this model of chronic immune activation to examine the impact of IFN-γ on RBC homeostasis in vivo. Although CD70TG mice have normal RBC numbers, hemoglobin levels and hematocrit at four weeks of age, they develop severe anemia over time, as is evident from the significant decrease of these parameters in twelve week old mice (Fig. 1a). Cytometric analysis indicated that CD70TG mice develop a normochromic (normal mean corpuscular hemoglobin concentration) and normocytic (normal mean corpuscular volume) anemia (Table I). Importantly, this anemia is fully dependent on IFN-γ, as it does not occur in IFN-γ-deficient CD70TG (CD70TG*IFN-γ-/-) mice. Mean corpuscular hemoglobin (MCH) is slightly increased in CD70TG mice, but not in CD70TG*IFN-γ-/- mice (Table I), which correlates with an IFN-γ-dependent increase in reticulocytes in peripheral blood (Fig. 1b-c).

As mice typically respond to anemia and hypoxic stress by inducing extramedullary stress-erythropoiesis in the spleen, we examined the spleens of CD70TG mice in more detail. CD70TG mice develop a severe IFN-γ-dependent splenomegaly (Fig. 1d-e). This increase in spleen size can not be attributed to a leukocyte expansion, as CD70TG mice have reduced splenocyte numbers due to the progressive depletion of B cells. Therefore, we examined the splenic erythroid compartment, based on the differential expression of TER119 and CD71. We found that CD70TG mice have a dramatic increase of all erythroblast subsets, whereas CD70TG*IFN-γ-/- mice do not (Fig. 1f-g). Finally, CD70TG mice also displayed an
Figure 1. CD70TG mice develop IFN-γ-dependent anemia.

Analysis of WT, CD70TG, IFN-γ-/- and CD70TG*IFN-γ-/- mice. (a) Hemocytometric analysis at 4 and 12 weeks of age: RBC, red blood cell counts; Hgb, hemoglobin; Hct, hematocrit; Mean ± SD is shown in bar graphs for 3-5 mice per group. * indicates a significant difference (p<0.05) between CD70TG mice and all other groups, using a two-way ANOVA with Bonferroni correction. (b, c) Representative dot plots and bar graph depicting the percentage of blood reticulocytes, i.e. CD71⁺ and Thiazole Orange (TO) positive cells. (d) Picture of spleens and (e) bar graph depicting spleen weight. (f) Representative dot plots of the splenic erythroid compartment. Numbers I, II, III and IV refer to the respective erythroblast subsets defined by Socolovsky et al.22. (g) Absolute numbers of indicated erythroblast subsets per spleen. Mean ± SD is shown in bar graphs for three mice per group; results are representative from three independently performed experiments. * indicates a significant difference (p<0.05) between CD70TG mice and all other groups, using a one-way ANOVA. (h) Plasma EPO levels. Mean ± SD is shown in bar graphs for 5-8 mice per group; * indicates a significant difference (p<0.05) between CD70TG mice and all other groups, using a one-way ANOVA with Bonferroni correction. For (b-h) mice were used in the age range of 10-16 weeks old.
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IFN-γ-dependent increase in plasma EPO levels (Fig. 1h), consistent with the notion that these mice have hypoxia. These data demonstrate that CD70TG mice develop an IFN-γ-dependent anemia and concomitant stress erythropoiesis in the spleen. We used this model to investigate the mechanism by which IFN-γ contributes to ACD.

IFN-γ enhances RBC turnover and increases macrophage erythrophagocytosis

As normocytic anemia in combination with reticulocytosis is indicative of accelerated RBC destruction, we examined whether IFN-γ affected the lifespan of RBCs in CD70TG mice. Therefore, mice were injected intravenously with biotin, which stably labels all RBCs in the circulation23 and allowed us to determine the turnover of RBCs (Fig. 2a). Whereas WT mice lost 50% of their biotinylated RBCs in ~23 days, CD70TG mice reached this point already after 12 days (Fig. 2b). This increased loss of biotinylated RBCs was fully IFN-γ-dependent, as RBC removal was normal or even slightly delayed in CD70TG*IFN-γ-/- mice (Fig. 2b and Fig. S2a).

Since IFN-γ is a potent activator of macrophages and the hemophagocytic system, we determined whether IFN-γ accelerated RBC turnover in CD70TG mice in an extrinsic manner. Therefore, we transferred ex vivo-biotinylated WT RBCs to the four different groups of mice and followed the fate of these cells over time. This approach revealed that WT RBCs were also removed more rapidly from the circulation of CD70TG mice, which was not seen in CD70TG*IFN-γ-/- mice (Fig. 2c and Fig. S2b). This increase in hemophagocytic capacity correlated well with the finding that splenic red pulp macrophages (F4/80hi, CD11blo) were activated in CD70TG mice, but not in CD70TG*IFN-γ-/- mice, based on the increased expression of MHCII (Fig. 2d). To establish the erythrophagocytic capacity of these cells, splenic red pulp macrophages were isolated, cocultured for 2 hours with CFSE-labeled WT RBCs, followed by lysis of the remaining non-phagocytosed RBCs. These experiments indicate that, compared to those from WT mice, red pulp macrophages from CD70TG mice phagocytose more erythrocytes, while red pulp macrophages from CD70TG*IFN-γ-/- mice phagocytose fewer (Fig. 2e). This correlates well with the degree of RBC turnover observed in vivo (Fig. 2b). Taken together, these data indicate that IFN-γ produced during chronic inflammation activates the splenic hemophagocytic compartment, thereby enhancing the turnover of RBCs.

Table I. IFN-γ-dependent normochromic and normocytic anemia in CD70TG mice.

Hemocytometric analysis of 10–16 week old WT, CD70TG, IFN-γ-/- and CD70TG*IFN-γ-/- mice. RBC, red blood cell counts; Hgb, hemoglobin; Hct, hematocrit; RDW, red cell distribution width; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration. Values indicate the average (SD) from 10–18 mice. * indicates a significant difference (p<0.05) between CD70TG mice and all other groups, using a one-way ANOVA with Bonferroni correction.
**IFN-γ inhibits erythroid output from BM**

As IFN-γ can also negatively regulate erythropoiesis of BM progenitor cells, we analyzed the erythroid compartment in BM from CD70TG mice and found significantly fewer erythroblasts (Ery II, III and IV) than in WT BM (Fig. 3a). For the early subsets this was to a certain extent also seen in CD70TG*IFN-γ−/− mice, but the final stage of orthochromatophilic erythroblasts (Ery IV) was not affected in these mice (Fig.3a). This correlated well with the observations that BM of CD70TG mice was paler compared to WT, IFN-γ−/− and CD70TG*IFN-γ−/− mice (data not shown), which is also a clear sign for a reduction in hemoglobinized cells in CD70TG mice. Furthermore, we found a relative accumulation of pro-erythroblasts in CD70TG mice (Fig. 3b), which, together with the decrease in absolute number of mature erythroid cells (Fig. 3a), indicates that erythropoiesis in CD70TG BM is hampered. We found no evidence for increased apoptosis of erythroid precursor populations in CD70TG mice, based on Annexin-V stainings of bone marrow (Fig. S3a). Yet, reduced erythropoiesis was confirmed when the erythroid forming capacity of the BM was functionally tested using colony-forming assays, as the number of BFU-e was strongly reduced in CD70TG BM compared to WT BM. This reduction was dependent on IFN-γ (Fig. 3c). Formation of more mature CFU-e was not affected, but the negative impact of IFN-γ on erythroid formation was evident from the fact that both IFN-γ−/− and CD70TG*IFN-γ−/− mice had more CFU-es than WT and CD70TG mice (Fig. 3d). Moreover, purified CMPs from CD70TG mice formed fewer erythroid colonies compared to WT mice, but more myeloid colonies (Fig. 3e), indicating a decreased commitment of hematopoietic progenitors to the erythroid lineage. Subsequently, we examined BM of CD70TG mice for the presence of IFN-γ-producing T cells, which revealed that CD70TG mice have 7-fold more IFN-γ-producing CD4+ T cells and 4-fold more IFN-γ-producing CD8+ T cells than WT mice (Fig. 3f-g). Serum IFN-γ levels were undetectable (data not shown and ) strongly suggesting that a local increase in IFN-γ-production is responsible for the decreased BFU-e capacity in CD70TG mice. Moreover, we demonstrate that IFN-γ is sufficient to inhibit the outgrowth of BFU-e from WT BM (Fig. 3h), whereas CFU-e are less sensitive to this cytokine (Fig. 3i), which is consistent with previous findings. In conclusion, these data imply that prolonged exposure to high IFN-γ levels causes ACD, not only by an increased turnover of RBCs, but also by a reduction in the erythroid forming capacity of hematopoietic progenitor cells in BM.

**IFN-γ induces expression of PU.1**

To investigate the molecular mechanism by which IFN-γ affects erythropoiesis, we performed microarray analysis on CD71+ erythroblasts from BM of WT, CD70TG, IFN-γ−/− and CD70TG*IFN-γ−/− mice. Unsupervised cluster analysis showed that erythroblasts from CD70TG mice clustered separately from the other mice, independently of their ability to produce IFN-γ (Fig. 4a). However, we also found a set of 191 transcripts that was differentially expressed in CD70TG mice compared to CD70TG*IFN-γ−/− as well as WT and IFN-γ−/− mice, and we decided to further focus on these genes, as they were directly or indirectly regulated by IFN-γ. Comparison with other datasets revealed that 128 out of these 191 genes (67%) are potential targets of the transcription factors PU.1 or GATA-1 (Fig. 4b and Fig. S4), which are the key regulators of myeloid and erythroid differentiation, respectively. During normal erythroid differentiation, PU.1 is downregulated, while GATA-1 upregulation mediates differentiation of erythroid precursors. Expression of these factors has to be tightly controlled during hematopoiesis, as PU.1 and GATA-1 physically interact and thereby

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Figure 2. Constitutive IFN-γ exposure in vivo enhances RBC turn-over and increases macrophage erythrophagocytosis.
(a) Flow cytometric analysis of in vivo biotinylated peripheral blood using fluorescently labelled streptavidin and Ter119. Representative dot plots of day 17 after transfer are depicted. (b) Turnover of in vivo biotinylated RBCs from WT, CD70TG, IFN-γ−/− and CD70TG*IFN-γ−/− mice as measured by flow cytometry. (c) Turnover of adoptively transferred ex vivo biotinylated WT RBCs in WT, CD70TG, IFN-γ−/− and CD70TG*IFN-γ−/− mice as measured by flow cytometry. (d) MHCII surface expression of splenic red pulp macrophages (F4/80+ CD11b low) expressed as geometric MFI. (e) Uptake of CFSE-labelled erythrocytes by splenic red pulp macrophages (F4/80+CD11b+) expressed as the ratio of CFSE geometric MFI compared to background. Data in (e) is obtained from two independent experiments. Mean ± SD is depicted for 4 mice (b, c), 3 mice (d) or duplicate analysis (e) per group. * indicates a significant difference (p<0.05) between CD70TG or CD70TG*IFN-γ−/− mice and all other groups, using a one-way ANOVA with Bonferroni correction.
Figure 3. IFN-γ-production in CD70TG mice inhibits erythroid BM output.

(a) Absolute numbers of various erythroblast subsets or (b) relative contribution of pro-erythroblasts (percentage Ery I (Ter119medCD71high) from all erythroid (Ter119med/high cells)) in BM (isolated from two femurs and two tibiae) of WT, CD70TG, IFN-γ-/- and CD70TG*IFN-γ-/- mice.
(c, d) BFU-e and CFU-e numbers of unfractionated BM from WT, CD70TG, IFN-γ-/- and CD70TG*IFN-γ-/- mice. (e) Colony assay of FACS-sorted CMPs from WT and CD70TG mice. (f, g) Absolute numbers of IFN-γ-producing CD4 and CD8 T cells in BM of WT and CD70TG mice, measured after PMA/ionomycin stimulation. (h, i) Effect of IFN-γ on the in vitro BFU-e and CFU-e colony forming capacity of BM cells from WT mice. Mean ± SD is depicted for 3 mice per group (a-d, f-g) or triplicate analysis (e, h-i); results are representative from three (a-d) or two (e-i) independently performed experiments. *p<0.01 (a) or p<0.05 (b-h) using a one-way ANOVA with Bonferroni correction (a-d), or a non-paired Student’s t-test (e-i).
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block each others function \(^{26-28}\). Protein expression analysis of these transcription factors on Western blot revealed that PU.1 was highly upregulated in CD71\(^+\) cells of CD70TG mice, which was fully dependent on IFN-γ, as it can be seen in IFN-γ\(^{-/-}\) samples where PU.1 levels are even lower than in WT, whereas expression of GATA-1 was not altered between the different groups of mice (Fig. 4c). Flow cytometric analysis of earlier progenitors revealed that PU.1 was also upregulated in megakaryocyte/erythrocyte lineage-restricted progenitors (MEPs) of CD70TG, but not of CD70TG*IFN-γ\(^{-/-}\) mice (Fig. 4d). Finally, we found that IFN-γ treatment of purified CD71\(^+\) cells (Fig. 4e) or MEPs (Fig. 4f) was sufficient to upregulate PU.1 expression, whereas it did not affect GATA-1 (Fig. 4f). Since PU.1 is a known inhibitor of erythroid differentiation \(^{27-29}\), these data strongly suggest that IFN-γ blocks erythropoiesis through the induction of PU.1.

**IFN-γ upregulates PU.1 expression through induction of IRF-1**

Next, we set out to determine the molecular mechanism by which IFN-γ-signaling induces the expression of PU.1. The microarray analysis revealed that IFN-γ also induced expression of the transcription factor IRF-1 (interferon regulatory factor 1) and its downstream target genes (Table II and Fig. S4). We validated by quantitative PCR that IRF-1 was upregulated in CD71\(^+\) BM cells of CD70TG mice in an IFN-γ-dependent manner (Fig. 5a). Moreover, incubation with IFN-γ was sufficient to induce IRF-1 in CD71\(^+\) cells and MEPs (Fig. 5b). Examination of the PU.1 promoter revealed a potential IRF-1/2 binding site ~15kb upstream of the transcription start site, which was highly conserved among mammals (Fig. 5c). To determine whether IRF-1 protein indeed interacted with this domain, we performed chromatin immune-precipitation (ChIP) experiments, using expanded primary human erythroid progenitor cells; these cells can be expanded to sufficient numbers required for these experiments \(^{18}\) and they enabled us to validate the effect of IFN-γ also on human erythroid precursors. We found that IFN-γ inhibited the outgrowth of BFU-e and CFU-e of human erythroid progenitor cells *in vitro* (Fig. 5d), which corroborates comparable experiments with progenitor cells from human BM \(^{14-16}\). Moreover, IFN-γ induced the expression of both IRF-1 and PU.1 also in these primary human cells (Fig. S5a). Subsequent ChIP analysis revealed that IFN-γ treatment led to increased IRF-1 binding to the highly conserved IRF-1/2 motifs in the PU.1 locus (Fig. 5e). IFN-γ treatment also increased binding of IRF-1 to an IRF-1 binding site in the Tap1 promoter \(^{21}\), which served as a positive control. No significant enrichment was observed when a negative control antibody (anti-CD71) was used for the immunoprecipitation.

Finally, to demonstrate that IRF-1 and PU.1 were responsible for the IFN-γ-induced inhibition of erythroid colony formation, we transduced human erythroid progenitor cells with lentiviruses expressing a shRNA to either IRF-1 or PU.1, stimulated these cells with IFN-γ and tested their ability to form erythroid colonies. These constructs strongly inhibited the upregulation of their cognate target mRNAs upon IFN-γ stimulation (Fig. S4b). In addition, inhibition of IRF-1 expression efficiently prevented the IFN-γ-mediated upregulation of PU.1 and vice versa (Fig. S5b), indicating that IRF-1 and PU.1 reciprocally enhance each others expression. Importantly, subsequent colony assays revealed that inhibition of either IRF-1 or PU.1 expression was sufficient to overcome the IFN-γ-induced reduction in CFU-e and BFU-e numbers (Fig. 5f). These data demonstrate that IFN-γ blocks erythroid differentiation by inducing the expression of PU.1 in an IRF-1 dependent manner.
Figure 4. IFN-γ induces expression of PU.1 in erythroblasts.
(a) Microarray heat map of unsupervised cluster analysis for genes expressed in CD71+ erythroblasts from BM of WT, CD70TG, IFN-γ−/− and CD70TG*IFN-γ−/− mice. Red; high expression, green; low expression. Three mice were analysed per group. (b) Venn-diagram displaying potential PU.1 and GATA-1 targets amongst the IFN-γ-regulated genes in CD70TG mice. (c) Western blot analysis of PU.1 and GATA-1 expression on purified CD71+ BM cells from 2 mice per experimental group. (d) Flow cytometric analysis of PU.1 expression in MEPs of WT, CD70TG, IFN-γ−/− and CD70TG*IFN-γ−/− mice. Horizontal line represents the value of the isotype control. Mean ± SD is depicted for three mice per group; results are representative from two independently performed experiments, * indicates a significant difference (p<0.05) between CD70TG mice and all other groups, using a one-way ANOVA with Bonferroni correction. (e) QPCR analysis of PU.1 mRNA on WT CD71+ erythroblasts cultured overnight with or without IFN-γ. (f) QPCR analysis of PU.1 and GATA-1 mRNA on WT MEPs cultured overnight with or without IFN-γ. (e, f) Data is presented as the fold induction of expression compared to the medium control for seven (e), nine or six (f) mice per group, pooled from at least two independently performed experiments; * p<0.01 (e) or p<0.05 using a paired Student’s t-test.
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Discussion

Next to its well-established pro-inflammatory and anti-microbial role, IFN-γ can also influence the hematopoietic process in the BM, as it affects HSC quiescence and inhibits the formation of B cells and eosinophilic granulocytes. IFN-γ also has a strong inhibitory effect on erythroid colony formation in vitro and it is therefore assumed that IFN-γ plays a major role in the development of ACD in patients suffering from cancer or chronic inflammatory conditions. However, the impact of chronic IFN-γ production on erythroid homeostasis in vivo as well as the molecular mechanism by which IFN-γ inhibits erythroid differentiation has been poorly investigated. This is due to the fact that most methods used to induce chronic inflammation not only elicit the production of IFN-γ, but a whole plethora of pro-inflammatory cytokines, which can also act synergistically with IFN-γ on erythroid differentiation, as has been shown for TNFa and type I IFNs. Of interest in this respect is the recent finding that the anemia accompanying the acute phase of Toxoplasma gondii infection is dependent on the production of IL-15, which is regulated by IFN-γ. Yet, the underlying molecular mechanism linking IFN-γ to anemia in either this model or in ACD remains unclear. Using a sterile form of chronic inflammation elicited by enhanced and sustained T cell activation, we describe here that the ensuing increase in IFN-γ production induces anemia due to the concomitant inhibition of BM erythropoiesis and the enhanced turn-over of circulating RBCs. Although T cells in CD70TG mice do not have increased production of IL-2, TNFa or type I IFNs (data not shown), we cannot exclude that other pro-inflammatory mediators are induced in CD70TG mice that contribute to the severity of the anemia. Importantly, we could not detect any significant changes in the serum levels of IL-10, IL-1α, IL-6 nor TNFa in these mice (data not shown) Yet, the phenotype of CD70TG IFN-γ-/- mice demonstrates that even if the (local) production of such factors would be increased, they are either not sufficient to induce anemia and/or stress erythropoiesis or they are induced by IFN-γ itself.

Table II. Relative expression of IFN-γ-inducible genes in erythroblasts of CD70TG mice and their relationship with IRF-1.

Selection of IRF-1 related genes (based on the microarray data described in Figure 4 and Figure S3) that were significantly upregulated in CD70TG mice compared to WT as well as CD70TG IFN-γ-/- mice. These IFN-γ inducible genes were categorized as being either IRF-1 dependent (top half) or independent (bottom half), based on indicated literature references (right column).
Figure 5. IFN-γ upregulates PU.1 expression in erythroblast through IRF-1.
(a) QPCR analysis of the expression of IRF-1 mRNA in CD71+ erythroblasts from WT, CD70TG, IFN-γ−/− and CD70TG*IFN-γ−/− mice. Mean ± SD is depicted for 3 mice per group, * indicates a significant difference (p<0.05) between CD70TG mice and all other groups (using a one-way ANOVA with Bonferroni correction). (b) QPCR analysis of IRF-1 mRNA in CD71+ erythroblasts or MEPs from WT mice cultured overnight with or without IFN-γ. Data is presented as the fold induction of expression compared to the medium control for seven (CD71+) or six (MEPs) mice per group, pooled from two independently performed experiments; * p<0.001 (e) or p<0.05 using a paired Student's t-test. (c) Sequence analysis of the promoter region of the human PU.1 (SPI1) gene, displaying a conserved putative IRF-1/2 binding site ~15kb upstream of the transcription start site (based on the UCSC Genome Browser). (d) Effect of IFN-γ on the in vitro BFU-e and CFU-e colony forming potential of human erythroid precursor cells cultured overnight with or without IFN-γ. Mean ± SD from triplicate analysis; results are representative from two independently performed experiments, *p<0.05 (non-paired Student’s t-test). (e) Chromatin immunoprecipitation analysis of the binding of IRF-1 to the putative IRF-1/2 binding site in the PU.1 promoter of human erythroid precursor cells cultured overnight with or without IFN-γ. RFE, relative fold enrichment. Mean ± SD from 3 independent experiments, *p<0.05 (non-paired Student’s t-test). (f) Effect of IFN-γ on the BFU-e and CFU-e potential of human erythroid precursor cells transduced with shRNA directed against IRF-1 or PU.1. Mean ± SD from 3 independent experiments, *p<0.05 (one-way ANOVA with Bonferroni correction).
Although we demonstrated that IFN-γ negatively affects both the formation and the lifespan of RBCs, it has been described that IFN-γ induced by in vivo administration of CpG-oligodeoxynucleotides does mediate erythroid suppression, but not reduction of RBC survival. However, this study addressed the role of IFN-γ in acute inflammatory responses, while we have used a valid model for chronic inflammation. We hypothesized that a chronic response could result in macrophage activation and thereby affect RBC uptake. Concordantly, we found an IFN-γ-dependent increase of RBC uptake by activated splenic macrophages in vitro, as well as increased iron-storage in the spleens of CD70TG mice (data not shown). The latter observation suggests potential defects in iron release, which also impedes the course of erythropoiesis. However, we did not find altered expression of the iron-regulating hormone hepcidin in the liver of CD70TG mice (Fig. S2a-b), indicating that hepcidin is not causally involved in the anemic phenotype of these mice.

Next to the clear impact on RBC lifespan, we also found that IFN-γ impaired BM erythropoiesis, as was evident from strongly reduced BFU-e numbers (Fig. 3c) and fewer mature erythroblasts (Fig. 3a) in CD70TG mice, but not CD70TG*IFN-γ-/- mice. It could though well be that the observed decrease is an underestimation and that this defect was already partly compensated in vivo by the increased EPO levels in these mice, since EPO is sufficient to increase erythropoiesis not only in the spleen, but also in BM. Regarding the spleen, CD70TG mice suffered from splenomegaly due to the induction of stress erythropoiesis, which is a physiological response to anemia in rodents. Yet, the fact that CD70TG mice still become progressively anemic at least demonstrates that this form of “emergency” erythropoiesis is not sufficient to prevent anemia in these mice. It could well be that stress erythropoiesis is negatively affected by the combination of increased local production of IFN-γ and enhanced RBC destruction in the spleen. This issue is currently under investigation.

At a molecular level, we demonstrate that IFN-γ inhibits erythropoiesis by activation of an IRF-1-PU.1 axis. Since type I IFNs can also induce expression of IRF-1 and efficiently suppress erythroid colony formation in vitro, we expect that chronic production of IFNa or IFNb can elicit a comparable degree of inhibition of BM erythropoiesis. This has been indirectly achieved in mice by knocking out IRF2, a suppressor of type I IFN signaling. These mice become anemic, which is rescued in the absence of IFNAR1, a member of the type I IFN receptor complex. However, type I IFNs are less potent activators of macrophages, which suggests that chronic production of type I IFNs has a less significant impact on RBC turnover and does not induce anemia as efficiently as IFN-γ. In steady state conditions, EPO levels in IRF2-/- mice are not increased to the same extent as in our mouse model of sterile chronic inflammation.

