Conditional Deletion of Ferritin H in Mice Reduces B and T Lymphocyte Populations

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Abstract

The immune system and iron availability are intimately linked as appropriate iron supply is needed for cell proliferation, while excess iron, as observed in hemochromatosis, may reduce subsets of lymphocytes. We have tested the effects of the ferritin H gene deletion on lymphocytes. Mx-Cre mediated conditional deletion of ferritin H in bone marrow reduced the number of mature B cells and peripheral T cells in all lymphoid organs. FACS analysis showed an increase in the labile iron pool. Reduced synthesis of heme-related oxygen species formation and mitochondrial depolarization. The findings were confirmed by a B-cell specific deletion using Fthlox/lox; CD19-Cre mice. Mature B cells were strongly under-represented in bone marrow and spleen of the deleted mice, whereas pre-B and immature B cells were not affected. Bone marrow B cells showed increased proliferation as judged by the number of cells in S and G2/M phase as well as BrdU incorporation. Upon in vitro culture with B-cell activating factor of the tumor necrosis factor family (BAFF), ferritin H-deleted spleen B cells showed lower survival rates than wild type cells. This was partially reversed with iron-chelator deferiprone. The loss of T cells was also confirmed by a T cell-specific deletion in Fthlox/lox;CD4-Cre mice. Our data show that ferritin H is required for B and T cell survival by actively reducing the labile iron pool. They further suggest that natural B and T cell maturation is influenced by intracellular iron levels and possibly deregulated in iron excess or deprivation.

Citation: Vanoaica L, Richman L, Jaworski M, Darshan D, Luther SA, et al. (2014) Conditional Deletion of Ferritin H in Mice Reduces B and T Lymphocyte Populations. PLoS ONE 9(2): e89270. doi:10.1371/journal.pone.0089270

Editor: Troy A. Baldwin, University of Alberta, Canada

Received April 9, 2013; Accepted January 18, 2014; Published February 21, 2014

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Funding: This work was supported by the Swiss National Science Foundation (http://www.snf.ch/E/Pages/default.aspx) grants 3100-065435 to LCK and 31003A-130488 to SAL. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

All cells need iron for the de novo synthesis of heme-, iron-, or iron-sulfur cluster containing proteins. This requires a cytoplasmic “labile iron pool” (LIP) of about 1 μM divalent iron [1]. The LIP is a transit pool at the cross-road of import and export of iron across the plasma membrane, of iron transport to mitochondria, and of iron deposition or release to and from the storage compartment of ferritin. The LIP can be measured by the quenching of the fluorescent probe calcein or by reversing the quenching with iron-specific chelators [2]. Besides of being essential, divalent iron in conjunction with side-products of mitochondrial respiration, hydrogen peroxide and superoxide ion, catalyzes the formation of radicals, collectively called “reactive oxygen species” (ROS). To escape damage by ROS, cellular defense mechanisms include a permanent feedback control over the LIP. In particular the syntheses of transferrin receptor 1 (TfR1), which functions in iron uptake, and ferritin H and L, which form the iron storage compartment to capture excess cytoplasmic iron, are adjusted to the LIP. This is achieved by the iron regulatory proteins 1 and 2, which bind to iron responsive elements on the respective mRNAs to control RNA translation and stability [3,4,5,6]. As a result, the steady state level of the LIP is maintained within a range that prevents damage, but ensures sufficient iron supply for biosynthetic pathways in the cytoplasm and mitochondria.

Ferritin is an assembled hollow protein shell composed of 24 subunits of ferritin H (Fh) and L, at variable stoichiometry that store iron [7]. Storage of iron into ferritin requires the ferroxidase activity of Fh protein [8,9]. Ferritin is thought to have a role in providing iron stores to the cytoplasm when cells have to cope with cell divisions, such as in embryos or during the immune response [10,11], to ensure de novo synthesis of iron-containing proteins. On the other hand, the role of Fth as a regulator of the LIP has been the subject of several investigations in cell culture [1,9,12,13]. Reduction of Fth expression by antisense mRNA, siRNA, or genetic ablation, increased the LIP and initiated ROS production. Although Fth synthesis is mainly translationally regulated, Fth gene transcription can also be induced by cytokines, such as TNFα, through NF-κB activation [12,14]. TNFα primarily activates the MAPK pathway ending in JNK activation and ROS accumulation, which provokes ultimately caspase-dependent cell death. The ROS-dependent death is counteracted by parallel activation of NF-κB. The Fth gene was revealed as an essential NF-κB target with an anti-apoptotic effect similar to iron chelation or ROS inhibitors [12]. Only Fth with an active ferroxidase activity protected cells, indicating that TNFα-induced ROS accumulation involves the LIP and sequestering of iron into ferritin is required to prevent cell death [12].

During their development, B and T cells undergo various steps of cell proliferation, as well as positive and negative selection to generate the immune repertoire [15,16]. The MAPK and JNK
Figure 1. Fth deleted mice show reduced number of mature B and T cells. 10–18 week old Mx-Cre transgenic Fthlox/lox mice or non-transgenic Fthlox/lox mice were injected 5 times with poly-IC over 8 days and analyzed on day 30. Results for Fthlox/lox (white) or FthΔ/Δ mice (grey) are shown as % of each cell population normalized to the average in Fthlox/lox mice (100%). E. Deletion efficiency of Fth mRNA measured in bone marrow, thymus and spleen (n = 9). F–H. Suspensions of bone marrow and spleen cells were stained with antibodies, analyzed by flow cytometry and plotted as numbers in experimental versus control mice (n = 8–9). F. Bone marrow subpopulations were identified as follows: granulocytes (Ter119−CD11b−GR1high), monocytes (Ter119−CD11b−GR1low), nucleated erythroid cells (Ter119+CD42CD82), T cells (CD4+ or CD8+) and B cells (CD19+CD45+; pool of precursor and mature B cells). G. Bone marrow B-cell populations (CD19−CD45+) were stained with relevant antibodies and gated into prepro-/-pro-B cells (IgD−IgM−), pre-/-immature B cells (IgD−μ− or IgD−IgM+) and mature B cells (IgD+IgM+) as shown in panel A. H. Splenic B-cell populations (CD19−CD45+) were stained with relevant antibodies and gated into transitional (T)1 B cells (IgDintIgMhi), T2 B cells (IgDhiIgMhi) and mature B cells (IgDhiIgMint) as shown in panel B. I–J. Suspensions of thymocytes were stained with antibodies, analyzed by flow cytometry and plotted as numbers in experimental versus control mice (n = 8–9). I. Analysis of the four major thymocyte subpopulations: double-negative (DN; CD4−CD8−CD3−), double-positive (DP; CD4+CD8+), CD4单 positive (CD4 SP; CD4−CD8−) and CD8 single positive (CD8 SP;
CD4 ‘CD8’) as shown in panel C. % of each cell population was normalized to the average in Fthlox/lox mice. J. Analysis of the four earliest, double negative (DN) thymocyte subsets (CD4+ CD8+ CD3−), DN1 (CD4+CD25−), DN2 (CD4−CD25−), DN3 (CD4−CD25+) and DN4 (CD4+CD25+) as shown in panel D. Results are compiled from three independent experiments with each having 2–3 mice per group. **p<0.0005; ***p<0.005; *p<0.05.

