Defective Iron Homeostasis in β2-Microglobulin Knockout Mice Recapitulates Hereditary Hemochromatosis in Man

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Summary

Previously, hepatic iron overload resembling that in hereditary hemachromatosis (HH) has been found in β2-microglobulin knockout ($\beta2m^{-/-}$) mice. We have now characterized iron metabolism in $\beta2m^{-/-}$ mice. The mutant mice fail to limit the transfer of iron from mucosal cells into the plasma. Transferrin saturation is abnormally high. Pathologic iron depositions occur predominantly in liver parenchymal cells. Reconstitution with normal hematopoietic cells redistributes the iron from parenchymal to Kupffer cells, but does not correct the mucosal defect. We conclude that (*a*) iron metabolism is defective in the gut mucosa as well as the liver of $\beta2m^{-/-}$ mice; and (*b*) a β2m-dependent gene product is involved in iron homeostasis. Recently, a novel gene of the major histocompatibility complex class I family, HLA-H, has been found to be mutated in a large proportion of HH patients. Our data provide functional support for the proposed causative role of HLA-H mutations in HH.

Hereditary hemochromatosis (HH)[1], an autosomal recessive, HLA-linked disease, is one of the most common genetic disorders in man. It is characterized by a failure in regulation of iron absorption, an increase of transferrin saturation, and progressive iron deposition predominantly in the parenchymal cells of several organs (1). Increased iron absorption in HH homozygotes leads to accumulation of iron, with eventual tissue damage and organ dysfunction. When the disorder remains untreated, premature mortality resulting from hepatocellular carcinoma, cirrhosis, cardiomyopathy, or diabetes mellitus is common (2, 3). Removal of iron and the prevention of its reaccumulation by phlebotomy treatment dramatically improves life expectancy in these patients (4–7). The discovery of a tight linkage between HH and the HLA-A locus has led to the clarification of the genetic nature and the autosomal recessive inheritance of the disease (8, 9). A similar disease is not known in animals, and although many attempts have been made to develop an appropriate animal model by dietary application of different iron compounds, severe organ damage as seen in HH could not be reproduced in these models (10).

In a previous study, we have revealed the existence of hepatic iron overload in β2-microglobulin (β2m)-deficient mice similar to that found in HH (11, 12). This was based on a histological and quantitative study of the steady-state iron distribution and content in tissues of $\beta2m^{-/-}$ mice. These results were confirmed more recently by others (13). In addition, the latter study demonstrated that increased levels of dietary iron led to accelerated iron accumulation, and reported an increased incidence of hepatocellular carcinoma in β2m knockout mice. Such carcinomas are frequently seen in HH patients.

In this report, we characterize the nature of the iron metabolism defect found in $\beta2m^{-/-}$ mice. β2m is required for the normal surface expression of classical and nonclassical MHC class I molecules (14, 15). Thus, mice lacking a functional β2m gene fail to express MHC class I products (16, 17). Such mice lack $CD8^+$ lymphocytes. To analyze a possible involvement of $CD8^+$ cells, we also analyzed iron metabolism in $CD8^{-/-}$ mice. Mice that are mutant in the TAP-1 peptide transporter do not express classical class I molecules on the surface of their cells (18). Some nonclassical MHC-I molecules can be expressed independently of TAP function, however (19, 20). Therefore, to extend our analysis of the nature of the β2m-dependent molecules involved in iron metabolism, we included the $TAP1^{-/-}$ mice in our study. $\beta2m^{-/-}$ mice failed to reduce intestinal iron absorption in the face of iron overload and had abnormally high (>80%) values of transferrin saturation. This defect

[1]*Abbreviations used in this paper.* β2m, β2-microglobulin; $\beta2m^{-/-}$, β2m knockout (mice); Hb, hemoglobin; HCT, hematocrit; HH, hereditary hemachromatosis; IR, iron retention; MCV, mean corpuscular volume; MT, mucosal transfer fraction of iron; MU, mucosal uptake of iron; RE, reticuloendothelial; TIBC, total iron-binding capacity; TS, transferrin saturation.

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was specific for $\beta 2m^{-/-}$ mice, since the iron status of $TAP1^{-/-}$ mice and $CD8^{-/-}$ mice was comparable to that of controls. Additional results are presented that suggest that the iron metabolism in $\beta 2m^{-/-}$ mice is affected at the level of the gut mucosa, as well as at the level of iron storage in Kupffer cells.

The results presented here reveal a remarkable similarity between iron metabolism in $\beta 2m^{-/-}$ mice and in HH patients, and support the unexpected involvement of MHC class I–like molecule(s) in the regulation of iron homeostasis.

Materials and Methods

Mice. C57BL/6 (B6) female mice aged 6–8 wk were purchased from IFFA Credo (Brussels, Belgium), and used as controls. The β2-microglobulin "knockout" ($\beta 2m^{-/-}$) mice (16) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) and further bred in our animal facility; $CD8^{-/-}$ mice (21) were obtained from Dr. T. Mak (University of Toronto, Toronto, Canada), and further bred in our animal facility. Both mutant mice had been back-crossed onto the C57BL/6 background. Liver samples from $TAP^{-/-}$mice (18) were obtained from Dr. S. Tonegawa (Massachusetts Institute of Technology, Cambridge). All animals were given a commercial diet (RMH-B®; Hope Farms, Woerden, The Netherlands) or, when indicated, an iron-enriched diet containing 2.5% (wt/wt) carbonyl iron (Sigma Immunochemicals, St. Louis, MO) for 14 d.

For all animal experiments, written consent was obtained from the local Animal Experiments Committee of Utrecht University (Utrecht, The Netherlands).

Transferrin Saturation and Hematological Measurements. Heparinized blood was obtained by orbital puncture under diethylether anesthesia. Hemoglobin (Hb), hematocrit (HCT), and mean corpuscular volume (MCV) were determined using a Coulter-S counter (Coulter Electronics, Hialeah, FL). Plasma iron and total iron-binding capacity (TIBC) were determined by the Ferrozine method (Iron FZ Test; Roche, Basel, Switzerland) with the CO-BAS-BIO autoanalyzer (Hoffman-La Roche BV, Mijdrecht, The Netherlands). Transferrin saturation (TS) was calculated from the TIBC and plasma iron values.