PU.1 is a well known transcriptional regulator of myelopoiesis. In the erythroid lineage, PU.1 activity is repressed by GATA-1, which is essential for normal erythropoiesis. The interplay of PU.1 and GATA-1 is relevant for lineage commitment and high expression of PU.1 is known to represses GATA-1 activity and thereby erythroid differentiation. We identified a highly conserved IRF-1 binding site in the distal promoter of the PU.1 gene locus and showed that IFN-γ induces PU.1 expression via IRF-1. IFN-γ can induce apoptosis of erythroid progenitors through TRAIL, TWEAK and CD95 and indeed we found that TRAIL (TNFSF10), a target of PU.1, is upregulated by IFN-γ in CD71+ cells of CD70TG mice (Fig. S4). We have found that IRF-1 and PU.1 are interdependent, since shRNA-mediated downregulation of each factor resulted in downregulation of the expression levels of the other. These findings are
supported by the fact that IRF-1−/− mice have defective myelopoiesis, which is partly caused by downregulation of PU.138, and that infection of neutrophil precursors with Anaplasma phagocytophilum results in downregulation of both IRF-1 and PU.139. A cooperative function between IRF-1 and PU.1 has also been reported during ATRA-mediated granulopoiesis40 and in IFN-γ-stimulated myeloid cells41. Furthermore, IRF-1 is naturally downregulated towards the final stages of erythroid maturation42, which overall sustains the notion that suppression of the IRF-1-PU.1 axis is a prerequisite for normal erythropoiesis. Our findings provide the molecular mechanism by which IFN-γ affects erythropoiesis, which implicates transcriptional regulation of hematopoietic-lineage differentiation in response to inflammation.

Regarding the physiological rationale behind the impact of IFN-γ on erythropoiesis, we postulate that during immune activation, IFN-γ temporarily shifts the balance of hematopoietic differentiation towards myeloid cells in order to combat an infection. Activated T cells might well play an important role in this process, as these cells can migrate to the BM and modulate hematopoiesis10,12,43. A temporary increase of myelopoiesis at the cost of erythropoiesis will not be detrimental to the host and will benefit the ongoing immune response, as the lifespan of RBCs is 10-50 fold longer than that of neutrophils or monocytes44,45. However, during chronic immune activation, the prolongation of such a shift can lead to anemia, as observed in CD70TG mice, which also have increased monocyte formation12. Our findings not only emphasize the importance of IFN-γ as a potent regulator of hematopoiesis, but also reveal the potential molecular mechanism of inflammation-induced anemia that occurs frequently in patients suffering from chronic inflammatory diseases, such as HIV-infection and rheumatoid arthritis. Interestingly, BFU-e colony formation is strongly reduced in BM of anemic patients suffering from chronic idiopathic neutropenia, which is significantly increased when the cells are cultured with IFN-γ-neutralizing antibodies46. It will be important to determine the contribution of this cytokine to the development of anemia in several chronic inflammatory diseases and whether neutralization of IFN-γ could be an effective treatment for different forms of ACD.

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Reference List


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Supplementary Figures

Supplementary Figure 1. Altered sorting strategy of precursor cells due to Sca-1 upregulation.
(a) In comparison to the traditional sorting strategy, in this study Sca-1 could not be used in the sorting strategy of CMPs and MEPs due to Sca-1 upregulation in CD70TG mice. Sca-1 was therefore excluded from the sorting strategy. (b) To look at functional capacity, WT BM CMPs or MEPs were FACS-sorted using the traditional method and the method used in this study and cultured in M3434 medium (Stemcell Inc) at 250 cells per 35 mm culture dish. After 8 days of culture, colony forming units (CFU) were scored according to the type of cells present. Mean ± SD from triplicate analysis is depicted.
IFN-γ induces anemia by reducing erythrocyte lifespan and inhibiting erythropoiesis.

Supplementary Figure 2. IFN-γ enhances RBC turn-over.
(a) Determination of the area under the curve of the turnover of biotinylated RBCs of WT, CD70TG, IFN-γ-/- and CD70TG* IFN-γ-/- mice as displayed in Figure 2b. (b) Determination of the area under the curve of the turnover of adoptively transferred biotinylated WT RBCs in WT, CD70TG, IFN-γ-/- and CD70TG* IFN-γ-/- mice as displayed in Figure 2c. Mean ± SD is depicted, * indicates a significant difference (p<0.05) between CD70TG mice and all other groups, using a one-way ANOVA with Bonferroni correction.

Supplementary Figure 3. Annexin-staining on erytroid precursor cells in the bone marrow reveals no direct link between IFN-γ and apoptosis.
(a-b) FACS-analysis for Annexin V-positivity was performed on total bone marrow samples of WT, CD70TG, IFN-γ-/- and CD70TG* IFN-γ-/- mice directly ex vivo. Mean percentage ± SD is depicted for 2 mice per group in experiment 1 and 3 mice per group in experiment 2. * indicates a significant difference (p<0.05) using a one-way ANOVA with Bonferroni correction.
Supplementary Figure 4. Microarray analysis: IFNγ dependent genes in CD71+ erythroblasts isolated from CD70TG mice.

Grey colour tab, potential PU.1 target genes. Cream colour tab, potential GATA-1 target genes. White, neither PU.1 nor GATA-1 target genes.

The shape of each symbol represents the function of the encoded protein, whereas the color indicates whether a gene is upregulated (red) or downregulated (green) by IFN-γ.
Supplementary Figure 5. Transcription factor expression and suppression in human erythroid precursor cells.
(a) Western blot analysis of PU.1, GATA-1 and IRF-1 expression on human erythroid precursor cells cultured overnight with or without IFN-γ. Actin and nucleophosmin (NPM) are used as loading controls. (b) QPCR analysis of the expression of PU.1 and IRF-1 mRNA of human erythroid precursor cells transduced with either a short hairpin against PU.1 or IRF-1 and cultured overnight with or without IFN-γ. Data is presented as mean of the fold enrichment IFN-γ/untreated ± SD.

Supplementary Figure 6. Unaltered hepatic hepcidin expression.
Q-PCR analysis of the expression of hepcidin mRNA levels in liver cells of WT, CD70TG, IFN-γ−/− and CD70TG*IFN-γ−/− mice. Mean ± SD is depicted for 3 mice per group.
IFN-γ induces a Noxa-dependent alternative pathway of cell death in erythroid precursors

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Abstract
Besides the well-known stimulatory and regulatory effects on the immune system, the pro-inflammatory cytokine IFN-γ also has profound effects on hematopoiesis. IFN-γ contributes to the development of anemia of chronic diseases as it enhances the breakdown of erythrocytes and diminishes erythropoiesis in the bone marrow by upregulation of the myeloid transcription factor PU.1. The cellular fate of IFN-γ stimulated erythroid precursors however is unknown. We here show that IFN-γ induces programmed cell death in erythroid precursor cells that are expanded or differentiated. This process is mediated by the BH3-only member Noxa, as its knockdown in IFN-γ stimulated cells prevents cell death. The underlying mechanism by which IFN-γ stimulated cells die is exceptional, as blocking apoptosis, autophagy, reactive oxygen species, necroptosis and ferroptosis did not prevent induction of cell death. This thus suggests the existence of an alternative programmed pathway of death in which Noxa is involved. Inhibiting mTOR signaling, which normally controls cell growth and proliferation by modulating translation, transcription and protein synthesis upon environmental cues, allows survival of erythroid precursors stimulated with IFN-γ by blocking Noxa upregulation.
Introduction

Anemia of chronic disease (ACD) is the second most prevalent form of anemia, just after anemia caused by iron deficiency, and is the most prevalent form of anemia found in hospitalized patients\(^1\). ACD develops in patients suffering from chronic inflammatory disorders such as chronic infections, cancer and autoimmune diseases like rheumatoid arthritis, systemic lupus erythematosus and inflammatory bowel disease\(^2\). The etiology of ACD is still under debate as it is a multi-factorial disease with alterations in erythropoiesis, erythrocyte breakdown as well as iron sequestering. Pro-inflammatory cytokines however, are believed to play an essential role in its development\(^1;2\).

Especially the role of the pro-inflammatory cytokine interferon-gamma (IFN-γ) in this respect has been well studied. Although first recognized for its important stimulatory and modulatory capacities on both innate and adaptive immune cells to fight viral and intracellular bacterial infections, IFN-γ was also found to have profound effects on hematopoiesis\(^3\). IFN-γ induces proliferation of hematopoietic stem cells (HSCs) and negatively affects their self-renewal capacity\(^4;5\). Furthermore, IFN-γ alters the myeloid output from the bone marrow\(^6;7\), while also inhibiting the outgrowth of burst-forming unit-erythroid (BFU-e) and colony-forming unit-erythroid (CFU-e)\(^8;11\). Various mechanisms have been described by which IFN-γ can affect proliferation and differentiation of erythroid precursor cells. Exposure to IFN-γ \textit{in vitro} down-regulates stem cell factor (SCF) and erythropoietin (EPO)-receptor expression on colony forming cells, thereby impeding the cell to respond to survival and differentiation signals\(^12\). Furthermore, IFN-γ induces expression of death receptors CD95/CD95-ligand (Fas/Fas-ligand), TWEAK and TRAIL\(^13;14\), indicating that IFN-γ de-sensitizes erythroid cells for growth factors and induces apoptosis. Fas/Fas-L interactions have also been described to be physiologically active during erythropoiesis and play an important role in the control of the rate of erythropoiesis\(^15;16\). Programmed cell death decisions during stress conditions are most often carried out by members of the highly conserved B-cell lymphoma (Bcl-2) family as they prevent or promote cytochrome c release from the mitochondria and ultimately the activation of caspases that execute apoptosis\(^17;19\). Whereas the anti-apoptotic Bcl-2 family members Bcl-2, A1 and Mcl-1 have been shown to play minor roles in erythropoiesis\(^20;21\), Bcl-\(X\_i\) is required in erythropoiesis as its expression is induced during erythroid maturation and prevents apoptosis during terminal differentiation into erythrocytes\(^22;25\). Although having weak pro-apoptotic potential, Noxa is important in fine-tuning cell death decisions by targeting the anti-apoptotic protein Mcl-1 for proteasomal degradation during stress-conditions\(^26;27\).

More recently, we showed that chronic exposure to IFN-γ negatively affects erythropoiesis both \textit{in vitro} and \textit{in vivo} by induction of the transcription factor PU.1 via IRF-1\(^11\). PU.1 is essential for myelopoiesis and can bind to and repress the function of GATA-1, the most important transcriptional regulator of erythropoiesis\(^28;29\), thereby directing differentiation at the progenitor level towards myelopoiesis. Elevated levels of PU.1 in the erythroid lineage are known to repress GATA-1 function and thereby block erythropoiesis\(^29;31\). Although the effects of IFN-γ on erythropoiesis are well studied, it is largely unknown what the cellular fate is of erythroid-committed cells that are stimulated with IFN-γ. We here show that IFN-γ induces cell death during expansion and erythroid differentiation of erythrocyte precursor cells. IFN-γ induced cell death does not involve Fas-Fas-ligand interactions, apoptosis, autophagy, induction of reactive oxygen species (ROS), necroptosis or ferroptosis, but involves a yet
unidentified mechanism that is Noxa-dependent. Interestingly, the IFN-γ induced cell death can largely be overcome by blocking the mammalian target of rapamycin (mTOR) pathway as it prevents upregulation of Noxa.

**Material and Methods**

**Cell Lines and Cultures**
The pre-myeloid cell line TF-1 was maintained at 37°C and 5% CO₂ in RPMI 1640 (Lonza) supplemented with 10% fetal calf serum, 100 μg/ml gentamycin (Invitrogen), 2 mM L-Glutamin (Invitrogen), 0.5 mM β-mercapto-ethanol (Sigma-Aldrich) and 0.2 ng/ml human recombinant IL-3 (R&D Systems). To induce differentiation IL-3 was replaced by 1 U/ml EPO. Where appropriate, 100 ng/ml recombinant human IFN-γ (Sanquin PeliKine), 5 μg/ml cycloheximide (Calbiochem), 5 μM MG132 (Calbiochem), 20 μM pan-caspase inhibitor Q-VD-OPh (R&D Systems), 30 μM Necrostatin-1 (Enzo Life Sciences), 5 mM 3-methyladenine (Sigma-Aldrich), 5 mM N-acetyl cysteine (Sigma-Aldrich), 100 nM H₂O₂ (Merck), 10 μM U0126 (Calbiochem) or 10 ng/ml Rapamycin (Wyeth Laboratories) was added. Cell numbers were determined using a Bürker counting chamber (LO - Laboroptik Ltd.).

**Mice**
For experiments wild-type C57BL/6J (WT), Fas⁻/⁻ and Noxa⁻/⁻ mice were used which were maintained in the animal facilities of the Academic Medical Center (University of Amsterdam, Amsterdam, The Netherlands) in specific pathogen-free conditions. Mice were given standard chow and acidified drinking water ad libitum. All animal experiments were approved by the Experimental Animal Committee of the Academic Medical Center, according to institutional and national guidelines.

**Lentiviral-mediated shRNA targeting**
Lentiviral shRNA clones (Sigma Mission RNAi) targeting NOXA and a scrambled non-targeting control (SHC002) were obtained from Sigma. Vectors were co-transfected with the packaging vectors psMD2G, pMDLg/pRRE and pRSV-Rev into 293T cells using Eugene6 (Roche Applied Science) to produce virus. After 48 hours, lentiviral vector particles were harvested, virus supernatants were filtered with 0.22-µm filters and stored at -80°C. Efficiency of different lentiviral shRNA clones in cells was determined by Western blot. TF-1 cells were transduced by a single round of infection for 24 hours, followed by puromycin (Sigma-Aldrich) selection (1 μg/mL) for 1 week.

**Flow Cytometry**
For determining the differentiation of TF-1 cells the following monoclonal human-antibodies were used: CD34-peridinin chlorophyll protein (Per-CP; 8G12), CD71-fluorescein isothiocyanate (FITC; M-A712), CD235a-phycocerythrin (PE; HIR-2). Dead/live discrimination was achieved by addition of 20 nM TO-PRO-3 Iodide (Invitrogen) to cells directly before measuring. Single cell suspensions for flow cytometric analysis of the erythroid composition of mice were obtained by mincing spleens directly through 40 μm cell strainers, while single cell suspensions of bone marrow were collected by first crushing femurs and tibiae using a
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Cells were incubated with anti-CD16/CD32 blocking antibodies (2.4G2; a kind gift of Louis Boon, Bioceros). Monoclonal mouse-antibodies used were: C-kit-allophycocyanin (APC; 2B8), CD71-PE (R17217), Ter119-PE-Cy5.5 (Ly-76). Data acquisition was done using FACSCalibur, (BD Biosciences), FACSCanto I (BD Biosciences) or FACSCanto II (BD Biosciences). Data were analyzed using Flowjo Version 9.2 for Mac OS X or Version 7.6.5 for Windows (TreeStar).

Reverse transcriptase multiplex ligation-dependent amplification (RT-MLPA)
Total RNA for RT-MPLA was isolated from TF-1 cells using Trizol (Invitrogen). mRNA levels of pro- and anti-apoptotic genes were analyzed with the Apoptosis Human RT-MLPA kit R011 (MRC-Holland) according the manufacturers instruction. Sample data was obtained using Genescan and subsequently analyzed with Genemapper (Applied Biosystems) and Excel (Microsoft), as described previously.

Hanging Drop Assays
Hanging drop assays were performed as described previously. Briefly, total bone marrow cells were resuspended in Hanging Drop medium (DMEM supplemented with 20% FCS, 0.1% β-mercaptoethanol, 2 x 10^-9 M hemin (Sigma), 1% penicillin/streptomycin, 2 U/mL EPO and 5 μg/mL insulin) at a concentration of 1.25 x 10^6 cells/mL and approximately 20 drops were pipetted on the inner side of the lid of a Petri dish. The lid was gently inverted and placed on the PBS-filled Petri dish. Cultures were then maintained at 37°C and 5% CO_2 and harvested for FACS-analysis at day 1 and 3 by washing cells in PBS supplemented with 10% FCS.

Semisolid Colony Assays
Assays for determining burst-forming unit-erythroid (BFU-e) and colony-forming unit-erythroid (CFU-e) were performed as described earlier. Briefly, 3.75 x 10^5 total bone marrow cells were taken up in methylcellulose medium (Methocult M3234; StemCell Technologies) supplemented with 4 U/ml human recombinant EPO (Aranesp, Amgen), 100 ng/mL recombinant murine stem cell factor, 20 μg/mL human holo-transferrin (SCIPAC, T101-5), 2 x 10^-4 M hemin (Sigma-Aldrich) and 1% gentamycin (Invitrogen) and plated in triplo. CFU-e colonies were scored at day 3, BFU-e colonies at day 8. When appropriate 100 ng/mL recombinant murine IFN-γ (PeproTech) was added to the medium.

Western Blot
Total protein extracts were made according to standard methods. Proteins were separated on 12.5% SDS-PAGE-gels and subsequently transferred onto Immobilon-P membranes (Millipore) by electroblotting. Membranes were blocked with PBS containing 2% low-fat-milk (ELK, Campina) and 0.25% Tween. Blots were incubated overnight at 4°C with primary antibodies, thoroughly washed with PBS containing 0.25% Tween and subsequently incubated with IRDye680 or IRDye800 labeled secondary antibodies (LI-COR Biosciences) for 1 hour. Detection and quantification of proteins was done using the Odyssey Imager (LI-COR Biosciences) according to the manufacturer's protocol. Primary antibodies used were: PU.1 (Santa Cruz Biotechnology, T21, sc-352), IRF-1 (Santa Cruz Biotechnology H8, sc-74530), Mcl-1 (BD Pharmingen), Noxa (Imgenex), Bcl-XL (Transduction Laboratories), PUMA (Cell Signaling), LC3 (MBL, clone 51-11) or β-Actin (Santa Cruz Biotechnology, I-19, sc-1616).
Statistical Analysis
Results are expressed as mean ± SD. Statistics on groups was performed using Graphpad Prism 5, using a one-way ANOVA with Bonferroni correction. P<0.05 was considered statistically significant.

Results

**IFN-γ induces cell death of erythroid committed precursors**
To elucidate the fate of erythroid precursors after stimulation with IFN-γ, we applied two in vitro systems. The hanging drop culture method described by Gutierrez et al. allows the study of the effect of IFN-γ on terminal erythroid differentiation of bone marrow cells. Stimulation with IFN-γ revealed a significant loss of cell viability as measured with the nuclear DNA-stain TO-PRO-3. The loss of viability was accompanied with the loss of immature erythroid cells based on forward scatter (FSC) (Fig. 1a). More mature erythroid cells (FSC<sup>lo</sup>) seemed largely unaffected. Similar results were obtained with the pre-myeloid TF-1 cell line. TF-1 cells still have erythroid and myeloid differentiation capacity and can be expanded with IL-3 and differentiated to erythroid cells with erythropoietin (EPO). Upon differentiation towards the erythroid lineage, CD71 and CD235a expression is induced while CD34 expression is lost (Fig. 1b). After 4 days of culture in the presence of IFN-γ, the percentage of differentiated cells (CD34<sup>-</sup> CD71<sup>+</sup>) increased in the presence of IFN-γ, independently of culturing with IL-3 or EPO (Fig 1b). However, total cell numbers deteriorated in time when cultured in the presence of IFN-γ and cell viability was lost after 3 days of culture (Fig. 1c-d), revealing that IFN-γ blocks proliferation and differentiation of erythroid precursors due to induction of cell death in early erythroid precursors. More mature erythroid cells are less responsive to IFN-γ and survive.

**Regulation of apoptosis in erythroid committed precursors**
To gain better insight into what mechanism might be involved in the induction of cell death of erythroid precursors, TF-1 cells were cultured in the presence of IL-3 or EPO and with or without IFN-γ. Protein lysates were analyzed after 1 and 2 days of culture. We first checked whether IRF-1 and PU.1 expression was induced by IFN-γ. While IRF-1 was highly expressed after 1 and 2 days of culture in the presence of IFN-γ, surprisingly, PU.1 expression was unaltered (Fig. 2a), suggesting that alterations in PU.1 cannot explain the observed changes induced by IFN-γ in TF-1 cells.

Initiation of apoptosis is the most common form of programmed cell death and can be mediated via both the extracellular pathway, which involves triggering of death receptors, and the intracellular pathway, which is regulated by balancing pro- and anti-apoptotic members of the B-cell lymphoma 2 (Bcl-2) family. In addition to the death receptor Fas having a physiological role in the regulation of erythropoiesis, IFN-γ has been found to sensitize erythroid cells to Fas-induced cell death. We therefore tested whether absence of Fas on total bone marrow cells from mice influenced the erythroid output. Using colony assays we found that the CFU-e and BFU-e outgrowth from Fas<sup>−/−</sup> mice was comparable to that of WT controls. Addition of IFN-γ to cultures affected WT and Fas<sup>−/−</sup> bone marrow equally. As the BFU-e output from both WT and Fas<sup>−/−</sup> was significantly reduced (Suppl. Fig. 1a), while CFU-e output was unaltered (Suppl. Fig. 1b), these data argue that IFN-γ does not induce cell
IFN-γ induces a Noxa-dependent alternative pathway of cell death in erythroid precursors

To determine whether IFN-γ is capable of inducing cell death via the intracellular pathway, TF-1 cells were cultured for 8 or 24 hours in the presence of IFN-γ after which mRNA expression of pro- and anti-apoptotic proteins were determined using reverse transcriptase-multiplex ligation-dependent probe amplification (RT-MLPA). mRNA expression levels of the pro- or anti-apoptotic members of the Bcl-2 family were not significantly altered upon IFN-γ stimulation (Fig. 2b). Expression of some Bcl-2 family members is however subjected to post-translational regulation and modification, thereby altering their function37. We therefore next checked whether IFN-γ affects protein expression of various Bcl-2 family.

Figure 1. IFN-γ induces cell death of erythroid committed precursors
(a) Representative dot plots of flow cytometric analysis of the effects of IFN-γ on WT BM cells cultured for 1 or 3 days in a hanging drop assay. Results are representative for 2 independently performed experiments. (b) Representative dot plots of flow cytometric analysis of expanding and differentiating TF-1 cells cultured for 4 days with or without the presence of IFN-γ. (c) Quantification of cell numbers in time of expanding and differentiating TF-1 cells cultured with or without the presence of IFN-γ. (d) Quantification of cell viability in time of expanding and differentiating TF-1 cells cultured with or without the presence of IFN-γ using flow cytometric analysis of TO-PRO-3. (b-d) Results are representative for 3 independently performed experiments.
Figure 2. IFN-γ induces Noxa-expression in erythroid committed precursors
(a) Western blot analysis of IRF-1 and PU.1 expression on TF-1 cells cultured with either IL-3 or EPO and with or without the addition of IFN-γ for 1 or 2 days. β-Actin is used as loading control. Data is representative for 2 independently performed experiments. (b) mRNA expression analysis of pro- and anti-apoptotic proteins in TF-1 cells after 8 or 24 hours of culture with IL-3 alone or with the addition of IFN-γ. Data represents 1 experiment using 1 sample measured in duplo. (c) Western blot analysis of IRF-1, PU.1, Bcl-XL, PUMA, Noxa and Mcl-1 expression on TF-1 cells cultured with either IL-3 or EPO and with or without the addition of IFN-γ for 1 or 2 days. β-Actin is used as loading control. Data is representative for 2 independently performed experiments.
members that are involved in erythropoiesis or the induction of stress-related cell-death. The anti-apoptotic Bcl-X\textsubscript{L}, whose expression is important during erythroid maturation and prevents apoptosis during terminal differentiation\textsuperscript{21,22,25}, was unaltered by IFN-\textgamma and also the expression of the pro-apoptotic protein PUMA, which can directly be induced by IRF-1\textsuperscript{38}, was found unchanged (Fig. 2c). IRF-1 has also been found to induce the expression of the pro-apoptotic protein Noxa\textsuperscript{39,40}. Noxa was expressed at higher levels in cells cultured with IL-3 compared to cultures containing EPO, but IFN-\textgamma increased its expression under both culture conditions (Fig. 2c). Expression of Mcl-1, the main binding partner of Noxa, remained relatively unaffected after 1 day of culture in the presence of IFN-\textgamma. After 2 days it is slightly upregulated in these cultures, however not to the extent as the upregulation of Noxa (Fig. 2c), suggesting that IFN-\textgamma induces cell death via deregulating the Noxa-Mcl-1 balance.

Figure 3. IFN-\textgamma induced cell death of erythroid precursors is NOXA-dependent
(a) Western blot analysis of NOXA expression on TF-1 cells transduced with a control shRNA (Cntrl) or shRNA directed against Noxa (Noxai) and cultured for 1, 2 or 3 days with IL-3 and with or without the presence of IFN-\textgamma. \(\beta\)-Actin is used as loading control. (b) Quantification of cell numbers in time of expanding Cntrl and Noxai TF-1 cells cultured with or without the presence of IFN-\textgamma. (c) Quantification of cell viability in time of expanding Cntrl and Noxai TF-1 cells cultured with or without the presence of IFN-\textgamma using flow cytometric analysis of TO-PRO-3. (d) Representative dot plots of flow cytometric analysis of expanding Cntrl and Noxai TF-1 cells cultured for 4 days with or without the presence of IFN-\textgamma. (b-d) Results are representative for 3 independently performed experiments.
IFN-γ induced cell death of erythroid precursors is Noxa-dependent

To investigate whether IFN-γ indeed induces cell death in TF-1 cells via a Noxa-dependent mechanism, cells were transduced with lentivirus expressing a shRNA targeting Noxa mRNA (TF-1 Noxai). Cells were subsequently cultured with IL-3, stimulated with IFN-γ and cell viability was measured over time. Compared to IFN-γ stimulated cells transduced with a nontarget control (TF-1 Cntrl), knockdown of Noxa mRNA inhibited IFN-γ induced expression of Noxa protein (Fig. 3a). Noxa knockdown did not alter the growth of TF-1 cells (Fig. 3b), but it was sufficient to overcome IFN-γ induced cell death as measured with TO-PRO-3 (Fig. 3c-d). These experiments thus confirm that IFN-γ induces cell death of TF-1 cells via a Noxa-dependent mechanism.

