BRIEF REPORT

Transplantation of Allogeneic T-cells Alters Iron Homeostasis in NOD/SCID Mice

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Short running title: Iron Homeostasis after Allogeneic T-cells

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Abstract

Iron overload is common in patients undergoing allogeneic hematopoietic cell transplantation (HCT), but the mechanisms leading to overload are unknown. Here, we determined iron levels and the expression of iron regulatory proteins in the liver and gut of NOD/SCID mice transplanted with syngeneic (histocompatible) or allogeneic (histoincompatible) T lymphocytes. Infusion of histoincompatible T cells resulted in a significant rise in serum iron levels and liver iron content. Iron deposition was accompanied by hepatocyte injury and intestinal villous damage. Feeding of low or high iron diet was associated with appropriate ferroportin 1 and hepcidin responses in mice given histocompatible T cells, while mice given histoincompatible T cells showed inappropriate upregulation of duodenal ferroportin 1 and a loss of expression of hepatic hepcidin. These findings suggest that alloreactive T cell-dependent signals induced dysregulation of intestinal iron absorption, which contributed to liver iron overload after HCT.
Introduction

Iron overload is common in patients undergoing hematopoietic cell transplantation (HCT).\(^1\)\(^-\)\(^3\) Many of these patients develop iron overload even without heavy red cell transfusion support; the mechanisms are largely unknown. High-dose conditioning preceding HCT may result in hyperferremia and increased non-transferrin-bound iron, in association with the cessation of erythropoietic activity.\(^4\)\(^,\)\(^5\) In many patients hyperferremia persists after HCT,\(^1\)\(^,\)\(^2\) suggesting aberrant iron release into the circulation.

The major regulator of iron release into the circulation is hepcidin (Hamp), secreted mainly by the liver.\(^6\) Hepcidin binds to the iron transporter ferroportin 1 (Fpn), expressed on enterocytes and macrophages, which delivers iron from inside the cell to the circulation.\(^7\) Hepcidin binding results in internalization and cytoplasmic degradation of ferroportin. As both the intestinal tract and liver are targets of graft-versus-host disease (GVHD),\(^2\)\(^,\)\(^8\)\(^,\)\(^9\) we hypothesized that one effect of allogeneic transplantation may be direct or indirect interference by T lymphocytes with the expression or function of iron regulatory proteins in liver and gut, thereby contributing to iron overload. Here we characterized Hamp and Fpn expression and dietary iron uptake in a murine model of histoincompatible allogeneic T lymphocyte transplantation.
Materials and Methods

Female NOD/LtSz-scid/scid (NOD/SCID [H-2d]), C57BL/6J (H-2b), and BALB/cJ mice (H-2d) were purchased from Jackson Laboratories (Bar Harbor, ME). NOD/SCID mice were maintained in a pathogen-free environment and kept on normal chow (iron content ~35 ppm) or chow with low (≤ 1 ppm) or high (30,000 ppm) iron content (Test Diets, Richmond, IN), for 14–28 days before transplantation. Histocompatible (syngeneic; H-2^d^) and histoincompatible (allogeneic; H-2^b^) T lymphocytes were prepared from spleens obtained from BALB/c(H-2^d^) and C57BL/6J(H-2^b^) mice, respectively. Single-cell suspensions of non-adherent cells were adjusted to a concentration of 5×10^6^ cells/mL. Contamination by CD3 negative cells was 8–12%. Recipient mice received 1.0×10^7^ or 3.0×10^7^ histocompatible or incompatible T-cells intravenously via the lateral tail vein (3–8 recipient mice per experiment). The use of mice in this study was approved by the IACUC of the Fred Hutchinson Cancer Research Center.

Mice were euthanized at 7 or 14 days post-transplant (3–6 weeks after initiating a particular diet) by CO2 inhalation. Blood was collected via cardiac puncture and total serum iron was measured. Hepatic iron content was determined using a colorimetric assay as described.\(^1^0\) Tissue for histology was fixed in 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin and with Perl’s Prussian blue to detect iron deposition.

Hepatocyte suspensions were generated by mincing and straining liver tissue through a 75 μm mesh. Enterocytes were obtained by scraping the mucosa off the inverted duodenum and mincing and straining through a 75 μm mesh. Total RNA was isolated and cDNA synthesized using the μMACS One-Step cDNA Synthesis kit (Miltenyi Biotec, Auburn, CA) as instructed and as previously described.\(^1^1\) Quantitative-PCR was carried out using the comparative C\(_i\)
method as described by Livak and Schmittgen\textsuperscript{12} and as used previously.\textsuperscript{13} Gene Expression Assays (Applied Biosystems, Foster City, CA) were used to analyze expression of the murine hepcidin gene \textit{hamp1} (Mm00519025\_m1) and FPN1 (\textit{fpn1}; Mm00489837\_m1). Murine $\beta$-actin (Mm0060793\_s1) served as endogenous control. Tissues from each dietary group not transplanted with T lymphocytes were used as reference for gene expression, and experimental results were expressed as values relative to the reference.

