Haptoglobin Enhances Cardiac Transplant Rejection

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Rationale: Early graft inflammation enhances both acute and chronic rejection of heart transplants, but it is unclear how this inflammation is initiated.

Objective: To identify specific inflammatory modulators and determine their underlying molecular mechanisms after cardiac transplantation.

Methods and Results: We used a murine heterotopic cardiac transplant model to identify inflammatory modulators of early graft inflammation. Unbiased mass spectrometric analysis of cardiac tissue before and ≤72 hours after transplantation revealed that 22 proteins including haptoglobin, a known antioxidant, are significantly upregulated in our grafts. Through the use of haptoglobin-deficient mice, we show that 80% of haptoglobin-deficient recipients treated with perioperative administration of the costimulatory blocking agent CTLA4 immunoglobulin exhibited >100-day survival of full major histocompatibility complex mismatched allografts, whereas all similarly treated wild-type recipients rejected their transplants by 21 days after transplantation. We found that haptoglobin modifies the intra-allograft inflammatory milieu by enhancing levels of the inflammatory cytokine interleukin-6 and the chemokine MIP-2 (macrophage inflammatory protein 2) but impair levels of the immunosuppressive cytokine interleukin-10. Haptoglobin also enhances dendritic cell graft recruitment and augments antigen donor T-cell responses. Moreover, we confirmed that the protein is present in human cardiac allograft specimens undergoing acute graft rejection.

Conclusions: Our findings provide new insights into the mechanisms of inflammation after cardiac transplantation and suggest that, in contrast to its prior reported antioxidant function in vascular inflammation, haptoglobin is an enhancer of inflammation after cardiac transplantation. Haptoglobin may also be a key component in other sterile inflammatory conditions. (Circ Res. 2015;116:1670-1679. DOI: 10.1161/CIRCRESAHA.116.305406.)

Key Words: immunology ■ inflammation ■ rejection ■ transplantation

Sterile inflammation occurs in several medical conditions, including after organ transplantation.1-3 In the latter, the harvest and subsequent preservation of an organ induces ischemic injury to the transplant, which is exacerbated at organ implantation by restoration of blood flow, a condition known as ischemia reperfusion injury (IRI).4 IRI, in turn, induces intragraft inflammation, which can have detrimental effects on long-term allograft function.5

In organ transplantation, several putative inflammatory triggers including cellular chaperones, nuclear proteins, and components of the extracellular matrix have been associated with acute allograft rejection.6-8 These putative triggers may be released by the cellular necrosis that accompanies organ implantation and reperfusion. However, it is unclear what role these triggers play in inflammation induction after organ transplantation. Importantly, inhibition of some of these triggers only modestly extends cardiac allograft survival,9 indicating that other yet undiscovered factors may contribute to inflammation after cardiac transplantation.

As graft inflammation has detrimental effects on graft outcomes,9 the identification of novel inflammatory mediators is therefore key for identifying lead candidates for the development of novel therapies to reduce inflammation after organ transplantation. Such lead candidates are particularly relevant to organ transplantation as the harvest and preservation of an organ provides a therapeutic window of opportunity to treat the graft before implantation while avoiding the inhibition of host defense pathways in recipients, an important strategy given that organ transplant recipients are intentionally immune suppressed.

Here, we used an unbiased proteomic screen to identify novel inflammatory modulators in transplanted tissue after...
cardiac transplantation in a murine model. Our study revealed that by 72 hours after transplantation, 22 proteins are significantly upregulated within the graft including haptoglobin, a heme-binding protein with antioxidative properties. In our murine cardiac transplant model, haptoglobin remarkably prevents the effects of costimulatory blockade therapy to induce indefinite allograft survival. We also found the protein in human cardiac allograft specimens undergoing acute graft rejection. Haptoglobin therefore may be a potential candidate for the development of novel therapeutics to reduce inflammation within cardiac allografts and possibly other organ transplants.

Methods

Note that detailed experimental procedures and methods are reported in the Methods section in the Online Data Supplement.

Mice Heart Transplant Model

C57BL/6J (stock# 000664), BALB/cJ (stock# 000651) mice were purchased from Jackson Laboratories (Bar Harbor ME). C57BL/6 haptooglobin deficient (Hp−/−), C57BL/6 MyD88−/− (each backcrossed ten times) were purchased from Jackson Laboratories (Bar Harbor ME). C57BL/6J (stock# 000664), BALB/cJ (stock# 000651) mice were purchased from Jackson Laboratories (Bar Harbor ME). C57BL/6J (stock# 000664), BALB/cJ (stock# 000651) mice were purchased from Jackson Laboratories (Bar Harbor ME). C57BL/6J (stock# 000664), BALB/cJ (stock# 000651) mice were purchased from Jackson Laboratories (Bar Harbor ME). C57BL/6J (stock# 000664), BALB/cJ (stock# 000651) mice were purchased from Jackson Laboratories (Bar Harbor ME). C57BL/6J (stock# 000664), BALB/cJ (stock# 000651) mice were purchased from Jackson Laboratories (Bar Harbor ME). C57BL/6J (stock# 000664), BALB/cJ (stock# 000651) mice were purchased from Jackson Laboratories (Bar Harbor ME).

To determine whether cardiac transplantation led to an increase in inflammatory modulators, we procured cardiac lysates before and after transplantation and cultured them with bone marrow–derived dendritic cells (DCs) as previously reported.11 Cardiac lysates were inflammatory modulators, we procured cardiac lysates before and after transplantation. Dictatorial haptoglobin deficient (Hp−/−), C57BL/6MyD88−/− (each backcrossed ten times) and relevant wild type (WT) littermate controls 2 to 4 months of age, both males and females, were maintained in our colony. The Hp−/− mice were initially provided by Dr Maffei (Dulbecco Telethon Institute, University Hospital of Pisa, Italy). The use of vertebrate animals was approved by the Yale School of Medicine IACUC.

A murine heterotopic heart transplant model was used to assess intragraft inflammatory responses12 (detailed in the Online Data Supplement). Anesthesia was induced by isofluorane via a pressurized vaporizer and maintained with ketamine (0.1 mg/g body weight) and xylazine (0.1 mg/g body weight).

In Vitro Culture, Cytokine Measurement, and Flow Cytometry

To determine whether cardiac transplantation led to an increase in inflammatory modulators, we procured cardiac lysates before and after transplantation and cultured them with bone marrow–derived dendritic cells (DCs) as previously reported.11 Cardiac lysates were free of lipopolysaccharide (LPS) (Limulus assay) and did not contain any microbial peptides as assessed by mass spectrometry. Lysates were treated with pronase (0.5 U/mL; Calbiochem-Behring Corp, La Jolla, CA), DNase I (60 U/mL, Roche), or RNase (10 μg/mL, Thermo Scientific) at 37°C for 1 hour and then added to the DC cultures overnight as indicated. Target cytokines and chemokines analytically were decreased in the cardiac lysates by ELISA for interleukin (IL)-6, IL-10, monocyte chemotactic protein 1 (MCP-1) (eBioscience), macrophage inflammatory protein 2 (MIP-2) (R&D Systems), heme-oxigenase-1 (Enzo Life Sciences, Farmingdale, NY), and haptoglobin (Immunology Consultants Laboratory, Tampa, FL). We enriched subpopulations of cells within cellular suspension from cardiac tissue with a serial magnetic enrichment protocol (magnetic beads and columns from Miltenyi Biotec, Inc, CA) as detailed in the Online Data Supplement. Purified human haptoglobin was obtained from Sigma and found to contain ≤40 pg/mL of LPS. Mixed lymphocyte cultures consisted of magnetically enriched T cells (Stem Cell Technologies, Vancouver, British Columbia) cultured in a 1:5 ratio with irradiated donor spleen cells. Antidonor T-cell responses were assessed either by measuring cytokine production by ELISPOT (enzyme-linked immuno spot), ELISA or proliferation by thymidine incorporation. Cellular suspensions (ie, spleen cells and cells harvested from cardiac tissue) were stained with relevant fluorescent-tagged antibodies and data acquired on an LSR II (BD Bioscience) flow cytometer and analyzed with FlowJo software (Treestar, Ashland, OR).

