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Heme Exporter FLVCR Is Required for T Cell Development and Peripheral Survival

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All aerobic cells and organisms must synthesize heme from the amino acid glycine and the tricarboxylic acid cycle intermediate succinyl CoA for incorporation into hemoproteins, such as the cytochromes needed for oxidative phosphorylation. Most studies on heme regulation have been done in erythroid cells or hepatocytes; however, much less is known about heme metabolism in other cell types. The feline leukemia virus subgroup C receptor (FLVCR) is a 12-transmembrane domain surface protein that exports heme from cells, and it was shown to be required for erythroid development. In this article, we show that deletion of FLVCR in murine hematopoietic precursors caused a complete block in αβ T cell development at the CD4+CD8+ double-positive stage, although other lymphoid lineages were not affected. Moreover, FLVCR was required for the proliferation and survival of peripheral CD4+ and CD8+ T cells. These studies identify a novel and unexpected role for FLVCR, a major facilitator superfamily metabolite transporter, in T cell development and suggest that heme metabolism is particularly important in the T lineage. The Journal of Immunology, 2015, 194: 000–000.

The role of heme as a prothetic group in proteins involved in oxygen transport, electron transfer, and catalysis has been long appreciated. Heme is critical for mitochondrial oxidative phosphorylation, and heme deficiency impairs assembly of the electron chain subunits (1). Heme also has important regulatory functions. It regulates erythroid lineage differentiation by binding transcriptional (2) and translational regulators of globin synthesis (3). On the organismal level, heme synthesis and the circadian clock are reciprocally regulated (4), and heme plays a role in integrating the internal circadian clock with metabolic processes (5). Although the enzymatic steps of heme synthesis and degradation have been well characterized (Supplemental Fig. 1), less is known about intra- and intercellular heme trafficking (7). Free heme causes lipid peroxidation and oxidative damage and must be regulated carefully (8). The feline leukemia virus subgroup C receptor (FLVCR), a 12-transmembrane domain protein in the major facilitator superfamily (MFS) (9), was shown by our group to export heme (10). The gene encoding FLVCR is referred to as FLVCR1 in humans and Mfsd7b in mice; to avoid confusion and maintain consistency with the existing literature, we refer to the gene and protein as FLVCR and FLVCR in this article. Conditional deletion of FLVCR in neonatal or adult mice caused severe anemia (11), similar to the erythroid failure seen in cats viremic with feline leukemia virus subgroup C (FeLV-C), in which cell surface expression of FLVCR is inhibited by viral interference (12, 13). Older studies noted that cats viremic with FeLV-C had thymic aplasia and lymphopenia (14), although it was not known whether lymphopenia was due to cell-intrinsic loss of FLVCR or secondary to chronic viremia and/or anemia. To answer this question, we developed and studied several models in which FLVCR function could be knocked out in lymphoid cells or more specifically in T cells during development.

Materials and Methods

Mice

Flvcr<sup>flox/flox</sup>;Mx-cre mice and controls were described previously (11) and were backcrossed to C57BL/6 mice (The Jackson Laboratory) for 10 generations. C57BL/6, CD45.1 (Pep3b), and Rag1<sup>−/−</sup> mice (15) were obtained from The Jackson Laboratory. Homozygous Lck-cre and CD4<sup>+</sup>cre mice (16) were obtained from Taconic and crossed to Flvcr<sup>flox/flox</sup> mice to generate Flvcr<sup>flox/flox</sup>;Lck-cre and Flvcr<sup>flox/flox</sup>;CD4<sup>+</sup>cre mice. OT-I (17) and OT-II (18) mice were crossed to Flvcr<sup>flox/flox</sup>;Mx-cre mice. OT-I;Flvcr<sup>flox/flox</sup>;Mx-cre mice were bred to Lck-cre mice to obtain OT-I;Flvcr<sup>flox/flox</sup>;Lck-cre. All mice were bred and maintained in a specific pathogen–free barrier facility at the University of Washington. Animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of Washington.