doi:10.1371/journal.pone.0089270.g001

pathways activated by Toll-like or T cell receptors contribute to negative selection by apoptosis, while NF-xB promotes cell survival [17,18]. Thus, as in 3T3 cell cultures, NF-xB-mediated Fth synthesis is potentially important to prevent lymphocyte death by blocking ROS formation [12].

There exist various reports that a deregulation of cellular iron supply may perturb the immune system. Cell proliferation requires iron [19] and intracellular iron stores in ferritin are thought to sustain mitogen-stimulated proliferation of immune cells [10,11]. Iron-deficiency reduces T-lymphocyte numbers and impairs natural killer cell activity [20]. Similarly, loss of iron uptake in TBJR deleted mice impairs T-cell development at an early CD4+3−3+ stage and reduces mature T-cell numbers [21]. Patients with iron-overload in hemochromatosis patients show a trend to lower CD8a+ cells [22], while idiopathic hemochromatosis patients show a trend to lower CD8a+ T cells depending on the HLA haplotype [23,24,25]. It was therefore of interest to test whether deletion of ferritin iron stores would alter lymphocyte proliferation or survival.

We have analyzed the conditional deletion of Fth by the interferon regulated Mx-Cre allele in mice. Bone marrow and peripheral lymphocyte compartments showed a partial loss of mature B and T cells. We have characterized the B and T cell subsets with respect to iron-mediated alterations and found an increased LIP and mitochondrial degranulation as hallmarks correlating with the loss of lymphocytes. Short-term cultures of splenic B cells with B-cell activating factor of the tumor necrosis factor family (BAFF) indicated that Fth was necessary for the survival of mature B cells. These findings were confirmed in vivo with B- and T-cell specific Fth deletions. The results highlight that ferritin controls the LIP and is required to prevent ROS formation and cell death.

Materials and Methods

Animals

Mice were maintained at the EPFL animal facility under pathogen free conditions and housed in individually ventilated cages. Animal experimentation was performed according to protocols approved by the Swiss Veterinary Office, authorization 1802. Fthlox/lox control mice in C57BL/6J background [9] were crossed with Mx-Cre transgenic mice [26] to obtain Mx-Cre;Fthlox/lox mutant mice. To study the conditional Fth deletion in bone marrow, thymus and spleen, 10-week old control and mutant mice were injected i.p. with polyaninosine-polyctydilic acid (poly-IC) (InvivoGen, San Diego, CA), Pacific Blue-conjugated anti-CD4 (GK1.5, BioLegend, San Diego, CA), Pacific Blue-conjugated anti-CD4 Fluor 700-conjugated anti-CD8a (55-6.7, eBioscience), biotin-conjugated anti-CD11b (M1/70, BioLegend), PE-Cy7- and Alexa Fluor 700-conjugated anti-CD19 (6D5, eBioscience), biotin-conjugated anti-CD25 (PC61.5, eBioscience), PE- and biotin-conjugated anti-CD45 (eBioR2/62, eBioscience), FITC-conjugated anti-CD44 (KM81, Immunotools, Friesoythe, Germany), PE-Cy7-conjugated anti-CD45 (30-F11, BioLegend), PE-Cy7-conjugated anti-CD45R (B220) (RA3-6B2, eBioscience), APC-conjugated anti-CD3 (AA4.1, eBioscience), FITC-conjugated anti-Gr1 (RB6-8C5, BioLegend), PE-Cy5-conjugated anti-CD11b (M1/70, Biogend), PE-Cy7-conjugated anti-CD11c (B20) (RA3-6B2, eBioscience), APC-conjugated anti-CD3 (AA4.1, eBioscience), PE- and biotin-conjugated anti-CD45 (eBioR2/62, eBioscience), biotin-conjugated anti-CD8a (55-6.7, eBioscience). Secondary antibodies were APC-Cy7- or eFluor450-conjugated streptavidin (eBioscience).

Antibodies

Primary anti-mouse antibodies to surface markers were: PerCP-Cy5.5-conjugated anti-CD3e (145-2C11, eBioscience, San Diego, CA), Alexa647-conjugated anti-CD4 (H129.19.6, BD Pharmingen, San Diego, CA), Pacific Blue-conjugated anti-CD4 (GK1.5, BioLegend, San Diego, CA), PE-Cy7- and Alexa Fluor 700-conjugated anti-CD8a (55-6.7, eBioscience), biotin-conjugated anti-CD11b (M1/70, BioLegend), PE-Cy5-conjugated anti-CD19 (6D5, eBioscience), biotin-conjugated anti-CD25 (PC61.5, eBioscience), PE- and biotin-conjugated anti-CD45 (eBioR2/62, eBioscience), FITC-conjugated anti-CD44 (KM81, Immunotools, Friesoythe, Germany), PE-Cy7-conjugated anti-CD45 (30-F11, BioLegend), PE-Cy7-conjugated anti-CD45R (B220) (RA3-6B2, eBioscience), APC-conjugated anti-CD3 (AA4.1, eBioscience), FITC-conjugated anti-Gr1 (RB6-8C5, BioLegend), PE-Cy5-conjugated anti-CD11b (M1/70, Biogend), PE-Cy7-conjugated anti-CD11c (B20) (RA3-6B2, eBioscience), APC-conjugated anti-CD3 (AA4.1, eBioscience), PE- and biotin-conjugated anti-CD45 (eBioR2/62, eBioscience), biotin-conjugated anti-CD8a (55-6.7, eBioscience). Secondary antibodies were APC-Cy7- or eFluor450-conjugated streptavidin (eBioscience).