IFN-γ induces an alternative pathway of programmed cell death in TF-1 cells

Although the role of Noxa in the IFN-γ induced block of erythropoiesis does not seem to be of major importance in murine bone marrow, understanding the fundamental mechanisms involved in IFN-γ induced cell death via Noxa can be of importance for other cell types and for the development of therapy. Noxa is traditionally involved in the induction of apoptosis and the prediction would be that caspase inhibitors could overcome IFN-γ induced cell death of TF-1 cells. Therefore, we assessed whether addition of the pan-caspase inhibitor Q-VD-OPh (Q-VD) could block IFN-γ induced cell death of TF-1 cells. Addition of Q-VD to control cultures did prevent steady-state apoptosis and prevented nutrient deprivation induced cell death, which is known to induce apoptosis through Noxa41 (Fig. 4a, Suppl. Fig. 1c). However, addition of Q-VD could not overcome the IFN-γ induced cell death (Fig. 4b), which demonstrates that IFN-γ does not induce classical apoptosis in TF-1 cells, but rather induces cell death via another mechanism.

Under steady-state conditions, autophagy can be found at low levels in cells as it plays an important role in maintaining intracellular homeostasis by targeting unwanted proteins, cytoplasmic components and organelles for removal42-44. Enhancing autophagy during stress conditions, like i.e. nutrient-deprivation or immune activation, promotes cell survival by generating energy from breakdown of targeted intracellular components and allows trafficking of cellular proteins to MHC molecules45;46. Excessive autophagy however can induce cell death47. To test whether IFN-γ induces autophagy-related cell death in TF-1 cells, cells were cultured in the presence of the autophagy inhibitor 3-MA and cell numbers and cell viability were assessed. Unfortunately, 3-MA treatment was incompatible with proper cell expansion (Fig. 4c) and lowered cell viability (Fig. 4d). Although this did not allow us to determine whether IFN-γ induced cell death through autophagy, we did not find indications that IFN-γ did induce autophagy based on the formation of autophagosomes by measuring conversion of LC3-I to LC3-II (Suppl. Fig. 1d)48.

Although apoptosis and autophagy are the best-studied mechanisms involved in programmed cell death, more recent findings point to the existence of multiple non-apoptotic regulated cell death mechanisms49. Necroptosis, or programmed necrosis, can lead to non-apoptotic cell death when caspases are inhibited. Necroptosis is critically dependent on RIP-1 kinase activity50, which together with RIP-3 alters the metabolic rate of mitochondria and induces the expression of reactive oxygen species (ROS), ultimately leading to mitochondrial dysfunction and initiation of necroptosis51;52. To see whether IFN-γ induces cell death of TF-1 cells by means of necroptosis, cells were cultured with or without
IFN-γ induces a Noxa-dependent alternative pathway of cell death in erythroid precursors

Figure 4. IFN-γ induces an alternative pathway of programmed cell death in TF-1 cells

(a,c,e,g,i) Quantification of cell numbers in time and (b,d,f,h,j) cell viability at day 4 of expanding TF-1 cells cultured with or without the presence of IFN-γ and (a-b) Q-VD, (c-d) 3-MA, (e-f) Nec-1, (g-h) U0126 or (i-j) NaC. Data are representative for (a-b) 5 independently performed experiments, (e-f) 3 independently performed experiments or (c-d, g-i) 1 experiment.
Figure 5. Blocking the mTOR pathway prevents IFN-γ induced cell death of TF-1 cells
(a) Quantification of cell numbers in time of expanding TF-1 cells cultured with or without the presence of IFN-γ and rapamycin. (b) Quantification of cell viability in time of expanding TF-1 cells cultured with or without the presence of IFN-γ and rapamycin using flow cytometric analysis of TO-PRO-3. (c) Representative dot plots of flow cytometric analysis of expanding TF-1 cells cultured for 4 days with or without the presence of IFN-γ and rapamycin. (a-c) Results are representative for 3 independently performed experiments. (d) Western blot analysis of Noxa, Mcl-1, IRF-1 and PU.1 expression on TF-1 cells cultured with IL-3 and with or without the addition of IFN-γ and rapamycin for 1 or 2 days. β-Actin is used as loading control. Data represents 1 experiment.

The presence of Necrostatin-1 (Nec-1), which specifically blocks RIP-1 kinase activity and overcomes necroptosis\(^\text{53}\). After 4 days of culture in the presence of both IFN-γ and Nec-1 cell growth was comparable to cell cultured with IFN-γ alone (Fig. 4e). Addition of Nec-1 did not save cells from IFN-γ-induced cell death as cell viability was comparable to cells cultured in the presence of IFN-γ alone (Fig. 4f). Furthermore, addition of Nec-1 combined with QVD
to IFN-γ stimulated TF-1 cells also did not overcome induction of cell death (Suppl. Fig. 1e), indicating that IFN-γ induced cell death of TF-1 cells does not involve necroptosis.

More recently, an iron-dependent programmed cell death mechanism, termed ferroptosis, was described which is genetically, morphologically and biochemically distinct from apoptosis, necrosis and autophagy\textsuperscript{54}. Ferroptosis is induced when cysteine uptake is inhibited and lipid ROS accumulate. This process can be specifically blocked with Ferrostatin-1 or by the less-specific compounds DFO, trolox and U0126\textsuperscript{54}. Given the fact that erythropoiesis is dependent on iron sequestering and IFN-γ might induce iron dependent ROS-formation, we next checked whether addition of the MEK-inhibitor U0126 could overcome the IFN-γ induced cell death of TF-1 cells. Blocking ferroptosis impaired cell proliferation, while cell viability was maintained, but could not prevent induction of cell death by IFN-γ (Fig. 4g-h).

Both necroptosis and ferroptosis rely on the formation and accumulation of ROS for the induction of cell death\textsuperscript{51;52;54}. Although blocking the specific upstream signaling pathways of these processes could not overcome the IFN-γ induced cell death, a direct role for ROS-induced cell death was not excluded. As IFN-γ has also been shown to induce cell death via the formation of ROS\textsuperscript{55}, we next checked whether directly scavenging ROS could prevent IFN-γ induced cell death. Where addition of the ROS-scavenger N-acetylcysteine (NaC) to cultures could prevent H\textsubscript{2}O\textsubscript{2}-induced cell death, it could not rescue the decline in cell growth and prevent induction of cell death induced by IFN-γ (Fig. 4i-j, Suppl. Fig. 1f ). These data indicate that IFN-γ does not induce lethal levels of ROS, thereby further supporting that induction of necroptosis and ferroptosis in TF-1 cells by IFN-γ is highly unlikely. Taken together, these data thus show that IFN-γ induces cell death in TF-1 cells not via apoptosis, autophagy, induction of ROS, necroptosis, or ferroptosis, but by a yet unidentified mechanism, which is Noxa-dependent.

Blocking mTOR activity prevents IFN-γ induced cell death of TF-1 cells

Interferons enhance the formation of immunoproteasomes that prevent accumulation of harmful newly generated proteins defective in folding, translation or assembly during immune activation\textsuperscript{56-59}. These so-called defective ribosomal products (DRiPs) are formed by ROS upon activation of the mammalian target of rapamycin (mTOR) pathway by IFN-γ\textsuperscript{59}. Although blocking ROS does not overcome the IFN-γ induced cell death of TF-1 cells (Fig. 4i-j) and Noxa and Mcl-1 breakdown is not altered by IFN-γ (Suppl. Fig. 2), cell death could be the consequence of accumulation of newly synthesized proteins, including Noxa. We therefore determined whether blocking the mTOR pathway using rapamycin could prevent induction of cell death. Although addition of rapamycin significantly decreased cell numbers after 4 days of culture even without IFN-γ (Fig 5a), it did prevent IFN-γ induced cell death (Fig. 5b-c). Next, we examined whether this increased survival was correlated to Noxa and Mcl-1 protein and found that rapamycin didn’t affect IFN-γ induced IRF-1 and Mcl-1 protein expression, but that the upregulation of Noxa protein was reduced in the presence of rapamycin (Fig. 5d). These data indicate that blocking the mTOR pathway upon IFN-γ stimulation lowers the formation of newly synthesized Noxa and thereby blocks induction of cell death.
Discussion
Prolonged exposure to elevated levels of the pro-inflammatory cytokine IFN-γ severely affects erythropoiesis and is associated with the development of ACD. We here provide evidence that IFN-γ induces cell death during both expansion and differentiation of erythroid precursors via a Noxa-dependent mechanism (Fig. 3). Targeting Noxa mRNA for degradation by shRNAs or lowering Noxa-expression by blocking mTOR signaling overcomes IFN-γ induced cell death (Fig. 3/6). Although Noxa expression is induced during erythroid maturation of human CD34+ peripheral blood mononuclear cells (PBMCs) (Geest/Wensveen, unpublished data), we found that Noxa does not play a role in steady-state erythropoiesis in vivo using Noxa−/− mice (Suppl. Fig. 3a-b). In contrast with the human TF-1 cell line, Noxa ablation in mice does not appear to affect the IFN-γ induced block of erythropoiesis (Suppl. Fig. 3c-d). Human and mouse Noxa however differ significantly in gene structure, protein sequence and the number of BH3 domains present within the Noxa protein. Species differences could thus account for differences found in the involvement of Noxa in the IFN-γ induced block of erythropoiesis between human and mouse. Determining functional differences however are impeded by the lack of proper antibodies directed against mouse Noxa.

Noxa is traditionally involved in the induction of apoptosis by targeting the anti-apoptotic protein Mcl-1 for proteasomal degradation upon cellular stress like DNA damage and oxygen and glucose deprivation. Although IFN-γ did not induce Noxa and Mcl-1 mRNA expression (Fig. 2b), we found that protein synthesis of Noxa was enhanced, while Mcl-1 protein expression remained stable (Fig. 2c). Depletion of the 19S regulatory subunit of 26S, which degrades Noxa, leads to loss of proteasome function and induces accumulation of Noxa and subsequent Noxa-dependent apoptosis. Under metabolic stress conditions the half-life of Noxa is short and unaffected, whereas the half-life of Mcl-1 reduces. By blocking protein synthesis we however did not find any evidence for major alterations in the breakdown of both Noxa and Mcl-1. Whereas the half-life of Noxa appears to be longer than that of Mcl-1, the breakdown kinetics of Noxa and Mcl-1 were not influenced by IFN-γ after 1 or 3 days of culture (Suppl. Fig. 2). Dependent on the cell type and other signals involved in the induction of cell death, Noxa can relieve inhibition of the pro-apoptotic effector proteins Bax and Bak, which induce mitochondrial fragmentation and subsequent cytochrome c release, ultimately leading to the formation of the apoptosome and activation of proteolytic caspases. Whereas inhibiting the proteolytic function of caspases can prevent apoptosis, the pan-caspase inhibitor Q-VD could not overcome the IFN-γ-induced cell death of TF-1 cells (Fig. 5a-b), thereby excluding involvement of both the intrinsic and extrinsic pathway of apoptosis. As cell death appears to take place in a controlled manner and does not occur up until 3 days of culture in the presence of IFN-γ, this suggest a role for Noxa in a programmed cell death pathway other then apoptosis.

Induction of autophagy is an important step in erythropoiesis, as cellular organelles need to be cleared during reticulocyte maturation. Importantly, GATA-1, the essential transcription factor for erythropoiesis, induces transcription of the essential autophagy component LC3 and activates genes involved in the formation of autophagosomes. Induction of autophagy and autophagy-induced cell death by IFN-γ has been found in a variety of cells during immune activation. Here however, we found no signs of induction of autophagy by IFN-γ in TF-1 cells based on LC3 conversion (Suppl. Fig. 1d). Interpreting
LC3 immunoblotting however involves difficulties as LC3-II itself is degraded by autophagy and longer starvation periods induce breakdown of both LC3-I and LC3-II. Measuring LC3 conversion 24 and 48 hours after the start of cultures therefore might underestimate the role of IFN-γ in the induction of autophagy cell death in erythroid precursors. We cannot fully exclude that IFN-γ induces cell death using autophagy as 3-MA prevented proliferation of TF-1 cells and induced cell death (Fig. 5c-d), making interpretation of these data difficult. As 3-MA can prevent autophagy-related cell death by inhibiting phosphatidylinositol 3-kinase (PI3K), this argues for an essential role of PI3K in the survival of TF-1 cells. To what extent IFN-γ induces or alters autophagy during maturation of erythroid precursors was not checked. Interestingly, the Bcl-2 family member Nix plays a role in autophagy by targeting mitochondria to autophagosomes during erythroid maturation, indicating that other members from the Bcl-2 family might also be involved in induction or regulation of autophagy.

Necroptosis can be induced by death receptors like Fas-Ligand and tumor necrosis factor-α (TNF-α) when the machinery that executes apoptosis is blocked or absent. Also IFN-γ can induce necroptosis, however only in the absence of NFκB-signaling, which induces a survival response that preserves mitochondrial integrity by suppressing ROS. Necroptosis, but also ferroptosis, are thus induced upon the formation of ROS. Blocking necroptosis (Fig. 3 e-f, Supp. Fig. 1c) and ferroptosis (Fig. 3g-h) specifically, or the formation of ROS in general (Fig. 3i-j), however, could not overcome IFN-γ induced cell death of TF-1 cells. The involvement of necroptosis and ferroptosis can nevertheless not be fully excluded, as we have not formally demonstrated that Nec-1 and U0126 were functionally active in our system. Yet, supplementation of NaC after 2 days of culture however had no positive effect on survival (data not shown), making it highly unlikely that the formation of ROS and the involvement of necroptosis and ferroptosis account for the induction of cell death by IFN-γ.

Another possible mechanism in which IFN-γ could induce cell death in TF-1 cells is by interfering with cytokine signaling. This can either be by downregulation of SCF and EPO-receptors, or by interfering with downstream cytokine signaling via induction of suppressor of cytokine signaling (SOCS)-proteins by IFN-γ. Elevated levels of EPO have been described to overcome the negative effects of IFN-γ on CFU-e formation, but to what extent elevating the levels of IL-3 and EPO can overcome the IFN-γ induced cell death of TF-1 cells remains to be elucidated. Supplementation of additional IL-3 after 2 days of culture could however not overcome IFN-γ induced cell death, nor could the addition of SCF (data not shown). We recently showed that prolonged exposure to IFN-γ in vivo induces the expression of PU.1, thereby enhancing the myeloid output from CMPs at the expense of erythropoiesis. In contrast to primary erythroid precursors from mouse and human, IFN-γ does not induce expression of PU.1 in TF-1 cells. IFN-γ induced cell death of erythroid precursors could thus be the consequence of lack of growth factors and signals inducing re-programming of cells.

Interferons are known for the activation of the mTOR pathway and interferon-α has been described to induce cell death via a PI3K/mTOR pathway. Downstream of PI3K, mTOR controls cell growth by integrating growth factor, nutrient, energy and stress signaling. Although we provide no evidence that mTOR signaling is activated or enhanced by IFN-γ, blocking mTOR function reduces IFN-γ induced cell death of TF-1 cells (Fig. 6b-c), most probably by preventing the induction of Noxa-expression (Fig. 6d). As induction of mTOR by interferon signaling enhances the production of harmfull newly synthesized proteins, the
damage caused by these proteins might be most pronounced upon cell division and trigger induction of Noxa-dependent cell death during cell cycle. Interestingly, blocking mTOR signaling inhibited cell proliferation (Fig. 6a), indicating that arresting cell cycle by inhibiting mTOR might account for survival of IFN-γ stimulated erythroid precursors. Although mTOR inhibitors (Sirolimus, Everolimus and other derivatives) are nowadays widely applied in clinical trials for the treatment of autoimmune disorders and prevention of allograft rejection, their immunosuppressive effects may also be shadowed by the induction of metabolic, hematological and dermatological side effects. Induction of anemia is commonly found during treatment with mTOR inhibitors, and our and other data suggest that this might be caused by growth inhibition of erythroid precursors (Fig. 6a). Precaution should thus be taken when starting mTOR inhibitor treatment in patients with an underlying anemia.

**Acknowledgements**
We thank the staff of the animal facility of the AMC for excellent animal care.
## Reference List


24. Gregoli PA, Bondurant MC. The roles of Bcl-X(L) and apopain in the control of erythropoiesis by erythropoietin. Blood 1997;90:630-640.


IFN-γ induces a Noxa-dependent alternative pathway of cell death in erythroid precursors


Supplementary Figures

Supplementary Figure 1. IFN-γ induces an alternative pathway of programmed cell death in TF-1 cells

(a-b) Effect of IFN-γ on the in vitro CFU-e (a) and BFU-e (b) colony forming capacity of unfractioned WT and Fas-/- bone marrow samples. Data are expressed as mean +/- SD for 3 mice per group. * indicates a significant difference (P<0.05) between medium and IFN-γ-treated samples using a paired 2-tailed Student t-test. (c) Quantification of cell viability of IL-3 deprived TF-1 cells cultured with or without the presence of QVD using flow cytometric analysis of TO-PRO-3. Data represents one experiment. (d) Western blot analysis of LC3-I and LC3-II expression on TF-1 cells cultured with IL-3 and with or without the addition of 3-MA for 1 or 2 days. β-Actin is used as loading control. Data represents 2 independently performed experiments. (e) Quantification of cell viability at day 4 of expanding TF-1 cells cultured with or without the presence of IFN-γ, Nec-1 or Q-VD. Data represents 2 independently performed experiments. (f) Quantification in time of cell viability of expanding TF-1 cells cultured with or without the presence of IFN-γ and NaC, using H₂O₂ as a control. Data represents 2 independently performed experiments.
IFN-γ induces a Noxa-dependent alternative pathway of cell death in erythroid precursors.

Supplementary Figure 2. Noxa and Mcl-1 turnover upon IFN-γ stimulation
(a) Western blot analysis of Noxa and Mcl-1 breakdown and synthesis in TF-1 cells cultured for 1 day with IL-3 with or without the addition of IFN-γ, at the start of the experiment and 1, 2 and 6 hours after addition of CHX and MG132 respectively. β-Actin is used as loading control. (b) Quantification of Noxa and Mcl-1 protein breakdown in TF-1 cells cultured for 1 day with IL-3 and with or without addition of IFN-γ, at the start of the experiment and 1, 2 and 6 hours after addition of CHX. Protein expression at the 0 hour time-point are taken as start value for the medium and IFN-γ stimulated condition. (c) Quantification of Noxa and Mcl-1 protein synthesis in TF-1 cells cultured for 1 day with IL-3 and with or without addition of IFN-γ, 1, 2 and 6 hours after addition of MG132. Protein expression at the 0 hour time-point are taken as start value for the medium and IFN-γ stimulated condition. (d) Western blot analysis of Noxa and Mcl-1 breakdown and synthesis in TF-1 cells cultured for 3 days with IL-3 with or without addition of IFN-γ, at the start of the experiment and 1, 2 and 6 hours after addition of CHX and MG132 respectively. β-Actin is used as loading control. (e) Quantification of Noxa and Mcl-1 protein breakdown in TF-1 cells cultured for 1 day with IL-3 and with or without addition of IFN-γ, 1, 2 and 6 hours after addition of CHX. Protein expression at the 0 hour time-point are taken as start value for the medium and IFN-γ stimulated condition. (f) Quantification of Noxa and Mcl-1 protein synthesis in TF-1 cells cultured for 1 day with IL-3 and with or without addition of IFN-γ, 1, 2 and 6 hours after addition of MG132. Protein expression at the 0 hour time-point are taken as start value for the medium and IFN-γ stimulated condition.
Supplementary Figure 3. Erythropoiesis in Noxa-/- mice is equally affected by IFN-γ
(a-b) Quantification of the cellularity of the bone marrow (a) and splenic (b) erythropoietic compartment from WT and Noxa-/- mice using flow cytometry. Ery I, II, III, IV refer to the respective erythroblast populations as defined by Socolovsky et al\textsuperscript{87}. Data are expressed in bar graphs as mean +/- SD, including 3 mice per group. Bone marrow samples reflect cellularity of 2 femurs and 2 tibiae. (c-d) Effect of IFN-γ on the in vitro CFU-e (c) and BFU-e (d) colony forming capacity of unfractioned spleen from WT and Noxa-/- animals. (c-d) Data are expressed in bar graphs as mean ± SD for 3 mice per group. * indicates a significant difference (P<0.05) between medium and IFN-γ-treated samples using a one-way ANOVA with Bonferroni correction.
IFN-γ induces a Noxa-dependent alternative pathway of cell death in erythroid precursors
Differential effects of IFN-γ on erythropoiesis in bone marrow versus spleen

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Abstract

Inflammatory diseases are often associated with anemia. Although the etiology is multifactorial, pro-inflammatory cytokines are described to be involved in the onset and persistence of anemia of chronic diseases. We and others have shown that IFN-γ impairs erythropoiesis in the bone marrow, but whether IFN-γ also affects the anemia-induced, compensatory response of stress erythropoiesis is still unknown. Despite the expression of IFN-γR1 and their ability to respond to IFN-γ, we here demonstrate that the erythroid output of murine splenic erythroid precursors is not affected by this pro-inflammatory cytokine. As splenic erythropoiesis remains unaffected during both stress- and steady-state conditions, the effect of IFN-γ seems organ-specific rather than stress-specific. We show that glucocorticoids, that are required for the induction of stress-erythropoiesis, can overcome the negative effects of IFN-γ observed on bone marrow erythropoiesis. In contrast, hedgehog and BMP-4 signaling, that induce the stress fate of erythroid precursors migrated to the spleen after hypoxic stress, cannot overcome IFN-γ effects. These results not only give important leads for the treatment of anemia of chronic disease by means of glucocorticoid supplementation, they also indicate that there is a critical role for the erythropoietic niche during steady-state and stress-erythropoiesis regarding the sensitivity to stimulatory and inhibitory signals.
**Introduction**

Erythropoiesis is characterized as a multi-step process involving differentiation of hematopoietic stem cells through several lineage-committed progenitor stages to erythroid precursors that subsequently proliferate and differentiate towards terminally differentiated erythrocytes\(^1\). Steady-state erythropoiesis occurs primarily in the bone marrow\(^3\), while extramedullary stress-erythropoiesis is induced during hypoxic stress conditions such as acute anemia due to blood loss to augment the output of erythrocytes\(^3;4\). The mechanisms that regulate induction of stress-erythropoiesis are distinct from those involved in steady-state erythropoiesis\(^4;5\). While steady-state erythropoiesis in the bone marrow requires erythropoietin (EPO)- and stem cell factor (SCF)-signaling to generate erythroid output, stress-erythropoiesis in mice is mainly found in the spleen and additionally requires signaling by hedgehog, bone morphogenic protein 4 (BMP4) and glucocorticoids\(^6;10\). Transplantation experiments showed that bone marrow short-term reconstituting hematopoietic stem cells (STR-HSC) are capable of migrating to the spleen to replenish the pool of mobilized stress-erythroid progenitors after stress conditions\(^1\). Hedgehog signaling is required to adapt these cells to a stress-fate and allow them to respond to BMP4\(^8\). Hypoxia in combination with BMP4-, SCF- and glucocorticoid-signaling initiates rapid expansion of the stress-erythroid progenitor pool\(^7;12;13\), while accompanying EPO-signaling promotes terminal differentiation towards erythrocytes\(^1\).

Inflammation can have profound effects on erythropoiesis as patients suffering from diseases involving chronic immune activation often develop anemia\(^14\). Although it is still debated which processes are involved in the development of anemia of chronic diseases (ACD), pro-inflammatory cytokines are found to play an important role in its etiology\(^14;15\). Particularly IFN-γ has a direct suppressive effect on erythropoiesis both in vitro and in vivo\(^16-19\). Using a mouse model in which mice develop an IFN-γ dependent anemia due to chronic immune activation, we showed that IFN-γ not only enhances breakdown of erythrocytes from the blood, but also blocks erythropoiesis in the bone marrow through upregulation of the myeloid transcription factor PU.1. In response to the progressive anemia, mice displayed extensive stress-erythropoiesis in the spleen and concomitant splenomegaly\(^19\).