Sections of duodenum for immunohistochemistry for Fpn were prepared by standard procedures. The deparaffinized and rehydrated sections were blocked, labeled with primary rabbit antibody, washed, and labeled with secondary swine anti-rabbit antibody (Dako Inc., Carpinteria, CA) as described by Canonne-Hergaux et al.\textsuperscript{14} Non-immune rabbit serum served as control. The procedure was carried out on a Leica MicroSystems Bond Polymer Refine Detection kit (Bannockburn, IL).

**Results and Discussion**

NOD/SCID mice were used to allow for engraftment of donor cells without cytotoxic conditioning of the recipient, which could induce interleukin (IL)-6 and affect regulation of hepcidin. In NOD/SCID mice fed normal chow and infused with $3.0 \times 10^7$ histoincompatible T lymphocytes, liver sections obtained on day 14 showed iron deposition in hepatocytes (Figure 1A). In contrast, no iron deposition was observed in mice infused with histocompatible syngeneic T cells or in control mice not given T lymphocytes (Figure 1A). Quantitative analysis of liver tissue confirmed elevated levels of hepatic iron in mice transplanted with histoincompatible T cells (Figure 1B). Mice given histoincompatible T-cells also showed histological evidence of injury of the gut mucosa (Figure 1C) and the liver (Figure 1D),
associated with serum transaminase elevations (Figure 1E). Control mice showed slight increases in body weight over 14 days, whereas the weight declined progressively in mice given histoincompatible T cells, as classically observed in murine GVHD. The weight loss was prevented or reversed in mice pre-treated with apoTf (Figure 1F). We had shown previously that Fas-initiated hepatocyte injury was attenuated or prevented by apotransferrin. Since alloactivated T lymphocytes express Fas-ligand, and Fas signaling is involved in GVHD, we postulated that pre-treatment of NOD/SCID mice with apotransferrin before histoincompatible T-cell infusion would interfere with hepatic injury and liver iron deposition. Results illustrated in Figures 1C-F support this hypothesis by showing substantial reduction in hepatocyte apoptosis and serum transaminase elevations. Thus, chelation of iron by apotransferrin, or possibly transferrin-mediated antiapoptotic signals, counteracted iron overload and protected tissues against allogeneic histoincompatible T cell dependent injury. These data support earlier reports of a GVHD prophylactic effect of apoTf.

To determine whether NOD/SCID mice properly regulated intestinal iron absorption, we tested the effect of diets with different iron content on iron uptake in the absence of T cell transplantation. Serum iron levels on all diets remained in the range of 260–350 μg/dL (Figure 2). There was a slight increase in duodenal Fpn mRNA expression (relative to house keeping genes) in mice on low iron, and a significant decrease in mice on high iron diet. In comparison to mice on normal chow, Hamp expression in the liver decreased in mice on low iron, and increased in mice on high iron diet (Figure 2B and C). This pattern was consistent with appropriate responses of NOD/SCID mice to variations in dietary iron content.

Responses differed strikingly in mice infused with histoincompatible allogeneic T lymphocytes. While mice transplanted with histocompatible syngeneic T cells showed a pattern
of serum iron levels, and hepcidin and Fpn expression that was similar to that in mice not injected with T lymphocytes, the infusion of histoincompatible T cells resulted in significant dysregulation: mice on all diets showed significant increases in serum iron levels, which was most marked in mice on high iron diet (p< 0.001; Figure 2A). Fpn expression in the duodenal mucosa increased 10-100-fold following the infusion of allogeneic cells, even in mice on high iron diet (Figure 2B). Upregulation of Fpn message was reflected in enhanced expression of Fpn protein in enterocytes, as illustrated in Figure 2D. Conversely, Hamp expression in the liver declined consistently, including mice on high iron diet and documented liver iron overload (Figure 2C).

These findings suggest that alloreactive (histoincompatible) T cells cause dysregulation of intestinal iron absorption, and thereby contribute to liver iron accumulation. Both the strikingly increased Fpn expression in the duodenum and the suppressed hepcidin expression in the liver would be expected to considerably increase intestinal iron uptake, in line with the increased serum iron levels observed in the NOD/SCID mice after histocompatible allogeneic T cell transplantation.