Histopathology

To determine the presence of intragraft haptoglobin after human heart transplantation, we stained for haptoglobin in human, archived endomyocardial specimens from the Pathology Department of the Yale New Haven Hospital. The samples had been histologically scored as either no evidence of cellular graft rejection or moderate cellular graft rejection according to the diagnostic criteria of the International Society for Heart and Lung Transplantation.13 These specimens were paraffin embedded at the time of routine protocol biopsy after heart transplantation and were deparaffinized and counterstained with an antihuman haptoglobin antibody (AbCam, Cambridge, MA) or iso-type control via immune histochemistry. Slides were read blindly. The use of clinical specimens and accessing patient data from de-identified medical records were approved by the Human Regulatory Board at Yale School of Medicine.

To assess histological inflammation after cardiac transplantation in mice, native and transplanted hearts were harvested and fixed by immersion in 10% neutral buffered formalin, bisected lengthwise, processed, sectioned, and stained for hematoxylin and eosin by routine methods. An antimurine haptoglobin antibody (Proteintech, Chicago, IL) was used to stain for haptoglobin in murine hearts. Murine histological data were read in a blinded fashion.

Statistics

Statistical analysis is detailed in the Online Data Supplement.

Results

Characterization of Early Intragraft Inflammation

After organ implantation, graft inflammation occurs within hours in mice and humans.13 To establish a model to assess early intragraft inflammation after cardiac transplantation, we subjected murine BALB/c hearts to increasing cold storage and then implanted these organs into C57BL/6J murine recipients. This model differs by a full major histocompatibility complex mismatch between the donor and the recipient.

When we measured inflammatory cytokines and chemokines within the transplant 24 hours after implantation, we found that regardless of the exposure of the organ to cold storage, the inflammatory mediators MCP-1, MIP-2, and IL-6 were increased 2- to 10-fold over those in nontransplanted hearts (Online Figure IA), indicating an early phase of intragraft inflammation after organ implantation.

Characterization of the intragraft levels of cytokines and chemokines demonstrated similar levels between syngeneic and allogeneic transplants at 24 hours after transplantation (Online Figure IB). At this time point the T-cell response to transplantation was not yet evident within the transplant (Online Figure II). Our observations also held true when the hearts were exposed to 4 hours of cold storage before implantation (Online Figure II), indicating that the early inflammatory phase within the graft after cardiac transplantation is antigen independent.

To determine the kinetics of the early, antigen-independent (ie, syngeneic transplants), inflammatory phase for cardiac transplants, we analyzed the levels of inflammatory mediators including IL-6, MCP-1, and MIP-2. These three mediators...
peaked within the graft between 6 and 48 hours after transplantation and then subsided by 72 hours after transplantation (Online Figure IC). In parallel, the levels of inflammatory mediators within the recipient’s native heart (denoted as native heart), which was exposed to perioperative stressors, but not to the effects of organ procurement and implantation, were comparable with those in the donor heart before implantation (Online Figure IB and IC), indicating that the early antigen-independent phase of graft inflammation is mainly driven by the effects of organ procurement and implantation.

Proteins Are Contributors to Inflammation After Cardiac Transplantation

To characterize intragraft inflammation further after cardiac transplantation, we adapted an in vitro assay in which DCs are cultured with tissue lysates.11 We then assessed the production of the proinflammatory cytokine IL-6 by DCs during the culture to determine the degree of DC activation induced by the lysates. We measured IL-6 in the assay, as there are prior reports that IL-6 contributes to the tempo of cardiac allograft rejection.14–16 The IL-6 response by DCs was increased after the culture with the lysates obtained from cardiac transplants at 24 and 48 hours after transplantation but not with the lysates obtained from nontransplanted hearts or recipient native hearts (Online Figure IIIA). These findings indicate that cardiac transplantation increases the concentration of inflammatory mediators within the graft.

To evaluate the nature of the inflammatory mediators within cardiac grafts, we added RNase, DNase, or pronase separately to lysates from syngeneic cardiac grafts at 24 hours after transplantation to degrade RNA, DNA, and proteins, respectively. Our findings suggest that proteins are the major contributors to the early antigen-independent phase of inflammation after cardiac transplantation as digestion of proteins abrogated the induced IL-6 response of DCs (Online Figure IIIB). In contrast, digestion of RNA or DNA did not reduce the IL-6 response (concentrations of RNase and DNase used in our experiments effectively digested known RNA and DNA-based DC activators, and the concentration of pronase used in the assay did not impair DCs to respond to LPS; Online Figure IIIC and IIID).

Proteomic Screen of Cardiac Tissue Before and After Transplantation

Based on the results from our in vitro DC assay, we identified proteins that are differentially regulated after cardiac transplantation by comparing nontransplanted cardiac tissue with tissue from syngeneic cardiac transplants at various time points after transplantation via mass spectrometry. By 6 hours after transplantation, only 1 protein, NADP transhydrogenase was differentially (down) regulated in cardiac grafts, whereas by 24 to 72 hours after transplantation, several proteins were differentially down- and upregulated within the transplant (Online Figure IVA and IVB). The entire proteomic screen identified a total of 1318 up- and downregulated proteins (Online Table I). Among these proteins, bioinformatic analyses indicated enrichment for biological and molecular processes including immune response, phagocytosis, and cytoskeletal reorganization (Online Tables II and III) and cellular components including mitochondrial components and myofilaments (Online Table IV), reflective of the complex alterations that occur within the graft after cardiac transplantation.

Upregulation of Proteins After Cardiac Transplantation

Our proteomic screen identified 22 proteins that were significantly upregulated in the graft after cardiac transplantation (Online Table V). The only significantly upregulated proteins at both 24 and 72 hours after transplantation were calgranulin A, haptoglobin, chitinase-like 3 protein, and vimentin (Online Table V). We had previously used an unbiased proteomic screen of skin transplants and noted that haptoglobin, a protein with antioxidant and immune modulatory properties,17–19 was upregulated in the graft after skin transplantation.11 Our earlier findings indicated that haptoglobin increases the tempo of minor mismatched skin graft rejection.11 Given this result, we explored the role of haptoglobin in cardiac transplantation.
We confirmed the upregulation of intragraft haptoglobin in the syngeneic heart transplant model via an ELISA test (Figure 1A), and found that haptoglobin was highly induced in the serum 3 hours after cardiac transplantation in mice (Figure 1A). At 24 hours after transplantation, haptoglobin levels were 6-fold higher in the cardiac transplant than in the native heart (Figure 1B), indicating that haptoglobin preferentially enters sites of inflammation.