As stress-erythropoiesis is induced during chronic inflammatory conditions, this raises the question whether stress-erythroid precursors in the spleen are equally sensitive to inhibitory signals provided by the immune system as their steady-state counterparts in the bone marrow. Here we demonstrate that, in contrast to the burst forming unit-erythroid (BFU-e) capacity of the bone marrow, splenic BFU-e capacity in stress, as well as in during steady-state conditions, is not affected by IFN-γ. Unresponsiveness of stress-erythroid precursors could not be explained by the absence of the IFN-γ receptor or hampered IFN-γ receptor signaling. We show that glucocorticoid signaling, but not Hedgehog or BMP4 signaling plays an important role in overcoming the detrimental effects of IFN-γ on steady-state bone marrow erythropoiesis. These data imply that the environment in which erythroid precursors reside is thus critical for their responsiveness to stimulatory and inhibitory signals.
Material and Methods

Mice
For experiments wild-type (WT) C57BL/6J mice were used which were maintained in the animal facilities of the Academic Medical Center (University of Amsterdam, Amsterdam, The Netherlands) in specific pathogen-free conditions. Mice were given standard chow and acidified drinking water ad libitum. Acute anemia in mice was induced by a single injection of 60 mg/kg phenylhydrazine (Sigma-Aldrich) intra-peritoneally. All procedures involving mice were approved by the Experimental Animal Committee of the Academic Medical Center, according to institutional and national guidelines.

Peripheral Blood Analysis
Determining RBC counts, hemoglobin content, red cell distribution width, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and mean corpuscular volume of heparinized full blood was performed using an automated hemocytometer (Coulter AcT Diff 2, Beckman Coulter). Hematocrit was determined by taking up heparinized full blood in capillaries, sealing them with Critoseal (Krackeler Scientific) and subsequent spinning down at 10000 RPM for 5 minutes in an Eppendorf centrifuge, after which the volume percentage of RBCs was determined.

Cell Line and Cultures
The erythroleukemic cell line TF-1 was maintained at 37°C and 5% CO₂ in RPMI 1640 supplemented with 10% fetal calf serum, 100 μg/ml gentamycin (Invitrogen), 2 mM L-Glutamin (Invitrogen), 0.5 mM β-mercapto-ethanol (Sigma-Aldrich) and 0.1 ng/ml human recombinant IL-3 (R&D Systems). For differentiation IL-3 was replaced by 1 U/ml EPO and to mimic stress-conditions 10⁻⁶ M dexamethasone (Sigma-Aldrich) was added to the medium.

Flow Cytometry
Single cell suspensions for flow cytometric analysis were obtained by mincing spleens directly through 40 μm cell strainers, while single cell suspensions of bone marrow were collected by first crushing femurs and tibiae using a mortar and pestle and subsequent filtering through 40 μm cell strainers. Where appropriate, cells were incubated with anti-CD16/CD32 blocking antibodies (2.4G2; a kind gift of Louis Boon, Bioceros). Monoclonal mouse-antibodies used were: C-kit-allophycocyanin (APC; 2B8), CD71-phycoerythrin (PE; R17217), Ter119-PE-Cy5.5 (Ly-76), IFN-γR1-PE (2E2). For identification of megakaryocyte-erythroid precursors (MEPs) in mice, cells were first incubated with a lineage directed biotin-conjugated antibody cocktail containing antibodies against CD4 (GK1.5), CD8α (53-6.7), B220 (RA3-6B2), CD11b (M1/70), Gr1 (RB6-8C5) and Ter119 (Ly-76), then washed and subsequently incubated with streptavidin-PE, CD34-fluorescein isothiocyanate (RAM34), CD127-peridinin chlorophyllprotein-Cy5.5 (A7R34), CD16/32-PE-Cy7 (93) and C-kit-APC (2B8). For cell sorting of CD71⁺ cells, total bone marrow or spleen cells were incubated with CD71-PE, washed and subsequently sorted using a FACSAria (BD Biosciences). Sorted cells were subsequently cultured overnight in X-vivo medium (Lonza) with or without the addition of recombinant murine IFN-γ (Peprotech), 10⁻⁶ M dexamethasone or recombinant human EPO (Aranesp, Amgen). Reticulocyte analysis was done by washing 3-5 μL heparinized full blood in FACS-buffer and subsequent incubation...
with CD71-PE. Cells were washed, taken up in FACS-buffer and directly before measuring Thiazole Orange (Sigma-Aldrich) was added to a final concentration of 1 ng/ml. Monoclonal human-antibodies used were: CD34-PE-Cy5 (BD; 581), CD71-PE (Miltenyi; AC102), CD235a-FITC (Dako;JC159). Dead/Live discrimination was achieved by addition of 20 nM TO-PRO-3 Iodide (Invitrogen) to cells directly before measuring. To determine phosphorylation of STAT1, cells were starved for 4 hours and where appropriate 10^{-6} M dexamethasone or 100 ng/ml human recombinant IFN-γ (PeliKine, Sanquin Blood Supplies) was added. Cells were then stimulated with 0.1 ng/ml IL-3 or 100 ng/ml IFN-γ, fixed with Fix/Perm solution (BD Biosciences) for 10 minutes at 37°C, chilled on ice, washed with FACS-buffer and fixed in 90% methanol for 30 minutes on ice. Subsequently, Permeabilization buffer (BD Biosciences) was added and cells were incubated with anti-pSTAT1 (pY701) Alexa Fluor 488 (BD Biosciences) for 60 minutes at RT. Cells were then washed and acquired. Data acquisition was done using FACS Calibur, FACS Canto I or FACS Canto II (all BD Biosciences). Data were analyzed using Flowjo Version 9.2 for Mac OS X or Version 7.6.2 for Windows (TreeStar).

**Quantitative PCR**

Total RNA was isolated using Trizol reagent (Invitrogen) and reverse-transcribed to cDNA using random hexamer primers (Invitrogen) and RevertAID H Minus Reverse Transcriptase (Fermentas). Quantitative PCR was performed using the StepOne-Plus Real Time PCR system (Invitrogen) using Express SYBR GreenER (Invitrogen).

Primer sequences used for mouse samples are:

- **18s fwd:** CGGCTACCACATCAGGAAGGA
- **18s rev:** GCTGGAATTACCGCGGCT
- **irf-1 fwd:** CAGAGGAAAGAGAGAAAGTCC
- **irf-1 rev:** CACACGTTGACAGTGCTGG
- **GR fwd:** TGCAGGAGTCTCAACAAGACA
- **GR rev:** CATGTTGACGCTGTCATAA
- **Fkbp5 fwd:** TCGACAAAGCCCTGGGTGAA
- **Fkbp5 rev:** AAAACCATAAGGTGTTCAA
- **Dusp1 fwd:** GTGCTGACAGTGCAAGAAAT
- **Dusp1 rev:** CCACAGTACAGGAAGGACAGG

Primer sequences used for human samples are:

- **18S fwd:** GGACAACAAGCTCCGTGAAGA
- **18S rev:** CAGAAGTGACGCAGCCCTC
- **IRF-1 fwd:** CAAAGGAGCAGCAGCCCTC
- **IRF-1 rev:** CCAAGCAGGTGAAATGTGAAGG
- **IRF-3 fwd:** AAAGCAGGGAGGACGGGATGA
- **IRF-3 rev:** AGAGGTAGGAGGTGCGAGTTCAGGTC
- **SOCS1 fwd:** TTTGAGGAGGCGGATGGGTGAG
- **SOCS1 rev:** ATCTTCTGAGGCGGCAGTAC
- **SOCS3 fwd:** ACTGAGGTCTGGACGCTGAG
- **SOCS3 rev:** ACTGAGGTCTGGACGCTGAG

**Semisolid Colony Assays**

Assays for determining burst-forming unit-erythroid (BFU-e) and colony-forming unit-erythroid (CFU-e) were performed as described earlier. Briefly, 3.75 x 10^5 total bone marrow...
or 7.5 x 10⁵ total spleen cells were taken up in methylcellulose medium (Methocult M 3234; StemCell Technologies) supplemented with 4 U/ml human recombinant EPO, 100 ng/mL recombinant murine stem cell factor (Peprotech), 20 μg/mL human holo-transferrin (SCIPAC, T101-5), 2 x 10⁻⁴ M hemin (Sigma-Aldrich) and 1% gentamycin (Invitrogen) and plated in triplo. CFU-e colonies were scored at day 3, BFU-e colonies at day 8. When appropriate, 100 ng/mL recombinant murine IFN-γ (PeproTech) or 10⁻⁸ M dexamethasone was added to the medium.

**Statistical Analysis**

Results are expressed as mean ± SD. Statistics are performed with Graphpad Prism 5, using either a paired or unpaired 2-tailed Student t-test when comparing 2 groups or a repeated measures ANOVA with Bonferroni correction when comparing different treatments on similar samples. P<0.05 was considered statistically significant.

**Results**

**Steady-state and stress-erythropoiesis in the spleen is unaffected by IFN-γ**

Stress-erythropoiesis in mice was induced with a single intra-peritoneal injection of 60 mg/kg of phenylhydrazine (PHZ), which resulted in an acute hemolytic anemia with a significant decline of RBCs and a reduced hematocrit 36 hours after injection (Table 1). This was accompanied by an increase of reticulocyte numbers in the blood, indicative for the induction of stress-erythropoiesis (Table 1). Classification of erythroid precursor cells, based on differential expression of CD71 and Ter-119, indicated a reduced number of orthochromatophilic erythroblasts (Ery IV) in both spleen and bone marrow of PHZ-treated mice 36 hours after injection (Fig. 1a-d). The EPO-dependent polychromatic erythroblasts (Ery III) were increased in bone marrow (Fig. 1c-d), whereas all stages of erythropoiesis (pro-, basophilic- and polychromatic erythroblasts) were highly upregulated in spleen (Fig. 1a-b). This indicates that erythropoiesis is enhanced in bone marrow and stress-erythropoiesis is induced in the spleen.

To examine whether IFN-γ impairs erythropoiesis to the same extent in spleen as it does in the bone marrow, under both steady-state and stress conditions, total bone marrow and spleen samples from control and PHZ-treated mice were subjected to erythroid colony forming assays in the presence or absence of IFN-γ. Compared to steady-state conditions, PHZ-injection significantly increased the erythroid output both the colony forming units-erythroid (CFU-e) and burst forming units-erythroid (BFU-e) numbers in the spleen, but not in bone marrow (Fig. 1e-f). Erythroid precursors in the spleen however were insensitive to the inhibitory effects of IFN-γ as both the CFU-e and BFU-e colony formation from PHZ-treated spleens are unaffected by IFN-γ (Fig. 1e). This is in sharp contrast with bone marrow, where IFN-γ impaired BFU-e and to a certain extent also CFU-e outgrowth (Fig. 1f). Interestingly, IFN-γ decreased erythroid colony outgrowth from bone marrow independently of PHZ injection, whereas outgrowth of CFU-E and BFU-E colonies from spleen was always insensitive to IFN-γ. This suggests that the effect of IFN-γ on erythropoiesis is not stress-dependent, but rather organ-specific.
Figure 1. Steady-state and stress-erythropoiesis in the spleen is unaffected by IFN-γ.

Flow cytometric analysis of the erythropoietic compartment of spleen and bone marrow 36 hours after a single i.p. injection with 60 mg/kg phenylhydrazine. Representative dot plots and bar graphs of the splenic (a-b) and bone marrow (c-d) erythropoietic compartment. Ery I, II, III, IV refer to the respective erythroblast populations as defined by Socolovsky et al.20 (e) Effect of IFN-γ on the in vitro CFU-e and BFU-e colony forming capacity of unfractioned spleen from control and PHZ-treated animals. (f) Effect of IFN-γ on the in vitro CFU-e and BFU-e colony forming capacity of unfractioned bone marrow from control and PHZ-treated animals. (a-d) Bone marrow samples reflect cellularity of 2 femurs and 2 tibiae. Results are representative for 3 independently performed experiments containing 3 or 5 mice per experimental group. * indicates a significant difference (P<0.05) between control and PHZ-treated animals using a unpaired 2-tailed Student t-test. (e-f) Data are expressed as mean ± SD for 3 mice per group. Results are representative for 3 independently performed experiments. * indicates a significant difference (P<0.05) between medium and IFN-γ-treated samples using a paired 2-tailed Student t-test.

Splenic erythroid precursors are capable of responding to IFN-γ
To test whether the non-responsiveness of splenic erythroid precursors to IFN-γ could be due to the absence of the IFN-γ receptor (IFN-γR) on the cell membrane, expression of IFN-γR1 was checked on splenic and bone marrow erythroid precursors of control and PHZ-treated
mice. BFU-e are enriched in the megakaryocyte erythroid precursor (MEP) population that were isolated as Lin- C-kit+ Sca-1- CD34 FcyRlll/IIIlo cells, while CFU-e are enriched in the C-kit+ CD71+ erythroblast population. IFN-γR1 expression was found on MEPs isolated from both spleen and bone marrow, although expression is slightly downregulated during hypoxic stress-conditions (Fig. 2a-b). IFN-γR1 is also found expressed on C-kit+ CD71+ erythroblast, however downregulation of IFN-γR1 expression in PHZ-treated animals was only found in the spleen (Fig. 2c-d). In contrast to these early erythroid progenitor populations, we hardly detected any expression of IFN-γR1 on CD71+ erythroblasts isolated from bone marrow or spleen (data not shown). We previously showed that expression of the transcription factor IRF-1 was induced by IFN-γ in both MEPs and CD71+ cells isolated from bone marrow. Therefore, we tested whether the IFN-γR on splenic erythroid precursors of control and PHZ-treated mice can be induced to signal. Due to the low numbers of erythroid precursors in the spleen during steady-state conditions we isolated total splenic and bone marrow CD71+ cells, cultured them overnight with or without IFN-γ and analyzed IRF-1 expression. IRF-1 expression was similarly upregulated in BM and splenic CD71+ cells from both control and stress-induced animals (Fig. 2c-d). These data demonstrate that spleen and bone marrow derived erythroid precursors respond equally well to IFN-γ, although the effect of IFN-γ on colony forming capacity by BFU-E is different. This suggests that spleen-specific factors or signals may be able to overcome or block the effects of IFN-γ on splenic erythroid precursors.

**BMP4 and Hedgehog cannot overcome the IFN-γ induced block of BM erythropoiesis**

Stress-erythropoiesis in the spleen is controlled by hedgehog and BMP-4 signaling. To test whether hedgehog or BMP4 signaling could overcome the IFN-γ induced block of erythropoiesis in bone marrow, total bone marrow samples were subjected to semi-solid colony assays supplemented with either BMP4 and Sonic hedgehog (Shh) alone, or with the combination of the two factors. Similar to previous results, BFU-e outgrowth is severely diminished by IFN-γ in control cultures, while CFU-numbers remain unaffected (Fig. 3a-b). Addition of BMP4, Shh or the combination did not affect BFU-e or CFU-e outgrowth either in presence or absence of IFN-γ. Pre-incubating total bone marrow cells with Shh or BMP4

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**Table I. Development of hemolytic anemia in PHZ-treated mice 36 hours after injection.**

<table>
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<th>Control</th>
<th>PHZ-treated</th>
<th>*</th>
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<td>Reticulocytes (%)</td>
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<td>MCH (fmol)</td>
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<td>1.68 (0.05)</td>
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</tr>
<tr>
<td>MCV (fL)</td>
<td>56.09 (2.32)</td>
<td>52.59 (2.15)</td>
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<tr>
<td>MCHC (mmol/L)</td>
<td>18.37 (0.70)</td>
<td>32.19 (1.43)</td>
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Hemocytometric analysis of control and PHZ-treated mice. RBC, red blood cell counts; Hgb, hemoglobin; Hct, hematocrit; RDW, red cell distribution width; MCH, mean corpuscular hemoglobin; MCV, mean cell volume; MCHC, mean corpuscular hemoglobin concentration. Data are presented as mean ± SD using 5 mice per group. Data are representative for 2 independently performed experiments. * indicates a significant difference (p<0.05) between control mice and PHZ-treated mice, using a non-paired 2-tailed Student t-test.
Figure 2. Splenic erythroid precursors are capable of responding to IFN-γ.

(a-b) Flow cytometric analysis of IFN-γR1 expression on MEPs from bone marrow and spleen from control mice and mice 36 hours after injection of PHZ. (c-d) Flow cytometric analysis of IFN-γR1 expression on C-kit+ CD71+ cells from bone marrow and spleen from control mice and mice 36 hours after injection of PHZ. (a-d) Overlays are representative for 3 different mice. Data in bar graph are expressed as mean ± SD for 3 mice per group; the horizontal cut-off line represents background fluorescence based on an all minus one staining. * indicates a significant difference (P<0.05) between control and PHZ-treated animals using a unpaired 2-tailed Student t-test. (e-f) Quantitative PCR analysis of IRF-1 mRNA expression on FACS-sorted CD71+ cells from spleen (c) and bone marrow (d) cultured overnight with or without IFN-γ. Data are expressed as mean ± SD for 3 mice per group and results are representative for 3 independently performed experiments. * indicates a significant difference (P<0.05) between medium and IFN-γ-treated samples using a paired 2-tailed Student t-test.
before plating erythroid colony assays also did not affect the outgrowth of colonies or overcome the block in erythropoiesis induced by IFN-γ (data not shown). Taken together, these data thus show that BMP4 and Hedgehog signaling do not affect the erythroid output from the bone marrow and cannot overcome the IFN-γ induced block in bone marrow erythropoiesis.

Glucocorticoids can overcome the negative effects of IFN-γ on erythroid precursors
Besides the need for the growth factors SCF, EPO and BMP4, expansion of the erythroid compartment requires glucocorticoid signaling\(^9\). Because glucocorticoid signaling is dispensable for steady-state erythropoiesis, a differential sensitivity between spleen and bone marrow derived erythroblasts may explain the difference in sensitivity to IFN-γ. We first checked to what extent bone marrow and spleen erythropoiesis differ in glucocorticoid sensitivity by measuring glucocorticoid receptor (GR) expression and its downstream target genes on sorted CD71\(^+\) cells from steady-state bone marrow and spleen. Compared to the expression of 18S rRNA, mRNA expression of the GR and its downstream targets Fkbp5 and Dusp1 are significantly lower in spleen compared to bone marrow (Fig. 4a), indicating that bone marrow erythropoiesis is potentially more sensitive to glucocorticoid signaling. To determine whether IFN-γ signaling interferes with glucocorticoid signaling and vice versa, total bone marrow from WT mice was cultured in colony assays with or without IFN-γ and the glucocorticoid dexamethasone (Dex). In contrast to spleen, where addition of Dex to cultures significantly elevated the output of CFU-e and BFU-e (Fig. 4d–e), addition of Dex to bone marrow cultures did not raise the erythropoietic output (Fig. 4b–c). However, Dex partially relieved the IFN-γ induced block of BFU-e colony formation (Fig. 4c), suggesting that glucocorticoid signaling can render erythroid precursors insensitive to IFN-γ in both spleen and bone marrow.

**Figure 3. Hedgehog and BMP4 signaling cannot overcome the IFN-γ induced block of bone marrow erythropoiesis.**
(a–b) Effect of IFN-γ on the *in vitro* CFU-e (a) and BFU-e (b) colony forming capacity of unfractioned WT bone marrow samples cultured in the presence of BMP4, Shh or the combination of BMP4 and Shh. Data are expressed as mean ± SD for 3 mice per group. * indicates a significant difference (P < 0.05) between control and IFN-γ-treated samples using a repeated measures ANOVA with Bonferroni correction. ns indicates not significant using a paired 2-tailed Student t-test.
Figure 4. Glucocorticoids can overcome the IFN-γ induced block of bone marrow erythropoiesis.

(a) Quantitative PCR analysis of GR, Fkbp5 and Dusp1 mRNA expression on FACS-sorted CD71+ cells from spleen and bone marrow. Data are expressed as mean ± SD for 3 mice per group. * indicates a significant difference (P<0.05) between BM and spleen using a 2-tailed Students t-test. (b–c) Effect of IFN-γ on the in vitro CFU-e (b) and BFU-e (c) colony forming capacity of unfractioned WT bone marrow samples cultured in the presence of Dexamethasone. (d–e) Effect of IFN-γ on the in vitro CFU-e (d) and BFU-e (e) colony forming capacity of unfractioned WT spleen samples cultured in the presence of Dexamethasone. (b–e) Data are expressed as mean ± SD for 3 mice per group. * indicates a significant difference (P<0.05) between control and IFN-γ-treated samples using a repeated measures ANOVA with Bonferroni correction. ns indicates not significant.
Glucocorticoids can overcome the negative effects of IFN-γ by a yet unidentified mechanism

To investigate the underlying mechanism by which glucocorticoids can overcome the IFN-γ induced block in erythropoiesis, we made use of the pre-myeloid cell line TF-1. This cell line, isolated from a patient with erythroleukemia, can be grown with IL-3 and differentiated towards the erythroid lineage in presence of EPO. When cultured with IL-3, cell numbers expanded and cells remained viable, while proliferation was blocked in the presence of IL-3 plus IFN-γ (Fig. 5a-c). Furthermore, TO-PRO-3 staining revealed induction of cell death after 3 to 4 days of culture in the presence of IFN-γ (Fig. 5a,c). Addition of Dex to these cultures inhibited proliferation of cells, but largely prevented the induction of cell death by IFN-γ (Fig. 5a-c). Taken together, these data show that glucocorticoids can alleviate the inhibiting effects of IFN-γ on erythropoiesis.

To gain further insight into how glucocorticoids can account for the discrepancy between signaling of IFN-γ and sensitivity to IFN-γ of erythroid precursors we determined whether glucocorticoids interfere with downstream signaling of IFN-γ. Upon binding its receptor, IFN-γ induces phosphorylation of STAT1, which subsequently transduces signals to the nucleus. Because prolonged exposure to Dex has been described to block IFN-γ induced phosphorylation of STAT1 in PBMCs, we cultured TF-1 cells with or without the presence of Dex. Cells were then deprived of IL-3 in absence or presence of Dex, stimulated with IFN-γ and STAT1 phosphorylation was analyzed. Phosphorylation of STAT1 showed a biphasic response with a peak at 10 minutes, followed by a rapid decline and subsequent steady phosphorylation (Fig. 5d). TF-1 cells pre-cultured with Dex followed the same kinetics of STAT1 phosphorylation, but overall phosphorylation following IFN-γ stimulation was lower (Fig. 5d). Furthermore, prolonged exposure to IFN-γ in the presence of IL-3 and Dex showed decreased mRNA expression of IRF-1 at day 2 and 3 of culture (Fig. 5e). Although this suggests that Dex may impair IFN-γ signaling, it had no consequences for induction of downstream IFN-γ-inducible negative regulators of cytokine signaling genes such as the suppressor of cytokine signaling 1 (SOCS1) and SOCS3 (Fig. 5f-g). SOCS1 expression peaked after 1 day of culture and then decreased in time, irrespective of the presence of Dex (Fig. 5f). SOCS3 mRNA expression was gradually induced after prolonged IFN-γ stimulation, but was not altered by addition of Dex (Fig. 5g). Taken together these data show that also in an in vitro model system, glucocorticoids can overcome the negative effects of IFN-γ on erythropoiesis. This is, however, not by altering the expression of downstream negative regulators of cytokine signaling SOCS1 and SOCS3, but through a still unidentified mechanism.

Discussion

Complementary to having important pro-inflammatory and immune-modulatory functions during intracellular bacterial and viral infections, IFN-γ has profound effects on hematopoiesis in general, and erythropoiesis in particular. IFN-γ severely inhibits the erythroid colony formation from bone marrow in vitro and causes a compensated anemia in mice, characterized by impaired bone marrow erythropoiesis combined with enhanced erythropoiesis in the spleen (stress-erythropoiesis). Whereas bone marrow erythropoiesis is severely affected by IFN-γ, we here show that erythropoiesis in the spleen is not functionally affected by IFN-γ during both steady-state and stress conditions (Fig. 1e-f).
Figure 5. Glucocorticoids can overcome the negative effects of IFN-γ by a yet unidentified mechanism. 
(a) Representative dot plots of flow cytometric analysis of TF-1 cells cultured for four days in the presence of IL-3, Dex and IFN-γ. (b) Growth curve of TF-1 cells cultured in the presence of IL-3, Dex and IFN-γ, expressed as absolute numbers per ml of culture medium. (c) Cell viability of TF-1 cells cultured in the presence of IL-3, Dex and IFN-γ in time. Data is expressed as the percentage of live cells compared to their respective control culture. (d) Flow cytometric analysis of STAT1 phosphorylation in TF-1 cells. (e-g) Quantitative PCR analysis of IRF-1 (e), SOCS1 (f) and SOCS3 (g) mRNA expression of TF-1 cells cultured in the presence of IFN-γ and Dex. (a-c, e-g) Results are representative for two independently performed experiments. (d) Experiment performed once.
Although splenic erythroid precursors express IFN-γR and are able to transduce signals upon ligand binding (Fig. 2), they are not sensitive to the inhibitory effects of IFN-γ (Fig. 1e) or IFN-γ induced cell death (Fig 5a). Our data are in accordance with those of Millot et al., who used a zymosan-induced general inflammation (ZIGI) mouse model where mice developed a long lasting anemia with high serum levels of IFN-γ. In this model bone marrow BFU-e's were inhibited by IFN-γ, while the erythroid output from the spleen was unaffected. In this study however we uncoupled the effect of IFN-γ from chronic inflammation and uncovered that the insensitivity to IFN-γ is not due to BMP4 and hedgehog signaling (Fig 3), which is important for inducing a stress-erythroid fate within the spleen, but might be due to the stimulatory and protective effects of glucocorticoids (Fig. 4b-e, Fig. 5a).