Flow Cytometry

Cells were acquired on a FACScan®, FACSCanto® or LSRII® flow cytometer (Becton Dickinson, Franklin Lakes, NJ) and analyzed with FlowJo software (TreeStar, Ashland, OR). Cells were isolated from bone marrow, spleen, thymus and lymph nodes were isolated and passed through a 40 μm mesh, washed and resuspended in RPMI-1640 medium, 10% FCS. Isolated cells were resuspended in 10 ml DMEM, 2% FCS (with Pen/Strep and HEPES diluted 1/100) on ice. Living cells were counted using trypan blue exclusion. Antibody staining was performed with 1 to 1.5×106 cells in 96-well V-bottom plates (Falcon). Cells were blocked with 1% normal mouse serum and antibodies added in 25 μl PBS containing 2% FCS, 2 mM EDTA and 1% NaN3. Cells were washed twice with 200 μl of this buffer after each step.

Splenic B-cell Survival Assay

Splenocytes were filtered through a 40 μm-mesh Falcon cell strainer (Becton-Dickinson) and centrifuged in 8 ml complete RPMI 1640 medium (Invitrogen), 10% FCS, with antibiotics penicillin-streptomycin-neomycin (PSN) (Invitrogen), 50 μM 2-
Figure 2. Increased LIP and mitochondrial depolarization in bone marrow B-cell populations of Fth−/− mice. B220+ B cells in bone marrow were selected with PE-Cy7-conjugated anti-B220 antibody. Cells were stained with TMRM to assess their mitochondrial polarization and
mercaptoethanol over a 5 ml cushion of Ficoll-Paque (GE Healthcare, Pollards Wood, UK) at 700 × g for 30 min at 22°C. After washing in medium, the lymphocyte layer was resuspended at 10^7 cells per 100 μl MACS buffer (PBS, 0.5% BSA, 2 mM EDTA) containing 10% anti-CD19 antibody beads (Miltenyi, Bergisch Gladbach, Germany) and incubated on ice for 15 min before separating on MACS LS column (Miltenyi). CD19-positive B cells were cultured in 96-well round bottom plate at 3 × 10^5 cell per well in 200 μl complete media for 1–3 days at 37°C and 5% CO2 before assaying for viability by the cytometric scatter profile using a CyAn cytometer. Survival assays were carried out in the presence or absence of 20 ng/ml BAFF in the His-tagged, recombinant 60-mer configuration [30] (courtesy by P. Schneider). Where indicated, the iron chelator 3-hydroxy-1,2-dimethyl-4(1H)-pyridone (deferiprone) (Sigma-Aldrich) was added to cell cultures as a 10 mM stock solution in DMSO. Following antibody staining and washing in PBS, 1% BSA, 2 mM EDTA, TMRM and calcein AM, either together or separately, for 40–60 min at 37°C. In some instances 0.5 μg/ml 7-aminoactinomycin D (7AAD) was added at room temperature shortly prior to analysis. Events were acquired directly without washing using a LSRII flow cytometer or a CyAn cytometer (Dako, Glostrup, Denmark). To test depolarization, the protonophore m-chlorophenylhydrazone (CCCP) (Sigma-Aldrich) was prepared as a 100 mM stock solution in DMSO and added to the staining solution at 100 μM. For the analysis of the LIP, deferiprone was prepared as a 50 mM stock solution in 1% NaCl and added to the staining solution at 300 μM.

**Statistics**

Data are presented as average values ± SD. All FACS experiments were analyzed once for each mouse without replicates. Cell culture experiments were done in duplicates. Statistic significance was assessed by paired or unpaired Student's t-tests or by single factor Anova in case of comparison of more than two series.

**Results**

**Effects of Mx-Cre Mediated Fth Deletion on the Immune System**

To test whether the development and homeostasis of hematopoietic cell lineages depend on ferritin, we analyzed bone marrow, thymus and spleen 30 days after Mx-Cre induced Fth deletion. The deletion efficiency based on Fth mRNA was 88±2% in the bone marrow, 71±9% in thymus, and 82±7% in spleen (Fig. 1E), in the range of previous studies [26,31,32]. In the bone marrow of experimental versus control mice, no difference was found in the number of granulocytes and monocytes/macrophages, while the number of nucleated erythroid cells was increased, and that of mature T cells and B-lineage cells significantly decreased (Fig. 1F). Analysis of the developmental stages of B cells revealed a 50% reduction in mature B cells, while pre- and immature B cells were not affected (Fig. 1G). As B cells leave the bone marrow at the immature B cell stage to complete their maturation in the spleen [33], the number of the two transitional immature B cell populations (T1 and T2) as well as of mature B cells in the spleen was assessed. While T2 B-cell numbers were not altered, the number of T1 B cells was increased and that of mature B cells decreased (Fig. 1H). This finding is consistent with the reduction of mature B cells in the bone marrow.