### Haptoglobin Enhances Acute Cardiac Allograft Rejection in Mice

Although intragraft haptoglobin levels were similar between allografts and syngeneic grafts 1 day after transplantation, by 3 days after transplantation, haptoglobin levels were 2-fold higher in allografts than in syngeneic grafts (Figure 1C), although by day 7, after transplantation levels in allografts declined to the levels of syngeneic grafts (Figure 1C). We also observed that haptoglobin levels increased in the liver of murine heart transplant recipients and that recipients of allografts exhibited higher levels of haptoglobin in the liver than recipients of syngeneic grafts (Figure 1D). These results indicate that intragraft haptoglobin is highly induced in both allografts and syngeneic grafts but peaks at a higher level in allografts during the first week after transplantation. Furthermore, concentrations of haptoglobin in the liver, a known site of haptoglobin production, increase after cardiac transplantation.

Given these findings, we examined the effect of haptoglobin on the tempo of acute allograft rejection after cardiac transplantation. For this purpose, we transplanted WT or Hp−/− recipient mice with a WT BALB/c cardiac allograft with or without perioperative CTLA4 Ig. CTLA4 Ig is related to belatacept, a drug that is used currently in clinical organ transplantation and which inhibits costimulation between T cells and antigen-presenting cells, thus increasing transplant survival.\(^2\) Without CTLA4 Ig treatment, Hp−/− recipients exhibited a 3-day significant extension of allograft survival compared with WT recipients (Figure 2A). With CTLA4 Ig treatment, this difference was dramatically increased as Hp−/− recipients displayed significantly longer cardiac allograft survival (median survival time >100 days) than WT recipients (median survival <21 days; Figure 2B), indicating that recipient haptoglobin impairs the ability of CTLA4 Ig to induce prolonged allograft survival. Haptoglobin expression in the donor allograft did not affect the tempo of acute allograft rejection in our model (Online Figure V). At 21 days after transplantation, the majority (8/9) of Hp−/− recipients treated with CTLA4 Ig exhibited beating heart grafts and thus allograft survival, whereas all similarly treated WT recipients had rejected their allografts (Online Figure VI and Online Movies I and II). At this time point, Hp−/− recipients treated with CTLA4 Ig also exhibited reduced histological evidence of graft necrosis (Figure 2C). Immune histochemical staining for haptoglobin within the allografts of WT recipients showed that haptoglobin costained with some cardiomyocytes, macrophages and exhibited scant perivascular staining (Figure 2D).

### Haptoglobin Alters the Intragraft Inflammatory Milieu After Cardiac Transplantation and Treatment With CTLA4 Ig

We previously demonstrated that haptoglobin activates bone marrow–derived DCs in vitro to induce the production of pro-inflammatory cytokines, specifically the production of IL-6.\(^1\) Therefore, we evaluated how recipient haptoglobin affected the intragraft inflammatory milieu after cardiac transplantation.
and perioperative treatment with CTLA4 Ig. During the first 3 weeks after transplantation, we noted lower intragraft levels of IL-6 and MIP-2 in Hp−/− than in WT recipients treated with CTLA4 Ig (Figure 3A and 3B). In addition, graft levels of the immunosuppressive cytokine IL-10 were higher in the allografts of Hp−/− recipients treated with CTLA4 Ig than in similarly treated WT recipients (Figure 3C).

This elevation of IL-10 in the grafts of Hp−/− recipients was surprising given that prior work found that haptoglobin–hemoglobin complexes are internalized in macrophages to induce hemeoxygenase-1 and IL-10 production.19,21 We determined that intragraft levels of hemeoxygenase-1 were not altered by haptoglobin after cardiac transplantation and treatment with CTLA4 Ig (Online Figure VII). Furthermore, Hp−/− recipients of WT and thus haptoglobin sufficient allografts treated with CTLA4 Ig exhibited abrogated intragraft levels of haptoglobin (Figure 3D), further supporting the idea that haptoglobin, which impairs the ability of CTLA4 Ig to enhance graft survival (Figure 2B), originates from the recipient. Finally, we found that haptoglobin levels were significantly increased in the livers of WT cardiac transplant recipients and treatment with CTLA4 Ig but not in nontransplanted recipients (Online Figure VIII), indicating that the liver is a contributing source to haptoglobin after cardiac transplantation.

We further characterized how haptoglobin altered the inflammatory response of specific cells within heart allografts by enriching immune cells, fibroblasts, and endothelial cells (ECs) from allografts at day 21 after transplantation from WT and Hp−/− recipients treated with CTLA4 Ig. The total numbers of these different cell types were obtained from the allografts of WT or Hp−/− recipients and the cells were then cultured ex vivo. As IL-6 and MIP-2 were reduced in the allografts of Hp−/− recipients (Figure 3A and 3B), we measured IL-6 and MIP2 in the supernatants after 12 hours of cell culture. Compared with cells obtained from nontransplanted WT BALB/c hearts, immune cells enriched from BALB/c allografts from WT recipients produced significantly elevated (ie, 5- to 10-fold) levels of both IL-6 and MIP-2 compared with immune cells enriched from Hp−/− recipients (Figure 3E and 3F). We also noted that there were significantly more immune cells (ie, 4-fold higher) in the allografts of WT recipients as compared with Hp−/− recipients at this time point after transplantation (Online Figure IX). These data suggest that infiltrating immune cells are a likely source of proinflammatory cytokines within the allograft and that haptoglobin enhances this response by increasing the recruitment of immune cells into the allografts.

Fibroblasts and ECs from allografts did not produce IL-6 and MIP-2 above levels from cells obtained from nontransplanted hearts (Online Figure X). Immune cells enriched from allografts also produced more IL-10 after culture than cells obtained from nontransplanted hearts (Online Figure XI). Furthermore, immune cells enriched from the allografts of Hp−/− recipients exhibited somewhat lower levels (ie, 50% reduced) of IL-10 than immune cells enriched from the allografts of WT recipients (Online Figure XI). ECs, but not

Figure 3. Recipient haptoglobin alters the intragraft inflammatory environment after cardiac transplantation and treatment with perioperative CTLA4 Ig. Wild-type (WT) and Hp−/− recipients were implanted with a WT BALB/c cardiac allograft and were treated with perioperative CTLA4 Ig (200 μg days 0, 2, and 4 after transplantation). At day +14 or +21 after transplantation, cardiac allografts were obtained and intragraft interleukin (IL)-6 (A), MIP-2 (B), IL-10 (C), and haptoglobin (D) were measured via ELISA. *P<0.01 (t test). Arrows in D indicate the Hp−/− groups. Figures represent pooled data from three independent experiments. Error bars, SEM, n=6 to 9 mice per time point. E and F, Immune cells (ie, CD45+ cells) were enriched from hearts of recipients at day +21 after transplantation and treatment with CTLA4 Ig and cultured for 12 hours. IL-6 and MIP-2 were measured in the culture supernatant. Pooled data from 3 independent experiments, n=2 per experiment, *P<0.01 (t test).
fibroblasts, enriched from allografts had higher levels of IL-10 production than cells obtained from nontransplanted hearts, although we did not observe significant differences between ECs enriched from the allografts of Hp−/− recipients and WT recipients. These results suggest that in addition to immune cells, ECs contribute to the graft levels of IL-10 after cardiac transplantation and treatment with CTLA4 Ig.