Noncompetitive and competitive transplants

Flvcr<sup>+/+</sup>, Flvcr<sup>−/−</sup>, and Flvcr<sup>+/−</sup> mice were generated by mating 6–12-wk-old Flvcr<sup>+/+</sup>;Mx-cre, Flvcr<sup>flox/flox</sup>;Mx-cre, or Flvcr<sup>flox/flox</sup>;Mx-cre mice with polyinosinic-polyctidylic acid (polyI:C; 0.15 mg i.p., three doses every other day; Amersham). Eight or nine days later, bone marrow mononuclear cells (BMCs) from the femurs and/or tibias of polyI:C-treated mice were harvested, and 2.5 × 10<sup>6</sup> BMCs were injected i.v. into sublethally irradiated (6.5 Gy) Rag1<sup>−/−</sup> mice. For competitive transplants, Flvcr<sup>+/−</sup>;Mx-cre,
Flvcr<sup>−/−;Mx-cre</sup>, Flvcr<sup>+/−;Mx-cre</sup>, and CD45.1 mice were treated with the same polyclonal protocol as described above. Eight or nine days later, BMs from the femurs and/or tibias of polyclonal-treated mice were harvested, and 5 × 10<sup>6</sup> BMs from Flvcr<sup>+/−;Mx-cre</sup> or control mice were mixed with 5 × 10<sup>6</sup> CD45.1 BMs and injected i.v. into lethally irradiated (11 Gy) Rag1<sup>−/−</sup> mice or C57BL/6 × Peplb F1 (CD45.1/CD45.2) mice.

**Blood cell analysis**

Mice were bled retro-orbitally into EDTA anti-coagulated microtainer tubes (Becton Dickinson). Complete blood counts were performed on a Hemavet 950FS analyzer (Drew Scientific) programmed for mouse blood.

**Flow cytometric analysis and sorting**

Flow cytometric analysis was performed using a FACSCanto II and an LSR II (BD Biosciences); cells were sorted using a BD FACSaria (BD Biosciences) at the Cell Analysis Facility, Department of Immunology, University of Washington. Flow data were analyzed with FlowJo 7.6 (TreeStar).

**Abs and reagents**

Fluorochrome-conjugated Abs were purchased from BD Biosciences and eBioscience.

**Tissue staining**

Whole thymus was fixed in 10% buffered formalin. Fixed thymus was paraffin embedded, sectioned, and stained with H&E by the Experimental Histopathology Facility at the Fred Hutchinson Cancer Research Center.

**Quantitative RT-PCR analysis**

RNA was isolated from sorted cells using an RNeasy Plus Mini Kit (Qiagen) and reverse transcribed using iScript reverse transcription (Biorad), per the manufacturer's instructions. Quantitative RT-PCR (qRT-PCR) was performed on CDNA or genomic DNA using either a TaqMan assay or a SYBR Green assay with gene-specific primers obtained from Integrated Source at the Fred Hutchinson Cancer Research Center.

**Results**

Thymocytes from Flvcr<sup>−/−;Cdx4-cre</sup> or Flvcr<sup>+/−;Cdx4-cre</sup> mice were mixed 2:1 with CD45.1 thymocytes and incubated with 10 μM CFSE (Invitrogen) in serum-free HBSS for 15 min at 37°C. The reaction was quenched with pure FCS, and the cells were washed twice with serum-free medium; 1.5 × 10<sup>6</sup> CFSE-labeled thymocytes were injected i.v. into sublethally irradiated Rag1<sup>−/−</sup> mice (6.5 Gy). A sample of the CFSE-labeled mix also was analyzed by flow cytometry for CD4 and CD8 staining so the starting CD45.2:CD45.1 ratio of CD4 single-positive (SP) cells and CD8SP cells could be determined. Recipient mice were sacrificed at the specified time points, and splenocytes were counted and analyzed by flow cytometry.

**Microarray sample preparation**

Three separate competitive transplant cohorts were generated as described above using lethally irradiated Rag1<sup>−/−</sup> mice (11 Gy) transplanted with polyclonal-treated Flvcr<sup>+/−;Mx-cre</sup> or Flvcr<sup>+/−;Cdx4-cre</sup> BMs mixed with polyclonal-treated CD45.1 BMs. At 12 wk posttransplant, thymocytes were harvested from three recipients/group and pooled, and CD45.2<sup>+</sup>CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) cells were sorted. This was repeated for the three cohorts, resulting in three Flvcr<sup>+/−</sup> and three Flvcr<sup>−/−</sup> replicates. After sorting, cells were washed twice with cold PBS, and cell pellets were frozen and stored at −80°C until all samples were collected. RNA was isolated using a QIAGEN RNasey Plus Mini Kit, per the manufacturer’s instructions, and the yield was determined on a NanoDrop ND-1000 spectrophotometer (Thermo Scientific). All samples had an appropriate yield (>100 ng total RNA) and were analyzed for RNA integrity using an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA determined to be of high quality was converted to cDNA and labeled with biotin.