The role of glucocorticoids in erythropoiesis has long been debated as addition of glucocorticoids to colony assays with BM-derived hematopoietic cells increased, decreased or did not affect erythroid potential. However, glucocorticoids appeared to be required for long term in vitro expansion of erythroblasts and for stress-erythropoiesis in the mouse spleen following hypoxia induction. Our data are in accord with both observations. Dexamethasone did not affect BFU-e numbers from BM, but specifically increased BFU-e derived from spleen (Fig. 4c,e). Strikingly however, we found that expression of the GR and downstream target genes is significantly lower in splenic erythroid precursors compared to bone marrow (Fig. 4a). This may suggest that splenic erythroid cells are less sensitive to glucocorticoids and depend more on an increased dose of glucocorticoids. When added to colony assays from in vitro expanded erythroblasts, dexamethasone increases the number of BFU-e and thus increases the renewal capacity of erythroblasts. It is also possible that the spleen erythroblasts are at a developmental stage between BFU-e and CFU-e. Addition of dexamethasone to cells at this stage shifts their colony forming capacity to BFU-e, whereas BFU-e from BM may be derived from a more immature fraction that will form BFU-e size colonies independent of the presence of dexamethasone. In any case, it would be conducive to further determine possible differences between developmental pathways of erythroid progenitors, erythroid precursors and maturing erythroid-committed cells within bone marrow and spleen. It is unclear which cells exactly give rise to erythroid offspring in colony assays, as these could be both HSCs, multipotent progenitors (MPPs), CMPs, MEPs and later stage erythroid precursors. Theoretically, it could even be that IFN-γ and glucocorticoids act on different progenitor cells and that this could reflect the observed differences in erythropoiesis in bone marrow vs spleen. Differences in environmental factors may also be important in this respect and may account for the inter-experimental variations observed for the IFN-γ responsiveness of bone marrow CFU-e's (Fig. 1f vs Fig. 3a, 4b). Inhibition of CFU-e formation by IFN-γ can in fact be overcome by increasing the EPO-levels in the assay, indicating that a rather subtle balance exists in this system between pro- and anti-erythroid differentiation cues. More indepth experiments are required to elucidate the underlying molecular mechanism.

A subsequent question is whether it is glucocorticoid signaling that renders splenic erythropoiesis insensitive to IFN-γ. We did find that inhibition of glucocorticoid receptor signaling by addition of mifepristone to erythroid colony assays did not affect the insensitivity of splenic erythroid precursors to IFN-γ (data not shown). The cooperative signaling of glucocorticoids with EPO- and SCF-signaling enhances the expansion of erythroid progenitors. Possibly its effects are indirect as activation of the GR upregulates...
Differential effects of IFN-γ on erythropoiesis in bone marrow versus spleen

expression of the SCF receptor gene cKit and the transcription factor cMyb. On the other hand, enhanced glucocorticoid signaling is known to overcome inhibitory IFN-γ signaling by downregulating STAT-1 expression in PBMCs. Addition of Dex to TF-1 cultures did not enhance the growth of TF-1 cells, but in line with the latter data, downregulated IFN-γ-induced STAT-1 and IRF-1 expression (Fig. 5d-e). This, however, had no consequences for the downstream expression of the IFN-γ-inducible negative regulators of cytokine signaling SOCS1 and SOCS3 (Fig 5f-g). Interestingly, expression of SOCS1 is also an example for the synergistic action of STAT5 and the GR on common target genes. Maybe other targets are however more critically dependent on the level of IFN-γ signaling and STAT1 phosphorylation in erythroid precursor cells. As IFN-γ induces the expression of PU.1 via IRF-1 in erythroid precursors, glucocorticoids might prevent upregulation of PU.1, thereby explaining the protective effects of glucocorticoids on the inhibitory effects on erythropoiesis in general and the IFN-γ induced cell death in particular. To what extent glucocorticoid signaling is involved in the insensitivity of splenic erythroid precursors to IFN-γ is however unknown and what the exact mechanisms by which glucocorticoids can overcome the IFN-γ induced block of bone marrow erythropoiesis remain to be further elucidated.

Our finding that splenic erythropoiesis in both stress- and steady-state conditions is unaffected by IFN-γ, while bone marrow erythropoiesis is (Fig. 1e-f), indicates the presence of organ-specific niches for erythropoiesis. This hypothesis is supported by the fact that following recovery of anemia, bone marrow STR-HSCs migrate to the spleen and adapt to an erythroid stress-fate by hedgehog signaling, thereby making these cells responsive to BMP4. In contrast to earlier studies where Sonic hedgehog (Shh) has been described to induce the stress-fate of bone marrow cells in vitro, in our hands culturing total bone marrow in the presence of Shh alone or in the combination with BMP4 did not alter the output of erythropoietic colonies (Fig 3a-b), arguing that, just as with the effect of glucocorticoids on erythropoiesis, the chosen culture method and the added supplements are of significant importance. However, not Shh, but Desert hedgehog (Dhh) and Indian hedgehog (Ihh) expression is found in the spleen upon recovery of anemia, indicating that rather these members of the hedgehog family have a niche specific role in erythropoiesis. Interestingly, Dhh is co-expressed with BMP4 on non-hematopoietic stromal cell in the spleen during recovery of anemia, while BMP4 expression is furthermore induced on macrophages during stress-erythropoiesis. Macrophages are considered the key-player in the erythropoietic niche as central macrophages in erythroblastic islands are important for proliferation, differentiation and enucleation of the erythroid cells surrounding them. To what extent however the macrophages of the erythroblastic islands in bone marrow and spleen differ from each other in terms of function and cell surface expression of signaling molecules is unknown, but may be the key to the differences found between the spleen and bone marrow erythropoietic niche.

With respect to the physiological rationale behind the impact of IFN-γ on erythropoiesis we postulated earlier that during immune activation IFN-γ shifts hematopoiesis temporarily in favor of myelopoiesis to combat infection, however, during chronic infection this leads to the development of ACD. To avoid exhaustion of the complete erythroid pool by prolonged exposure to IFN-γ, the induction of extramedullary stress-erythropoiesis in mice, which is IFN-γ insensitive, could circumvent this and might serve as an evolutionary conserved
backup system for survival during a prolonged state of immune activation. This is particularly relevant in the spleen where many immune cells can produce IFN-γ. Although it is tempting to speculate on potential therapies for ACD that induce or manipulate stress-erythropoiesis in human, experiments that confirm the presence of stress-erythropoiesis are impossible to conduct. That stress-erythropoiesis might be present in humans and could resemble stress-erythropoiesis in mice comes from the discovery of CD34+ C-Kit+ CD71+ CD235a+ cells in the blood of patients with sickle cell anemia\textsuperscript{42}, but further conclusive data is lacking. To what extent glucocorticoids have an effect on erythropoiesis in humans is largely unknown, but for decades however glucocorticoids have been widely used to treat or dampen inflammatory conditions as they inhibit pro-inflammatory gene expression and induce anti-inflammatory responses. Our data implies that glucocorticoid therapy might also have a dual beneficial effect in patients suffering from ACD as besides its anti-inflammatory effects it could also overcome the IFN-γ induced block in erythropoiesis and induce stress-erythropoiesis.

**Acknowledgements**
We would like to thank Berend Hooibrink (AMC), Erik Mul, Floris van Alphen and Tomasz Poplonski (Sanquin) for cell sorting and the staff of the animal facility of the AMC for excellent animal care.
Reference List


Chapter 7

General Discussion
Introduction
All cell lineages from the hematopoietic system find their origin in one population of common progenitor cells, the hematopoietic stem cells (HSCs). Because of the therapeutic potency of HSCs in the treatment of inherited blood disorders, leukemias, lymphomas, cancers and autoimmune diseases amongst others, significant effort is put in understanding HSC behavior in vivo and optimizing the isolation and culture methods needed for the expansion, differentiation and manipulation of hematopoietic stem and progenitor cells (HSPCs) ex vivo.

Until recently, induction of hematopoietic lineage fate was generally thought to be stochastic and independent of extrinsic factors. However, current research revived Schofield’s HSC niche concept and stresses the importance of the microenvironment in regulating HSC behavior. Schofield described the HSC niche as an anatomical location that affects stem cell number and behavior by inducing self-renewal in proximity of this location or inducing differentiation at a distance. After birth, HSCs are found in two distinct anatomical locations of the bone marrow. One site is located near the endosteal lining of the bone and is termed the endosteal niche, the other is found in proximity of the sinusoidal blood vessels of the bone marrow and is termed the vascular niche. The HSC niche, which comprises both hematopoietic and non-hematopoietic cells, as well as physical and mechanical stimuli, does not only provide anatomical space for HSCs, but also supplies HSCs with signals for their maintenance, quiescence, self-renewal, proliferation and differentiation. Osteoblasts, mesenchymal stem cells (MSCs), endothelial cells, stromal cells, reticular cells, Schwann cells and macrophages have all been implemented into the HSC niche.

By cell-cell interaction, the production of soluble factors, or a combination of both, these cells have been shown to contribute to the maintenance of the HSC pool and keep HSCs quiescent during steady-state hematopoiesis. Loss of these stimuli in most cases results in mobilization, proliferation and loss of HSCs.

Differentiation of multipotent HSCs towards more mature progenitors and eventually fully mature cells of all hematopoietic lineages requires expression of particular transcriptional programs and extrinsic signals which instruct, permit or restrict differentiation. Although the transcriptional programs and stimuli guiding hematopoietic differentiation are generally known, the signals that initiate transcription or the cells that can affect lineage commitment are not fully elucidated yet. They are however most probably provided by the microenvironment in which HSPCs reside. Interestingly, during pathogenesis or cellular stress situations like anemia or embryogenesis, the hematopoietic output needs to be rapidly enhanced or shifted to cope with the body’s needs. Although it was generally thought that adaptation of hematopoiesis during inflammation was induced by systemic responses, more and more evidence is accumulating that activated immune cells can actively influence hematopoiesis by providing feedback to HSPCs via inflammatory signals. As both innate and adaptive immune cells have been found to reside in the bone marrow and can be activated in close proximity of hematopoietic niches, we here discuss how the niche for immune cells may overlap with the hematopoietic niche and how inflammatory signals can alter HSPC function and affect lineage commitment. As lineage commitment shows parallels with the 3-signal model of T cell activation, we in addition provide a related 3-signal model for lineage commitment.
Chapter 7

Immune Cells as Hematopoietic Niche Constituents

Although bone marrow serves as the primary organ for the development of innate immune cells and can act as a primary lymphoid organ by the production of lymphoid precursor cells, full maturation and activation of immune cells largely takes place outside of the bone marrow. Whereas innate immune cells get recruited from the bone marrow by chemokine gradients and are fully activated by cytokines at sites of infection, activation of adaptive immune cells primarily takes place in secondary lymphoid organs like lymph node and spleen. These organs contain highly structured microenvironments for efficient activation, clonal expansion and differentiation of both T and B cells, which is driven by specific recognition of their cognate antigen that is derived from the environment. However, bone marrow does not only serve as the primary site for hematopoiesis, it also contains a wide variety of resident immune cells from the innate and adaptive immune system and can act as a microenvironment where immune cells can be retained during steady-state conditions and can be recruited and activated during immune activation. Interestingly, innate and adaptive immune cells that reside in the bone marrow are generally found in close proximity of HSCs and largely rely on the same retention factors as HSCs, indicating that they might support or influence hematopoiesis and that they share and compete for niche space with HSCs. Furthermore, immune activation in the bone marrow most often takes place in close proximity of HSCs, suggesting that activation of bone marrow immune cells can alter hematopoiesis directly via interaction with hematopoietic progenitors or indirectly by signaling to hematopoietic niche constituents.

Dendritic Cells

By processing pathogens and presenting antigens to naive T cells, dendritic cells (DCs) are specialized antigen presenting cells (APCs) that bridge the gap between innate and adaptive immunity. DCs are mainly found in tissues that have a high antigenic pressure and are in close contact with the external environment. Although the bone marrow has a low antigenic pressure and has no direct contact with the external environment, approximately 1-3% of immune cells in the bone marrow are DCs. The exact origin of bone marrow DCs is unclear, but most likely consists of a mixed population of bone marrow resident DCs that differentiated from common dendritic precursors (CDPs), and a population of DCs that after activation in peripheral tissues migrated to the bone marrow. To what extent resident bone marrow DCs recirculate through the body is unknown, but activation of Toll-like receptor (TLR)-2, -4, and -9 on CDPs causes downregulation of C-X-C chemokine receptor 4 (CXCR4), whereas at the same time C-C chemokine receptor 7 (CCR7) is upregulated, allowing migration of the activated CDPs to lymph nodes. On the other hand, both immature and differentiated DCs in the blood are found to migrate to spleen, liver and lung, but not to lymph nodes. They home preferentially to the bone marrow nevertheless, where their retention is dependent on the expression of vascular cell adhesion molecule-1 (VCAM-1) and P- and E-selectins on the endothelium of bone marrow microvessels. Bone marrow resident DCs have however been found to not directly bind to endothelium, but they cluster in the perivascular space around blood vessels, where also HSCs reside in their vascular niche.

The fact that migrated DCs express CXCR4 and depend on chemokine (C-X-C) motif ligand 12 (CXCL12) signaling for their maintenance and reside in close proximity of HSCs argue that DCs share a common docking cell for their support. CXCL12 is expressed on a variety
General Discussion

of bone marrow cells, including CXCL12-abundant reticular (CAR) cells, which are important for the maintenance of restricted hematopoietic progenitor cells and B-cell progenitors, mesenchymal stem and progenitor cells, perivascular stromal cells and endothelial cells, that harbor HSCs, and osteoblasts that maintain common lymphoid progenitors\(^{36,37}\). As these cells express CXCL12 most abundantly, they are also likely to allow DC retention in the bone marrow. In addition to CXCL12, Fms-like tyrosine kinase 3 ligand (Flt3L)-signaling, a cytokine that positively affects the number of myeloid and lymphoid-primed progenitor cells\(^{38}\) and which receptor, Flt3, is broadly expressed on HSPCs\(^{38,39}\), is also required for development of DCs in the bone marrow and homeostatic division in peripheral tissues\(^{40,41}\). Lack of Flt3-signaling reduces the number of DCs not only in peripheral organs like spleen, lymph nodes and thymus\(^{42}\), but also in bone marrow\(^{40}\). However Flt3-signaling is dispensable for steady state maintenance of HSCs\(^{43}\), the fact that more mature progenitor cells and DCs depend on Flt3 and CXCL12 for their maintenance and function in the bone marrow makes it likely that DCs and hematopoietic progenitor cells can interact and influence each other. This hypothesis is further supported by the observation that DCs are found in the same anatomical locations in the bone marrow as HSCs\(^{40}\), thereby further suggesting interactions between hematopoietic progenitors and DCs.

Bone marrow DCs are very well capable of mounting both primary and secondary immune responses. Circulating antigen-laden DCs have been found to migrate from peripheral tissues to the bone marrow and effectively mount memory T cell responses\(^{28}\). Furthermore, using adoptive transfer techniques of OT-I T cells and subsequent OVA-stimulation, resident bone marrow DCs were found to induce activation, proliferation and differentiation of naive CD4\(^+\) and CD8\(^+\) T cells, independently from secondary lymphoid organs. Whereas in steady-state situations adoptively transferred T cells are scattered through the bone marrow parenchyma, interaction between transferred T cells and resident bone marrow DCs upon antigen recognition lead to the formation of multicellular clusters containing approximately 1-3 DCs and 10-30 T cells. Within these clusters clonal expansion and the development of immunological memory was demonstrated\(^{27,35}\), indicating that immune activation takes place in close proximity of HSCs. In addition, also adoptively transferred mature B cells migrate to the perivascular clusters of bone marrow DCs. DCs support the survival of recirculating B cells in the bone marrow by the production of macrophage migration-inhibitory factor (MIF)\(^{10}\). As ablation of bone marrow DCs results in the loss of mature B cells in the bone marrow\(^{10}\), DCs can thus generate microenvironments favorable for the maintenance of B cells and T cells within the bone marrow. To what extent bone marrow DCs further support hematopoiesis by the production of maintenance factors or cell adhesion molecules remains to be elucidated. The fact that DCs are capable of mounting immune responses in close proximity of HSCs in the perivascular niche and express inflammatory molecules that have been described to modulate hematopoiesis, however strongly suggests that DCs can influence the function of HSCs, either directly or indirectly by affecting the niche cells.

**Macrophages and Monocytes**

As their primary function is to phagocytose cellular debris and pathogens and to a lesser extent to serve as an APC to initiate adaptive immune responses, it was unexpected that bone marrow macrophages play an important role in the maintenance of HSCs in both the
endosteal and the perivascular niche. Perivascular bone marrow macrophages are rare, but are tightly associated with vascular endothelium of the bone marrow\textsuperscript{30}. Depletion of mononuclear phagocytes from the bone marrow using clodronate-laden liposomes leads to reduced CXCL12 levels and concomitant HSPC mobilization from the bone marrow\textsuperscript{13,44}. Using detailed flow cytometric analysis of the mononuclear phagocytic compartment in combination with conditional depletion models, Chow et al. identified CD169\textsuperscript{+} macrophages as cells that induce the expression of HSC retention factors CXCL12, stem cell factor (SCF), Angiopoetin-1 (Ang-1) and VCAM-1 on Nestin\textsuperscript{+} mesenchymal stem cells (MSCs) within the perivascular niche. The exact signals performing the communication between macrophages and Nestin\textsuperscript{+} MSCs are unknown, but tumor necrosis factor (TNF), interleukin -1 (IL-1), IL-10 and insulin-like growth factor-1 (IGF) were excluded\textsuperscript{13}.

Interestingly, granulocyte-colony stimulating factor (G-CSF), which is commonly used in the clinic to mobilize HSPCs from the bone marrow to the blood also specifically depletes a population of endosteal macrophages (osteomacs) that support osteoblast function within the osteoblastic HSC niche\textsuperscript{44}. Endosteal osteoblasts, just like Nestin\textsuperscript{+} MSCs in the perivascular niche, provide HSPCs with niche retention-factors like CXCL12, SCF and Ang-1\textsuperscript{3,4}. Depletion of macrophages from the endosteal niche thus leads to diminished osteoblast numbers and HSC retention factors, ultimately leading to the mobilization of HSPCs into the blood\textsuperscript{44}. As G-CSF can be produced in copious amounts upon inflammation to induce granulopoiesis, it is likely that systemic G-CSF can affect bone marrow mononuclear phagocytes to locally affect the hematopoietic lineage fate of HSCs. Gaining insight in which signals bone marrow macrophages give to their microenvironment in both steady-state conditions as well as upon activation by inflammatory mediators or upon engulfing pathogens could provide valuable leads for how immune cells can directly or indirectly regulate hematopoiesis.

Granulocytes

As described previously, G-CSF is capable of mobilizing HSPCs from the bone marrow. Mobilization of HSPCs is not a direct effect of G-CSF signaling as the G-CSF-receptor on HSPCs is not required for mobilization\textsuperscript{45}. The primary function of G-CSF in the bone marrow is to stimulate survival, proliferation and differentiation of granulocytic precursors and concomitant mobilization of granulocytes to sites of infection.

Granulocytes can be found in copious amounts in the bone marrow and their granules contain a plethora of effector molecules, cytotoxic molecules, antimicrobial proteins and enzymes that are capable of fighting pathogens, but that also can physically alter their direct microenvironment. In fact, the capacity of granulocytes to alter their environment has been found to be indispensable for HSPC mobilization\textsuperscript{46}. In particular proteases have been implicated to play a crucial role in the cytokine-induced mobilization of HSPCs upon G-CSF treatment by generating a highly proteolytic environment in the bone marrow\textsuperscript{47}. Matrix metalloprotease 9 (MMP-9) was found elevated in serum after G-CSF treatment in humans and antibodies directed against MMP9 prevent the mobilization of HSPCs upon IL-8 treatment in rhesus macaques\textsuperscript{38,49}. In mice lacking MMP-9 however, cytokine induced HSPC mobilization was found unaffected upon G-CSF or IL-8 stimulation\textsuperscript{50}. More recently, the importance of MMP-8 in HSPC mobilization became apparent as next to a rise of serum MMP-9, also MMP-8 was found elevated in human serum upon G-CSF treatment\textsuperscript{51}. In vitro studies with MMP-9 and activated MMP-8 showed that these strongly influence the contact
between osteoblasts and HSPCs, most probably by altering cell-cell contact. As activated MMP-8 is capable of specifically degrading the N-terminus of CXCL12, this strongly suggests that activated MMP-8 can mobilize HSPCs by affecting the interaction of osteoblasts and HSPCs in the endosteal niche. These data are strengthened by the fact that G-CSF inhibits osteoblast function by suppressing CXCL12 expression.

MMP-8 needs to be activated before it can exert its proteolytic function. As this is most likely done by other proteases that are released from activated immune cells in the bone marrow, it remains unclear to what extent granulocytes also contribute to HSPC behavior during steady-state conditions. Regarding granulopoiesis in the bone marrow, it is interesting that inflammatory signals like G-CSF, TNF-α and IL-1β significantly affect CXCL12 and SCF levels in the bone marrow. CXCR4/CXCL12 interaction is not only needed to preserve HSPCs in the bone marrow it is also crucial for retaining granulocytes in the bone marrow. Elevated levels of TNF-α and IL-1β induced by immunization were found to decrease CXCL12 and SCF levels to such an extent that lymphopoiesis was impaired, while granulopoiesis was still maintained. Hereby lymphocytes get mobilized to the blood, generating space for the induction of granulopoiesis and arguing for a common microenvironment where lymphocytes and granulocytes compete for survival factors in a common niche. As also HSPCs rely on CXCL12-signaling and just like lymphocytes and granulocytes can get recruited to the blood stream upon inflammatory stimuli, this again argues that immune cells and HSPCs share a common niche where pro-inflammatory signals can directly alter hematopoietic progenitors and their environment.

Besides being the primary site for granulopoiesis and harboring a pool of immature granulocytes, under steady state conditions bone marrow also serves as an important site for clearance of senescent granulocytes. By expressing high levels of CXCR4, senescent granulocytes home back to the bone marrow where they are phagocytosed by bone marrow macrophages. Phagocytosis of senescent granulocytes induces expression of G-CSF, which thus provides a positive feedback loop for the production of new granulocytes. Strikingly, a recent paper showed that neutrophils can act as virus carrier cells that capture viral particles in the dermis and migrate to the bone marrow. Here they interact with resident myeloid APCs that induce the development of a virus-specific CD8+ T cell response and the formation of memory CD8+ T cells. As depletion of macrophages from the bone marrow using clodronate-laden liposomes prevented the development of the T cell response in the bone marrow, macrophages most probably serve a dual role in granulocyte turn-over by both depleting senescent granulocytes and adapting immune responses by the signals delivered from phagocytosed senescent granulocytes. Given the fact that depletion of macrophages from the bone marrow also mobilizes HSPCs, it is likely that macrophages support both HSPCs and granulocytes within the bone marrow via expression of common retention and survival factors.

B cells
Whereas T cell progenitors migrate to the thymus to further develop the T cell pool outside of the bone marrow, B cell progenitors remain in the bone marrow, where they further differentiate. In specific niches for B cell development, osteoblasts, osteoclasts, CAR-cells and IL-7 producing stromal cells play essential roles in the maturation of pre-pro B cells to immature B cells that eventually migrate to the spleen to continue their maturation.
Depletion of osteoblasts and osteoclasts results in the decrease of B cell precursors and the re-localization of B cell progenitors to spleen, respectively. As the depletion of early B cells upon osteoblast ablation shows striking similarity with the depletion of HSCs upon osteoblast ablation, it is feasible that early B cells are in close contact with HSCs and have osteoblasts as mutual niche constituents that provide CXCL12-signaling. Furthermore, and in line with HSPCs, early B cell precursors are associated with CAR-cells, while more mature B cells reside at greater distance of CXCL12-expressing cells and adjoin IL-7 expressing cells. Depletion of CAR-cells leads to diminished numbers of common lymphoid progenitors (CLPs) and early B-cells, further indicating that CXCL12 signaling is essential for B cell lymphopoiesis.