Histology. Samples of liver, spleen, kidney, heart, and pancreas were fixed in 4% buffered formaldehyde. After routine histology processing, the paraffin sections were stained by hematoxylin and eosin. For the detection of ferric iron (Fe^{3+}), the Prussian blue stain was used (22).

Measurement of Liver Iron Concentration. Liver samples were weighed wet, then dried overnight at 106°C and weighed again. The dried samples were ashed in an oven at 500°C for 17 h, then fully solubilized in 6 M HCl, and the final solution was adjusted with demineralized water to a final HCl concentration of 1.2 M. Iron concentration of the samples was determined by flame atomic absorption spectrometry (Varian SpectrAA 250 Plus; Varian, Mulgrave, Victoria, Australia).

Electron Microscopy. Small pieces of liver were fixed in a modified Karnovsky fixative consisting of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M Na-cacodylate buffer, supplemented with 2 mM $CaCl_2$, for at least 24 h at 4°C. The tissue was washed twice with the same buffer, postfixed in 1% OsO_4, and embedded in Epon 812. 1-mm semithin sections were stained with methylene blue and pararosanilin (23). Ultrathin sections (60 nm) were cut and contrasted with 3% uranyl magnesium acetate for 45 min at 63°C followed by Reynolds' lead citrate for 10 min. Stained and unstained sections were viewed in an electron microscope (201c; Philips Technologies, Chesire, CT).

Gastrointestinal Iron Absorption. For iron absorption tests, the mice were fasted for 6 h and housed for 3 d in cages equipped with grates to minimize coprophagy.

All test doses were freshly prepared and were administered in aqueous solution using demineralized water. $^{59}Fe(III)$ citrate was added to Fe(II) as ferrous sulfate and a 20× molar excess of L-ascorbic acid solution to reduce the Fe(III). $^{51}CrCl_3$ was added as a nonabsorbable indicator. The total amount of Fe(II) per test dose was 5 μg per mouse and had a final volume of 0.3 ml. Each mouse received ~50 kBq of ^{59}Fe and 200 kBq of ^{51}Cr.

The test dose was orally applied with the use of an olive-tipped oroesophageal needle. Total body radioactivity was measured with a whole-body gamma counter (Automatic Scanner DS4/4S; Tracelab Ltd., Weybridge, Surrey, UK) with separate detection windows for ^{59}Fe and ^{51}Cr peaks. The values were corrected for radioisotope decay and day-to-day fluctuations of the scanner with the use of a radium source. The ^{51}Cr peaks were corrected for the Compton effect of ^{59}Fe.

Mucosal uptake of iron (MU) was calculated from the activity of ^{59}Fe and ^{51}Cr administrated (measured immediately after test dose administration and considered as 100%), as well as from the activity of ^{59}Fe (F1) and ^{51}Cr (C1) found within the body 22 h later, using the formula $MU = 100 \times (F1 - C1)/(100 - C1)\%$. F1 and C1 were expressed as the percentage of the amount of ^{59}Fe and ^{51}Cr administered. ^{59}Fe retention (IR) was determined by whole-body counting 7 d after administration of the test dose. The mucosal transfer fraction of iron (MT) was determined as the ratio IR/MU.

When the animals were tested twice for iron absorption, the second test dose was administered 16 d after the first dose. Background values of the first test dose were corrected for radioisotope decay.

Fetal Liver Cell Transfer. Recipient animals were lethaly irradiated (9.5 Gy) and reconstituted with $5–10 \times 10^6$ day 13 fetal liver cells by intravenous injection. Chimeras were killed at 4, 8, and 12 wk after reconstitution, and chimerism was monitored by FACS® (Becton Dickinson, Mountain View, CA) using CD4, CD8 (PharMingen, San Diego, CA), and H141.31.10 (anti-K^b) antibodies.

Statistical Analysis. Results are presented as the mean + SD. Student's *t* test was used for comparison between the control and knockout mouse groups. For individual comparisons between two measurements, the paired *t* test was used. The level of significance was preset at $P < 0.05$.

Results

Increased Plasma Iron and TS in $\beta 2m^{-/-}$ Mice. Under normal circumstances, the vast majority of plasma iron is bound to transferrin. Transferrin-bound iron is transported to various sites of utilization, where it is delivered to cells via transferrin receptors (24). In HH homozygotes, the earliest laboratory abnormalities are elevated plasma iron concentrations and increased TS (25, 26). Therefore, we measured plasma iron and TIBC, and calculated transferrin saturation from that in 2- and 12-mo-old mice. At both ages, $\beta 2m^{-/-}$ mice had persistently higher plasma iron concentrations (Fig. 1 *A*) and TS (Fig. 1 *B*) than either B6 or $CD8^{-/-}$ control mice.