These observations provide novel data on iron dysregulation and support the notion that histoincompatible allogeneic transplantation may contribute to iron overload. They also raise new questions. For example, does the immunodeficient condition of NOD/SCID mice contribute to iron accumulation? Is the sterile milieu in which these mice are raised a contributing factor? Studies in immunocompetent mice with the same genetic background and investigations in wild type mice rendered gnotobiotic should provide some answers.
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Authors’ Contributions. Steven Bair carried out in vivo experiments and wrote the manuscript; Emily Spaulding assisted with in vivo experiments and carried out molecular analysis; Jaakko Parkkinen co-designed the experiments and co-wrote the manuscript; Howard M. Shulman carried out all histologic analyses; Vladimir Lesnikov co-designed experiments, assisted with in vivo and in vitro studies, and provided the apo-transferrin data; Mary Beauchamp carried out immunohistochemistry studies; Francois Canonne-Hergaux provided anti-Fpn antibody and technical expertise on its use; Kris V. Kowdley critically evaluated the experimental design and determined liver iron content; H. Joachim Deeg co-designed the experiments, evaluated all results, and edited the manuscript.

Conflicts of Interest: There are no conflicts of interest to declare by any author.
References


Figure Legends

Figure 1. Protective effect of apotransferrin against tissue injury induced by allogeneic T cells. Iron accumulation in the liver and hepatic and intestinal injury after transplantation of allogeneic T cells to NOD/SCID mice. (A) NOD/SCID mice [H-2d] were transplanted with 3×10^7 allogeneic lymphocytes from C57BL6 mice [H-2b], following injection of saline (middle panel) or human apotransferrin (1 mg/mouse) (right panel) 24 and 2 hours before T cell infusion. Livers from untreated mice served as additional controls (left panel). (B) Hepatic iron content (HIC) as determined by colorimetric assay (mean ± 1 SD) on day 14 after the infusion of saline and either no cells or syngeneic or allogeneic cells, respectively (3-8 mice per group). (C) Histology of the duodenum and (D) liver, representative for the three groups of mice described in (A). (E) Serum transaminase levels (mean ±1 SD) in controls (blue column) and mice on day 14 after infusion of allogeneic T lymphocytes after pretreatment with saline (red column) or apotransferrin (green column). (F) Weight changes in mice from panel E over the 14 day course of the experiment. Beyond day 7 body weight was significantly higher in ApoTf pre-treated than in saline pre-treated mice (p<0.05 to p<0.02 by Student’s t-test, calculated on days 7 and 14).

Figure 2. Effects of T lymphocyte transplantation on iron homeostasis. Serum iron (mean ± 1 SD) (A), duodenal ferroportin 1 (Fpn) (B), and hepatic hepcidin (hamp) mRNA expression (C), on day 14 after T cell infusion. Mice not injected with T lymphocytes served as controls. Mice were fed normal chow (normal Fe) or low Fe or high Fe diets. Values for (B) and (C) were
obtained in each mouse by expressing the levels of Fpn1 and hamp, respectively, relative to the levels of β-actin; shown are the means ± 1 SD of the relative values in 3 mice (normal Fe) or 5 mice (low and high Fe groups). (D) Fpn protein expression in the duodenum of mice on high iron diet. The upper panel shows intense staining of enterocytes, most prominently in baso-lateral localization, in a mouse transplanted with allogeneic histoincompatible T cells; the lower panel from a mouse transplanted with histocompatible (syngeneic) T cells shows only very faint staining for Fpn. [Image for (D) created using Leica Dm 3000, Wetzlar Germany; type, magnification, and numerical aperture of the objective lenses temperature: N plan dry 63x NA 0.80, LED lighting; imaging medium: digital camera photograph of paraffin embedded formalin fixed glass slide, photos 64% (RGB/8; no fluorochromes; camera make and model (with manufacturer location): Leica DCF 280 camera, Herrbrug Switzerland; acquisition software (with manufacturer location): Leica microsystems, Leica application suite LAS 3.1.0 (build: 1983-) CMS GmbH, Photoshop elements 4, headquarters located in Adobe, San Jose, CA 95110-2704.)
Figure 1

A

control  T cells + saline  T cells + ApoTf

B

HIC (µg Fe/g dry weight)

No cells  Saline  Syngeneic Saline  Allogeneic Saline

C Gut

D Liver

control  T cells + saline  T cells + ApoTf

E

AST levels (U/L)

control (n=3)  T cells + saline (n=8)  T cells + ApoTf (n=8)

* p<0.05 vs "saline"

F

% Body Weight (gain or loss)

Days
Figure 2

(A) Total Serum Fe Concentration 14 Days Post-transplant

(B) Duodenal Fpn1 Expression 14 Days Post-transplant

(C) Hepatic Hamp1 Expression 14 Days Post-transplant

(D) Micrograph images of tissue samples.
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