**Microarray data analysis**

The dataset consisting of all arrays was processed using the Bioconductor package lumi by using quantile normalization (22). The data were initially filtered by flagging probes that were below a defined signal “noise floor,” which was calculated as the 75th percentile of the negative-control probe signals within each array. A probe was retained if at least two of three samples in at least one condition were not flagged by the intensity filter. We filtered the data further through the application of a variance filter, using the “short” function of the Bioconductor package genefilter. Differential gene expression was determined using the Bioconductor package limma (23), and a false discovery rate method was used to correct for multiple testing (24). Significant differential gene expression was defined as |log<sub>2</sub>(ratio)| ≥ 0.585 (≥ 1.5-fold), and false discovery rate = 5%.

**Statistical analyses**

Statistical analyses were performed with Prism version 5.0 (GraphPad), using an unpaired two-tailed Student t test. A p value < 0.05 was considered statistically significant.

**Accession data**

The microarray data have been submitted to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE50202.

**Results**

**FLVCR is required for T cell development beyond the DP stage**

To determine whether FLVCR plays a role in lymphocyte development, we transplanted BMs from polyclonal-treated Flvcr<sup>−/−;Mx-cre</sup>, Flvcr<sup>+/−;Mx-cre</sup>, Flvcr<sup>+/−;Mx-cre</sup> mice, Flvcr<sup>−/−;Cdx4-cre</sup> mice, or Flvcr<sup>+/−;Cdx4-cre</sup> mice into sublethally irradiated Rag1<sup>−/−</sup> mice, referred to henceforth as Flvcr<sup>−/−</sup>, Flvcr<sup>+/−</sup>, or Flvcr<sup>+/−</sup> mice. Cells with the Mx<sup>−/−</sup> transgene express cre-recombinase after exposure to IFN-α or IFN-β, and the double-stranded synthetic RNA polyclonal induces IFN production and cre-mediated gene excision in Mx<sup>−/−</sup> BMs (25). The use of sublethal irradiation permitted reconstitution of endogenous Rag1<sup>−/−</sup> hematopoiesis; therefore, the mice that received Flvcr<sup>+/−</sup> BMs were not anemic (data not shown), and any
T or B cells present derived from the transplanted marrow. Strikingly, we found very few T cells in the blood (Fig. 1A, 1B), spleen, and lymph nodes (Supplemental Fig. 2A, 2B) of Flvcr$^{−/−}$ mice compared with controls. Both CD4$^{+}$ and CD8$^{+}$ T cells were similarly affected. B cells were present in normal numbers (Fig. 1B), suggesting that FLVCR loss selectively blocked development downstream of the common lymphoid progenitor stage.

We next examined the thymuses in Flvcr$^{−/−}$ mice and found that they appeared grossly normal (Fig. 1C, left panel). Early thymic progenitors derived from bone marrow hematopoietic stem cells enter the thymus as CD4$^{−}$CD8$^{−}$ double-negative (DN) cells that subsequently progress through four substages (DN1–DN4) (26). Mice had similar total numbers of DN cells (Fig. 1D, 1E). Because Rag1$^{−/−}$ mice have DN thymocytes, we examined Flvcr$^{−/−}$ DN thymocytes obtained from mice that were lethally irradiated and transplanted with a mixture of congenically marked control or Flvcr-deleted BMs and WT BMs (described in greater detail in the next section) to analyze the DN1–DN4 subsets. Although there was a deficit of Flvcr$^{−/−}$ DN1 cells, similar numbers of Flvcr-deleted and control cells were present at each of the subsequent three stages (Supplemental Fig. 2C, 2D). During DN3, thymocytes that have successfully rearranged the TCR $\beta$-chain ($(\beta$-selection checkpoint) undergo a proliferative burst before becoming CD4$^{+}$CD8$^{+}$ DP cells (26). The number of CD4$^{+}$CD8$^{+}$ DP thymocytes was unaffected by FLVCR loss (Fig. 1D, 1E); thus, FLVCR is not required for $\beta$-selection or subsequent late DN cell proliferation.

DP thymocytes rearrange their TCR$\alpha$-chain and undergo positive and negative selection to identify thymocytes capable of recognizing peptide–MHC complexes on APCs without excessive autoreactivity before finally differentiating into CD4$^{+}$ or CD8$^{+}$ SP cells (27). CD4$^{+}$ and CD8$^{+}$ SP thymocytes were severely decreased in Flvcr$^{−/−}$ mice (Fig. 1D, 1E). On thymic histology, there were fewer medullary regions in Flvcr$^{−/−}$ mice (lighter regions in Fig. 1C, right panel), correlating with the decreased number of SP cells, typically found in the thymic medulla.