After antigenic stimulation in secondary lymphoid organs, memory B cells can migrate back to the bone marrow and upon proper stimulation mature into antibody-producing plasma cells. Plasma cells make up approximately 0.5% of bone marrow cells and may maintain long-term immunity to specific antigens for more than 300 days. For their development and maintenance they require cytokines like IL-5, IL-6 and TNF-α and cell-cell interactions which involve CXCL12, a proliferation-inducing ligand (APRIL) and B cell activating factor of the TNFR family (BAFF)-signalling. All of these signals are directly provided by the microenvironment in which plasma cells reside.

Plasma cells are indeed found in close proximity of CAR cells that provide CXCL12-signaling and stromal cells that besides providing IL-6 and APRIL-stimulation, harbour plasma cells via VCAM-1/VLA-4 interactions. In fact, plasma cells and HSPCs localize to the same areas due to their mutual responsiveness to CXCL12. Furthermore, megakaryocytes and eosinophils are also thought to be part of the plasma cell niche. Megakaryocytes produce APRIL and IL-6, which supports the survival of plasma cells. Accumulation of plasma cells after immunization is disturbed in mice with impaired megakaryopoiesis due to the lack of survival factors, indicating the importance of megakaryocytes for maintaining humoral memory. Eosinophils also express high levels of APRIL and have been found in close proximity of plasma cell supporting stromal cells. Here they are most likely drawn to by CXCR4/CXCL12 interactions. Mice deficient for eosinophils have reduced APRIL-levels and a concomitant reduction of plasma cells in the bone marrow, thus indicating that eosinophils also play an important role in maintaining plasma cells in the bone marrow.

Megakaryocytes have also been implemented into the hematopoietic niche as they are located adjacent to sinusoids and rely for their expansion on the hormone thrombopoietin (TPO). TPO however is not only involved in megakaryopoiesis and the production of platelets, it also regulates HSC quiescence and has been found to interact with the osteoblastic HSC niche. Since CXCL12 is a strong chemoattractant for megakaryocytes it is plausible that megakaryocytes harbor the same docking cell for their retention as HSCs and are capable of affecting hematopoiesis by providing direct or indirect signals to HSPCs. The fact that plasma cells are directly associated with CAR-cells, are found in close proximity of HSC and rely on the support of megakaryocytes and eosinophils, suggests that all these immune cells mutually influence each other during both steady-state and inflammatory conditions.
T cells
Activation of T cells is commonly believed to take place in secondary lymphoid organs like lymph nodes and spleen upon migration of antigen-laden DCs and presentation of antigenic peptides to cognate T cells. More and more evidence is however accumulating that also the bone marrow acts as an organ that is capable of priming T cells. Approximately 1-5% of nucleated cells in the bone marrow are T cells with a CD4/CD8 ratio of 1:2. This is in sharp contrast to secondary lymphoid organs where the CD4/CD8 ratio is generally 2:1. Bone marrow should be considered an important organ in T cell trafficking, as T cells can home to the bone marrow, reside for longer periods in the bone marrow parenchyma or move back to the blood to migrate to secondary lymphoid organs. Whereas in secondary lymphoid organs the majority of T cells display a naive phenotype, about two-thirds of T cells in the bone marrow display a memory phenotype and have thus previously seen their cognate antigen. Interestingly, using adoptive transfer studies naive, effector and memory T cells were found to migrate to the bone marrow, however memory T cells were found to preferentially accumulate to this organ.

Using intravital confocal imaging, adoptively transferred mature T cells isolated from spleen and lymph node were found to migrate to CXCL12-rich perivascular regions of the bone marrow. Interestingly, also adoptively transferred HSPCs migrated to these same regions as also they rely on CXCR4-signaling for their retention. Two weeks after transfer, adoptively transferred mature T cells were located in the same perivascular regions as adoptively transferred HSPCs, indicating that they share common niches for their retention. Although not specified whether the transferred T cells had a naive, effector or memory phenotype, it is likely that they are memory T cells as those have been found to preferentially migrate to the bone marrow upon adoptive transfer. In addition to the need for CXCR4-signaling to migrate to the bone marrow perivascular regions, both CD4+ and CD8+ T cells rely for homing and retention in the bone marrow on the expression of adhesion molecules (ICAM-1, MadCAM-1, VCAM-1, integrins, selectins). As CAR-cells express high levels of CXCL12 and are known to maintain HSCs in the vascular niche via VCAM-1, CAR-cells are likely candidates that harbor both HSCs and T cells in the bone marrow. Memory CD4+ T cells are found in close proximity of IL-7 secreting stromal cells, which most likely keep them quiescent up until reactivation. As for B-cell development IL-7 and CXCL12-expressing cells are found in distance from each other, this would however suggest that CD4+ T cells locate, at least in part, to niches devoid of CXCL12 signaling or need additional CXCL12 expressing cells in their vicinity for their maintenance. In addition to IL-7 signaling, IL-15 is necessary for the homeostatic proliferation of memory CD8+ T cells and the retention and survival of memory CD4+ T and CD8+ T cells in the bone marrow after infection. Both CD4+ and CD8+ memory T cells have been found in the vicinity of IL-15 producing bone marrow cells. A variety of cells in the bone marrow can express IL-15, including DCs, monocytes, stromal cells, and interestingly, CAR-cells. This latter observation thus further supports the hypothesis that CAR-cells can harbor both T cells and HSPCs.

Tripp et al. were the first one who described the possibility of priming T cells in the bone marrow. These data were generated in mice where lymphocyte trafficking to secondary lymphoid organs was disrupted and thereby artificially rerouted to the bone marrow. More recent data describes the priming of both CD4+ and CD8+ T cells in bone marrow during both primary and secondary infection. Bone marrow T cells are often detected in...
close proximity of bone marrow resident DCs, which together form follicle-like structures in BM parenchyma, indicating that the initiation of adaptive immune responses in the bone marrow is just as efficient as in other secondary lymphoid organs. Bone marrow memory CD4+ T cells can upon antigen-driven reactivation swiftly release a plethora of cytokines and can provide efficient help to B cells to produce high-affinity antibodies. Primed bone marrow CD8+ T cells have been found to display a different cytokine-producing and gene-expression profile than their lymph node counterparts, suggesting that bone marrow CD8+ T via signaling from their microenvironment can more efficiently generate memory cells. Collectively, these data suggest that bone marrow can serve as a niche for T cells that preferentially allows the maintenance of and the maturation into memory cells. Bone marrow could thus be the preferential site for maintaining long-term immunological memory. The fact that T cells and HSCs are both located in perivascular regions and induction of T cell responses can take place in follicle-like structures within the perivascular regions of the bone marrow, strongly suggests that pro-inflammatory signals generated upon activation of lymphoid cells can affect hematopoiesis.

Of particular interest in regulating hematopoiesis upon inflammation are regulatory T (Treg) cells. Treg cells make up approximately 25% of CD4+ T cells in human bone marrow. Using a mouse transplantation model, host Treg cells that reside in close proximity of the endosteal surface of the bone marrow were found to protect transplanted HSC from allorejection. As these Treg cells are in close proximity of HSCs and their depletion from the endosteal lining leads to allorejection, it is likely that Treg cells share a common niche with HSCs and either directly or indirectly influence the hematopoietic lineage fate of HSCs. More evidence for this hypothesis comes from the fact that Treg trafficking to the bone marrow is mediated by CXCR4/CXCL12 signaling. In addition, G-CSF not only mobilizes HSPCs and depletes osteomacs in the bone marrow, it also decreases Treg numbers by downregulation of CXCL12. Cell-cell contact between Treg cells and osteomacs in the endosteal niche and CAR-cells in the perivascular niche are thus likely to help retaining Treg cells in the bone marrow. Additional factors needed for Treg cell retention in the bone marrow are up until now unknown, but IL-2 would be a likely candidate, as it stimulates CXCR4-expression on Treg cells and is required for the maintenance and suppression function of Treg cells. During inflammatory conditions Treg cells presumably only indirectly influence HSC behavior by dampening inflammation by the production of IL-10. To what extent Treg cells can also directly influence HSCs is unknown, but gaining insight in the expression of cytokines and cell-surface molecules of Treg cells that are capable of directly influencing HSCs during both steady-state conditions and during immune activation would gain useful insights in this matter.

Natural killer cells and Natural killer T cells
In contrast to B and T cells, natural killer (NK) cells are lymphocytes that only have limited recognition for antigens as they rely on activating and inhibitory invariant receptors for their activation. Generation of fully mature NK cells takes place in the bone marrow and requires IL-15 and Flt3-signaling, which are, as discussed previously, also essential for DC and B-cell retention within the bone marrow. Using deletion models for CXCR4 and CXCL12, it was found that CXCR4/CXCL12 interactions are essential for NK cell development in adult mice, but not in the embryo. Developing NK cells were found to be in contact with CAR-cells
as there CXCL12 and IL-15 production was found highest89. Although controversial, NK cells might also rely on SCF for their development and maintenance101;102. It is currently unknown where mature NK cells exactly reside in the bone marrow, but the fact that NK cells share common niche factors with HSPCs for their retention, presumes that they can be found in close proximity of HSPCs.

Natural killer T (NKT) cells are a heterogeneous group of T lymphocytes that bear the expression of the common NK cells marker NK1.1 in mice or CD161 in humans, IL-2 receptors and an invariant T cell receptor α chain103. NKT cells normally develop in the thymus and get positively selected by CD1d-expressing bone marrow cells, but can also be generated in the bone marrow upon thymectomy104;105. In adult mice, between 2-4% of immune cells (20-30% of T cells) in the bone marrow are NKT cells104;106, and they are thought to play essential roles in guiding immunity upon bone marrow transplantation, during tumor development and in the development of autoimmunity107-111. Which factors are required for the maintenance of NKT cells in the bone marrow is unknown; however, as NKT cells display features of both T cells and NK cells, it is feasible that they rely on similar retention factors as lymphocytes and that they are retained in corresponding niches. Activation of NKT cells increases production of IgG upon interaction with B cells112 and provides signals to enhance or affect T cell responses113;114. Furthermore, NKT cells enhance DC maturation115 and cause NK cells to proliferate and enhance their cytotoxicity116. When activated in the bone marrow, NKT cells can thus instruct a variety of immune cells and could potentially alter hematopoiesis by the production of inflammatory cytokines.
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Table 1. Immune cells in the bone marrow and their relation with, and effect on, the HSC niche.
Parallels between differentiation of T cells and HSPCs: “the 3-signal model”

Lineage commitment and differentiation of HSCs is a well-described process involving a variety of intrinsic and extrinsic signals. Besides the well-known stimuli and differentiation factors that drive lineage commitment in steady-state hematopoiesis, recent data reveal that pathogen recognition receptors (PRRs), pro-inflammatory cytokines and co-stimulatory molecules that are involved in guiding adaptive immune responses, are also expressed on HSPCs. As immune activation can take place in close proximity of HSPCs in the bone marrow, these data indicate that pathogens and immune modulating signals expressed on, or secreted by, activated immune cells in the bone marrow can directly affect hematopoiesis. These signals thereby might quickly adapt the output of hematopoietic progenitors to generate specific offspring capable of fighting infections and cope with the loss of specific immune cells.

Strikingly, lineage differentiation of HSPCs upon inflammation shows great overlap with the three-signal model described for T cell activation (Figure 1). During immune activation T cells rely for their full activation on three extrinsic signals that are provided by APCs. Signal one is provided when the highly specific T cell receptor (TCR) interacts with its cognate antigen peptide presented by major immunohistocompatibility complex (MHC) molecules on APCs. Co-stimulatory molecules expressed on APCs provide a second signal that drives survival, proliferation and differentiation of T cells upon activation. Lack of co-stimulatory signaling during T cell activation may lead to T cell anergy and hyporesponsiveness to infections. The third signal that drives differentiation of the various effector types of T cells is provided by cytokines present in the microenvironment during T cell activation. We have come to realize that hematopoietic lineage commitment during immune activation has an interesting analogy with these three steps during T cell activation, since differentiation of quiescent HSCs requires their activation, after which they can also be modulated by costimulatory molecules and inflammatory cytokines. How these different signals are incorporated in lineage commitment will be discussed in the following part.
During steady-state hematopoiesis, hematopoietic niche cells carefully regulate dormancy, quiescence, proliferation and differentiation of hematopoietic stem cells (HSCs) via cell-cell interactions, chemotactic signals and by the production of soluble factors. During inflammatory conditions, interaction of activated immune cells or pathogens directly with HSCs, or indirectly with hematopoietic niche cells, induces loss of niche signaling (Signal 1). Thereby HSCs loosen from the niche, allowing proliferation and differentiation of HSCs by induction of lineage specific transcriptional programs. Activated immune cells can via co-stimulatory molecule signaling (Signal 2) and inflammatory cytokine signaling (Signal 3) further affect HSC proliferation by inducing or inhibiting lineage specific transcription factors.

Figure 1. Schematic overview of the 3-signal model for hematopoiesis during immune activation.
Signal 1: Activation

To preserve long-term self-renewal and life-long formation of blood and immune cells, HSCs are kept dormant by signals received from its niche. Several soluble factors, including SCF, Ang-1 and TPO, have been identified as niche factors that maintain quiescence of HSCs and assure long term repopulating capacity. In addition, chemotactic signals like CXCR4-CXCL12 interactions and cell-cell contact via adhesion molecules have been implied to keep HSC dormant. The effect of TGF-β on hematopoiesis is contradictory. Whereas HSCs from mice deficient for the TGF-β receptor display increased proliferative capacity in vitro, they have impaired long-term repopulating capacity in vivo. Furthermore, TGF-β has also been shown to negatively regulate HSC proliferation in vitro by inhibiting the cytokine-mediated clustering of lipid rafts needed for entering cell cycle. TGF-β thereby induces HSC hibernation. More recently, nonmyelinating Schwann cells were found to be essential for this process, as they activate the latent form of TGF-β that is produced by HSC niche cells. Recent literature suggests that besides the presence of HSC niches there are several hematopoietic niches that harbor distinct hematopoietic progenitors with either full or restricted hematopoietic capacities. This indicates that quiescent HSCs either detach from their niche, lose dormancy signals and directly get activated by local signals to induce proliferation and differentiation, or that HSCs migrate from their dormancy niche towards other microenvironments that activate them and induce proliferation and differentiation. Although some signals that are involved in keeping HSCs quiescent and viable are known, the exact mechanisms and signals that drive activation and lineage commitment of HSCs are largely unknown and are an important subject of ongoing research.

In T cell activation signal 1 is provided by activation of the TCR upon binding its cognate antigen. However HSPCs do not express TCRs, the essential signal that induces lineage commitment within HSPCs can be considered activation of lineage specific transcriptional programs. Targeted disruption of the transcription factors GATA-1 and PU.1 results in early embryonic and neonatal death, respectively, due to defective hematopoietic development. Whereas disruption of GATA-1 results in red cell crisis, deletion of PU.1 results in impaired myelopoiesis and lymphopoiesis, indicating that the induction of transcriptional programs is essential for lineage fate decisions. Using GFP-reporter mice, PU.1 and GATA-1 were found to be expressed in the earliest HSPC population. Whereas GATA-1+ HSPCs have myeloerythroid potential and lack lymphoid potential, PU.1+ HSPCs harbour myeloid and lymphoid potential but no erythroid potential. Except for the fact that the transcription factor GATA-3 maintains HSC quiescence, no other transcription factors then PU.1 and GATA-1 are known that affect lineage fate decision in HSPCs. Further differentiation of progenitor cells into specific hematopoietic lineages is dependent on the order of lineage specific transcription factor expression and might be marked by overlapping and graded expression and repression of lineage specific transcription factors. Despite the fact that the transcriptional programs for lineage commitment are largely known, the exact mechanisms involved in induction of lineage fate commitment at the HSC level by means of transcription factor expression are still not fully understood and cause controversy in the field.
cell survival), instructive (induce lineage commitment) or restrictive (inhibiting commitment into specific lineages). Although expression of their receptors varies between the several populations of HSPCs, cytokines like GM-CSF, G-CSF, M-CSF, IL-3, IL-6 and IL-7 are implicated to induce lineage commitment during steady-state hematopoiesis. IL-3 serves as an HSC expansion factor, but also acts on a broad range of progenitors and drives HSCs to myeloid progenitors. GM-CSF induces the formation of granulocytes and monocytes in general, while IL-6 controls proliferation and differentiation of progenitor cells and synergizes with SCF to induce proliferation and survival of HSCs. Whereas G-CSF directly drives neutrophil production from the GMP, M-CSF induces the formation of monocytes from GMPs. G-CSF however does not only drive granulopoiesis, it also affects hematopoiesis by effectively inducing proliferation and mobilization of quiescent HSCs. While all the aforementioned cytokines mainly enhance the output of myeloid cells, IL-6 and IL-7 are primarily involved in the induction of production, maintenance, survival and differentiation of lymphoid-primed progenitors, B cells and T cells. During infection stromal derived IL-6 in addition also expands the GMP population, while erythropoiesis is blocked. This indicates a dual role for IL-6 in both lymphopoiesis and myelopoiesis. Of note, in response to hypoxic stress, such as after blood loss, enhanced erythrocyte turnover or diminished erythropoiesis, hypoxia-inducible factor 1 (HIF-1) is stabilized, and activates both local and systemic production of EPO. EPO enhances the erythroid output from the bone marrow and drives stress-erythropoiesis within the spleen of mice to maintain tissue oxygenation levels.

The discovery that HSPCs express pathogen recognition receptors (PRRs) further indicates that, at least during immune activation, extrinsic signals play an important role in directing lineage differentiation. Although HSPCs have no direct immune function, they do express various PRRs like Toll like receptor (TLR) 2, -4, -7 and -9. Injection of LPS into mice activates quiescent HSCs and turns them into self-renewing and proliferating HSCs. Whereas lineage restriction is not altered in LPS-stimulated HSCs, their repopulation capacity is impaired. Furthermore, triggering of TLR2 and TLR4 on LKS-cells induces proliferation and myeloid differentiation in the absence of lineage instructing cytokines, indicating that TLR signaling on HSPCs can directly alter expression of lineage specific transcription factors. Triggering of TLR2 and TLR4 on GMPs drives the development of monocytes and macrophages at the cost of granulocytes. Upon TLR9 triggering CLPs differentiate to DC’s, while the outgrowth of B-cells is diminished. Apart from a direct effect on hematopoietic progenitors, PAMPs can also influence hematopoiesis indirectly as TLR-expression is also found on mesenchymal stem, stromal cells and resident immune cells within the hematopoietic niche. Upon triggering of TLRs on hematopoietic niche cells, a variety of pro-inflammatory cytokines, chemokines and signaling molecules can be produced or expressed that are capable of affecting hematopoiesis. More data that argues for extrinsic regulation of lineage differentiation by altering transcriptional programs comes from a recent study that shows that in co-cultures osteoblasts not only induces proliferation of HSCs, they also increase the number of monocyte/granulocyte precursors, while erythroid precursor numbers are diminished.
Signal 2: Co-Stimulation

Up until recently, co-stimulation was primarily known for its involvement in immune activation of lymphocytes and its modulatory actions on immune response. CD27, which belongs to the TNF receptor superfamily, is the most extensively studied co-stimulatory molecule expressed on HSPCs. Whereas in mouse CD27 is expressed on a variety of adult HSPCs, including long-term self renewing HSCs (LT-HSC), short-term reconstituting HSCs (ST-HSCs), multipotent progenitors (MPPs), common lymphoid progenitors (CLPs), common myeloid progenitors (CMPs) and granulocyte-monocyte progenitors (GMPs), but not on megakaryocyte-erythroid progenitors (MEPs) (De Bruin, unpublished data), the expression of CD27 on adult human HSPCs remains controversial. In line with these data, we describe in chapter 3 the initiation of CD27 expression on arising HSPCs within the aorta-gonad-mesonephros (AGM)-region and fetal liver in mice. CD27 expression on human HSPCs from fetal liver, fetal bone marrow and cord blood was found either very low or absent. The observation of early initiation of CD27 expression on mouse embryonic HSPCs led to the thought that CD27-sigaling might play a role in the ontogeny of the hematopoietic system. However, the presence or absence of CD27 on fetal HSPCs neither affect ontogeny of the hematopoietic system in mouse, nor did it affect the functional output of HSPCs as measured by semi-solid colony assays. In addition, overexpression of the type II TNF-related transmembrane glycoprotein CD70, the solely known ligand for CD27, did not affect fetal hematopoiesis. Given the fact that CD70 in adult mice is only transiently expressed on activated DCs, B cells and T cells upon immune activation, this indicates that CD27 is not of major importance in the development of the hematopoietic system and does not affect lineage commitment during ontogeny, but rather exerts its hematopoietic effects during immune activation in adult mice.

Indeed, the effect of CD27-binding on lineage commitment of HSPCs upon immune activation is more pronounced and indicates that activated adaptive immune cells can actively give feedback to the hematopoietic system to alter the output in favor of cells capable of fighting specific pathogens. Constitutive binding of CD27 by CD70 enhances HSC self-renewal and results in the accumulation of LT-HSCs and ST-HSCs in the bone marrow, whereas the MPP, lymphoid-primed MMP (LMPP) and CLP numbers are declined. In agreement with the in vitro colony-forming potential of CD27-triggered HSPCs, adoptively transferred CD27-triggered HSCs were found to be myeloid biased, while their lymphoid potential was diminished (De Bruin; unpublished data). Importantly, constitutive CD27-triggering on HSCs did not only induce enhanced self-renewal and a myeloid-biased gene expression, it also induced expression of genes associated with HSC aging (De Bruin; unpublished data). As enhanced self-renewal, loss of lymphoid potential and enhanced myeloid potential have been associated with HSC aging, these data strongly suggests that CD27-triggering on HSC accelerates HSC aging. For most of these experiments however CD70 transgenic (CD70TG) mice were used, which, in contrast to physiological inflammatory conditions, constitutively express CD70. Although being indicative for the effects of CD27-CD70 signaling and studying the effects of CD27-signaling on hematopoiesis, continuous CD27-stimulation will only take place during chronic inflammatory conditions. It will therefore be interesting to also examine transient CD27-triggering on hematopoiesis during acute infection, when activated lymphocytes in the bone marrow briefly express CD70, to...
determine the impact of CD27-mediated “co-stimulation” on HSC proliferation, myelopoiesis and lymphopoiesis.

Further downstream of the hematopoietic progenitors, CD27 expression is found on T cells, B-cells and NK cells. CD27-triggering on activated T cells promotes their survival, induces their differentiation into interferon-gamma (IFN-γ) producing effector T cells and is required for the generation of T cell memory. To what extent CD27-signaling also could be instructive in polarization to particular T helper (Th) subsets during immune activation was unknown and prompted our study described in chapter 2 of this thesis. Using in vitro skewing assays we found that CD27 does not provide instructive signals for specific Th cells subsets, but based on the cytokine milieu and genetic background of mice, CD27-signaling supports the differentiation into Th1 cells without modulating the differentiation into Th2 cells and T regulatory (Treg) cells. Differentiation into Th17 cells however was inhibited upon CD27-signaling. This latter finding was recently confirmed in an in vivo model for multiple sclerosis, where CD27-signaling inhibited the formation of Th17 cells and the development of autoimmune responses. Although constitutive CD27-signaling inhibits lymphopoiesis in favor of myelopoiesis, within the lymphoid compartment CD27-triggering impairs the outgrowth of B cells and NK cells from the bone marrow, suggesting that CD27-triggering on CLPs shifts differentiation within the lymphoid lineage towards the T cell lineage.

In addition to CD27 expression, also the TNFR superfamily members CD40, CD154 (CD40L) and 4-1BB ligand (4-1BBL) are expressed on HSPCs. Although not extensively studied regarding their role in hematopoiesis, 4-1BB is expressed on HSCs, CMPs and GMPs, while 4-1BBL expression is found on activated myeloid progenitors. Targeted deletion of these molecules in steady-state conditions leads to a decrease of GMPs, myeloid cells and mature dendritic cells, indicating that 4-1BB and 4-1BBL limit myelopoiesis and the development of DCs. To what extent these molecules also alter hematopoiesis during inflammatory conditions was not addressed. CD40 and CD154 interactions were originally found to be critically involved in B-cell activation, T cell activation, germinal center formation and memory cell formation as they are expressed on a variety of immune cells. However, CD40 expression is also found on hematopoietic progenitors. Homeostatic levels of CD154 have been shown to play a critical role in the development of naive CD4+ T cells and B-cell precursors and a role for CD40-CD154 interaction has been suggested in the development of NK cells. Regarding affecting early hematopoiesis, the interaction of CD154 to CD40 on HSPCs results in their proliferation and induces the formation of DCs. Interestingly, it was hypothesized that CD40-CD154 interactions might also be involved in the induction of myelopoieses and the homing of HSPCs to their nice, as CD40-triggering on bone marrow stromal cells increases the expression of CXCL12, TPO and Flt3L. Although CD40-CD154 interactions thus affect hematopoiesis during steady-state conditions, CD154 expression is quickly induced upon activation of a variety of immune cells, including T cells, B cells, DCs, monocytes, macrophages, NK cells, basophils and eosinophils. As activation of adaptive immune cells can take place in close proximity of HSC in the bone marrow, this suggests that activated immune cells in the bone marrow can directly alter hematopoiesis via CD40-CD154 interactions.

