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ORIGINAL PRE-CLINICAL SCIENCE

Disturbances in iron homeostasis result in accelerated rejection after experimental heart transplantation

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BACKGROUND: Clinical data suggest that iron disturbances deleteriously affect graft survival after heart transplantation (HTx), but immunological mechanisms underlying this phenomenon have not yet been elucidated.

METHODS: To identify the mechanistic influence of iron in a murine model of HTx, fully allogeneic BALB/c donor organs were transplanted into iron-overloaded or iron-deficient C57BL/6 mice, and recipients were analyzed for functional and immunological parameters.

RESULTS: After HTx, iron overload accelerated acute rejection as observed by shortened graft survival (HTx vs HTx + iron; $p = 0.01$), elevated rejection score ($p < 0.01$), and induction of troponin T ($p < 0.01$). Compared with controls, allografts and recipient spleens derived from iron-overloaded recipients were characterized by a pronounced graft infiltration of $CD4^+$ T cells ($p < 0.01$), $CD3^-NKp46^+$ natural killer cells ($p < 0.05$), and reduced frequencies of regulatory T cells ($p < 0.01$). This was accompanied by lower mRNA expression levels of anti-inflammatory cytokines, including interleukin-10, transforming graft factor-β, and Foxp3. Cardiac allograft survival was further tested under co-stimulation blockade (CTLA4-Ig) showing that naïve grafts transplanted into iron-overloaded recipients illustrated restricted graft outcome compared with wild types ($p = 0.0051$), which was rescued after treatment with the iron chelator deferoxamine. Iron deficiency (ID) also resulted in enhanced intragraft infiltration of inflammatory cells and accelerated rejection in the acute setting (HTx vs HTx + ID; $p = 0.02$) and after co-stimulation blockade ($p = 0.0059$).

CONCLUSIONS: We provide novel insights into the understanding of disturbances in iron homeostasis and their consequences after HTX, allowing novel insights regarding improvements in personalized immunosuppression to prolong allograft survival.

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Solid-organ transplantation (SOT) has evolved as the therapy of choice for patients with end-stage organ failure. However, marked morbidity resulting from lifelong medication remains an important limitation for long-term

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graft survival.[1] The development of treatment schemes in terms of personalized immunosuppression could provide a future approach to overcoming these limitations. The basic prerequisite for personalized medication comprises a detailed pre-transplant risk evaluation based on molecular and immunological parameters in the recipient a priori.[2] Recently, in the search for novel bioparameters indicative for tolerance, alterations in iron metabolism were suggested to predict intragraft alloimmune responses.[3]

Because iron status is not routinely assessed in SOT allograft recipients, the exact impact of iron disturbances in these patients remains unknown.[4] However, accumulating studies have already shown that iron overload is present in a substantial proportion of SOT recipients. This is due to the fact that iron overload can result from hereditary diseases, such as hemochromatosis or β-thalassemia (primary iron overload),[5,6] and secondarily, such as from blood transfusions.[7,8] Each unit of transfused blood contains 220–250 mg of iron, an amount that by far exceeds the daily intestinal absorption of 1–2 mg of nutritional iron required for iron homeostasis.[9,10] Taking into account the body's inability to excrete excess iron, it becomes evident that chronic transfusions lead to progressive and pathologic iron accumulation. Accordingly, elevated iron levels are prevalent in patients with end-stage renal disease and patients with iron overload cardiomyopathy.[8] Iron deficiency (ID) is also a prevalent finding in SOT recipients.[11] For instance, 35% of heart transplant (HTx) recipients seem to display ID, which appears to be clinically associated with elevated inflammatory markers.[12–14] This is of particular note, as several clinical observations indicate detrimental outcomes of SOT in recipients with iron overload and ID. Several clinical studies have highlighted inferior outcomes after kidney transplantation in patients with increased iron levels,[7,15,16] and oral iron supplementation might be associated with higher rates of graft rejection.[17] Likewise, after liver transplantation, primary and secondary iron overload deleteriously affects both graft and patient survival rates,[18] and elevated iron levels have been suggested as an independent risk factor for mortality after transplantation.[19] Furthermore, iron accumulation in lung allografts was shown to be associated with acute rejection.[20] To date, only 1 report exists regarding HTx in patients with iron overload cardiomyopathy, indicating inferior survival rates in the long-term.[21]