We examined Flvcr gene expression in sorted normal thymic subsets and found that it peaked during the DN3 stage and then declined (Fig. 1F, middle panel). The expression of Flvcr during thymic development mirrored that of the aminolevulinic acid synthase1 gene (Alas1) (Fig. 1F, top panel), encoding the rate-limiting enzyme in heme synthesis, consistent with FLVCR’s role in maintaining intracellular heme homeostasis. Heme oxygenase 1 (Hmox1), which encodes an enzyme that breaks down heme, was present at high levels early during thymic development, but it was downregulated after the DN stage (Fig. 1F, bottom panel); thus, FLVCR is required for T cell development beyond the DP stage.

**FIGURE 1.** FLVCR is required for T cell development beyond the DP stage. (A) Flow cytometric analysis of peripheral blood leukocytes by B220 and CD3 staining (upper panels) and CD4 and CD8 staining (lower panels) of cells within the CD3$^{+}$ gate. Numbers indicate the percentage of cells within the gate. (B) Absolute numbers of B220$^{+}$, CD3$^{+}$, CD3$^{+}$CD4$^{+}$, and CD3$^{+}$CD8$^{+}$ PBLs. Bars show mean and SEM of three or four mice/group. *p < 0.03, **p < 0.009. (C) Representative gross thymuses from Flvcr$^{+/+}$, Flvcr$^{+/−}$, and Flvcr$^{−/−}$ mice (left panel) and representative thymus sections from Flvcr$^{+/+}$ and Flvcr$^{−/−}$ mice stained with H&E (original magnification $\times$100) (right panel). (D) CD4 and CD8 flow staining of Flvcr$^{+/+}$ and Flvcr$^{−/−}$ thymocytes. (E) Enumeration of thymocyte subsets. *p = 0.05, **p = 0.02. (F) Heme regulatory gene expression quantified by qRT-PCR in thymocyte subsets relative to peripheral splenic CD8$^{+}$ T cells. Mean and SEM from three mice are shown. Data are representative of two (C and F) or three (A, B, D, and E) separate experiments with three or four mice/group.
later-stage thymocytes may rely more on FLVCR to regulate intracellular free heme and prevent toxicity.

**Requirement for FLVCR is T cell intrinsic**

To determine whether the block was due to FLVCR loss in APCs (BM and non-BM derived) or stromal cells in the thymus, rather than to T cell–intrinsic FLVCR loss, we performed competitive-repopulation studies, transplanting polyI:C-treated Flvcr<sup>floox/flox</sup>; Mx-cre or Flvcr<sup>floox/+</sup>; Mx-cre (CD45.2) BMs mixed 1:1 with congenically marked (CD45.1) WT BMs into lethally irradiated Rag1<sup>−/−</sup> mice. Few CD45.2<sup>+</sup> Flvcr<sup>−/−</sup> CD4SP or CD8SP cells were found in recipients, although CD45.1<sup>+</sup> WT CD4SP and CD8SP cells were found in expected proportions (Fig. 2A). Therefore, the loss of SP T cells was not due to thymic stromal cell FLVCR deficiency. The proportion of Flvcr<sup>−/−</sup> DP thymocytes was increased, whereas CD4SP and CD8SP thymocytes were markedly decreased (Fig. 2B). We sorted CD45.2<sup>+</sup> (Flvcr<sup>flox/flox</sup>; Mx-cre-derived) B220<sup>+</sup>, Gr-1<sup>−</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> peripheral blood cells and quantified Flvcr deletion through qRT-PCR. Although donor BMs and donor-derived peripheral B220<sup>+</sup> and Gr-1<sup>−</sup> blood cells were nearly completely deleted (BM mean 97.5%, SD 2.1, n = 3; B220<sup>+</sup> mean 99.3%, SD 0.9, n = 5; Gr-1<sup>−</sup> mean 98.1%, SD 1.6, n = 5), donor-derived peripheral CD4<sup>+</sup> and CD8<sup>+</sup> cells were only partially deleted (CD4<sup>+</sup> mean 68.9%, SD 19.2, n = 5; CD8<sup>+</sup> mean 70.8%, SD 19.2, n = 5).