To test whether Fth also plays a role in T lymphocyte development, the various thymocyte subsets were investigated. Thymocytes go through a double-negative stage before turning on the expression of CD4 and CD8. Once both genes are expressed, double-positive thymocytes are positively and negatively selected giving rise to mature single-positive T lymphocytes expressing either CD4 or CD8 [34], which may then migrate to the periphery and further mature [16]. In Mx-Cre transgenic Fth^+/− mice the total thymocyte number was reduced on average by 20% relative to control mice, although the reduction in double-positive thymocyte numbers was not significant (Fig. 2). Interestingly, the iron chelator def.
Figure 3. Increased LIP and mitochondrial depolarization in thymocytes of Fth deleted mice. Thymocytes were stained with Pacific Blue-conjugated anti-CD4 and Alexa Fluor 700-A conjugated anti-CD8 to analyze their state of T-cell differentiation, followed by TMRM for mitochondrial depolarization and calcein AM for cell viability and LIP content. FACS analysis was carried out on cells from Fthlox/lox (A–E) and FthΔ/Δ mice (F–J). C–E and H–J show a representative FACS gating used to distinguish double-negative cells in lower left zone (DN; CD4−/CD8α−), double-positive cells in upper right zone (DP; CD4+/CD8α+), single-positive cells for CD4 in upper left zone (CD4 SP; CD4+/CD8α−), and single-positive cells for CD8α in lower right zone (CD8 SP; CD4−/CD8α+). Most T cells showed a high calcein staining in Fthlox/lox mice representing a low LIP (A). Only about 10% of cells with polarized mitochondria showed low calcein staining, which was unquenched by the iron chelator deferiprone (B). In contrast, in FthΔ/Δ mice the proportion of low LIP cells was significantly increased (C). The percentage of high LIP cells was significantly reduced in FthΔ/Δ mice compared to Fthlox/lox mice (K).
about 80% of cells with polarized mitochondria showed a low calcein staining representing a high LIP (F) that was unquenched by deferiprone (G). Double staining with mitochondrial depolarization marker TMRM showed a similar average staining in Fthlox/lox and FthD mice in spite of a very different LIP. Only a small fraction of cells showed depolarized mitochondria. Adding the protonophore CCCP depolarized mitochondria in all cells (not shown). For the analysis of T cell subsets (C–E and H–J), only cells with polarized mitochondria (red zone of A and F) or sub-fractions thereof with low LIP (above the blue line) or high LIP level (below the blue line) were analyzed. K. Percent thymocytes with polarized mitochondria with a high LIP in total T cells or T-cell subsets of Fthlox/lox (white) and FthD mice (grey). L. Percent cells with a low TMRM fluorescence indicating depolarization in each subset of Fthlox/lox (white) and FthD mice (grey). M. Graphical representation of all subset data obtained in C–E and H–J. T cells in each subset expressed as % of T cells with polarized mitochondria in the low LIP (white), total (median grey) or high LIP (dark grey) fraction of Fthlox/lox and FthD mice. Subsets for each color and separate genotype add up to 100%. Results are average values of 7 or 8 mice ± SD. **p<0.005; ***p<0.0005; **p<0.005; *p<0.05.

doi:10.1371/journal.pone.0089270.g003

the proportion of the four subsets was unaffected. TIR1 staining was not significantly altered (not shown). To investigate further the defect in early thymocyte development, double-negative (DN) thymocytes were stained for CD44 and CD25 to distinguish the four developmental stages from DN1 to DN4. The cell number of all four stages was reduced by 30–50% in experimental mice but the fraction of each stage did not change (Fig. 1J). Therefore, the thymic T cell development defect does not correlate with the phases of genetic recombination at the T cell receptor locus, its expression and cell proliferation.

Fth Deletion Increases the Labile Iron Pool and Selects against Mature B Cells in the Bone Marrow

To test the cause of the lymphocyte decline, bone marrow B cells were examined by flow cytometry with probes for the LIP and mitochondrial polarization in combination with surface marker antibodies. B220+ B cells of bone marrow were stained with calcein AM and trimethyl rhodamine methyl ester (TMRM) to define cells with low or high LIP and with polarized or depolarized mitochondria (Fig. 2A). The same cells were also characterized with respect to CD93 and CD43 antigen expression to distinguish three major subsets (Fig. 2B) [35]. Calcein, a FITC-fluorochrome, is quenched by binding cytoplasmic divalent iron, and used to detect differences in the LIP [36,37]. Strong quenching and hence less calcein fluorescence is observed at high LIP. Reversion of the quenching by iron chelators, such as deferiprone, serves as a proof that the iron was indeed labile and accessible (Fig. 2D, 2H). Moreover, staining with the non-fluorescent calcein AM derivative distinguishes viable from dead lymphocytes. Dead cells lack the hydrolase activity necessary to remove the acetyl group from calcein AM and are negative for calcein fluorescence. Finally, the fluorescent probe TMRM is selectively taken up by polarized mitochondria due to the proton gradient. In the early stages of cell death, mitochondria depolarize and decrease in TMRM fluorescence after loss of the gradient [38,39].

In the bone marrow of Fthlox/lox mice, an inconsistent number of B220+ B cells, usually less than 10%, had a high LIP (Fig. 2C). Most of these cells showed depolarized mitochondria and did not respond to iron chelation (Fig. 2D) indicating that they were primarily non viable. They were also permeable to 7-aminoactinomycin D (7AAD) (not shown). In contrast, in FthD mice, up to 86% showed a high LIP (Fig. 2G). This strong quenching of calcein fluorescence was readily reversed by iron chelation (Fig. 2H). Thus, a high LIP fraction could only be reliably defined in the cell fraction with polarized mitochondria of FthD mice (Fig. 2G). On average 44% of total B cells in FthD mice had a high LIP (Fig. 2L), with a strong variability of 16–86% that correlated with the degree of mRNA deletion (n = 7, p<0.05). In the pre-B and immature B-cell subsets, the high LIP fraction was on average 52% in FthD mice, whereas in mature B cells it was only 21% (Fig. 2L), suggesting the loss of mature B cells with high LIP in FthD mice. These results were supported by those for mitochondrial depolarization (Fig. 2M). The mature B-cell subset showed the highest fraction of cells with depolarized mitochondria in FthD mice suggesting that they were more readily damaged by high LIP. Most of the cells with high LIP in FthD mice showed no mitochondrial depolarization in comparison to protophophore-induced depolarization (Fig. 2E). However, 15–20% of high LIP cells in FthD had depolarized mitochondria (Fig. 2G) of which a substantial fraction was consistently unquenched by iron chelation (Fig. 2H). The prevalence of a high LIP and depolarization in FthD cells suggests a causative relation between the high LIP and cell death. In addition, a selection against mature B cells was apparent in cells with polarized mitochondria (Fig. 2F, I, N). Mature B cells decreased from 43% in Fthlox/lox to 19% in FthD mice with corresponding increases in the prepro–/pro- and pre–/immature B-cell subsets. Moreover, when only cells with polarized mitochondria and high LIP were analyzed, the frequency of mature B cells was 8% in FthD mice (Fig. 2K, N).