Haptoglobin Increases Accumulation and Activation of Intragraft DCs After Cardiac Transplantation and Treatment With CTLA4 Ig

As DCs have been shown to be critical for activating CD4+ T cells to induce acute cardiac allograft rejection,22,23 we next assessed whether there were alterations in the activation phenotype and accumulation of intragraft DCs (defined as CD11c+ major histocompatibility complex class II+ cells) in the cardiac allografts of Hp−/− compared with WT recipients treated with CTLA4 Ig at day +7 after transplantation. We found that the upregulation of the costimulatory molecule CD80 was reduced 3-fold on the surface of intragraft DCs in the Hp−/− recipients as compared with WT recipients treated with CTLA4 Ig (Figure 4A and 4B). There was also a lower number of DCs within the allografts of Hp−/− recipients treated with CTLA4 Ig than in WT recipients treated with CTLA4 Ig (Figure 4C). Similar to what we observed at day +21 after transplantation, we found a 2-fold lower total number of immune cells within the allografts of Hp−/− recipients than in WT counterparts at day +7 after transplantation (Figure 4D). Analysis at day +7 after transplantation also found reduced numbers of neutrophils and T cells in the allografts of Hp−/− recipients as compared with their WT counterparts (Online Figure XII).

MyD88 Expression Within the Allograft Alters the Intragraft Inflammatory Milieu

We previously demonstrated that haptoglobin activates bone marrow–derived DCs to produce IL-6 in vitro via MyD88.11 Furthermore, we and others have shown that MyD88 is critical for IL-6 production by DCs and macrophages in response to microbial stimulation.24 We therefore examined the impact of MyD88 expression within the allograft on the development of graft inflammation. For this purpose, WT or MyD88−/− C57BL/6 hearts were transplanted into WT BALB/c recipients that were treated with CTLA4 Ig. At day 14 after transplantation, the allografts were obtained and graft levels of IL-6, MIP-2, and IL-10 were measured. We found that the graft levels of IL-6 and MIP-2 were significantly reduced 3- to 5-fold in MyD88−/− allografts as compared with WT allografts (Figure 5A and 5B). IL-10 levels were significantly elevated 3-fold in the MyD88−/− allografts as compared with WT allografts (Figure 5C). These results phenocopy the graft inflammatory alterations observed in Hp−/− recipients of WT allografts (Figure 3A–3C).

To determine the cellular targets of haptoglobin within resident heart cells, we isolated immune cells, fibroblasts, and ECs from WT and MyD88−/− nontransplanted hearts and stimulated the cells in vitro with haptoglobin. We found that only immune cells enriched from hearts produced IL-6 in response to haptoglobin and this response was mostly abrogated in immune cells obtained from MyD88−/− hearts (Figure 5D; immune cells, fibroblasts, and ECs produced IL-6 in response to in vitro stimulation with LPS, indicating that these populations of cells enriched from hearts were capable of producing IL-6; Online Figure XIII). Overall, these data show that donor expression of MyD88 enhances allograft inflammation after cardiac transplantation and treatment with CTLA4 Ig and that immune cells are the likely targets within the heart that respond to haptoglobin in a MyD88-dependent fashion.

Haptoglobin Enhances Antidonor T-Cell Responses Without Affecting Intrinsic T-Cell Function

As prior in vitro studies have indicated that haptoglobin may affect T-cell function to nominal antigens or nonspecific stimulation,18,25 we assessed whether haptoglobin alters intrinsic T-cell responses to allostimulation in a mixed lymphocyte culture. We found that purified WT and splenic Hp−/− polyclonal T cells stimulated in vitro with irradiated allogeneic cells, enriched from the allografts of Hp−/− recipients and WT recipients, were capable of producing IL-6; Online Figure XIII). Overall, these data show that donor expression of MyD88 enhances allograft inflammation after cardiac transplantation and treatment with CTLA4 Ig and that immune cells are the likely targets within the heart that respond to haptoglobin in a MyD88-dependent fashion.

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**Figure 4.** Recipient haptoglobin enhances accumulation of mature dendritic cells (DCs) after cardiac transplantation and treatment with perioperative CTLA4 Ig. A, Representative flow cytometric plot showing surface expression of CD80 on CD11c+ major histocompatibility complex (MHC) class II+ cells within the graft at day +7 after transplantation in wild type (WT) and Hp−/− mice. Gray shadow shows expression on CD11c+ MHC class II+ cells in the allograft before implantation. Solid line, WT; dotted line, Hp−/−. B, Median fluorescent intensity (relative units) of CD80 expression on CD11c+ MHC class II+ cells in the allograft before implantation. Solid line, WT; dotted line, Hp−/−. C, Median fluorescent intensity (relative units) of CD80 expression on CD11c+ MHC class II+ cells in the allograft before implantation. Solid line, WT; dotted line, Hp−/−. D, Enumeration of DCs and immune cells within the allografts of WT and Hp−/− recipients treated with CTLA4 Ig at day +7 after transplantation. Pooled data from 2 independent experiments with n=3 per experiment. *P<0.01 (t test). Arrow in C indicates pretransplant group.
spleen cells exhibited similar production of the Th1 cytokine, interferon-γ, and similar IL-2 levels as WT T cells (Figure 6A and 6B). The levels of proliferation measured in the mixed lymphocyte culture were also similar between WT and Hp−/− T cells (Figure 6C). However, when we assessed antidonor T-cell responses in T cells obtained from the spleens of WT and Hp−/− recipients treated with CTLA4 Ig and transplanted with cardiac allografts, Hp−/− recipients exhibited a 2- to 3-fold reduction in splenic antidonor T-cell interferon-γ and IL-2 responses (Figure 6D and 6E). Recipient haptoglobin had no impact on the numbers of splenic CD4+FoxP3+ regulatory T cells either before or after transplantation and treatment with CTLA4 Ig (Figure 6F). Thus, haptoglobin seems to amplify intragraft inflammation (Figure 3A and 3B) and enhances antidonor Th1 T-cell alloimmunity to impair the graft-prolonging effects of costimulatory blockade.

**Intragraft Expression of Haptoglobin Associates With Acute Allograft Rejection in Humans**

To provide an initial determination of the presence of haptoglobin in human heart transplants, we performed a case-controlled study in which 10 endomyocardial biopsies that had no histological evidence of rejection on routine endomyocardial biopsy obtained at various time points after transplantation were compared with 9 biopsies that exhibited moderate histological evidence of rejection. We found that haptoglobin staining was present in 7 of 9 human cardiac transplant specimens that exhibited evidence of moderate allograft rejection but...
only in 2 of 10 cardiac transplant specimens that were free of acute rejection (P=0.02, Fisher exact test; Figure 7A and 7B). Examination of the 7 biopsies that exhibited moderate rejection and haptoglobin staining suggested that haptoglobin was present within some cardiomyocytes (Online Figure XIV) and within some scattered ECs (Figure 7B). In this small sample size, we did not observe haptoglobin staining with immune cells (the clinical characteristics of patients who had moderate rejection are compared with those of patients with no rejection in Online Table VI). These findings suggest that intragraft haptoglobin is also associated with acute allograft rejection in humans.