**FIGURE 2.** The requirement for FLVCR is T cell intrinsic. (A) Representative flow analysis of thymocytes from mice transplanted with a 1:1 mix of Flvcr<sup>−/−</sup> CD45.1 BMs with CD4 and CD8 expression shown on gated CD45.1 or CD45.2 thymocytes. (B) Quantification of CD45.2 thymocyte subsets from competitively repopulated (CR) mice that received Flvcr<sup>+/+</sup> CD45.1 (black circles) or Flvcr<sup>−/−</sup> CD45.1 (red squares). Each symbol represents an individual mouse. *p < 0.0001. (C) Representative flow analysis with TCR<sub>gd</sub> and TCR<sub>b</sub> expression shown on gated CD45.2 Flvcr<sup>+/+</sup> (left panels) and Flvcr<sup>−/−</sup> (right panels) DN cells (top panels) from CR mice. Numbers indicate the percentage of TCR<sub>gd</sub><sup>+</sup> cells within the gate. Enumeration of the proportion and absolute number of Flvcr<sup>+/+</sup> and Flvcr<sup>−/−</sup> TCR<sub>gd</sub>-expressing cells in the thymus (middle panels) and spleen (bottom panels) are shown. (D) Representative flow analysis with CD69 and TCR<sub>b</sub> expression on Flvcr<sup>+/+</sup> and Flvcr<sup>−/−</sup> thymocytes (gated on CD45.2) from CR mice with gates indicating successive stages of development (upper left panel). CD4 and CD8 expression on gated populations with the percentage of cells in the population shown in parentheses (remaining upper panels). CD45.2:CD45.1 ratios for the gated populations are shown for individual mice (lower panels). *p = 0.02, **p = 0.001. (E) CD24 and TCR<sub>b</sub> staining on gated CD4SP and CD8SP thymocytes from representative mice (left panel). Ratio of mature SP cells (CD24<sup>lo</sup> TCR<sub>b</sub><sup>hi</sup>) to immature SP cells (ISP, CD24<sup>hi</sup> TCR<sub>b</sub><sup>lo</sup>), which are precursors to the DP stage, is shown in the bar graphs (right panel). Data are representative of four separate experiments with four or five mice/group (A, B, and D) or two separate experiments with three mice/group (C). *p < 0.0001, **p = 0.0006.
CD8+ mean 58.7%, SD 12.6, n = 5). Thus, the few peripheral T cells present from polyI:C-treated donor Flvcr<sup>-/-</sup> Mx-cre BMs likely expanded from a few precursors in which only one Flvcr allele was deleted (resulting in 50% deletion), reinforcing the conclusion that FLVCR is absolutely required for CD4<sup>+</sup> and CD8<sup>+</sup> T cell development.

**FLVCR is not required for postnatal γδ T cell development.**

Given that FLVCR loss impacted T cell development specifically, we also looked at the numbers and early thymic development of γδ T cells. TCRβ, γ, and δ gene rearrangements occur during the DN stage, and thymocytes that express a functional TCRγδ receptor on the surface diverge from stage, and thymocytes that express a functional TCR on the surface diverge from stage, and there is little subsequent proliferation (30). Therefore, we predicted that thymic development in Flvcr<sup>-/-</sup> mice would proceed normally through to the SP stage. Flow analysis of Flvcr<sup>-/-</sup> Lck-cre and control thymocytes showed that early Flvcr deletion blocked development beyond the DP stage, and Flvcr<sup>-/-</sup> Lck-cre mice had reduced numbers of CD4SP and CD8SP cells (Fig. 4A). Few peripheral CD4<sup>+</sup> or CD8<sup>+</sup> T cells were seen in Flvcr<sup>-/-</sup> Lck-cre mice (data not shown). Thus, FLVCR was required for primary T cell development and not only for T cell development after bone marrow transplantation.

In contrast to Flvcr<sup>-/-</sup> Lck-cre mice, Flvcr<sup>-/-</sup> CD4<sup>+</sup> mice had relatively normal thymic T cell development (Fig. 4B). Despite normal thymic T cell development, Flvcr<sup>-/-</sup> CD4<sup>+</sup> mice had few peripheral CD4<sup>+</sup> or CD8<sup>+</sup> T cells (Fig. 4C, upper panel). The few CD8<sup>+</sup> T cells present were nearly all CD44<sup>hi</sup> (Fig. 4C, lower panel), when Flvcr<sup>-/-</sup> CD4<sup>+</sup> peripheral T cells were sorted and genomic Flvcr deletion was assessed, CD8<sup>+</sup> T cells showed 49.3% deletion (SD 0.03, n = 3). Thus, the few CD44<sup>hi</sup> CD8<sup>+</sup> T cells present in Flvcr<sup>-/-</sup> CD4<sup>+</sup> mice were likely due to homeostatic proliferation of a small number of incompletely deleted CD8<sup>+</sup> T cells.