Selection against Early and Mature T Cell Stages in Fth Deleted High LIP Cells of the Thymus

Parallel to B cells we analyzed thymocytes of the same mice for the LIP and mitochondrial depolarization (Fig. 3). Thymocytes of both Fthlox/lox and FthD mice showed distinct frequencies of high-LIP cells (Fig. 3A, F), that could be unquenched by chelation with deferiprone (Fig. 3B, G). Both Fthlox/lox and FthD mice showed some cells with complete mitochondrial depolarization unable to shift in calcein fluorescence with chelation. They were considered as dead and not included in the subset analysis. All thymocyte subsets of FthD mice showed a 3- to 8-fold increased number of high-LIP cells compared to Fthlox/lox mice (Fig. 3K). Mitochondrial depolarization (Fig. 3A, F) was increased in all T cell subsets of FthD compared to Fthlox/lox mice but reached significance only in the CD4 SP subset (Fig. 3L). Thymocytes with polarized mitochondria were further analyzed for T cell subsets, either in total or separated into low and high LIP sub-fractions (Fig. 3C–E, H–J, M). They showed a high LIP-dependent selection against the mature CD4 and CD8 SP as well as the DN subsets independent of the deletion (Fig. 3M).

B-cell Specific CD19-Cre Mediated Fth Deletion Confirms Reduction in Mature B Cells

The polyIC induced deletion by Mx-Cre leads to a complex phenotype as the Mx-promoter responds to interferon most effectively in the liver and bone marrow, but also several other tissues with lower efficiency [9,26]. To verify that Mx-Cre mediated effects were cell autonomous, we crossed Fthlox/lox mice with transgenic CD19-Cre mice to induce the Fth deletion in the earliest recognizable B-lineage cells during development [28,40]. In conjunction we made the mice homozygous for the Rosa-EYFP allele that serves as an indicator of active Cre recombination [29]. Enhanced yellow fluorescent protein (EYFP) is expressed from a retroviral enhancer that serves as an indicator of active Cre recombination [29]. In independent of the deletion (Fig. 3M). Therefore, the thymic T cell development defect does not correlate with the phases of genetic recombination at the T cell receptor locus, its expression and cell proliferation.
Figure 4. Reduced mature B-cell number in mice with a B-cell specific Fth deletion. $Fth^{+/+}, CD19$-Cre$^+$ (white) and $Fth^{+/+}$ (grey) mice carried the CD19-Cre allele for B-cell specific deletion and Rosa-EYFP allele as a marker for cells where CD19-Cre is active. B cells were in all instances identified as B220$^+$, CD19$^+$ and EYFP$^+$. A. Viable B cells among total cells were determined in different lymphoid tissues. B. B cells were stained with dihydroethidium to determine the % cells with ROS activity above background. C. Bone marrow B cells were divided into 3 subsets with antibodies against CD93 and CD43 as shown in Fig. 2B: CD93$^+$/CD43$^-$; prepro$^-$; preimmature; and CD93$^+$/$CD43^-$, mature B cells. Spleen
B cells were divided into 4 subsets with antibodies against CD21, CD23, and IgM, separating transitional stages 1 and 2, follicular, and marginal zone B cells. D. Staining with TMRM was used to determine mitochondrial polarization for each subset. Values represent the % of the parent population; CD19-Cre mice (Fig. 5A) indicating an increased S phase and side scatter in flow cytometry. Independently of the genotype, BAFF addition increased viability approximately 3-fold with respect to untreated control cells (Fig. 6A). The assay demonstrated that Fth was essential for B-cell survival. BAFF-mediated 3-day survival was reduced from 41% in Fth+/+ to 17% in FthΔ/Δ B cells of mice aged 15–20 weeks. This reduction was similar to the one observed with B cells of TACI-Fc mice transgenic for a secreted BAFF receptor, which acts as a dominant negative BAFF inhibitor [41]. With B cells of mice aged 50–70 weeks, the BAFF-mediated survival was similar to the 15–20 week group, and only slightly but not significantly lower for FthΔ/Δ B cells (Fig. 6A). This difference was, however, significant for FthΔ/Δ.EYFP+ B cells, the survival of which diminished from 54% at 15–20 weeks to 18% at 50–70 weeks (Fig. 6B). In control B cells, it was 2- to 5-fold higher (Fig. 6B) and unaffected by age (not shown). Thus, BAFF supports the survival of both deleted and undeleted cells, but the number of surviving Fth-deleted EYFP+ cells is lower and reduced further with age, indicating selection against the Fth recombination. This conclusion was supported by measuring the frequency of genomic Fth deletion and EYFP+ at 0 h and 72 h cell culture in the 50–70 week age group. At time 0 h, 81% of the viable CD19-Cre+ cells showed the Fth genomic deletion (Fig. 6C) and 54% were EYFP+ (Fig. 6D). At 72 h, the frequency of the genomic deletion was reduced to 53% and that of EYFP+ cells to 21%. Thus, the loss of approximately 30% B cells with the Fth genomic deletion and 30% EYFP+ B cells occurred concomitantly during the in vitro cell culture rather than in vivo. No negative selection against wild-type EYFP+ B cells was observed (Fig. 6D).