Although co-stimulatory molecules are mainly known for their role in immune responses, triggering of co-stimulatory molecules on HSPCs can thus significantly alter...
the hematopoietic output during both steady state and inflammatory conditions. Up until now only a few co-stimulatory molecules are found to be expressed on HSPCs, however, the TNF-receptor superfamily and B7/CD28 family comprise a variety of molecules of which it is unknown whether they could affect hematopoiesis. Thorough identification of co-stimulatory molecule expression on HSPCs is needed to sketch the full impact of immune activation on hematopoiesis. Although it is most likely that activated immune cells deliver co-stimulatory signals to hematopoietic cells, conclusive studies from which this can be concluded are largely lacking. Not only are further studies thus required to determine which cells affect hematopoiesis via co-stimulation, they also could gain useful insight into whether these signals are necessary and/or sufficient for lineage commitment during steady-state hematopoiesis and what their effects are during inflammatory conditions.

**Signal 3: Inflammatory Cytokines**

Like during T cell activation, where cytokines provide signal 3 to induce T helper subset differentiation and differentiation into memory and effector T cells, lineage commitment of hematopoietic progenitors can also be driven by inflammatory cytokines. As described earlier, cytokines like GM-CSF, G-CSF, M-CSF, IL-3, IL-6 and IL-7 induce lineage commitment during steady-state hematopoiesis. However, these cytokines are also released in copious amounts during immune activation or stress-responses, indicating that their impact on hematopoiesis then is greatest.

In addition to these well-known hematopoietic cytokines, more and more evidence has been gathered that pro-inflammatory cytokines produced by locally activated immune cells in the bone marrow can affect HSC function and lineage commitment. In fact, the majority of HSPCs express receptors for a plethora of pro-inflammatory cytokines. IL-1 induces proliferation of HSCs in vitro and is necessary for the induction of granulopoiesis in vivo. Proliferation and expansion of multipotent and myeloid progenitors by IL-1 in vivo is however indirect, as proliferation of HSCs, MPPs and GMPs, and accelerated output of granulocytes upon alum injection is dependent on IL-1R expressing radiation resistant, non-hematopoietic cells.

Whereas IL-1 enhances HSC proliferation, TNF-α negatively affects HSC function both in vitro and in vivo. Addition of TNF-α to in vitro colony assays significantly reduces the number of colonies generated from HSCs and although unclear via what mechanism, in vivo administration of TNF-α suppresses the function of actively cycling HSC. TNF receptor (TNFR) deficient mice have normal numbers of HSCs, but their long-term reconstitution in competitive repopulation assays is diminished, thereby further illustrating the inhibiting effects of TNF-α. As there are two different receptors for TNF-α, its effect on hematopoiesis is dual. TNFR-I (p55) mainly exerts the effects of TNF-α on GMPs, while TNFR-II (p75) is essential in signaling inhibition of primitive progenitors. However, both receptors are involved in the inhibitory effects seen on erythropoiesis, most likely by downregulating GATA-1 expression.

The most extensively studied pro-inflammatory cytokines that affect hematopoiesis belong to the family of interferons (IFNs). Treatment of mice with the type I interferon IFN-α awakens dormant HSCs to exit G₀ and enter cell cycle. Chronic stimulation with IFN-α however exhausts the HSC pool and in competitive repopulation assays IFN-α treated HSC are outcompeted by untreated HSCs. Of great interest is the type II interferon interferon-γ
(IFN-γ), which is typically produced in copious amounts by T cells, NK cells and NKT cells upon infection with intracellular pathogens. Although the effects on hematopoiesis of this cytokine are known for long in both humans and mouse, the exact mechanisms involved in affecting specific hematopoietic processes only became more clear recently. Regarding HSC biology, early studies indicated that IFN-γ negatively affects the in vitro colony forming capacity and self-renewal capacity of isolated HSCs either by inducing differentiation or apoptosis. A recent study by Baldridge et al. however proposed that the hematopoietic response to infection not only involves the activation of intermediate hematopoietic progenitors, but also the activation of quiescent LT-HSCs. They found that IFN-γ acted as a positive regulator of HSCs by inducing proliferation and thereby modulating self-renewal and lineage commitment to cope with the increased demand for immune cells in an in vivo model of chronic bacterial infection. Studies from our lab however provide evidence that IFN-γ both in vivo and in vitro impairs the maintenance of HSCs by interfering with signal transducer and activator of transcription (STAT)5 and thereby reducing their self-renewal capacity. These findings are at odds with the conclusions from Baldridge et al., but the latter are most likely resulting from indirect effects of IFN-γ on other cell types and an overestimation of the number of HSCs due to Sca-1 upregulation.

Next to the effects on HSCs, IFN-γ can have both inhibitory and stimulatory effects on commitment into specific hematopoietic lineages. Using both IFN-γ transgenic and CD70TG mice, of which the latter due to constitutive CD27-triggering have increased numbers of IFN-γ-producing effector T cells, we and others showed that IFN-γ severely affects B cell development in the bone marrow. The decrease in B-cells is most likely by inducing the expression of suppressor of cytokine signaling (SOCS)-molecules that interfere with IL-7 signaling. Besides suppressing B lymphopoiesis, IFN-γ is well known for its negative effects on erythropoiesis and it is suggested to play an important role in the development of anemia of chronic diseases (ACD). In chapter 4 we describe the molecular mechanism underlying the inhibitory effect of IFN-γ on erythropoiesis. The balance between the essential erythroid transcription factor GATA-1 and the myeloid transcription factor PU.1 determines whether a CMP will differentiate into a MEP or GMP, respectively. As PU.1 and GATA-1 physically interact with each other and inhibit each others function, deregulated levels of PU.1 or GATA-1 can thus affect erythropoiesis. We found that both in vivo and in vitro stimulation of erythroid progenitors with IFN-γ induces the expression of IRF-1 and PU.1 mRNA and protein. When tested in colony formation assays, both human and mouse IFN-γ stimulated erythroid progenitors were severely impaired in forming erythroid colony output. Inhibition of either IRF-1 or PU.1 was sufficient to overcome the IFN-γ-induced inhibition of erythropoiesis. These data thus indicate that when IFN-γ is produced during inflammatory conditions, it induces the expression of PU.1, thereby inhibiting erythropoiesis and shifting hematopoiesis towards myelopoiesis.

The microenvironment where erythropoiesis takes place however, determines whether IFN-γ has an inhibitory effect on erythroid output. In response to hypoxic stress, mice turn to stress-erythropoiesis in the spleen. In contrast to erythropoiesis the bone marrow, splenic erythropoiesis is not affected by IFN-γ (Chapter 6 and 204). We showed that glucocorticoid signaling, which is essential for induction of stress-erythropoiesis, overcomes the negative effects of IFN-γ on erythropoiesis. What molecular mechanism is involved in this process and whether this protective effect is given direct or indirect via signaling to the microenvironment
of the erythroid precursors remains to be elucidated (Chapter 6). In addition to the erythroid lineage, IFN-γ also affects the output within the myeloid lineage. By inducing expression of SOCS-molecules, IFN-γ suppresses granulopoiesis by interfering with G-CSF signaling and it suppresses the development of eosinophils by interfering with IL-5 signaling\textsuperscript{206,207}. IFN-γ does not impair M-CSF mediated proliferation and differentiation of CMPs and GMPs and induces expression of monocyte-inducing transcription factors PU.1 and IRF-8 in these cells, which explains why IFN-γ is in fact a very potent stimulator of monopoiesis\textsuperscript{207}.

Although pro-inflammatory cytokines have profound effects on the immune system and are important for the differentiation into T helper subsets, their effects on hematopoiesis in general, and hematopoietic lineage choices in particular, have only recently become a topic of research. While important progress is made regarding the effect of the most important pro-inflammatory cytokines on hematopoiesis, there remains a plethora of soluble immune mediators of which the hematopoietic effects remain to be explored. In addition, it is still largely unknown whether steady-state hematopoiesis also relies on low level signaling of pro-inflammatory signals. Furthermore, side effects of pro-inflammatory cytokines that are used as therapy in a variety of diseases can be serious\textsuperscript{208}, though studies investigating the short- and long-term effects on hematopoiesis are lacking. Gaining more insight into how affecting immune responses alters hematopoiesis could be beneficial for the treatment of hematological disorders during chronic immune activation.
### Table 2. The effect of different inflammatory molecules on hematopoietic processes.

<table>
<thead>
<tr>
<th>Inflammatory Molecule</th>
<th>Effect on hematopoiesis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pathogen Recognition Receptors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TLR2</td>
<td>Enhances self-renewal and proliferation of HSPCs.</td>
<td>21;144-146</td>
</tr>
<tr>
<td></td>
<td>Enhances monopoiesis, inhibits granulopoiesis.</td>
<td></td>
</tr>
<tr>
<td>TLR4</td>
<td>Enhances self-renewal and proliferation of HSPCs.</td>
<td>21;144-146</td>
</tr>
<tr>
<td></td>
<td>Enhances monopoiesis, inhibits granulopoiesis.</td>
<td></td>
</tr>
<tr>
<td>TLR9</td>
<td>Enhances DC development, diminishes B lymphopoiesis.</td>
<td>147</td>
</tr>
<tr>
<td><strong>Co-Stimulatory Molecules</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD27 / CD70</td>
<td>Enhances self-renewal and proliferation of HSCs.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Induces a myeloid biased gene expression in HSCs.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inhibits lymphopoiesis, induces myelopoiesis.</td>
<td></td>
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<tr>
<td></td>
<td>Within the lymphoid lineage enhances T cell development, while inhibiting NK cell and B cell development. Induces survival of effector T cells and development of T cell memory, affects Th cell differentiation.</td>
<td>De Bruin; unpublished 155;169-171</td>
</tr>
<tr>
<td>CD40 / CD154</td>
<td>Enhances HSC proliferation.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Enhances T cell, B cell, NK cell and DC lymphopoiesis.</td>
<td>175;177-179</td>
</tr>
<tr>
<td></td>
<td>Involved in myelopoiesis.</td>
<td></td>
</tr>
<tr>
<td>4-1BB / 4-1BBL</td>
<td>Limit formation of GMPs, myeloid cells and DCs.</td>
<td>176</td>
</tr>
<tr>
<td><strong>Inflammatory Cytokines</strong></td>
<td></td>
<td></td>
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<tr>
<td>IL-1</td>
<td>Induces HSC proliferation and granulopoiesis.</td>
<td>180</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Diminishes HSC output, inhibits HSPC proliferation.</td>
<td>181;182;187-189</td>
</tr>
<tr>
<td></td>
<td>Inhibits erythropoiesis.</td>
<td></td>
</tr>
<tr>
<td>IFN-α</td>
<td>Induces HSC proliferation, affects HSC fitness, inhibits HSC output.</td>
<td>190</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Diminishes HSC self-renewal and HSC output, enhances HSC proliferation.</td>
<td>170;191-196</td>
</tr>
<tr>
<td></td>
<td>Diminishes B cell lymphopoiesis and erythropoiesis, enhances myelopoiesis. Within the myeloid compartment enhances monopoiesis, while inhibiting granulopoiesis and eosinophil output.</td>
<td>197;199-201; 206;207</td>
</tr>
</tbody>
</table>
Conclusions and Implications

Although definitive studies that demonstrate direct cell-cell interactions between activated immune cells and HSPCs are still lacking, data is accumulating that immune cells reside, get activated, or migrate upon activation, to anatomical locations that either contain HSPCs or are in close proximity of HSPCs. In addition, immune cells have been found to largely rely on the same common retention factors and docking cells as HSPCs. As HSPCs express a variety of receptors for pathogen recognition and pro-inflammatory signals, these studies support the hypothesis that activated immune cells via direct cell-cell contact and the secretion of soluble factors can affect hematopoiesis in general, and hematopoietic lineage choices in particular. In this thesis we provide evidence for this hypothesis as the interaction between the co-stimulatory molecule CD27, which is expressed on HSPCs, and CD70, which is expressed on activated immune cells, can directly alter hematopoiesis by affecting hematopoietic lineage choices within the T cell lineage (Chapter 2). In addition, we show that the pro-inflammatory cytokine IFN-γ severely blocks erythropoiesis within the bone marrow by upregulating the myeloid transcription factor PU.1 (Chapter 4), thereby enhancing myelopoiesis. These effects are however largely dependent on the environment in which HSPCs reside, as CD27-triggering on HSPCs in the embryo does not have an effect on hematopoiesis (Chapter 3) and IFN-γ does not inhibit erythropoiesis in the spleen (Chapter 6). What mechanisms drive these differences are currently unknown, but can most probably be found in the signals provided by the microenvironment in which HSPCs reside. Ongoing research is currently focusing on unraveling these signals.

In line with the activation of T cells, we here propose that lineage commitment during immune activation follows a 3-signal model. Signal 1 is considered the loosening of the HSC from its niche, loss of its quiescent state, initiation of proliferation and the induction of lineage specific transcriptional programs that drive differentiation. This induction of lineage commitment can be directly influenced by signal 2, co-stimulation, and signal 3, inflammatory cytokines. These signals are either directly delivered to HSPCs by activated immune cells, or indirectly by the interaction of activated immune cells with hematopoietic niche cells. This model thus implies that hematopoiesis during immune activation is a dynamic system in which a variety of activating and inhibiting signals of homeostatic and inflammatory origin are integrated to induce a transcriptional program that allows the generation of appropriate offspring to fight invading pathogens. Up until now the expression and function of only a small number of co-stimulatory molecules and pro-inflammatory cytokines on HSPCs has been described, while during immune activation there is an abundance of molecules present of which the influence on hematopoiesis is still largely unknown. Additional studies should thus be undertaken to gain a full overview on which other inflammatory molecules can affect hematopoiesis and what their function is regarding the induction of lineage commitment. In addition, these studies could give useful insights in how pro-inflammatory molecules can be clinically used to boost immune responses upon infection with specific pathogens and may give leads to the development of therapies capable of modulating hematopoiesis in such a way that offspring is generated that can overcome chronic infections. As the effects of pro-inflammatory signaling regarding lineage commitment are dependent on the microenvironment in which HSPCs reside, elucidating how extrinsic stimulatory and inhibitory signals are integrated into one message within progenitor cells would not only yield insight in the complexity of lineage commitment it could also prove to be useful in generating therapies overcoming cytopenias.
Reference List


100. Ranson T, Vosshenrich CA, Corcuff E et al. IL-15 is an essential mediator of peripheral NK-cell homeostasis. Blood 2003;101:4887-4893.


147. Welner RS, Pelayo R, Nagai Y et al. Lymphoid precursors are directed to produce dendritic cells as a result of TLR9 ligation during herpes infection. Blood 2008;112:3753-3761.


Appendix

Nederlandstalige Samenvatting
List of Publications
PhD Portfolio
Curriculum Vitae
Acknowledgements
Nederlandstalige Samenvatting

Bloedcellen hebben slechts een bepaalde levensduur. Voor het goed functioneren van de mens dienen deze cellen dan ook constant, en gedurende het gehele leven, venieuwd te worden. Het proces waarbij nieuwe bloedcellen gemaakt worden, wordt hematopoïese genoemd en vindt plaats in het beenmerg. In het beenmerg bevindt zich een zeer kleine hoeveelheid speciale cellen, zogenaamde hematopoïetische stamcellen (HSCs), die de constante aanwas van nieuwe bloedcellen verzorgt. Stamcellen kunnen door celdeling, ook wel proliferatie genoemd, twee dochtercellen genereren die exact aan elkaar gelijk zijn en dezelfde eigenschappen bezitten als de moedercel. Dit proces wordt zelfvernieuwing genoemd en zorgt ervoor dat gedurende het leven van de mens er altijd voldoende stamcellen zijn die de hematopoïese in stand houdt. Naast zelfvernieuwing kunnen stamcellen middels celdeling en stapsgewijze uitruijping, differentiatie genoemd, alle verschillende soorten bloedcellen van het lichaam vormen. De mechanismen die zelfvernieuwing, proliferatie en differentiatie regelen worden strikt gereguleerd om zo de mate van zelfvernieuwing, alsmede de uitgroei van nieuwe bloedcellen in balans te houden. De signalen die deze hematopoïetische processen beïnvloeden kunnen zowel intrinsiek (signalen binnenin de cel) als extrinsiek (signalen van buitenaf) zijn. Intrinsieke regulatie omvat meestal het tot expressie brengen van regulatoire eiwitten, beter bekend als transcriptiefactoren, die de activiteit van genen en andere eiwitten beïnvloeden bij de hematopoïese beïnvloeden. Externe signalen die de hematopoïeze reguleren worden vaak door om de stamcel gelegen cellen afgegeven. Deze omliggende cellen vormen de zogenaamde hematopoïetische stamcel niche en kunnen middels de afgifte van boodschappereiwitten (cytokinen), die zich kunnen binden aan receptoren op de stamcellen, signalen overbrengen die de functie van de stamcellen beïnvloedt. Daarnaast brengen de niche cellen vaak moleculen tot expressie die als anker dienen voor de stamcel en daarmee de activiteit van de stamcel bepalen.

HSCs kunnen zoals gezegd alle verschillende bloedcellen genereren (Hoofdstuk 1, Figuur 1). Deze bloedcellen worden onderverdeeld in bloedplaatjes, die zorgen voor stolling bij bloedingen, rode bloedcellen, die voor de aanvoer van zuurstof en afvoer van koolstofdioxide in het lichaam zorgen, en witte bloedcellen, die ons beschermen tegen ziekteverwekkers en toxische stoffen. Witte bloedcellen kunnen vervolgens verder onderverdeeld worden in de aspecifieke afweercellen van het aangeboren immuunsysteem en de specifieke afweercellen van het verworven of adaptieve immuunsysteem. Granulocyten, monocyten en macrofagen (ook wel myeloïde cellen genoemd) behoren tot de aspecifieke afweer omdat zij slechts een aantal veel voorkomende moleculaire patronen (antigenen) van ziekteverwekkers herkennen. Na herkenning van deze patronen dragen zij door hun capaciteit tot het opeten van pathogenen, of het uitscheiden van voor pathogenen schadelijke stoffen, bij aan het beschermen van het lichaam tegen ziekteverwekkers. In tegenstelling tot de myeloïde cellen, kunnen de T en B cellen (of lymfocyten) van het adaptieve immuunsysteem elk een heel specifieke antigen herkennen, waarmee de totale pool aan lymfocyten samen dus een oneindig grote hoeveelheid antigenen kan herkennen. B cellen produceren na herkenning van hun specifieke antigen antilichamen (immuunglobulinen) die specifiek aan de ziekteverwekker binden en daarmee de ziekteverwekker neutraliseren en myeloïde cellen aanzetten tot het opruimen ervan. Waar B cellen een zogenaamde humorale immuunrespons
opzetten, vormen de T cellen enerzijds de cellulaire afweer doordat zij met pathogeen geïnfecteerde cellen herkennen en doden. Afhankelijk van het pathogeen dat ze herkennen brengen ze immuunrespons regulerende eiwitten tot expressie op het celoppervlak en scheiden ze cytokines uit waarmee ze ook in staat zijn andere immuuncellen aan te sturen en daarmee de immuunrespons in bredere zin reguleren. Naast de kenmerkende myeloïde en lymfoïde immuuncellen zijn er nog cellen die kenmerken van zowel het aangeboren als het adaptieve immuunsysteem hebben. Dendritische cellen (DCs) vormen een brug tussen het aangeboren en verworven immuunsysteem doordat zij opgeruimde ziekteverwekkers kunnen presenteren aan het adaptieve immuunsysteem en daarmee de adaptieve immuunrespons op gang zetten. Daarnaast zijn er natural killer cellen (NK cellen) die een lymfoïde origine hebben, maar doordat zij slechts een beperkt aantal antigenen herkennen, tot het aangeboren immuunsysteem behoren.

Recente literatuur heeft laten zien dat immuuncellen niet alleen in het beenmerg gemaakt worden en daarna migreren en aan het werk gezet worden op plekken waar ziekteverwekkers zich bevinden, maar dat zij ook zowel gedurende als na een infectie naar het beenmerg terug migreren en daar permanent kunnen verblijven. Ze migreren of verblijven dan vaak in de buurt van hematopoïetische stam- en voorlopercellen (HSPCs). Dit suggereert dan ook dat immuuncellen tijdens een infectie communiceren met het hematopoïetische systeem en feedback geven over hoe de output van het hematopoïetisch systeem aangepast dient te worden tijdens en na een infectie. Gedurende de strijd tegen ziekteverwekkers komen namelijk vele immuuncellen te overlijden en dienen specifieke pathogenen bestreden te worden door een specifieke set immuuncellen. Gedacht wordt dat geactiveerde immuuncellen deze informatie leveren aan het hematopoïetisch systeem en daarmee de uitgroei van bloedcellen in het beenmerg beïnvloeden. Onderzoek naar hoe dit proces verloopt zit momenteel in een stroomversnelling en in dit proefschrift beschrijven we dan ook het effect van twee door geactiveerde immuuncellen tot expressie gebrachte moleculen op verschillende hematopoïetische processen. Een van deze moleculen is het membraaneiwit CD70. CD70 komt alleen tot expressie op een aantal lymfoïde immuuncellen gedurende immuunactivatie en is voornamelijk betrokken bij de activatie van T cellen en het ontwikkelen van immunologische geheugen van deze cellen. De receptor voor CD70, CD27, komt echter niet alleen op immuuncellen voor, maar ook op HSPCs in het beenmerg. Binding van CD70 aan CD27 op HSCs induceert zelfvernieuwing en zorgt voor verminderde output van lymfoïde cellen, terwijl er meer myeloïde cellen gegenereerd worden.

Naïeve T lymfocyten zijn T cellen die nog niet eerder hun specifieke antigeen gezien hebben en onder invloed van antigeenherkenning (signaal 1), een secundaire stimulus (signaal 2) en cytokines (signaal 3) een bepaalde functie toebedeeld krijgen en daarmee differentiëren in een specifieke T helper (Th) subset. Th1 cellen bijvoorbeeld zijn betrokken bij het opzetten van een celgemedieerde immuunrespons, terwijl Th2 cellen helpen bij het opzetten van een humorale respons. Daarnaast zijn er nog Th17 cellen en regulatoire helper T (Treg) cellen, die respectievelijk betrokken zijn bij de afweer van bacteriën en schimmels in de slijmvlies en het onder controle houden van de immuunrespons. Binding van CD70 aan CD27 geeft een secundair signaal af aan T cellen, alleen is onbekend of dit signaal ook instructies kan geven om een bepaalde Th subset te vormen. In hoofdstuk 2 hebben we dan ook onderzocht hoe binding van CD70 aan CD27 de differentiatie van verschillende Th subsets beïnvloedt. Aangezien CD70 alleen tot expressie komt gedurende immuunactivatie
hebben we gebruik gemaakt van genetisch gemodificeerde muizen die constant CD70 tot expressie brengen op hun B cellen (CD70TG) en daarmee een model zijn voor chronische immuunactivatie. Afhankelijk van de genetische achtergrond van deze muizen leidt constante binding van CD70 aan CD27 op naïeve T cellen tot een verhoogde differentiatie tot Th1 cellen (in vivo). De aanmaak van Th2, Th17 en Treg cellen wordt niet beïnvloed door binding van CD70 aan CD27. Als we uit de muis geïsoleerde naïeve T cellen samen brengen met B cellen van de CD70TG muis in kweekschaltjes (in vitro), en ze vervolgens differentiëren tot de verschillende Th subsets door signaal 3 te veranderen, dan zorgt de continue binding van CD70 aan CD27 er voor dat voornamelijk Th1 cellen gevormd worden, terwijl de aanmaak van Th2 cellen gelijk blijft en die van Th17 cellen afneemt. Deze studie laat dan ook zien dat stimulatie van CD27 de differentiatie tot de verschillende Th subsets kan beïnvloeden maar dat dit erg afhankelijk is van het cytokine milieu en de genetische achtergrond.