To determine whether Flvcr<sup>-/-</sup> CD4<sup>+</sup> SP cells were maturing normally, we examined surface expression of CD24 and CD69 on Flvcr<sup>-/-</sup> CD4<sup>+</sup> and control SP cells. Few CD24<sup>hi</sup> cells (ISP, DP precursors) were found among either Flvcr<sup>-/-</sup> or CD4<sup>+</sup> T cells (Supplemental Fig. 2E). CD69 downregulation is required for sphingosine-1-phosphate (S1P) receptor 1 expression and function (31). The S1P<sub>1</sub>/S1P receptor 1 interaction mediates SP cell thymic emigration in response to S1P gradients from peripheral blood (31). Flvcr<sup>-/-</sup> CD4<sup>+</sup> and CD8<sup>+</sup> T cells evinced similar downregulation of CD69 compared with controls (Supplemental Fig. 2E). Thus, deletion of Flvcr late during the DP stage allowed for the production of normal numbers of phenotypically normal SP cells.

**FLVCR is required for peripheral T cell survival.**

To characterize the temporal requirement for FLVCR during T cell development, Flvcr<sup>+/-</sup> mice were crossed to Lck-cre- or CD4<sup>+</sup>-transgenic mice (16). Lck-cre mice express cre in the T cell lineage during the early DN stage, leading to early DN Flvcr deletion. The FLVCR protein has a long half-life (29), but because there is a strong proliferative burst at the end of the DN stage, we predicted that, in Flvcr<sup>+/-</sup> Lck-cre mice, FLVCR protein would be largely diluted by multiple cell divisions, resulting in little FLVCR at the DP stage and, consequently, a block in T cell development. In CD4<sup>+</sup>-cre mice, cre is expressed in the late DN/early DP stage, and there is little subsequent proliferation (30). Therefore, we predicted that thymic development in Flvcr<sup>floxflo</sup> CD4<sup>+</sup>-cre mice would proceed normally through to the SP stage.

Flow analysis of Flvcr<sup>floxflo</sup> Lck-cre and control thymocytes showed that early Flvcr deletion blocked development beyond the DP stage, and Flvcr<sup>floxflo</sup> Lck-cre mice had increased numbers of CD4SP and CD8SP cells (Fig. 4A). Few peripheral CD4<sup>+</sup> or CD8<sup>+</sup> T cells were seen in Flvcr<sup>floxflo</sup> Lck-cre mice (data not shown). Thus, FLVCR was required for primary T cell development and not only for T cell development after bone marrow transplantation.

In Figure 3, positive selection is impaired in Flvcr<sup>++</sup> transgenic TCR. CD4<sup>+</sup> and CD8<sup>+</sup> TCR expression on thymocytes from Rag<sup>1<sup>-/-</sup></sup> mice transplanted with Flvcr<sup>++</sup> and Flvcr<sup>++</sup> BMs expressing transgenes encoding the MHC class I–restricted OT-I TCR (A) or the MHC class II–restricted OT-II TCR (B). Enumeration of CD8SP and CD4<sup>+</sup> TCR thymocytes [lower left panel in (A) and (B)] and of CD8<sup>+</sup> vs<sup>+</sup> PBLs in OT-I mice [(A) lower right panel] and CD4<sup>+</sup> vs<sup>+</sup> PBLs in OT-II mice (B) lower right panel). Data are representative of two separate experiments with three or four mice/group. *p = 0.02, **p < 0.0001.
To determine the fate of Flvcr flox/flox;CD4-cre SP thymocytes in the periphery, we adoptively transferred CFSE-labeled CD45.2 Flvcr flox/flox;CD4-cre or Flvcr+/+;CD4-cre thymocytes mixed with WT CD45.1 thymocytes into sublethally irradiated Rag1−/− mice. CFSE analysis of recipient splenocytes was performed (Fig. 4D, 4E); each plot shows CFSE dilution by CD45.1 WT competitors above CD45.1 Flvcr+/+ (left panels) or Flvcr−/− (right panels) thymocytes. At days 4 and 8 posttransfer, Flvcr−/− CD8+ T cells showed little CFSE dilution compared with cotransferred CD45.1 WT CD8+ T cells or WT littermate Flvcr+/+ CD8+ T cells transferred into another host (Fig. 4D, 4F). These results suggest that FLVCR is required for the DP to SP transition during T cell development, as well as for the survival and/or proliferation of T cells in the periphery under lymphopenic conditions.