The importance of Fth in B cell survival was further examined by addition of the iron chelator deferoxamine (Fig. 6E). After 24 h of chelation, both BAFF-mediated and BAFF-independent viability was increased about two fold regardless of the Fth deletion. The effect of the chelator and BAFF together was additive. That chelation increased survival independently of the deletion might imply that Fth was needed for survival independently of chelatable iron in the LIP. However, chelation effectively blocked the selection against EYFP+ cells independent of the presence of BAFF, with a 3-fold increase in the viable fraction to more than 40%, almost the value obtained at the start of the experiment (Fig. 6F). Therefore, selection against survival after the Fth deletion is due to an increase of the LIP that can be rescued by addition of a chelator.

### Specific Deletion of Fth by CD4-Cre Reduces Cell Number of Primary and Peripheral T-Cell Subsets

In order to verify the observations on T cells made with Mx-Cre induced FthΔ/Δ mice, we crossed FthΔ/Δ mice with CD4-Cre mice. CD4-Cre initiates recombination as early as the DN3-TCRβ stage of T-cell development [42] and is complete in the DN4 and DP thymocytes [27,42]. T cells were analyzed in the thymus and spleen at adulthood with respect to subsets and maturation. The rate of the Fth genomic DNA deletion in thymocytes of FthΔ/Δ mice was 98 ± 1% (n = 6). FthΔ/Δ mice showed a significantly reduced cell number in the thymus, and a decrease in the spleen that was insignificant (Fig. 7A). Analysis of thymocytes showed that DN cells were unaffected, but interestingly, the CD4+CD8+ single positive and double negative subsets were greatly reduced.
Figure 6. Fth deletion blocks BAFF-supported survival of spleen B cells in vitro. B cells were isolated from either Fthlox/lox and FthD/D mice at 15–20 weeks (w) or CD19-Cre⁺Fth⁺/+ and Fth⁻/⁻ mice at 50–70 w, and compared in their response to BAFF. For this, CD19⁺ splenocytes were separated on magnetic beads and cultured in vitro in absence (white) or presence (grey) of BAFF (20 ng/ml) for 72 h. A. Cell viability was determined by FACS based on scatter 72 h after BAFF addition and expressed as % survival compared to plated cells. The additional strain secreting TACI-Fc
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whereas CD4+/CD8+ DP, and CD4 SP and to a slightly lesser extent CD6 SP cells were reduced in Fth+/+/ compared to control mice (Fig. 7B). Analysis of CD24, which is lost with maturation, was used to further distinguish immature and mature SP subsets. Mature SP subsets with low CD24 were reduced more than the less mature SP subsets with high CD24. There was a similar reduction of T cells in the periphery as measured in the spleen (Fig. 7B). The mediated Fth deletion strongly increased the LIP in all subsets of thymocytes (Fig. 7C). This increase was stronger for mature (CD24+/hi) than immature CD4 SP and CD8 SP thymocytes. In the spleen, a slight LIP increase could be detected in CD24+ cells but none in CD24− T cells (not shown). The increased LIP correlated with a slight decrease in DR3 expression (not shown). The loss of CD4 SP thymocytes and CD4 splenocytes in Fth+/+ mice was associated with increased depolarization relative to cells from control mice (Fig. 7D). In contrast, CD6 SP cells in Fth+/− thymus showed a decrease of mature CD24−/low cells with depolarized mitochondria, while CD8+ T cells in spleen showed no change. The results for these two subsets are qualitatively similar to those in the Mx-Cre induced deletion (Fig. 3L). Yet with good reproducibility, DN thymocytes in Fth+/− are EYFP+ at time 0 h and 72 h of cell culture. D. % of viable CD19+ B cells from CD19-Cre Rosa-EYFP:Fth+/− and CD19-Cre Rosa-EYFP:Fth+/+ that are EYFP+ at time 0 h and 72 h of cell culture. E. Viability of CD19+ B cells in absence or presence of iron chelator deferoxamine and BAFF after 24 h of culture. F. Viability of 15–20 w old EYFP+ B cells in absence or presence of 300 μM deferoxamine and BAFF (20 ng/ml) after 24 h of culture. In experiments A, B, E, and F, the 15–20 w old control mice were Fth+/+/ littermates without CD19-Cre, while the 50–70 w old control mice had a Fth−/−:CD19-Cre+ genotype. All cell cultures were analyzed in duplicates. Results are average values of 3 to 5 independent experiments ± SD.

**p<0.0005; **p<0.005; *p<0.05.

doi:10.1371/journal.pone.0089270.g006

Discussion

The present study shows that an Fth deletion in hematopoietic cell compartments reduced the number of B and T lymphocytes, while other cell lineages like granulocytes, monocytes and nucleated erythrocytes were not affected (Fig. 1). For B cells, the results were similar in mice deleted by Mx-Cre compared to the specific deletion with CD19-Cre (Figs. 1, 2, 4), demonstrating that the reduced development or survival was cell autonomous. Also for T cells, both Mx-Cre and CD4-Cre mice showed reduced T-cell subsets. This reduction was broader for the Mx-Cre induced deletion than the CD4-Cre mediated deletion (Figs. 1, 3, 7), possibly because CD4 is activated later in T-cell maturation. The analysis of relative subset frequencies indicates that the reduction in cell development or survival after the Fth deletion was the consequence of an increased LIP. It highlights that Fth and its ferroxidase activity are absolutely required for iron storage and scavenging of excess labile iron to protect cells from oxidative damage [8,9]. Yet, a high LIP alone may not be sufficient for cell death to occur as the overall reduction in B and T cell numbers was in the range of 30% (Figs. 1, 4, 7) while we observed rates of high LIP in the range of 50% or above (Figs. 2, 5, 7). Cell death appears to involve in addition the depolarization of mitochondria in B and T cells with a high LIP. The depolarization, associated with ROS production, starting at the pre−/−immature stage in B cells (Fig. 4B) is a characteristic of proton pump uncoupling that increases superoxide production [43]. In T cells, increased depolarization was detected in Mx-Cre deleted thymus already at the DN stage but was significant only at the CD4 SP stage (Fig. 3) while in CD4-Cre deleted thymus and spleen again only the CD4 SP stage cells were significantly different from control cells (Fig. 7). No increase in ROS activity was detectable (not shown) possibly due to the very rapid elimination of apoptotic T cells.