Discussion

Our study identified haptoglobin as a novel enhancer of cardiac graft inflammation that prevents the allograft-prolonging effects of CTLA4 Ig. Although haptoglobin has important physiological functions, such as an antioxidant in the setting of hemolysis,17 our study has found that it also enhances the levels of inflammatory cytokines, such as IL-6, which have been shown to impair cardiac allograft survival.14

In contrast to a skin transplant model,11 our current study found that only recipient, but not donor, expression of haptoglobin accelerates the tempo of acute cardiac allograft rejection (Figure 2; Online Figure V). In previous studies, the skin has been shown to be a source of haptoglobin.26 Unlike the skin, however, which undergoes neovascularization after transplantation, cardiac transplants are immediately vascularized and subjected to a rapid IRI. Our study suggests that the liver is a source of haptoglobin in our cardiac transplantation model. Haptoglobin production from the liver may be induced by the initial inflammation and hypoxia that occurs after graft implantation. In turn, haptoglobin enters the graft within 24 hours after transplantation via the vasculature and amplifies inflammation.

Prior work has indicated that haptoglobin enhances the clearance of Salmonella and increases inflammation in delayed-type hypersensitivity reactions of the skin.18 There is also evidence that haptoglobin affects intrinsic T-cell function in particular Th1 responses, at least to nominal antigens or nonspecific stimulation in vitro.19,25 Furthermore, haptoglobin increases the cellularity of secondary lymphoid organs in mice,18 which could affect immune responses including the tempo of cardiac allograft rejection. Other studies have found that haptoglobin dampens inflammation induced by innate immune activation via lipopolysaccharide.27 In addition, haptoglobin reduces oxidative stress within the myocardium and reduces the rate of late ventricular rupture after acute myocardial infarction induced by coronary ligation in mice.28 Moreover, when haptoglobin binds to hemoglobin, this complex induces the production of the immune suppressive cytokine IL-10 by macrophages.19 Given that innate immune activation is implicated in IRI,20 we expected haptoglobin to reduce inflammation after cardiac transplantation. However, our study found that haptoglobin enhanced graft inflammation and impaired the graft prolonging properties of CTLA4 Ig, although it did not affect intrinsic T-cell function during in vitro stimulation with allogeneic spleen cells (Figure 6A–6C). Together, our current study and prior reports imply that haptoglobin exhibits either anti- or proinflammatory effects, and the dominant phenotype may depend on the disease context or the model used.

Our current study indicates that in the context of organ transplantation haptoglobin enhances the recruitment of immune cells into the allograft including DCs to amplify graft inflammation. We think that the initial IRI after cardiac transplantation induces graft inflammation. This leads to the production of haptoglobin in the recipient, which is released into the circulation and subsequently enters the graft to amplify inflammation, possibly through activating intragraft immune cells, such as DCs. In turn, activated immune cells within the transplant release cytokines and chemokines to enhance immune cell recruitment and promote inflammation. Future studies will be needed to determine how haptoglobin affects other possible cellular sources of pro- and anti-inflammatory molecules within the heart, such as cardiomyocytes or vascular smooth muscle cells. Regardless of the source of inflammatory mediators, recipient haptoglobin enhances the intragraft inflammatory milieu and increases antidonor T-cell immunity after cardiac transplantation. Our study suggests that the inflammatory alterations mediated by haptoglobin renders T cells less susceptible to the effects of costimulatory blockade (Online Figure XV).

We previously demonstrated that haptoglobin activates bone marrow–derived DCs in vitro via MyD88, a signal adaptor downstream of TLR and IL-1 and 18 receptors, to induce inflammatory responses.11 In the current study, we found that the expression of MyD88 within the allograft enhances graft inflammation (ie, elevating levels of IL-6 and MIP-2 but impairing IL-10 levels) similar to the impact recipient haptoglobin has on graft inflammation. We also provide evidence that immune cells enriched from cardiac tissue respond to haptoglobin in vitro via MyD88. Together, these data imply that recipient-derived haptoglobin is sensed by immune cells in the

![Figure 7. Intragraft haptoglobin associates with acute allograft rejection in humans.](http://circres.ahajournals.org/)

Human heart transplant specimen with no cellular rejection and no staining for haptoglobin (A), and a specimen with evidence of rejection and positive staining for haptoglobin (black arrow; B). Blue arrow indicates circumferential staining consistent with endothelial cells. Scale bar, 50 μm.
graft to enhance alloimmunity. Future studies will be required to identify the MyD88 sensing cell within the allograft that is activated by haptoglobin in vivo to enhance intragraft inflammation after cardiac transplantation.

The cellular recognition mechanisms and pathways by which haptoglobin alters the intragraft inflammatory milieu after vascularized organ transplantation will require further investigations to discern detailed underlying molecular pathways. Moreover, haptoglobin was not the only upregulated protein in the graft after cardiac transplantation. Aside from haptoglobin, calgranulin A, chitinase-like 3 protein and vimentin were also significantly upregulated at both 24 and 72 hours after transplantation (Online Table V). Prior work has shown that calgranulin A suppresses DC priming and slows the tempo of major histocompatibility complex II mismatched cardiac allograft rejection in mice. A recent study identified that chitinase like 1 protein, which is similar to the chitinase-like 3 protein, correlates with the degree of graft injury after kidney transplantation, although the role of either protein in cardiac transplantation is not known. In addition, there is evidence that vimentin is an important antigen in organ transplantation and antibodies to it are associated with cardiac allograft rejection. These other proteins therefore warrant further exploration as they may potentially also contribute to inflammation after heart transplantation.

Aside from the murine data that implicate haptoglobin as an amplifier of inflammation after cardiac transplantation, our study also showed that there is an association between intragraft haptoglobin expression and acute allograft rejection in archived human biopsies that were obtained at various time points after heart transplantation. Our murine data indicate that without immune suppression, haptoglobin is upregulated within the graft during the first week after transplantation (Figure 1C) and is present in cardiac transplants 3 weeks after transplantation in recipients treated with CTLA4 Ig (Figure 3D). Human heart transplant biopsies are not routinely available before 1 week after transplantation because of patient safety issues. We therefore could not determine whether haptoglobin is upregulated within the immediate perioperative period after human heart transplantation. As our small case-controlled study did not determine where haptoglobin localizes within human cardiac transplants, future clinical studies will be required to identify the time course and localization of haptoglobin within human hearts transplants, although it will be challenging to assess whether haptoglobin is induced in the perioperative period.

In summary, we show that haptoglobin is an important amplifier of inflammation after cardiac transplantation. Our results from the murine experimental data combined with our human archived samples suggest that haptoglobin enhances allograft inflammation after cardiac transplantation. The protein and its associated pathways may be key to identifying potential candidates for future anti-inflammatory treatments and therapies.

Sources of Funding
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Disclosures
None.

References


Novelty and Significance

What Is Known?

- Cardiac transplantation leads to ischemia reperfusion injury and inflammation after graft implantation.
- Graft inflammation impairs the induction of transplantation tolerance and enhances acute and chronic transplant rejection.

What New Information Does This Article Contribute?

- Haptoglobin levels increase in the serum and the graft after cardiac transplantation in mice.
- Haptoglobin amplifies graft inflammation via activation of the innate immune system to impair indefinite cardiac transplant survival induced by costimulatory blockade.
- Haptoglobin expression in human heart transplants associates with acute transplant rejection.