Re-expression of FLVCR in deleted BMs rescues T cell development

To confirm that the failure of Flvcr-deleted hematopoietic cells to generate T cells was due to the loss of FLVCR expression and not to an off-target effect, we performed rescue transplants with Flvcr flox/flox;Lck-cre BMs transduced with vectors encoding full-length FLVCR-EGFP fusion protein (FLVCR-EGFP) or EGFP alone. Re-expression of FLVCR led to increased numbers of peripheral CD4+ and CD8+ T cells compared with EGFP-transduced controls (Fig. 5A), and thymic analysis showed that, although there were few CD4SP or CD8SP cells among the EGFP− Flvcr-deleted thymocytes, there were normal numbers of CD4SP and CD8SP cells among the EGFP+ thymocytes in the same mouse (Fig. 5B). A recent report suggested that an alternate splice form of Flvcr leads to mitochondrial targeting of a partial FLVCR (FLVCR1b) and that FLVCR1b exports heme from the mitochondria (32). The rescue construct that we used encodes full-length FLVCR without intronic sequences and is unlikely to be spliced; these data suggest that full-length FLVCR is necessary and sufficient for T cell development.

Flvcr loss does not cause global transcriptional changes in DP thymocytes

To confirm that the failure of Flvcr-deleted hematopoietic cells to generate T cells was due to the loss of FLVCR expression and not to...
cells, whose main function is to synthesize heme and hemoglobin, there is no known specific role for heme in T cell development or function. Heme binds and inhibits the transcription factor BACH2, a negative regulator of BLIMP1, the master regulator of plasma cell differentiation, thus affecting humoral immunity (33). A recent study showed loss of regulatory T cells in Bach2−/− mice (34), although other CD4+ and CD8+ T cell subsets were present in normal numbers. Thus, FLVCR function during T cell development is unlikely to be mediated by BACH2. However, this raised the question whether FLVCR loss/heme dysregulation alters transcription in DP thymocytes. Therefore, we carried out transcriptional profiling on sorted Flvcr−/− and Flvcr−/−/− DP thymocytes; surprisingly, there were very few transcriptional differences (Supplemental Fig. 3A). The increased expression of Cox6a2 seen by profiling (Supplemental Fig. 3B, Supplemental Table I) was validated in independent cohorts by qRT-PCR (Supplemental Fig. 3C). COX6A2 is a subunit of respiratory cytochrome c oxidase (complex IV) expressed in striated muscle, and it was shown to play a role in respiratory uncoupling in muscle (35). Studies in yeast demonstrated that COX6 expression is upregulated by heme (36); thus, the increase in Cox6a2 expression in Flvcr-deleted thymocytes may result from increased heme levels. The lack of transcriptional alterations in Flvcr-deleted DP subsets implies that FLVCR loss disrupts development in the late DP stage, rather than the block occurring at this stage as a result of cumulative deleterious effects during earlier stages.

**Discussion**

In this article, we demonstrate that FLVCR, an MFS protein previously shown to transport heme and to be required for erythroid development, is absolutely required for αβ T cell development beyond the DP stage. Through competitive transplantation, we showed that FLVCR’s role in developing thymocytes is T cell intrinsic. Flvcr-deleted thymocytes failed selection, as shown by the failure of FLVCR-deficient thymocytes to upregulate CD69, as well as by the failure of TCR-transgene expression to rescue development of Flvcr-deleted thymocytes. Through the use of mouse strains in which FLVCR could be deleted early or late during thymic T cell development, we showed that early deletion of Flvcr during the DN stage led to a block in T cell development, whereas late deletion of Flvcr during the early DP stage allowed for the formation of CD4 and CD8SP. However, these SP cells were unable to proliferate and/or survive in the periphery.

All aerobic cells require heme for oxidative phosphorylation and other key functions; however, excess intracellular free heme is toxic and must be carefully controlled. We analyzed expression of the genes encoding key heme regulators during T cell development and found that expression of Flvcr parallels that of Alas1, supporting the notion that FLVCR may act as a safety valve to export excess heme. Moreover, HMOX1, which breaks down free heme, is downregulated after the DN stage; therefore, FLVCR may be particularly critical for heme regulation after the DN stage in thymocytes.

The finding that FLVCR is required for T cell development beyond the DP stage and peripheral T cell survival is surprising, because it supports a novel and specific role for heme metabolism in T cell development. Because heme can regulate gene expression by binding directly to transcription factors, we carried out transcriptional profiling. One explanation for the paucity of transcriptional alterations in Flvcr-deleted DP cells compared with controls is that FLVCR loss blocks T cell development by non-transcriptional means. Metabolite profiling and heme measurement on whole thymus from deleted or control mice revealed no significant differences (data not shown), and metabolite and heme levels from sorted Flvcr-deleted DP and control thymus were below the limit of detection, likely because the DP subset is metabolically and transcriptionally quiescent as a result of c-myc downregulation (37). We are working to develop more sensitive reporter assays to measure heme in vivo and in ex vivo cells to determine heme levels during T cell development. Nonetheless, the increased expression of Cox6a2 seen in Flvcr-deleted DP cells suggests that heme levels are increased in Flvcr-deleted thymocytes. This raises the interesting question of why careful heme regulation might be required in T cells but not in other lymphoid lineages. One avenue for future studies is to leverage the differential requirement for FLVCR in αβ, but not γδ, T cells to identify specific functions or characteristics of αβ thymocytes/T cells that require more stringent heme metabolism.