The absence of intracellular iron stores did not inhibit cell proliferation as overall B-cell proliferation was actually stimulated by the Fth deletion in conjunction with the loss of mature B cells (Fig. 3). It suggests that proliferating cells had a sufficient amount of iron available for protein synthesis. This finding is not necessarily in contradiction with previous studies concluding that intracellular iron stores of ferritin are needed to support the more massive cell proliferation in the context of antigen or mitogen stimulation [11]. Here, the augmented LIP following Fth deletion (Figs. 2, 3, 7) could potentially increase iron availability for proliferation although leading ultimately to cell death. During cell proliferation cells might use up excess iron for de novo enzyme biosynthesis, while fully differentiated resting cells are expected to have less biosynthetic activity and would, therefore, be more exposed to excess iron and ROS. This might explain why mainly mature B cells are affected by the Fth deletion. Similarly, for T cells, immature cells undergo expansion through cell divisions, while those which complete their maturation become resting prior to antigen stimulation [44]. Thus, increased proliferation at immature SP stages with a characteristic high CD24 expression [45,46], in both the thymus and periphery [16], could explain their resistance to excess LIP compared to mature SP stages (Fig. 7).

The in vitro B cell culture assays with BAFF provide evidence that Fth is required for the survival of B cells (Fig. 6). The equivalent effect on survival by the BAFF negative inhibitor protein TACI-Fc [30] compared to the Fth deletion suggested Fth may be induced by BAFF as reported previously [12]. However, Fth transcription was unaffected by BAFF (not shown). As iron chelation almost compensated the negative effect of the Fth deletion, it appears that keeping the LIP in check by iron storage reduces the extent of ROS and cell damage [8,9,12,47]. We conclude that Fth and BAFF are both independently required for optimal B-cell survival.

The presence of a high LIP in T cells of Fth wild-type mice associated with depolarization suggests such a process may be operable in normal T cell (Fig. 3). This would explain why the selection against mature high-LIP cells was as important in wild-type as in deleted mice (Fig. 3M). NF-κB induces Fth transcription to prevent ROS formation and cell death in 3T3 fibroblasts [12], and it may similarly be protective in lymphocytes. After deletion of Fth, lymphocytes would loose this protection, and the high LIP might shift the normal sequence of clonal selection towards increased apoptosis. This hypothesis remains somewhat speculative. In the Fth-deleted mice with Ms-Cre we rather conclude that the loss of T cells occurs at an early developmental stage prior to clonal selection (Fig. 1). For the Fth deletion in B cells, or T cells
Figure 7. CD4-Cre mediated Fth deletion induces a reduction of T cells in thymus and spleen concomitant with high LIP and mitochondrial depolarization. Lymphocytes of thymus and spleen of 5–7 weeks old Fth−/−;CD4-Cre+ or Fth−/−; control mice (white) and Fth+/−; mice (grey) were stained with calcein AM and TMRM, and in addition with anti-CD4 and anti-CD8α antibodies as detailed in Fig. 3. They were further
separated into high- and low-level CD24 expressing cells. No significant differences were visible between 3 Fth−/−, CD4-Cre mice and 3 Fth+/+ control mice, and data were pooled. A. Total viable cell number in thymus and spleen. B. Number of cells in T cell subsets in thymus and spleen of Fth−/− mice relative to control mice, set as 100%. C. % cells with low calcein staining due to quenching by high LIP. D. % cells with low TMRE staining that is a sign of mitochondrial depolarization. Results are average values of 6 mice ± SD. **p<0.0005; ***p<0.005; ****p<0.05.

doi:10.1371/journal.pone.0089270.g007

by CD4-Cre, where we observe negative selection at rather late stages, close to terminal differentiation, the question remains open. It is noteworthy that TLR4+/− mice with a perturbed iron metabolism also show severe changes in lymphocyte survival [21]. There, thymocytes did not differentiate beyond the early DN stage and B cells did not reach full maturity, a phenotype that looks similar to the one described here. The authors conclude that the supply of extracellular transferrin iron is probably required at this stage and B cells did not reach full maturity, a phenotype that remains open. Our observations may provide a sign of mitochondrial depolarization. Results are average values of 6 mice ± SD.

Acknowledgments

The authors thank P. Schneider for the TACELFc mouse strain, the 60mer His-tagged BAFF and for valuable discussions, and Stephanie Favre for technical help.

Author Contributions

Conceived and designed the experiments: LV LR DD MJ SAL LCK. Performed the experiments: LV LR DD MJ. Analyzed the data: LV LR SAL LCK. Wrote the paper: LCK.