After organ transplantation, inflammation is induced by the innate immune system. However, the mechanisms that induce and maintain graft inflammation remain unclear. We performed a proteomic analysis of murine cardiac transplants and showed that the protein haptoglobin, which is known for its antioxidant properties, is upregulated in these grafts. Recipient, but not donor, expression of haptoglobin impairs the ability of immune modulators to induce indefinite cardiac allograft survival in mice. Furthermore, haptoglobin in cardiac allografts enhances the production of the proinflammatory cytokine interleukin-6, the chemokine MIP-2 but impairs the immune suppressive cytokine, interleukin-10. We also showed that haptoglobin activates resident immune cells in heart tissue via the innate immune adaptor, MyD88. Importantly, the presence of haptoglobin in human cardiac allograft biopsies correlates with acute allograft rejection. Together, these findings indicate a novel and surprising function for haptoglobin in cardiac allografts: activation of innate immunity to impair indefinite allograft survival. Our study reveals that the inflammatory pathway amplified by haptoglobin may be therapeutically targeted to reduced inflammation after organ transplantation and possibly in other sterile inflammatory conditions.
Haptoglobin Enhances Cardiac Transplant Rejection
Hua Shen, Elizabeth Heuzey, Daniel N. Mori, Christine K. Wong, Christopher M. Colangelo, Lisa M. Chung, Can Bruce, Ilya B. Slizovskiy, Carmen J. Booth, Daniel Kreisel and Daniel R. Goldstein

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Supplementary Material

Animals and cardiac surgery procedure
To discover and examine the role of novel inflammatory modulators after cardiac transplantation, we used a heterotopic heart transplant model as previously described \(^1\) to assess intra-graft inflammation and to perform a proteomic screen of transplanted heart tissue. The procedure was also employed to assess intra-graft inflammation and graft survival between wild type and haptoglobin deficient mice. In our heart transplant model, the pulmonary artery and ascending aorta of the heart transplant were anastomosed to the recipient’s inferior vena cava and abdominal aorta, respectively. Mice were given peri-operative penicillin and streptomycin (2000 units / mouse) via intra-peritoneal injection 1h after transplantation. Where indicated, transplants were placed in University of Wisconsin cold storage solution prior to implantation. For experiments in which allografts were implanted and graft survival was recorded, rejection was monitored by daily abdominal palpitation. Rejection was defined as loss of palpable heartbeat. At day 21 post transplantation, 9 mice/ group were anesthetized, their abdomens were incised and video was obtained to assess contraction of the donated heart. Mice were subsequently euthanized. CTLA4 Ig was obtained from Bio X cell (West Lebanon, NH) and was administered at 200μg day 0, 2, 4 post transplantation to recipient mice via intra-peritoneal injection.

Human endomyocardial specimens
We obtained archived endomyocardial specimens from the pathology department of the Yale New Haven Hospital. These specimens had been paraffin-embedded at the time of routine protocol biopsy after heart transplantation. The samples had been histologically scored as either no evidence of cellular graft rejection or moderate cellular graft rejection according to the diagnostic criteria of the International Society for Heart and Lung Transplantation \(^2\). The specimens were stained for haptoglobin by immune histochemistry (see below) in a blinded fashion. Use of clinical specimens and accessing patient data via de-identifying medical records was approved by the Human Regulatory Board at Yale School of Medicine.

Histopathology
Mice were euthanized by carbon dioxide asphyxiation before and after transplantation to obtain relevant tissue. Blood was collected by terminal cardiac puncture to obtain serum for haptoglobin measurement. Native and transplanted hearts were harvested and fixed by immersion in 10% Neutral Buffered Formalin, bisected lengthwise, processed, sectioned, and stained for hematoxylin and eosin (HE) by routine methods. Sections of hearts were examined by experimental manipulation by light microscopy on an Axio Imager A1 microscope (Zeiss Microsystems). Histological assessment was performed in a blinded fashion.

Immunohistochemistry
Sections of mouse heart were immuno-stained for T lymphocytes by CD3 using an anti-CD3 antibody (Abcam, Cambridge, MA). Anti-haptoglobin antibody (Abcam,
cat#13429) was used on human cardiac tissues and proteintech (cat #16665-1-AP) for murine cardiac tissue. Slides of tissue were de-paraffinized in xylene and rehydrated through graded ethanol to distilled water. Antigen retrieval was accomplished by placing slides in 0.01 M sodium citrate buffered water at 96-101°C for 20 min then cooling to room temperature. Slides were rinsed in distilled water, placed in Tris-buffered saline (Biocare Medical, Concord, CA), and immersed in 3% H₂O₂ and distilled water for 5 min to block endogenous peroxidase activity. After this, slides were rinsed in Tris-buffered saline, the primary antibodies were applied to the tissue sections, and subsequently, sections were incubated for 30 min at room temperature. Secondary antibody detection was performed (EnVision Kit, Dako Cytomation, Carpinteria, CA) by using diaminobenzedine tetrahydrochloride (DAB) (Dako Cytomation). The slides were then rinsed in distilled water, counter-stained in hematoxylin, dehydrated, cleared in xylene, and a cover slip with a resinous mounting media was added. For T cell staining, dark positive staining was considered positive in cells in the thymus and intestine. Staining with isotype control for human tissue exhibited no DAB positive staining (Supplemental Figure 11). Samples were read in a blinded fashion.

Dendritic Cell Culture and ELISA
To stimulate bone marrow derived dendritic cells in vitro, cardiac lysates were procured at indicated time point post transplantation and cultured with bone marrow-derived dendritic cells as previously reported. Cardiac lysates were free of LPS (Limulus assay) and did not contain any microbial peptides as assessed by mass spectrometry. Lysates were treated with pronase (0.5U/ml, Calbiochem-Behring Corp, La Jolla, CA), DNase I (60 unit/ml, Roche) or RNase (10ug/ml, Thermo Scientific) at 37°C for 1h and then added to the BMDC cultures overnight as indicated. IL-6 in the culture supernatant was measured by ELISA (eBioscience) according to manufacturer’s instructions. IL-6, IL-10, IL-12p40, MCP-1, (eBioscience), MIP-2, MIP-1 (R & D Systems) and HO-1 (Enzo Life Sciences, Farmingdale, NY), haptoglobin (ImmunoIdology Consultants Laboratory, Tampa, FL), ELISA kits were used to measure the target analytes in the cardiac lysates.