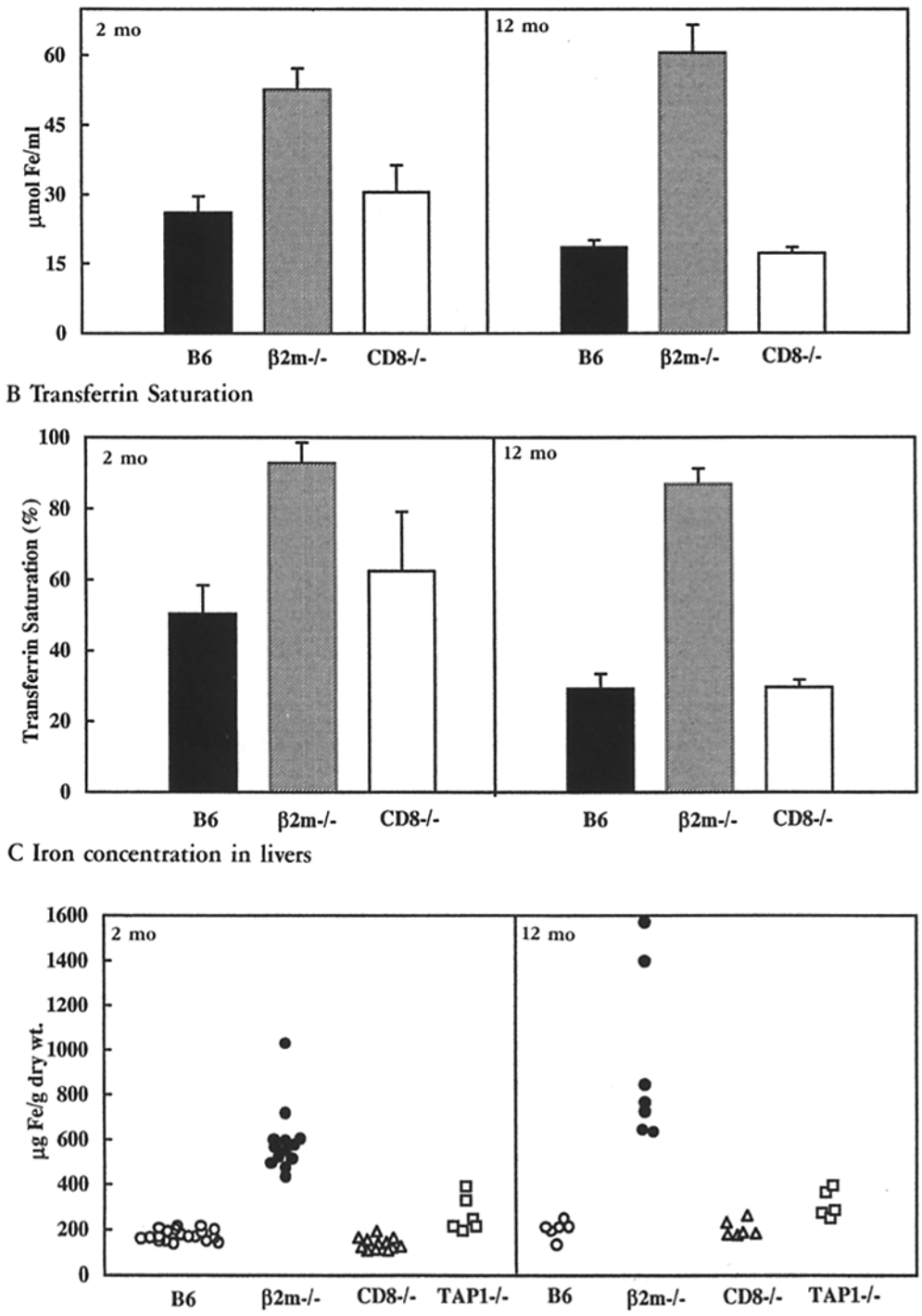

Figure 1. Increased iron levels in $\beta 2m^{-/-}$ mice. (*A*) Plasma iron and (*B*) TS in B6 (control), $\beta 2m^{-/-}$, and $CD8^{-/-}$ mice aged 2 and 12 mo. Plasma iron and TIBC were determined by the Ferrozine method. TS was calculated from the ratio of plasma iron and TIBC. Values represent mean ± SD. Mean values from $\beta 2m^{-/-}$ mice were significantly different from that for B6 or $CD8^{-/-}$ mice (Student's *t* test): $P < 0.001$. (*C*) Iron concentration in livers. Liver samples from B6, $\beta 2m^{-/-}$, $CD8^{-/-}$, and $TAP1^{-/-}$ mice aged 2 and 12 mo were analyzed by flame atomic absorption spectrometry for quantitative determination of iron. Individual values are represented. Mean values from $\beta 2m^{-/-}$ mice were significantly different from those of B6 or $CD8^{-/-}$ mice (Student's *t* test): $P < 0.0001$.

Increased Iron Concentrations in Livers from $\beta 2m^{-/-}$ Mice. Diagnosis of iron storage disease normally requires a biopsy of the liver, the major organ for excess iron storage (1). Further quantitative determination of the hepatic iron concentration, histochemical visualization of the cellular distribution of iron, and pathological examination of the extent of injury provide essential information about the type and degree of iron loading.

Confirming the finding reported earlier (11, 13), 8-wk-old $\beta 2m^{-/-}$ mice had hepatic iron concentrations that were more than twice as high as in B6 control mice. In contrast, iron concentration in the livers of $CD8^{-/-}$ mice