References

1. Kakhlon O, Gruenbaum Y, Cabantchik ZI (2001) Repression of ferritin expression increases the labile iron pool, oxidative stress, and short-term growth of human erythroleukemia cells. Blood 97: 2865-2871.
2. Kakhlon O, Cabantchik ZI (2002) The labile iron pool: Characterization, measurement, and participation in cellular processes. Free Radic Biol Med 33: 1037-1046.
3. Hentze MW, Kuhn LC (1996) Molecular control of vertebrate iron metabolism - mRNA-based regulatory circuits operated by iron, nitric oxide, and oxidative stress. Proc Natl Acad Sci USA 93: 8175-8182.
4. Hentze MW, Muckenthaler MU, Andrews NC (2004) Balancing acts: molecular control of mammalian iron metabolism. Cell 117: 295-297.
5. Kuhn LC (2009) How iron controls iron. Blood 102: 3711–3718.
6. Darshan D, Vanoaica L, Richman L, Beermann F, Kuhn LC (2009) How iron controls iron. Cell Metab 10: 439–441.
7. Nieminen AL, Saylor AK, Tesfai SA, Herman B, Lemasters JJ (1995) Flow cytometry measurement of the labile iron pool in mammalian cells. Anal Biochem 236: 18086–18092.
8. Hentze MW, Silverstone A, Nishiyama K, de Sostoa A, Munn G, et al. (1990) Mechanism of ferritin uptake: activity of the H-chain and deletion mapping of the ferro-oxidase site. A study of iron uptake and ferro-oxidase activity of human liver, recombinant H-chain ferritins, and of two H-chain deletion mutants. J Biol Chem 265: 19886–19892.
9. Darshan D, Vanoaica L, Richman L, Beermann F, Kuhn LC (2009) Conditional deletion of ferritin H in mice induces loss of iron storage and liver damage. Hepatology 50: 852–860.
10. Donner SM, Silverstone A, Nishiyama K, de Sostoa A, Munn G, et al. (1990) Ferritin synthesis by human lymphocytes. Science 209: 1019–1021.
11. Golding S, Young SP (1995) Iron requirements of human lymphocytes - Relative contributions of intracellular and extracellular iron. Scand J Immunol 41: 229–236.
12. Pham CG, Babici B, Zazzeroni F, Papa S, Jones J, et al. (2004) Ferritin heavy chain upregulation by NF-κB inhibits TNFα-induced apoptosis by suppressing reactive oxygen species. Cell 119: 529–542.
13. Xie CC, Zhang N, Zhou HM, Li JQ, Li QX, et al. (2005) Distinct roles of basal and IFN-γ-induced NF-κB in the T cell response to PRRS virus-like clusters is an intrinsic property of the tumor necrosis factor family member BAX (B cell activating factor). Biochem J 386: 31–40.
14. Rolink A, Andersson J, Melchers F (2004) Molecular mechanisms guiding late stages of B-cell development. Adv Immunol 84: 201–238.
15. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K (1991) Fluorescence analysis of the labile iron pool of mammalian cells. Anal Biochem 204: 31–40.
16. Petronilli V, Miotto G, Canton M, Brini M, Colonna R, et al. (1999) Transient increase of virus-like clusters is an intrinsic property of the tumor necrosis factor family member BAX (B cell activating factor). Biochemistry 45: 2006–2013.
17. Krop I, Shaffer AL, Fearon DT, Schlüsch MS (1996) The signaling activity of murine CD19 is regulated during B cell development. J Immunol 157: 48–56.
18. Schneider P, Takatsuka H, Wilson A, Mackay F, Tardivel A, et al. (2001) Maturation of marginal zone and follicular B cells requires B cell activating factor Dml1 and DNA methylation in T cell development, function, and survival. Immunity 15: 763–774.
19. Prus E, Fialch E (2006) Fluorescence microscopy measurement of the labile iron pool in human hematopoietic cells. Cytometry Part A 73A: 22–27.
20. Nieminen AL, Saylor AK, Tesfai SA, Herman B, Lernmeters JJ (1995) Contribution of the mitochondrial permeability transition to lethal injury after exposure of hepatocytes to t-butylhydroperoxide. Biochem J 307: 99–106.
21. Petronilli V, Kakhlon O, Glickstein H, Breuer W, Cabantchik ZL (1997) Repression of ferritin expression increases the labile iron pool, oxidative stress, and short-term growth of human erythroleukemia cells. Blood 97: 2865-2871.
22. Kakhlon O, Gruenbaum Y, Cabantchik ZI (2001) Repression of ferritin expression increases the labile iron pool, oxidative stress, and short-term growth of human erythroleukemia cells. Blood 97: 2865-2871.
23. Xie CC, Zhang N, Zhou HM, Li JQ, Li QX, et al. (2005) Distinct roles of basal and IFN-γ-induced NF-κB in the T cell response to PRRS virus-like clusters is an intrinsic property of the tumor necrosis factor family member BAX (B cell activating factor). Biochem J 386: 31–40.
24. Han H, Tanigaki K, Yamamoto K, Kuroda K, Yoshimoto M, et al. (2002) Inducible kinase gene knockout in mouse T cells reveals its essential role in T versus B lineage development. Int Immunol 14: 637–645.
25. Rolink A, Andersson J, Melchers F (2004) Molecular mechanisms guiding late stages of B-cell development. Immunol Rev 197: 41–50.
26. Krop I, Shaffer AL, Fearon DT, Schlüsch MS (1996) The signaling activity of murine CD19 is regulated during B cell development. J Immunol 157: 48–56.
27. Schneider P, Takatsuka H, Wilson A, Mackay F, Tardivel A, et al. (2001) Maturation of marginal zone and follicular B cells requires B cell activating factor Dml1 and DNA methylation in T cell development, function, and survival. Immunity 15: 763–774.
28. Prus E, Fialch E (2006) Fluorescence microscopy measurement of the labile iron pool in human hematopoietic cells. Cytometry Part A 73A: 22–27.
factor of the tumor necrosis factor family and is independent of B cell maturation antigen. J Exp Med 194: 1691–1697.

42. Wolfer A, Bakker T, Wilson A, Nicolas M, Ioannidis V, et al. (2001) Inactivation of Notch 1 in immature thymocytes does not perturb CD4 or CD8 T cell development. Nat Immunol 2: 233–241.

43. Johnson-Cadwell LI, Jekabsons MB, Wang A, Polster BM, Nicholls DG (2007) ‘Mild Uncoupling’ does not decrease mitochondrial superoxide levels in cultured cerebellar granule neurons but decreases spare respiratory capacity and increases toxicity to glutamate and oxidative stress. J Neurochem 101: 1619–1631.

44. Ernst B, Surh CD, Sprent J (1995) Thymic selection and cell-division. J Exp Med 182: 961–971.

45. Nielsen PJ, Lorenz B, Muller AM, Wenger RH, Bronnbacher F, et al. (1997) Altered erythrocytes and a leaky block in B-cell development in CD24/HSA-deficient mice. Blood 89: 1058–1067.

46. Boursalian TE, Golob J, Soper DM, Cooper CJ, Fink PJ (2004) Continued maturation of thymic emigrants in the periphery. Nature Immunol 5: 418–425.

47. Pourzand C, Watkins RD, Brown JE, Tyrrell RM (1999) Ultraviolet A radiation induces immediate release of iron in human primary skin fibroblasts: the role of ferritin. Proc Natl Acad Sci USA 96: 6751–6756.