Zoals gezegd wordt CD27 tot expressie gebracht op het celoppervlak van HSPCs en heeft binding van CD70 aan CD27 gevolgen voor de uitgroei van bloedcellen vanuit het beenmerg. In hoofdstuk 3 hebben we daarom gekeken of CD27 en CD70 al betrokken zijn bij de aanleg van het hematopoïetisch systeem in het embryo van de muis. Door gebruik te maken van gewone muizen (wildtypes) en muizen die geen CD27 tot expressie kunnen brengen op het celoppervlak (CD27KO) hebben we gevonden dat CD27 al zeer vroeg in de ontwikkeling van het embryo tot expressie komt op HSPCs. De afwezigheid van CD27 heeft echter geen invloed op de functie van HSPCs en de ontwikkeling van alle bloedcellen is normaal. Ondanks dat er in CD70TG embryo’s wel constant binding is van CD70 aan CD27 op HSPCs is de functie van CD27-getriggerde HSPCs normaal en is ook hier de uitgroei van alle bloedcellen normaal. In tegenstelling tot in de volwassen muis, heeft CD27 op embryonale HSPCs dan ook geen functie en krijgt dit molecuul pas een functie gedurende infecties met ziekteverwekkers. Aanvullend laten we in hoofdstuk 3 zien dat humane foetale HSPCs geen CD27 tot expressie brengen.

Een ander molecuul dat in grote hoeveelheden uitgescheiden wordt als reactie op pathogenen is het pro-inflammatoire cytokine interferon-γ (IFN-γ). IFN-γ wordt voornamelijk geproduceerd door geactiveerde T cellen en is betrokken bij de activering en regulering van andere immuuncellen. Daarnaast hebben eerdere studies al laten zien dat IFN-γ een effect heeft op de hematopoïese in algemene zin, en een remmend effect op de rode bloedcel aanmaak (erythropoïese) in het speciaal. Het onderliggende mechanisme van de rem op de rode bloedcel aanmaak was echter onbekend en dit hebben we verder ontleed in hoofdstuk 4. Door CD70TG muizen te kruisen met muizen die geen IFN-γ kunnen maken (IFN-γ KO) waren we in staat de rol van IFN-γ op de rode bloedcel aanmaak te onderzoeken gedurende langdurige immuunactivatie en vonden we dat CD70TG muizen een IFN-γ afhankelijke bloedarmoede (anemie) ontwikkelden. Door gelabelde rode bloed cellen in deze muizen in te spuiten en te volgen in de tijd bleek dat IFN-γ macrofagen in de milt aanwezig is en dat IFN-γ de rode bloedcel aanmaak in de milt remt. Voor de uitgroei van een bepaald celtype is een zogenaamd transcriptioneel programma nodig dat specifieke genen aan- of uitzet die essentieel zijn voor de uitgroei van deze cel. Voor voorlopercellen van de rode bloedcel te isoleren, in kweek te zetten samen met IFN-γ en daarna te kijken naar gen- en eiwitexpressie patronen bleek dat IFN-γ via IRF-1 de myeloïde transcriptiefactor PU.1 aanzet. Van PU.1
is bekend dat dit bindt aan GATA-1, de essentiële transcriptiefactor voor rode bloedcel aanmaak, en daarmee de functie van GATA-1 blokkeert en de rode bloedcel aanmaak remt. Deze data suggereert dus dat als we kijken naar de hematopoïetische stamboom en zien dat myeloïde en erythroïde cellen een gezamenlijke voorlopercel hebben dat IFN-γ door het aanzetten van PU.1 de aanmaak van myeloïde cellen stimuleert, terwijl dit de aanmaak van rode bloedcellen remt. Voor het bestrijden van infecties is dit geen probleem, aangezien rode bloedcellen een langere levensduur hebben dan myeloïde cellen. Is er echter langdurige immuunactivatie dan zal dit lijden tot een chronische remming van het aanmaken van rode bloedcellen en het ontwikkelen van anemie.

Aangezien het onbekend is wat er met erythroïd gecommitteerde beenmerg cellen gebeurt die gestimuleerd zijn met IFN-γ, hebben we getracht deze vraag te beantwoorden in hoofdstuk 5. Door de TF-1 cellijn, een cellijn van voorlopercellen die door het toedienen van de juiste stimuli kan uitgroeiën tot rode bloedcellen, te stimuleren met IFN-γ, bleek dat deze cellen na verloop van tijd minder goed groeiden en op een geprogrammeerde manier dood gingen. Geprogrammeerde celdood (apoptose) kan op verschillende manieren tot stand worden gebracht. Door te screenen op het expressiepatroon van verschillende pro- en anti-apoptotische eiwitten bleek IFN-γ te zorgen voor een verhoogde aanmaak van het pro-apoptotische eiwit Noxa en daarmee de balans tussen pro-apoptotische en anti-apoptotische eiwitten te verstoren. Waar Noxa normaal betrokken is bij inductie van apoptose, bleek dat bij toediening van specifieke apoptose remmers, de IFN-γ geïnduceerde celdood niet geremd kon worden en er in feite dus geen sprake is van apoptose. Naast apoptose zijn er echter nog enkele andere vormen van geprogrammeerde celdood, waaronder autofagie, necroptose en ferroptose, of celdood door vorming van schadelijke zuurstofradicalen. Echter het specifieke blokkeren van deze vormen van celdood voorkomt niet dat IFN-γ gestimuleerde TF-1 cellen doodgaan. Welke vorm van celdood dus aangezet wordt in IFN-γ gestimuleerde erytroïde cellen is dus onbekend, maar wordt de aanmaak van Noxa na IFN-γ stimulatie geremd door middel van silencing technieken of door toediening van rapamycine, dat transcriptie van genen en de aanmaak van eiwit remt, dan wordt celdood van deze cellen voorkomen. Rapamycine wordt veel in de kliniek gebruikt omdat het ook het immuunsysteem onderdrukt, maar onze data suggereren dat gebruik hiervan goed overdacht dient te worden aangezien het ook negatieve effecten kan hebben op de uitgroei van erytroïde cellen.

Ter compensatie van het verlies van rode bloed cellen is de muis in staat rode bloedcellen te genereren buiten het beenmerg. Dit proces wordt stress-erythropoïese genoemd en vindt plaats in de milt, en in sommige gevallen de lever. In hoofdstuk 4 beschrijven we dat CD70TG muizen vergrote milten hebben (splenomegalie) en dat dit komt door de aanwezigheid van grote hoeveelheden immature erytroïde voorloper cellen en de uitgroei daarvan tot mature rode bloed cellen. Aangezien de bloedarmoede in deze muizen zich mede ontwikkelt door de negatieve effecten van IFN-γ op de rode bloedcel aanmaak in het beenmerg, vroegen we ons in hoofdstuk 6 af of de stress-erythropoïetische respons in de milt ook gevoelig is voor dit pro-inflammatoire cytokine. Ondanks dat erytroïde voorlopercellen uit de milt wel de receptor voor IFN-γ tot expressie brengen en deze receptor ook signaleert bij binding van IFN-γ, wordt de uitgroei van rode bloedcellen, in tegenstelling tot het beenmerg, niet geremd. Deze data suggereren dus dat de omgeving waarin erytroïde voorlopercellen zich bevinden bepalen welk effect IFN-γ heeft op deze
cellen. Waar voor het verblijven van erythroïde voorlopercellen en het uittrijpen van rode bloed cellen in het beenmerg stamcel factor (SCF) en erythropoïetine (EPO) nodig zijn, is in de milt ook signalering nodig van bot morphogenetisch eiwit 4 (BMP4), Sonic hedgehog (Shh), en glucocorticoiden. Door beenmerg erythroïde voorlopercellen te isoleren en te kweken in de aanwezigheid van de additionele groeifactoren die essentieel zijn in de milt, bleek dat signalering door glucocorticoiden het negatieve effect van IFN-γ op beenmerg erythroïde voorlopercellen tegen gaat en de uitgroei van rode bloedcellen weer normaal wordt. Het precieze mechanisme van dit effect is echter nog niet bekend, maar het zou kunnen dat glucocorticoiden de signalering van IFN-γ in erythroïde voorlopercellen remt, de signalering van groeifactoren versterkt of het doodgaan van IFN-γ-gestimuleerde erythroïde voorlopercellen remt. Wel bieden deze resultaten meer inzicht in het gebruik van glucocorticoiden als therapie. Deze worden namelijk al veelvuldig toegepast als ontstekingsremmer, maar zou additioneel dus ook door ontsteking geïnduceerde anemie kunnen voorkomen.

Samenvattend hebben we in dit proefschrift de invloed van het geactiveerde immuunsysteem op de hematopoïese onderzocht en beschrijven we dat het molecuul CD70, dat alleen tot expressie komt gedurende immuunactivatie, differentiatie kan beïnvloeden door te binden aan naïeve T cellen in de volwassen muis, maar dat afwezigheid van, of binding van CD70 aan, CD27 op HSPCs in het embryo geen invloed heeft op de hematopoïetische output. Daarnaast beschrijven we dat het pro-inflammatoire cytokine IFN-γ, dat in grote hoeveelheden wordt uitgescheiden bij specifieke immuunreacties, de uitgroei van rode bloedcellen remt door te zorgen voor een verhoogde aanmaak van de myeloïde transcriptiefactor PU.1 en celdood te induceren in erythroïd gecommitteerde cellen. Het effect hiervan is echter specifiek voor de omgeving waarin deze cellen zich bevinden, aangezien IFN-γ wel een negatief effect heeft op de rode bloedcel aanmaak in het beenmerg, maar niet in de milt. Omdat CD27, wat bij T cel activatie zorgt voor signaal 2, en IFN-γ, dat zorgt voor signaal 3, van essentieel belang zijn voor het opzetten van een immuunrespons, maar ook betrokken zijn bij het bepalen van de hematopoïetische output gedurende immuunactivatie, stellen we in analogie met T cel activatie in hoofdstuk 7 een 3-signaal model voor voor hematopoëse. Signaal 1 is hierbij de activatie en het loslaten van HSCs uit hun niche en initiatie van celdeling en differentiatie tot mature cellen. Signaal 2 kan dus gegeven worden door stimulatoire moleculen als het membraangetijt CD27, terwijl signaal 3 ingenomen wordt door inflammatoire cytokinen als IFN-γ. Deze drie signalen worden geleverd door geactiveerde immuuncellen die terug migreren naar het beenmerg en kunnen hematopoësies beïnvloeden door interacties aan te gaan met HSPCs. Ondanks dat signaal 2 en 3 voor de hematopoëse niet essentieel zijn, kunnen zij wel de differentiatie van stamcellen tot mature bloedcellen beïnvloeden en daarmee de hematopoëtische output reguleren. Dit model laat dan ook zien dat de hematopoësische gedurende immuunactivatie een zeer dynamisch proces is waarbij grote hoeveelheden homeostatische en inflammatoire signalen geïntegreerd worden tot een transcriptioneel programma dat bepaalt wat de output moet zijn van hematopoëtische cellen.
List of publications


# PhD Portfolio

<table>
<thead>
<tr>
<th>Name PhD Student:</th>
<th>Sten Libregts</th>
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<tbody>
<tr>
<td>PhD Period:</td>
<td>September 2007 – June 2012</td>
</tr>
<tr>
<td>Name PhD Supervisor:</td>
<td>Dr. M.A. Nolte</td>
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## 1. PhD Training

<table>
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<tr>
<td>- The AMC World of Science</td>
<td>- Advanced Immunology</td>
<td>2007</td>
<td>20 / 0.7</td>
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<td>- Advanced Immunology</td>
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**Seminars, workshops and master classes**

| - Weekly Department Meetings | 2007 – 2012 | 126 / 4.5 |
| - Weekly Journal Clubs | 2007 – 2012 | 42 / 1.5 |
| - Lectures | 2007 – 2010 | 14 / 0.5 |
| - Masterclasses | 2007 - 2012 | 14 / 0.5 |

**Presentations**

| - Department Meetings (bi-annual) | 2007-2012 | 14 / 0.5 |
| - Journal Clubs (bi-annual) | 2007-2012 | 14 / 0.5 |
| - Molecular Meetings (triannual) | 2007-2010 | 12 / 0.4 |

| - Poster presentation Dutch Society Immunology (NVVI) | | |
| "CD27-Mediated Immune Activation Induces Anemia Via Two Distinct Mechanisms" | 2008 | 14 / 0.5 |
| "Chronic IFN-γ production induces anemia by inhibiting erythropoiesis through an IRF-1/PU.1 axis" | 2010 | 14 / 0.5 |

| - Oral presentation Dutch Society Immunology (NVVI) | | |
| "IFN-γ negatively affects erythropoiesis via upregulation of PU.1 during chronic immune activation" | 2009 | 14 / 0.5 |

| - Oral presentation Dutch Society Hematology (NVVH) | | |
| "CD27-mediated immune activation induces anaemia via two distinct mechanisms; a story on IFN-γ" | 2009 | 14 / 0.5 |
| "IFN-γ negatively affects erythropoiesis via upregulation of PU.1 during chronic immune activation" | 2010 | 14 / 0.5 |
| "Expression and function of CD27 on embryonic and adult hematopoietic stem cells" | 2011 | 14 / 0.5 |

**(Inter)national Conferences**

| - Dutch Society Immunology (NVVI) annual meeting | 2008-2010 | 14 / 0.5 |
| - Dutch Society Immunology Lunteren Meeting | 2010 | 14 / 0.5 |
| - Dutch Society Hematology (NVVH) annual meeting | 2009-2011 | 14 / 0.5 |

**Other**

- -

## 2. Teaching

<table>
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<th>Year</th>
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<table>
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<th>Lecturing</th>
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<td>Pieter Carrière, 9 months, Department of Experimental Immunology</td>
<td>2009 - 2010</td>
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| Other |  |

## 3. Parameters of Esteem

| Grants |  |
| Prizes |  |
Sten Libregts was born on the 28th of September 1983 in ‘s-Hertogenbosch, The Netherlands, and grew up in Vlijmen. After finishing primary school he attended high school at Het d’Oultremontcollege in Drunen. Here he followed pre-university education (VWO) and graduated in June 2001. Because of his interest in the functioning of the human body he in September 2001 started to study Biomedical Sciences at the Radboud University in Nijmegen. After performing an internship at the department of Experimental Rheumatology, where he under supervision of Dr. A.B. Blom investigated the role of synovial macrophages in MMP-induced cartilage damage during osteoarthritis, Sten obtained his Bachelor of Science degree in June 2004. He thereafter performed an internship at the department of Pathology of the University Medical Center Nijmegen. Here he set up a panel of disease markers to distinguish benign Spitz nevi from malignant Spitzoid Melanoma under the supervision of Dr. L. van Kempen. Additionally, he performed a second internship under supervision of Dr. A.D. Cook at the department of Medicine from the Royal Melbourne Hospital in Melbourne, Australia. Here he focused on determining the involvement of GM-CSF in the onset of immune-complex induced peritonitis. In June 2007 Sten obtained his Master of Science degree and decided to follow his passion for research, starting a PhD project under the supervision of Dr. M. A. Nolte in the group of Prof. Dr. R.A.W. van Lier at the department of Experimental Immunology of the Amsterdam Medical Center. Early 2011 this group moved to the Department of Hematopoiesis of Sanquin Blood Research in Amsterdam, where Sten continued his work. During his PhD, Sten focused on unravelling the mechanisms by which the activated immune system gives feedback to the hematopoietic system to alter the hematopoietic output. The results from these studies are described in this thesis. Since January 2013 Sten is working as a post-doc in the group of Prof. Dr. M.H. Wauben at the department of Cell Biology in the Faculty of Veterinary Medicine of the University of Utrecht.
Acknowledgements

‘Zweet prikt in zijn ogen, zijn hartslag zit op het omslagpunt en het zuur brandt in zijn met stof besmeurde benen als hij over de laatste kilometer kasseistroom richting finish dendert. Vijf jaar lang heeft hij naar dit moment toegeleefd. Alle minutieuze voorbereidingen en uren aan training brachten hem hier. Met de finish in zicht schieten de gedachten aan al diegenen die dit mede mogelijk hebben gemaakt door zijn hoofd…”

Ondanks dat het mijn naam is die prijkt op de voorkant van dit boekje, is het het ploegenspel van een peloton aan mensen geweest dat dit boekwerk tot stand heeft gebracht. Het peloton waarin ik me heb begeven de laatste 5 jaar was rijkelijk gevuld met groepleiders, PhD-studenten, post-docs, analisten, kamergenoten, samenwerkingen en andere labgenoten, allen met ieder met hun eigen invloed op mijn werk, en dus ook op dit boekje! Eenieder persoonlijk bedanken voor zijn bijdrage is lastig, maar het feit dat jij een boekje voor je neus hebt liggen, betekend dat ook jij je steentje hebt bijgedragen! Al vind je je naam hieronder misschien niet terug, weet dat ik je bijdrage, in welke vorm dan ook, enorm waardeer! Daarom dan ook met heel mijn hart:

“Dankjewell!”

Although it is my name that adorns the cover of this book, it is the work of a peloton of people that made this all possible. The peloton I moved in the last five years was filled with a variety of group leaders, post-docs, PhD-students, technicians, roomies, collaborations and other fellow lab members, all with their own particular influence on my work, and thus on this book! There are too many to name, but the fact that you are reading this, means that also you contributed to this work! Although you might not find your name here, know that I truly appreciate your contribution! Therefore, with all my heart:

“Thank you!”

Een aantal mensen wil ik echter in het bijzonder bedanken.

Martijn, ploegleider! Wijsheid komt met de jaren is mij altijd verteld. Wat me in 2007 te wachten stond en waar ik aan begon toen ik bij je solliciteerde was me toen nog niet helemaal duidelijk. Nu kan ik echter zeggen dat ik een excellente wetenschappelijke leerschool heb doorlopen. Fijn dat jij altijd positief bleef, het tempo er in hield en op zijn tijd een bidonnetje aanreikte, maar me af en toe ook aan het elastiek liet bungelen of in het rood liet rijden. Ik ben er alleen maar beter en wijzer van geworden en het voorkwam dat als het er om ging dat ik geparkeerd kwam te staan, een hongerklop kreeg of op de kant gezet werd. Martijn, Ik waardeer je aanhoudende enthousiasme voor de wetenschap, je immer kritische blik en oog voor detail. Bedankt voor de fijne samenwerking!

René, als Directeur Sportif hield jij de vorderingen van mijn project nauwlettend mee in de gaten. Fijn hoe jij met jouw overzicht en inzicht de puzzelstukjes zo nu en dan in elkaar liet vallen en mij weer op weg hielp als ik het koersboek net niet tot in detail had doorgekeken of de weg even kwijt was.
Appendix

Alex, meesterknecht! Aangezien jij als pionier van de BM-ploeg al een tijdje aan de slag was, kon ik in jouw slipstream snel aarden in het peloton en had jij al een weg gebaand om mij mee in het wiel te nemen. Je rust, relaxedheid en ogenschijnlijke onbezorgdheid heb ik als een fijne tegenhanger ervaren voor mijn soms wat zorgelijke karakter. Bedankt voor al je hulp tijdens experimenten en de fijn tijd gedurende bakjes, saffies, luistersessies, hakpartijen, congressen en andere fratsen. Jammer dat onze ‘free-hug’ actie nooit echt van de grond is gekomen… maar gelukkig hadden we elkaar nog!

Claudinha, soms pas dan je pas opmerkte wat iemand voor je betekende na zijn vertrek. Met plezier denk ik terug aan de vele koffies en gesprekken die we onder het zonnetje hadden. Dank je wel zo heel veel voor je praktische hulp in het laboratorium, maar vooral ook voor je ondersteuning buiten. Ik waarder je mooi optimistisch perspectief. Waar Fausto Coppi de legendarische soigneur Biagio Cavanni had die luisterde naar de klokkensignaal van zijn hart om zijn conditie te beoordelen, had ik jou. Daarom ben ik zodanig blij dat je bij ons zult zijn als paranymph! En ik heb die kleine flesje Port die je ooit voor mij gaven bewaard voor een speciale gelegenheid. Ik ben er van overtuigd dat het niet langer op zichzelf zal blijven!

Klaas & Natasja, wat een mooi duo! Ondanks dat jullie niet officieel bij de BM-ploeg horen, waren jullie wel altijd mee aan het acteren en behoren jullie in mijn ogen toch ook toe aan ons clubje. Klaas, klasbak, ik denk wel dat ik wel kan stellen dat je met jouw labervaring, bereidheid om te helpen en geduld een goede wegkapitein bent geweest. Veel succes met je VIDI! Ik wacht ondertussen rustig op de conferenties van de derykoersers van de Zesdaagse van Amsterdam probleemloos kan voorspellen. Natasja, wat konden we toch blijven vieren met onze kangoeroeburger en de kansen dat ook jij de winnaars van de dernykoersen van de Zesdaagse van Amsterdam kunt voorspellen. Hartelijk dank daarvoor!

Sulima & Felipe, jullie kwamen allebei als frisse bijdragen voor de BM en Hobit-groep tijdens het vorige jaar van mijn PhD. Sulima, je vrolijke, goedlachse en relaxte persoonlijkheid waren een mooie aanvulling voor ons ploegje. Felipe, dank je om het houden van de groep in vorm en voor jouw kritiek. Ik wens jullie allebei veel succes met jullie resterende jaar aan de universiteit!

Laura, in cycling vormen jullie soms een zo-called ‘combine’. In ons geval wilde niet alleen om een tegenstander te verslaan, maar om onze combinatie sterker te maken. Dat begon met enkele eenvoudige hangende cultuurgroepen in Amsterdam, maar spoedig werden die mooie experimenten gedurende een reeks mooie experimenten bij ons in Rotterdam, wat de erythropoëse beschreven in deel 4 heeft gedaan. Daarnaast heb ik jouw warme praatjes en open communicatie genoten, en ik denk dat hij mij in de winter van de meesten van de andere groepen heeft gedeeld. Dank je wel voor dat!

Zonder materiaal en mecaniciens is het lastig accelereren. Sandra, Lex & Suzan, als vaste kern van Algemeen 1 heb je veel gedaan voor een groot deel van het door mij gebrachte ‘materiaal’. Weet dat veel van wat je aangebracht hebt ook van jouw verdienste is. Dank je wel voor het verzorgen van onze muizen, jullie behulpaamheid en de gezelligheid die schuil ging achter de maskeren.
Dames en heren LLDM’ers! Jullie hebben je waarschijnlijk regelmatig afgevraagd waar ik nu eigenlijk mee bezig was en waarom ik soms schitterde in afwezigheid als er leuke dingen op het programma stonden… wel nu, vanwege dit boekje dus! Na de middelbare school hebben we allemaal onze eigen weg gekozen, maar zijn we onze vriendschap niet vergeten. Wat ben ik soms blij om gewoon weer even een weekendje op het oude vertrouwde nest te zijn voor een kaartspelletje, een filmje, een BBQ/kampvuur, een drankje, of gewoon een avondje relaxed op de bank met een versnapering. Lekker ouderwets lachen om slappe klets en bijdehante opmerkingen, heerlijk!

Lieve NMGN-crew! Ondanks dat we elkaar wat minder zien dan we zouden willen, is het altijd quality time als we er weer op uit trekken. Onze nachtelijke escapades in Doornroosje, muzikale trips in obscure panden in verre steden, luistersessies en chill-momentjes zorgden er voor dat de spanning even van de ketting af mocht. Het bracht mij precies de ontspanning die ik af en toe zo nodig had. Wat een boel mooie momenten, dwaze dansjes en fijne gesprekken zijn de revue gepasseerd in de afgelopen jaren! Dikke knuffel voor jullie allen! Mijn voetjes jeukten… op naar het volgende feestje!

Lieve familie, als supporters stelden jullie regelmatig de vraag: “maar wat doe je nu precies dan?” Dat blijft een lastige vraag om te beantwoorden, maar gelukkig bleven jullie hem stellen en bood het mij elke keer weer een kans om in Jip-en-Janneke-taal proberen uit te leggen waar ik me in mijn witte jas op het lab mee bezig hield. Ik ben jullie er dankbaar voor en heb er veel van geleerd.

Sweet Nina, as a scientist it is sometimes hard to comprehend that some things in life are and will remain a mystery. That however also makes life beautiful. When you walked into my life, it became more beautiful, but also a bit more complicated. But I wouldn't wish it any other way. Thanks for everything you did, everything you're doing and everything you will do!

Zuster Sanne, je directe bijdrage aan dit boekje is dan wellicht miniem, je persoonlijke invloed is van groter belang geweest. Door wat we de afgelopen jaren op het persoonlijke vlak allemaal gedeeld hebben, zowel de goede als mindere momenten, zijn we als broer en zus steeds meer naar elkaar toegegroeid. Bedankt voor al je steun en lieve woorden, je bent een schat!

Pa & Ma, dit boekje is voor jullie. Bedankt voor jullie onvoorwaardelijke steun en het warme nest dat ik elke keer weer vind als ik in Brabant thuis kom. Moeders, ik waardeer je zorgen, je luisterend oor en je mensenkennis. Blieb, met jouw rust, relativeringsvermogen en nuchterheid is er altijd een oplossing en dus een veilige haven. Al snapte ik je vroeger nooit, ik ben er nu achter wat de fiets kan betekenen; even alles van je af trappen, alles weer op een rijtje zetten en genieten van pure vrijheid!