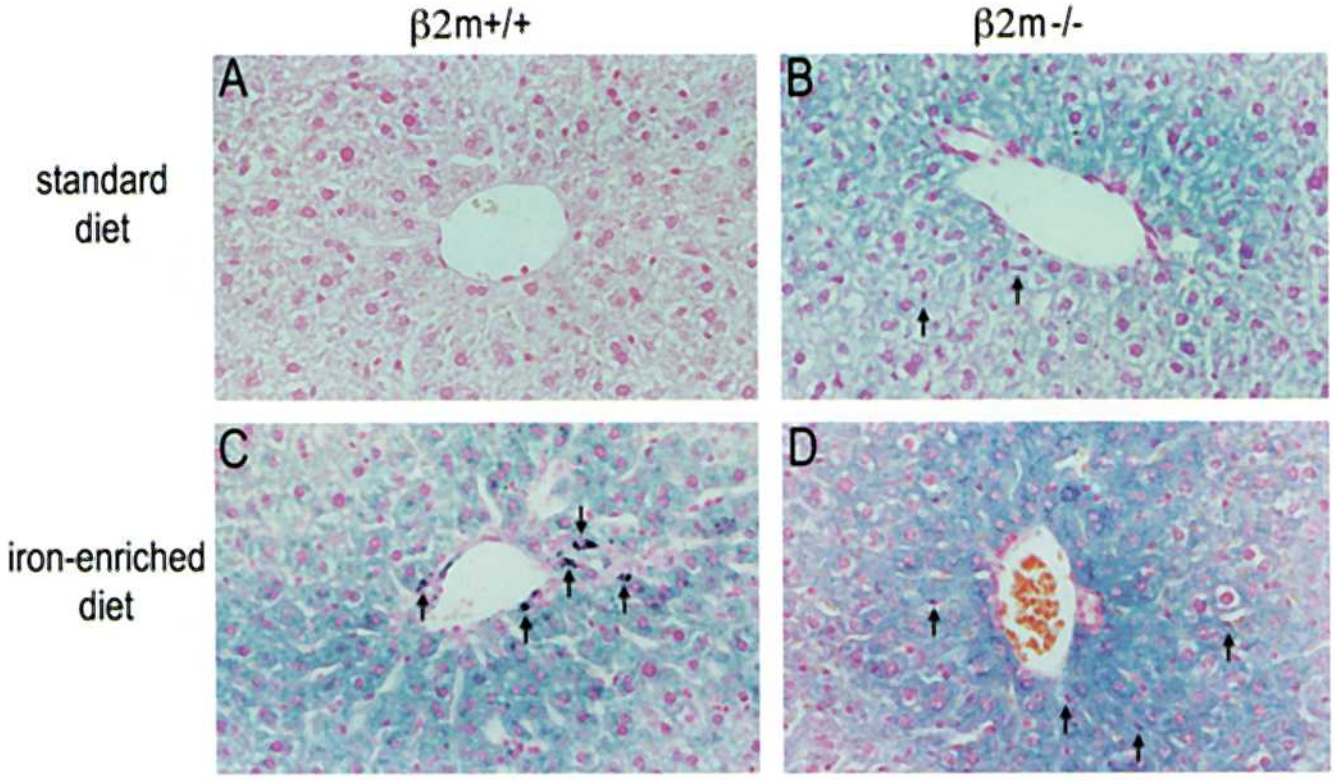

Figure 2. Storage of excess iron in hepatic cells of mice fed with different diets (Prussian blue staining). (*A* and *B*) Light micrograph of a liver section from B6 (indicated as $\beta2m^{+/+}$, *A*) and $\beta2m^{-/-}$ (*B*) mouse aged 2 mo and fed with a standard diet. In the liver of the $\beta2m^{-/-}$ mouse, liver parenchymal cells with an increased iron content are visible around portal spaces. (*C* and *D*) Light micrograph of a liver section from B6 (indicated as $\beta2m^{+/+}$, *C*) and $\beta2m^{-/-}$ (*D*) mouse aged 2 mo at the start of the experiment and fed with an iron-enriched diet (2.5% wt/wt carbonyl iron) for 2 wk. Beside parenchymal cells, heavily iron-loaded Kupffer cells are visible in the $\beta2m^{+/+}$ liver. Kupffer cells are indicated with arrows. Original magnification ×300.

and $TAP1^{-/-}$ mice were comparable to B6 control mice (Fig. 1 *C*). Similar observations were made at 12 mo of age, when $\beta2m^{-/-}$ mice had accumulated about three times more iron in their livers than age-matched B6, $CD8^{-/-}$, or $TAP1^{-/-}$ mice (Fig. 1 *C*).

Cellular Distribution of Storage Iron in Livers. One of the distinguishing features of the pathology in HH in humans is that the initial deposition of iron is predominantly in the parenchymal cells of affected tissues, with insignificant early involvement of macrophages of the reticuloendothelial system (27). No detectable iron was seen histologically in B6 (Fig. 2 *A*), $CD8^{-/-}$, and $TAP1^{-/-}$ mice (not shown) of various ages that were fed with a standard diet. Prussian blue staining of liver sections from >50 $\beta2m^{-/-}$ mice revealed a predominant presence of iron in parenchymal cells (Fig. 2 *B*), confirming the finding reported earlier (11, 13). At early stages, the iron-loaded hepatocytes were concentrated in periportal areas, but later they were distributed equally throughout all areas of the liver lobule. In older animals, iron depositions were also present in the pancreas, kidneys, and heart (not shown). Two out of seven 1-yr-old $\beta2m^{-/-}$ mice had a sevenfold increase in liver iron concentrations (Fig. 1 *C*) and had developed hepatic fibrosis (not shown), which was never seen in control mice of the same age.

Examination of livers from $\beta2m^{-/-}$ mice by electron microscopy revealed electron-dense lysosomal structures in hepatic parenchymal cells located in periportal areas. These lysosomes had a granular substructure (Fig. 3 *A*) and, in unstained sections, they were composed of more or less densely packed ferritin granules. Significant amounts of ferritin granules were also present in the cytoplasm of hepatic parenchymal cells (Fig. 3 *B*). Of interest, lysosomal deposits of ferritin and hemosiderin are prominent in the hepatocytes of patients with HH.

To study the effects of iron overloading on the iron storage in the liver, animals were fed a diet supplemented with 2.5% (wt/wt) of carbonyl-iron for 14 d. In B6 and $CD8^{-/-}$ mice, iron deposition was present in hepatocytes, but was particularly prominent in Kupffer cells (Fig. 2 *C*). In contrast, in dietary iron–loaded $\beta2m^{-/-}$ mice, the iron continued to be found predominantly in parenchymal cells (Fig. 2 *D*).

Erythroid Parameters. Excess storage iron can occur in a large group of hematological diseases, collectively termed iron-loading anaemias, in which ineffective erythropoiesis or abnormalities of hemoglobin synthesis are prominent (1). They are characterized by anemia and a subsequent increase in iron absorption. Ultimately, the inefficient use of the iron leads to iron overload. To rule out the possibility that anemia could account for the abnormal iron storage defect demonstrated in $\beta2m^{-/-}$ mice, we determined several erythroid parameters. The results demonstrated that RBC counts, Hb, HCT, and MCV values were even