ELISPOT, T Cell Enrichment, Cell Enrichment from Hearts, Graft Dendritic Cell Phenotyping and Flow Cytometry
T cells were enriched from suspensions of spleen cells with reagents from Stem Cell Technologies. The enriched T cells were cultured (1x 10⁵ cells / well) with irradiated donor (i.e., BALB/c) spleen cells (5 x 10⁵ cells / well) and IL-2 and IFNγ producing T cells were assessed via ELISPOT with reagents from BD Biosciences and spots read automatically on an AID Diagnostika ELISPOT analyzer or via ELISA as stated above. To measure cell proliferation, [3H]thymidine was added to the wells, and DNA was harvested and analyzed by a scintillation counter (PerkinElmer Life Science, Boston, MA). Single cell suspensions from mouse hearts were prepared according to the manufacturer’s instruction. Briefly, mouse hearts were placed in gentle MACS C Tubes (Miltenyi Biotec) containing PBS with collagenase II (600U/ml) and DNase I (60 U/ml). Tissues were dissociated by gentle MACS Dissociator (Miltenyi Biotec) and incubated for 30 min at 37°C. Cell suspensions were then applied to a cell
strainer (70 μm) and washed. We enriched subpopulations of cells within cellular suspension from cardiac tissue with a serial magnetic enrichment protocol (magnetic beads and columns from Miltenyi Biotec, Inc., CA) in which we positively selected for CD45+ve (i.e., immune cells) with CD45 microbeads. We then enriched for CD90+ve cells in the CD45−ve fraction to obtain fibroblasts (i.e., CD45−ve, CD90+ve) with CD90 microbeads and then we enriched for ECs (i.e., CD31+ve, CD45−ve, CD90−ve) by selecting for CD31+ve cells in the CD45−ve CD90−ve fraction with CD31 microbeads. Each of the subpopulations of cells were > 90% viable, as assessed by Zombie Aqua staining (BioLegend, CA) via flow cytometry. The purity of immune cells and endothelial cells enriched in this fashion was >90% and that of fibroblasts was > 80% as assessed by antibody staining and flow cytometric analysis. Cells from hearts were also resuspended and stained with relevant fluorescently tagged antibodies: anti-CD80 FITC, anti-MHC Class II PerCP, and CD11c Pacific Blue (eBioscience, CA). Spleen cells were also stained with anti-mouse fluorescently tagged mono-clonal antibodies (CD4+, CD25+, and FoxP3 [intracellular]). Data were acquired with a LSR II (BD Bioscience) flow cytometer and analyzed with FlowJo software (Treestar, Ashland, OR). To stimulate cells in vitro, cells were cultured with purified human (1x10⁴ immune cells / well, 2x10⁴ fibroblasts / well and 8 x 10⁴ ECs / well) haptoglobin obtained from Sigma containing <40pg/ml of LPS (Limulus assay).

Proteomics

Urea, ammonium bicarbonate, and iodoacetamide (IAA), methanol, LC-MS chromasolv acetonitrile (ACN), trifluoroacetic acid (TFA), formic acid (FA) and HPLC grade water were purchased from Sigma-Aldrich (St. Louis, MO). Chloroform and dithiothretitol (DTT) were from American Bioanalytical (Natick, MA). Sequencing grade modified trypsin was from Promega (Madison, WI). UltraMicroSpin columns (C18) were obtained from The Nest Group Inc. (Southborough, MA).

i) Heart Protein Extraction, Tryptic Digestion, and Sample Clean-up

To obtain protein lysates, murine heart tissue was homogenized using a Dounce tissue grinder in homogenization buffer (0.32 M sucrose, 20 mM HEPES, pH 7.4) with protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). Cardiac proteins were dissolved by sonication in urea buffer (7 M urea, 0.1 M Tris-HCl pH 7.5). Cysteines were reduced with 4.1 mM DTT (10 min at 60 °C) and the reaction was quenched on ice. Carbamidomethylation of cysteines was carried out for 30 min at room temperature in the dark using 8.3 mM IAA. Excess IAA was quenched with DTT and the reaction was diluted to ~1 M urea with 200 μl of 90 mM Tris-HCl buffer pH=8.0 (23 °C) containing 2 mM CaCl₂. Digestion with trypsin was carried out at 37°C for 15h using a trypsin to protein ratio of 1:15. The reaction was quenched with 12 μl 20% TFA. Peptides were desalted by reverse-phase chromatography using C₁₈ UltraMicroSpin columns. Peptides were dried in a vacuum centrifuge, dissolved in 20μl 3:8 v/v 70 % FA/0.1 % TFA, and protein quantitation was determined by hydrolysis and amino acid analysis (AAA). The peptide concentration was adjusted to 0.6 μg/μl for LC-MS.

ii) LC-MS/MS Protein Identification
LC-MS/MS was performed on a 5600 TripleTOF (AB Sciex, Framingham, MA) with a nanospray source connected to a nanoACQUITY UPLC (Waters, Milford, MA). The mobile phases for LC separation consisted of water (A) and acetonitrile (B) containing 0.1 % of FA, respectively. Three micrograms of peptides were injected onto a Waters Symmetry C18, 5 µm particle size, 180 µm ID x 20 mm nanoACQUITY UPLC trap column connected to a Waters BEH 30 C18, 1.7 µm particle size, 75 µm ID x 150 mm capillary column operated at 45°C. Peptides were trapped for 3 min at 1% B with a flow rate of 5 µl/min. Gradient elution was carried out using a flow rate of 0.5 µl/min with a two-step gradient from 5-40% B in 160 min and 40-85% B in 3.3 min, respectively. The mass spectrometer was operated in IDA mode with a single 250msec high resolution TOF MS survey scan ($m/z = 400-1250$) followed by up to 20 MS/MS scans ($m/z = 100-1500$) at > 15,000 resolution and 50 msec accumulation time. Precursor ions exceeding 125 counts were fragmented with a collision energy of 37 eV ± 15 eV using N2 as the collision gas. Fragmented peptides were set on an exclusion list for 10 sec.

iii) Database searching
All MS/MS spectra were searched in-house using the Mascot algorithm (version 2.4.0) 4 after using the AB Sciex MS Data Converter Software (1.0) program to generate Mascot compatible files. Each of the Mascot Compatible Files (mgf) was searched using the SwissProt database with a mouse taxonomy filter for the mouse heart samples. Search criteria were: enzyme: trypsin, allowing 1 missed cleavage. The precursor ion mass tolerance was set to 20 ppm for the precursor and 0.2 Da for fragment ions, respectively. Fixed modifications were Carbamidomethyl at cysteine and the variable modification was oxidation of Met. The overall false discovery rate estimated by MASCOT was < 1%. Mascot results files (*.dat) were transformed into an XML file employing the Mascot’s script export_dat_2.pl. The resulting XML files were processed using JAXB (http://jaxb.java.net/) and the Java StAX API (http://stax.codehaus.org/), and inserted into Yale Protein Expression Database 5.

iv) YPED Mouse Heart Proteome Spectral Library
To identify proteins from the 5600 TripleTOF Discovery Protein Identification Data, the data were filtered to only include peptides with MASCOT scores greater than or equal to the identifying score. The MS/MS spectra with the highest MASCOT score were chosen and all y- and b-ions were matched to an in-silico fragmentation and then sorted by peak height intensity. From there, the list was filtered so that each protein had two or more peptides and each peptide was distinct within a combined Mouse Taxonomy SwissProt database blast search 6. The remaining peptides were sorted based on the number of occurrences in the YPED database with the b- or y-ions for all peptides exported for downstream SWATH analysis 7. These SWATH peak extraction transitions along with their retention times were exported as a tab-delimited file (tsv).

v) Data-Independent SWATH Proteome Analysis
Each sample was processed through the Yale Protein Expression Database (YPED) pipeline and the “learned” peptide sequences were transformed into spectral libraries
that were used for data-independent SWATH analysis. Quantitation was accomplished using two (or more) of the most intense fragment ions from each of 2-3 tryptic peptides (e.g., 15 data points with 3 peptides x 5 transitions/peptide). The resulting data were integrated using Peakview and exported for data analysis where a comprehensive suite of custom bioinformatics tools (based on R scripts and that includes data metrics and normalization, PCA analysis, fold-change (with p-values), and volcano plots [Chung L and Colangelo C, personal communication]) calculate differences between sample groups. The raw data and fold-change calculations were then uploaded to YPED for web-accessible data dissemination and archiving in the YPED data repository.