Iron seems to exert a considerable influence on immune cell functions in vitro. The influence of iron overload has been best documented for monocytes[22] but also applies to other immune cells, including $CD8^+$ T cells,[23] natural killer (NK) cells,[24] and $CD4^+$ T cells.[25] Although less well understood, ID also impairs functions of innate and adaptive cell subtypes, such as the translocation capacity and blastogenesis of T lymphocytes.[26] Increasing evidence substantiates that iron overload and ID are frequent in SOT recipients and furthermore associated with inferior post-transplantation outcomes. However, the immanent precondition is the detailed understanding of the mechanisms underlying the clinically made observations.

Methods

Animals

Male C57BL/6 (H-2^b) and BALB/c (H-2^d) mice (12 weeks old) weighing 20–25 g were obtained from Charles River Laboratories (Sulzfeld, Germany). Animals were housed under standard conditions and received humane care in compliance with the "Principles of Laboratory Animal Care" (National Institutes of Health Publication No. 86-23, revised 1985). Animal experiments were performed in accordance with the guidelines of the Medical University of Innsbruck and the Austrian Ministry for Science and Education, Vienna (66.011/0078-II/3b/2012). Recipient mice were dichotomized, receiving standard diet either enriched with iron (25 g iron/kg; Altromin Spezialfutter GmbH, Lage, Germany) or deficient for iron (<10 mg/kg), whereas control mice received standard diet without iron supplementation for 4 weeks.

Heart transplantation

Fully allogeneic BALB/c− (donor) derived hearts were transplanted into C57BL/6 recipients using a heterotopic HTx model recently described by us[27,28] and others.[29] For long-term cardiac allograft survival experiments, a single injection of Abatacept (10 mg/kg; Bristol-Myers Squibb, Munich, Germany) was administered via intraperitoneal injection 48 hours after HTx in recipients with or without prior iron overload. To assess whether iron chelation can affect long-term outcomes in iron-overloaded recipients, daily intraperitoneal injections of deferoxamine mesylate (100 mg/kg; Desferal; Novartis, Basel, Switzerland) were performed for 2 weeks after HTx.

Flow cytometry

For the generation of single cell suspensions from murine hearts, whole organs were mechanically shredded and then digested with collagenase II (Invitrogen Corp., Carlsbad, CA) plus DNase (Invitrogen Corp.) in 10 ml of medium for 45 minutes at 37°C. Mononuclear cells from spleens were isolated by Histopaque-1083 (Sigma-Aldrich, St. Louis, MO) density gradient centrifugation. For flow cytometry, 1×10^6 cells were incubated for 15 minutes at 4°C with combinations of the antibodies listed in Table S1 (available in the online version of this article at www.jhltonline.org). Data were analyzed using FlowJo software 9.3 (Tree Star Inc., Ashland, OR).

Histology

Structural changes in hearts were evaluated at the end of the respective observation period by the same pathologist (G.S.), who was blinded to all experimental data. Specimens were fixed in 4% buffered formalin, paraffin-embedded, stained with hematoxylin-eosin, and assessed by light microscopy. Graft rejection was scored according to the standards of the International Society of Heart and Lung Transplantation (ISHLT).

Assessment of cardiac function

Serum samples were stored in aliquots at −80°C until laboratory measurements were performed. High-sensitivity cardiac troponin T was measured using a commercially available assay (hs-cTnT recalibrated assay, 5th generation) on an E170 instrument

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