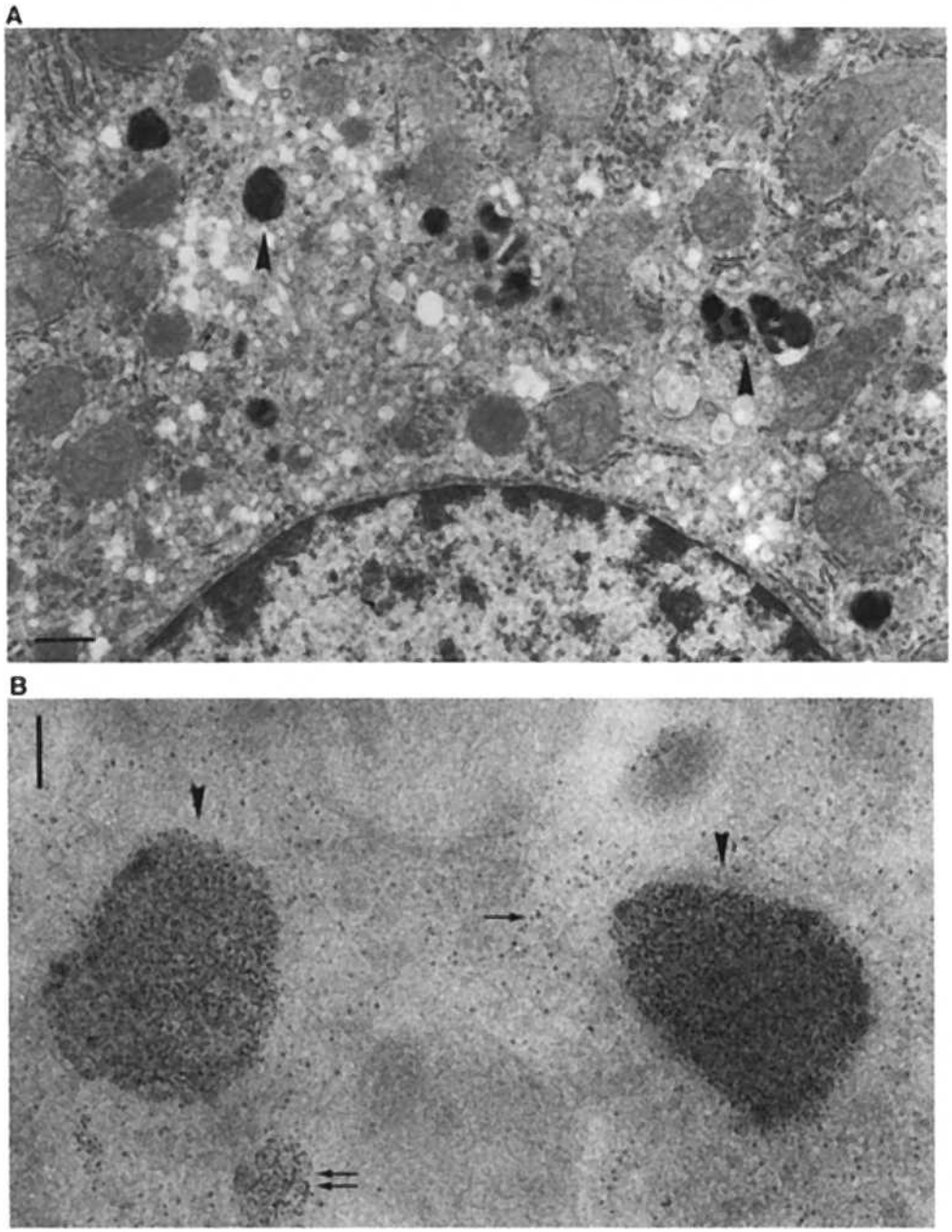

Figure 3. Intracellular distribution of excess iron in $\beta 2m^{-/-}$ hepatic parenchymal cells (*A*) Electron micrograph of a hepatic parenchymal cell from a 12-mo-old $\beta 2m^{-/-}$ mouse fed with a standard diet. Numerous iron containing lysosomes are present in the Golgi area (*arrowheads*). Bar, 0.5 mm. (*B*) Unstained section showing in detail lysomal structures (*arrowheads*) containing densely packed ferritin particles Ferritin particles are also present in the cytoplasm, scattered (*arrows*) or in small clusters (*double arrow*). Bar, 0.1 mm.

higher in the $\beta 2m^{-/-}$ mice than in the other animal groups (Table 1). Thus, the excess storage iron found in $\beta 2m^{-/-}$ mice could not be related to defective erythropoiesis or Hb synthesis.

Failure to Regulate Iron Absorption in $\beta 2m^{-/-}$ Mice. One of the hallmarks of iron homeostasis is its exclusive regulation at the sites of absorption (28, 29). The capacity to reduce or increase absorption of iron in response to increased or reduced iron stores is well documented. In HH, patients fail to decrease their iron absorption despite the accumulation of iron in the body (30–32).

Iron retention, here defined as the percentage of ^{59}Fe found in the body 7 d after the administration of an oral radioactive test dose, was plotted against iron concentration in livers (Fig. 4 *A*). Despite the fact that $\beta 2m^{-/-}$ mice have increased amounts of iron in livers when fed a standard diet, they did not downregulate their IR as compared to control B6 mice that were fed the same standard diet. Importantly, B6 mice that were fed an iron-enriched diet promptly downregulated IR as a response to the modest increase of iron in their livers.

To test whether $\beta 2m^{-/-}$ mice can downregulate iron absorption as a response to a similar overload induced by diet, iron retention was measured before and after feeding the animals with an iron-enriched diet for 14 d. Again, the downregulation of IR in response to moderately increased iron stores could readily be demonstrated in B6 and $CD8^{-/-}$ mice. In marked contrast to the results observed in these

Table 1. *Erythroid Parameters*

Mice	Age	*n*	RBC	Hb	HCT	MCV
	mo		*10^{12}/liter*	*mmol/liter*	*%*	*fl*
B6	2	19	9.4 ± 0.3	8.9 ± 0.3	45 ± 1	48 ± 1
B6	12	6	8.7 ± 0.5	7.9 ± 0.5	40 ± 2	45 ± 1
$\beta2m^{-/-}$	2	11	10.0 ± 0.4	10.2 ± 0.5	53 ± 2	53 ± 1
$\beta2m^{-/-}$	12	6	10.4 ± 0.3	10.2 ± 0.3	51 ± 1	49 ± 2
$CD8^{-/-}$	2	13	9.0 ± 0.3	8.6 ± 0.3	44 ± 1	49 ± 2
$CD8^{-/-}$	12	6	8.7 ± 0.7	8.0 ± 0.5	40 ± 1	46 ± 1

Data are presented as mean ± SD. *n*, number of animals.

mice, $\beta2m^{-/-}$ mice, even when challenged with high dietary iron, were unable to reduce their iron retention (Fig. 4 *B*).

A more specific indicator of the regulation of iron absorption is the so-called MT, here defined as the ratio of the iron ultimately retained in the body vs. the iron initially taken up by the gut mucosa. This is believed to be the critical step that is defective in HH patients (33). We used a double-isotope technique (32; Santos, M., K.J.H. Wienk, M.W. Schilham, H. Clevers, M. DeSousa, and J.J.M. Marx, manuscript in preparation) to discriminate between initial MU and subsequent iron retention, and hence calculate MT of iron. The MT in $\beta2m^{-/-}$ mice that were maintained on a standard diet was consistently higher than MT in normal or $CD8^{-/-}$ mice (Table 2). After experimental iron overload, MT in B6 and $CD8^{-/-}$ mice was strongly reduced, whereas MT in $\beta2m^{-/-}$ mice was only mildly decreased (Fig. 4 *C*).