There is growing appreciation that metabolites modify the epigenome, thereby regulating cell development and differentiation and contributing to oncogenesis (6, 38, 39). Recently, it was demonstrated that, in cancers with succinate dehydrogenase mutations, the tricarboxylic acid intermediate and heme building block succinate accumulates and acts as an oncometabolite, causing DNA hypermethylation and transcriptional alterations (Supplemental Fig. 1) (40, 41). Epigenetic changes often precede transcriptional changes in development (42), and one possibility is that FLVCR loss and heme accumulation cause epigenetic alterations in developing thymocytes. DP thymocytes are transcriptionally and metabolically quiescent but are epigenetically poised to reactivate transcription and initiate CD4+ or CD8+ T lineage differentiation once the cells pass positive and negative selection; FLVCR loss may disrupt epigenetic programming in these cells so that even DP cells with a TCR capable of selection cannot transition to the SP state. Even in a model in which Flvcr-deleted

![FIGURE 5. FLVCR re-expression in Flvcr flox/flox;Lck-cre BMs rescues T cell development. (A) Absolute numbers of CD4+ and CD8+ PBLs. Red bars show numbers of EGFP− (nontransduced) and EGFP+ thymocytes. Blue bars show numbers of EGFP+ thymocytes transduced (tx) either with EGFP only (open) or FLVCR-EGFP (filled). The bars show the mean and SEM of three or four mice/group. *p = 0.02, EGFP transduced versus FLVCR-EGFP transduced. (B) Representative flow plots of thymocytes from a recipient of Flvcr flox/flox;Lck-cre BMs transduced with FLVCR-EGFP. CD4 versus CD8 staining of the EGFP− (nontransduced) thymocytes (left panel). EGFP+ (FLVCR-EGFP-transduced) thymocytes from the same mouse (right panel). The data are representative of two experiments with three to five mice/group.](http://www.jimmunol.org/content/7/4/783/F1.large.jpg)
FLVCR REQUIRED FOR T CELL DEVELOPMENT

Thymocytes could develop to the SP stage, these SP cells were not able to survive or proliferate in the periphery in a lymphopenic host. This finding raises the possibility that FLVCR is required for T cells to transition between environments (e.g., between different thymic niches or between lymphoid niches). Although Flvcr-deleted SP cells appeared to mature normally, as determined by immunophenotyping, there may be underlying gene expression changes that lead to the demise of these cells in the periphery that could be interrogated through future microarray experiments. Our current models did not allow us to conclusively determine the role of FLVCR in mature peripheral T cells, but the requirement for FLVCR at the DP–SP transition and at the SP cell–peripheral T cell transition raises the question of whether FLVCR is required in mature T cells and, in particular, whether FLVCR is required for naive T cell differentiation upon Ag stimulation. We are developing models in which to address these questions.

Heme synthesis has been regarded as a generic housekeeping function that occurs in all cells (43); however, our finding that FLVCR is specifically required for αβ T cell and erythroid development, but not that of other hematopoietic lineages, suggests that there are important lineage-specific aspects of heme metabolism. Although there is now great interest in metabolic control of cell function and differentiation, much of the focus has been on enzymatic pathways, and less is known about the intra- and intercellular transport of metabolites. Recently, it was discovered that mutations in a ubiquitously expressed magnesium cation importer, MAGT1, caused T cell immunodeficiency and defects in T and NK cell cytotoxic function (44, 45). This revealed a specific role for Mg2+ in T cell and NK cell signaling, in addition to this cation’s universal role as a cofactor in nucleic acids and enzyme activity (46). Similarly, heme may have a specific and unique function in αβ T cells in addition to its other global functions. Our finding that FLVCR specifically blocks T cell development at the DP stage, together with the temporally regulated models of FLVCR function in T cell development that we generated, will provide the tools needed to determine the unique roles for FLVCR and heme in T cell development and, ultimately, lead to new insights into the metabolic regulation of T cell development and function.

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Disclosures

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