Taken together, the capacity of $\beta2m^{-/-}$ mice to regulate iron absorption in response to changes in iron stores was strikingly affected at the level of the MT of iron to the plasma.

Fetal Liver Cell Transfer. As an alternative site for the primary iron handling defect in HH patients, the reticuloendothelial (RE) system has been proposed. Specifically, it has been shown that the RE cells in HH patients are impaired in their capacity to store iron (34–36). A possible implication of these studies is that an abnormality in RE iron metabolism might affect the control of iron absorption by the intestinal mucosa (37). We attempted to address this issue by reconstituting lethally irradiated $\beta2m^{-/-}$ mice with hematopoietic cells from normal mice. It was expected that if RE cells play a role in regulating iron absorption, $\beta2m^{-/-}$-reconstituted mice should be able to correct their abnormalities in iron homeostasis. All $\beta2m^{-/-}$ mice reconstituted with $\beta2m^{-/-}$-derived cells had stainable iron in parenchymal cells, and they were indistinguishable from untreated $\beta2m^{-/-}$ mice (Figs. 2 *B* and 5, *A* and *C*). No stainable iron was detected in B6-reconstituted B6 mice (not shown). All attempts to reconstitute B6 mice with $\beta2m^{-/-}$ fetal liver cells failed, most likely because of rejection of the transplanted hematopoietic cells by host NK cells (38). A striking change in hepatocellular iron distribu-

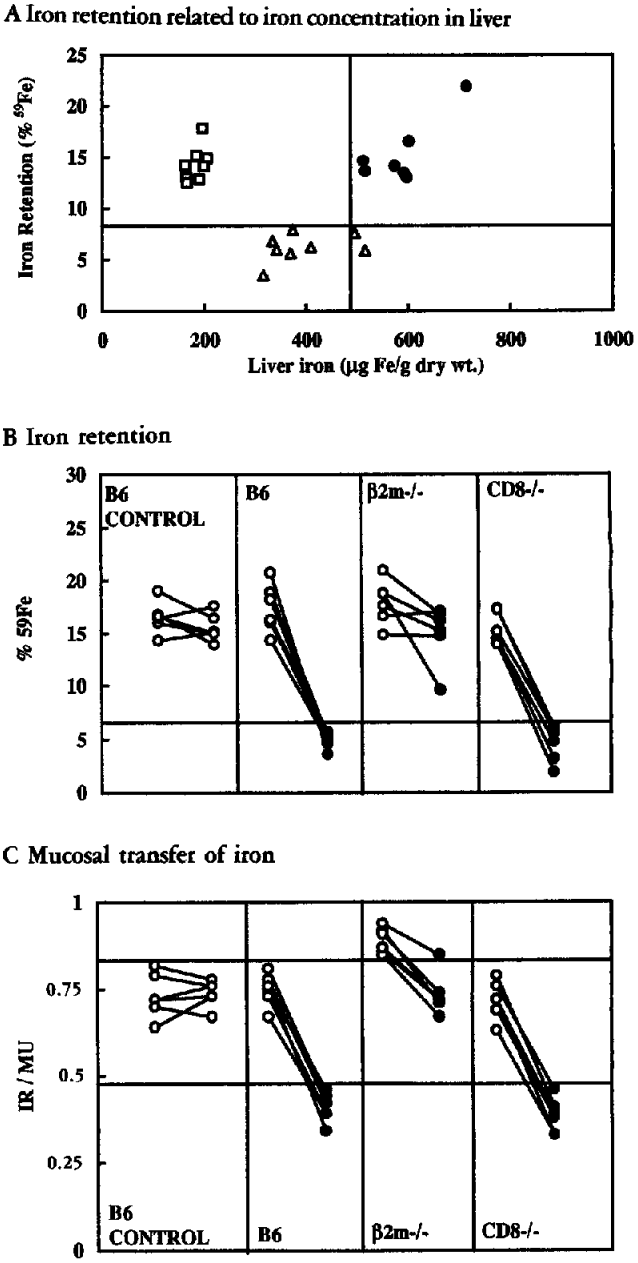

Figure 4. Impaired capacity to downregulate iron absorption in $\beta2m^{-/-}$ mice. All animals were 2 mo old at the start of the experiment. (*A*) IR vs. hepatic iron concentration in B6 control (□), B6 dietary iron overload (△), and $\beta2m^{-/-}$ (●) mice. B6 iron-overload mice were fed an iron-enriched diet containing 2.5% wt/wt carbonyl iron for 14 d. The remaining groups were maintained on a standard diet. Each group of mice ($n = 8$) was given a radioactive test dose solution. IR was determined at day 7 in a whole-body counter. Liver samples were analyzed by flame atomic absorption spectrometry for quantitative determination of iron. Individual values for each mouse are shown. (*B*) IR and (*C*) MT in the same mice before (○) and after iron loading (●) with carbonyl iron (2.5% wt/wt) for 14 d (six mice per group). Control B6 mice were maintained on a standard diet. IR was measured at day 7 after the administration of the test dose. Mucosal transfer of iron was calculated from the ratio IR/MU. Individual values for each mouse are shown.

Table 2. *Increased Intestinal MT in $\beta 2m^{-/-}$ Mice*

	B6		$CD8^{-/-}$		$\beta 2m^{-/-}$		*P*	
Age	n	MT	n	MT	n	MT	vs. B6	vs. $CD8^{-/-}$
mo								
2	12	0.74 ± 0.06	6	0.72 ± 0.06	6	0.89 ± 0.04	<0.0001	<0.0005
4	8	0.74 ± 0.04	5	0.64 ± 0.04	7	0.86 ± 0.05	<0.0001	<0.0001
6	7	0.70 ± 0.04	6	0.72 ± 0.05	6	0.82 ± 0.05	<0.001	<0.005
12	6	0.64 ± 0.05	6	0.65 ± 0.05	6	0.86 ± 0.06	<0.0001	<0.0001

MT was determined as the ratio of IR/MU. Each mouse received orally a double isotope test dose containing ^{59}Fe and ^{51}Cr. MU was calculated 1 d later. ^{59}Fe retention was determined by whole-body counting 7 d after administration of the test dose. Data are presented as mean ± SD. *n* = number of animals; *P* = Student's *t* test for comparison of $\beta 2m^{-/-}$ mice with B6 control and $CD8^{-/-}$ mice.

tion was observed in $\beta 2m^{-/-}$ mice at 1 (Fig. 5 *B*) and 2 mo (not shown) after reconstitution with B6 hematopoietic cells: the site of storage of excess iron shifted from parenchymal to Kupffer cells. Total liver iron concentrations, as measured by flame atomic absorption spectrometry, only showed a slight decrease (not shown). 3 mo after reconstitution, no stainable iron was observed in five out of seven treated mice (Fig. 5 *D*). Despite the decrease in iron deposition in the livers of B6-reconstituted $\beta 2m^{-/-}$ mice, hematological parameters and TS were still increased during the experimental period. This probably reflected a failure to correct the increased iron absorption by the intestinal mucosal cells. Indeed, mucosal transfer in $\beta 2m^{-/-}$ mice 3 mo after reconstitution with either $\beta 2m^{-/-}$ cells or B6

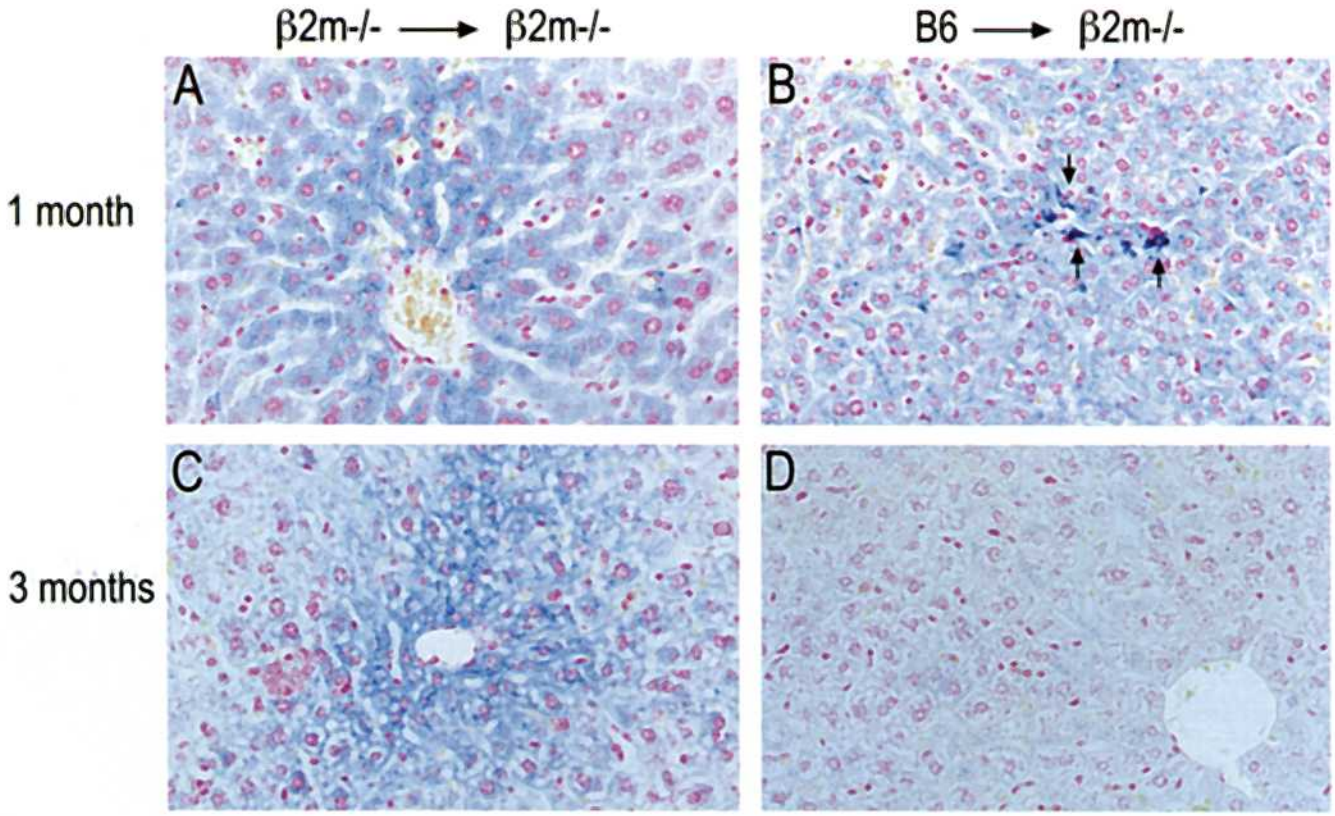

Figure 5. Liver iron in radiation chimeras (Prussian blue staining). All animals were 6 wk old at the start of the experiment. (*A* and *C*) Liver sections from $\beta 2m^{-/-}$ mice 1 mo (*A*) and 3 mo (*C*) after reconstitution with fetal liver cells from $\beta 2m^{-/-}$ donor mice. (*B* and *D*) Liver sections from $\beta 2m^{-/-}$ mice 1 mo (*B*) and 3 mo (*D*) after reconstitution with fetal liver cells from B6 donor mice. Heavily iron-loaded Kupffer cells (*arrows*) are visible 1 mo after reconstitution. After 3 mo, no iron depositions are present. Original magnification ×300.

hematopoietic cells was higher (MT = 0.86 ± 0.04; $n = 5$ and MT = 0.84 ± 0.05; $n = 5$, respectively) than in B6 mice reconstituted with B6 hematopoietic cells (MT = 0.67 ± 0.05; $n = 5$). These results suggest that hematopoietic cells have no influence on iron absorption at the level of the gut mucosa. They indicate, however, that such cells affect the pattern of cellular iron storage in the liver.

Discussion

Previously, we have reported the existence of hepatic iron overload in $\beta 2m^{-/-}$ mice similar to that found in HH (11). This was based on a histological and quantitative studies of the steady-state iron distribution and content in tissues of $\beta 2m^{-/-}$ mice. These results were confirmed more recently by others (13).

Now we report a detailed analysis of the nature of the iron metabolism defect of $\beta 2m^{-/-}$ mice. These mice recapitulate all central features of HH; i.e., increased TS, visible iron depositions, specifically in parenchymal cells of the liver, and a dysregulation of intestinal iron absorption. The defect appears to be caused by the lack of a class I–like, β2m-dependent molecule, and not by the lack of $CD8^+$ T cells or TAP-dependent MHC molecules. Moreover, although the storage abnormalities in the liver of $\beta 2m^{-/-}$ mice can be compensated by cell transfer, the increased intestinal iron absorption is not reduced by substitution of the hematopoietic compartment.

Intestinal Iron Absorption. The capacity of $\beta 2m^{-/-}$ mice to regulate intestinal iron absorption in the face of increased iron stores seems to be manifestly impaired. Similarly, in HH, patients fail to decrease their iron absorption despite the accumulation of iron in the body (30–32).

Iron retention is only the ultimate result of the iron absorption process, which can be divided into two steps: MU from the intestinal lumen and the subsequent transfer of iron through mucosal cells into the blood. Under normal conditions, not all the iron is transferred into the circulation, but remains inside the mucosal cell and is rapidly lost after desquamation of the epithelial cells. MU is influenced by many intraluminal factors: the state of the iron in the test dose (ferric or ferrous, heme or nonheme), the amount of iron, the composition of the test dose, gastric and intestinal secretions, and the state of the brush border of the mucosal cells. The next step, MT, is less dependent on these intraluminal factors and will better reflect the iron status of the body. In $\beta 2m^{-/-}$ mice, MT of iron into the plasma is invariably increased, even under normal dietary conditions. Importantly, MT is believed to be the critical step that is defective in HH patients (33). Under increased iron loading, the differences between normal and $\beta 2m^{-/-}$ mice become accentuated, as the $\beta 2m^{-/-}$ mice fail to downregulate MT and iron retention.

Cellular Iron Storage. The parenchymal iron accumulation in the livers of $\beta 2m^{-/-}$ mice resembles that of HH patients and contrasts with the histopathological findings in other iron storage disorders in man. The amount of demonstrable iron in macrophages in HH is minimal until the late stages of the disease (27, 39–42). By contrast, iron accumulation in the overload diseases Bantu siderosis (in Africa) and Kaschin-Beck (in Asia) is prominent both in mononuclear phagocyte system cells and in hepatic parenchymal cells (43–45). The organ damage that occurs in HH patients subsequent to the iron accumulation (e.g., liver fibrosis, cirrhosis, hepatocellular carcinomas, and diabetic hyperglycemia caused by pancreatic islet destruction) also occurs in old $\beta 2m^{-/-}$ mice (13, 46).

The relevance of the observed iron storage abnormalities in $\beta 2m^{-/-}$ mice emerges from numerous studies performed in animals in an attempt to mimic HH (47–51). In these studies, excess dietary iron alone failed to produce the typical parenchymal iron accumulation and the resulting organ damage, even when iron concentrations in the liver were three to four times higher than the critical iron level associated with fibrosis in HH (52). In accordance with these findings, we observed heavily iron-loaded macrophages in livers from control B6 mice after dietary manipulation. Apparently, surplus iron is redistributed from parenchymal cells to the mononuclear phagocyte system in liver of dietary iron–loaded animals (53). This notion is consistent with previous studies that show a decreased iron storage capacity of macrophages in HH (35, 36, 54). Similarly, it would appear that the Kupffer cells of $\beta 2m^{-/-}$ mice fail to store excess iron, even when surrounded by iron-loaded parenchymal cells. The apparent redistribution of stored iron from parenchymal to Kupffer cells upon transfer of hematopoietic cells might suggest that $\beta 2m^{-/-}$ Kupffer cells, like HH macrophages, carry an intrinsic defect in iron storage.

Molecular Nature of the Defect. β2m is required for the normal surface expression of classical and nonclassical MHC class I molecules (14, 15). Thus, mice lacking a functional β2m gene fail to express MHC class I products (16, 17), and consequently they lack CD8+ lymphocytes.

The TAP-1 and TAP-2 proteins are required for transport of cytosolic peptides into the endoplasmatic reticulum for association with classical class I molecules (55–57). TAP-1 mutant mice are deficient in peptide transport, and consequently do not express classical class I molecules on the surface of their cells (18). Recently, it was shown that some nonclassical MHC I molecules may be expressed independently of TAP function (19, 20). Livers from $TAP1^{-/-}$ mice had normal iron concentration values, implying that classical MHC class I molecules are not responsible for the observed iron storage phenotype in $\beta 2m^{-/-}$ mice. Rather, it would appear that a "nonclassical," β2m-associated molecule performs a function in iron metabolism. Such a molecule would be dependent on the presence of β2m, but independent of TAP function.

The gene responsible for HH has been located on the short arm of chromosome 6, in linkage desequilibrium with the HLA-A locus (8, 58), where several nonclassical MHC-I genes have been identified (59–61). While this paper was under submission, a novel MHC class I–like gene, termed *HLA-H*, was reported to be mutated in a large majority of HH patients (62). Two missense variants were found in 87% of HH patients. Importantly, the most preva-

lent mutation was inferred to disrupt the putative β2m-binding site on the HLA-H molecule. The current data lend independent, functional support for a causative role of HLA-H mutations in HH.

Iron Homeostasis. One of the hallmarks of iron homeostasis is its exclusive regulation at the sites of absorption (28, 29). It is not clear yet what mechanism regulates iron absorption from the gut, but two main hypotheses have been suggested from observations made in HH patients. One assumes a generalized defect in iron storage in the RE system and in the feedback regulation of iron absorption (37). The other hypothesis assumes a direct role of the intestinal mucosal cells in regulating iron absorption (63–68).

Our results in the reconstituted $\beta 2m^{-/-}$ mice supports the view that the primary iron metabolism defect is expressed both at the level of the gut epithelium and of Kupffer cells in the liver. The histopathological analysis indicated that the liver iron storage pattern was normalized, indicating the involvement of hematopoietic-derived cells, Kupffer cells or others, in the regulation of cellular iron storage in the liver. However, the iron absorption defect of the mucosal intestinal cells could not be corrected by the transferred cells. The defect appears not to reside in the liver parenchyma. Consistent with this view is the observation that inadvertently transplanted livers from HH patients into recipients with otherwise normal iron metabolism returned to normal iron concentrations (69–71). In these documented cases, a diseased liver was transplanted into recipients with normal hematopoietic compartment and gut mucosa. We propose that HLA-H is responsible for both primary metabolic defects in HH, namely the pattern of iron accumulation in the liver and the failure to regulate iron absorption.

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