Statistics Analysis:
Differences between groups using non-parametric measures were assessed by a 2-tailed Mann-Whitney, test otherwise differences between experimental groups were analyzed using a 2-tailed Student’s t-test. Transplant survival between groups was calculated using the log-rank method. Fisher’s exact test was employed to compare differences between samples from human heart transplant recipients. P < 0.05 was considered significant. All error bars and +/- in tables represent standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism Software.

Proteomics: Fragments that exhibited a signal-to-noise ratio of >5 from two or more samples were further considered. The data was transformed into a log2 scale and then normalized by matching the median intensities across samples. Protein-level intensity was obtained by following 2-stage calculation. First, a peptide-level summary was obtained by averaging the (log2) fragment-level intensities from each peptide. Second, the protein-level score was calculated by averaging the peptide-level summary for each peptide. Intensities from modified-peptides were not considered in this analysis.

Differential protein expression was evaluated to compare the means of protein-level scores between two experimental groups (e.g., 24hr vs 0hr). Moderated t-tests were performed by using R package Limma. P-values were further adjusted by Benjamini-Hochberg’s procedure to control false discovery rate (FDR). Differentially regulated proteins are shown in volcano plots in which log2-fold change on the x-axis is plotted against unadjusted p-values on the y-axis. In each graph, proteins were selected at 5% FDR and labeled in red. The full list of differentially regulated proteins between groups with fold changes, unadjusted and adjusted p-values, is shown in Supplemental Table 1.

Bioinformatics: GO term enrichment analysis of differentially regulated proteins was performed using MetaCore (Thompson-Reuters), using a background protein population that consists of 1342 proteins that were simultaneously detectable in our SWATH assay. P-values of enrichment relative to this background list were calculated by Fisher’s exact test.
On line References


Supplemental Figures and Legends I-XV
Online Figure I  Early inflammatory response after cardiac transplantation

**A**: BALB/c hearts were directly implanted into C57BL/6 recipients (denoted as 0h cold storage) or placed in cold storage solution for the indicated time before implantation into C57BL/6 recipients. Donor hearts were obtained 24h after transplantation and intra-graft chemokines and IL-6 were measured. *p<0.01 non-transplanted vs. 0h cold storage (Mann-Whitney test).

**B**: Cardiac allografts (i.e., C57BL/6 donor and BALB/c recipient) or syngeneic grafts (i.e., C57BL/6 donor and recipient) exposed to 4h cold storage prior to implantation. At 24h post transplantation, the indicated intra-graft cytokine or chemokine were measured.

**C**: C57BL/6 hearts exposed to 4h cold storage implanted into C57BL/6 recipients. At indicated time point after transplantation, cytokines and chemokines were measured from procured donor hearts.

Non-tx = non-transplanted donor heart. Native = native heart of the recipient procured after recipient received transplant.

Data are pooled from two to three independent experiments. N = 4-12 transplants / time point. Error bars = SEM
Online Figure II T cell infiltration in cardiac allografts at 24h post transplantation
Representative histological findings and CD3 immunohistochemistry for T cell recruitment into cardiac allografts (i.e., C57BL/6 hearts implanted into BALB/c recipients) or syngeneic cardiac transplants (i.e., C57BL/6 hearts implanted into C57BL/6 recipients) at 24h post transplantation with and without 4h cold storage. Positive control staining is shown by brown cells in the recipient thymus and small intestine. Scale bar = 20 µm.
Online Figure III Proteins contribute to inflammatory responses after cardiac transplantation  

**A:** Bone marrow derived dendritic cells were cultured with lysates of cardiac tissue procured at the indicated time point after transplantation or prior to transplantation (denoted as non-Tx) overnight. IL-6 was measured in culture supernatant. 24h native = native heart of the recipient obtained at 24h post transplantation. Lysates were free of LPS as measured via Limulus assay. Lysates did not contain microbial peptides as measured by mass spectrometry. * p<0.01 (Mann-Whitney test)

**B:** Cardiac lysates procured at 24h post transplantation were incubated with RNase, DNase or pronase for 1h at 37°C and added to the dendritic cell cultures. IL-6 was measured in the culture supernatant.

**C:** RNase and DNase used at the concentrations described in the methods section but dendritic cells stimulated with CpG with or without DNase or single stranded RNA with or without RNase. IL-6 was measured in the culture supernatant.

**D:** Pronase does not impair the ability of dendritic cells to produce IL-6 in response to LPS (50ng/ml) stimulation.

Representative of an experiment repeated with similar results. Each experiment included three biological replicates. Error bars = SEM
Online Figure IV Proteomic analysis after cardiac transplantation

A-B: Volcano plots comparing proteome of non-transplanted cardiac tissue with that of cardiac transplants after transplantation. P values were adjusted to limit the false discovery rate to 5%; red = p-values that are significant. (A) Non-transplanted cardiac tissue vs. cardiac transplants procured at 24h post transplantation; note insert of proteins (arrow), (B) non-transplanted tissue vs. cardiac transplants procured at 72h post transplantation. Hearts were exposed to 4h cold storage prior to transplantation. Data comprised of three biological replicates / time point. HPT = haptoglobin.
Online Figure V Impact of donor haptoglobin on the tempo of cardiac allograft rejection

Effect of donor haptoglobin on the tempo of cardiac allograft rejection. WT C57BL/6 donor or C57BL/6 Hp⁻/⁻ hearts were implanted into BALB/c recipients. The BALB/c recipients were treated with CTLA4 Ig (200μg/20gm body weight) at days 0, 2 and 4 post transplantation. Allograft survival was measured by abdominal palpation. Difference between the groups were not significant.
Online Figure VI Cardiac allograft survival times in WT and Hp⁻alk recipients treated with perioperative CTLA4 Ig

Allograft survival times in C57BL/6 WT and Hp⁻alk mice transplanted with a WT BALB/c allograft and treated with CTLA4 Ig (200μg day 0, 2, 4 post transplantation). Donor cardiac allografts were procured at day +21 post transplantation. Allograft survival up to this point was recorded and shown. The allograft survival times were significantly different, p <0.0001 (Log Rank).
Online Figure VII Similar intra-graft levels of HO-1 in WT and Hp⁻/⁻ recipients after transplantation and treatment with peri-operative CTLA4 Ig. WT and Hp⁻/⁻ recipients were implanted with a WT BALB/c cardiac allograft and were treated with peri-operative CTLA4 Ig (200μg day 0, 2, 4 post transplantation). At day +14 or +21 post transplantation cardiac allografts were obtained and intra-graft HO-1 levels were measured by ELISA.
Online Figure VIII Haptoglobin concentrations increase within the liver of WT recipients of WT allografts treated with CTLA4 Ig (200μg day 0, 2, 4 post transplantation) relative to the levels found in the livers of non-transplanted mice. Haptoglobin levels measured by ELISA. Livers from transplanted mice procured at day +21 post transplantation. N = 6 mice / group. *p = 0.02 (Mann-Whitney)
Online Figure IX Increased number of immune cells within the allografts of WT recipients treated with CTLA4 Ig (200μg day 0, 2, 4 post transplantation) as compared to the allografts of Hp⁻/⁻ recipients. Allografts obtained at day +21 post transplantation. Results are pooled from 3 independent experiments with 3 biological replicates / experiment. *p<0.01 (t-test).
Online Figure X Fibroblasts and ECs do not contribute to the intra-graft proinflammatory environment after cardiac transplantation and treatment with CTLA4 Ig. Fibroblasts and ECs were enriched from hearts of recipients at day +21 post transplantation and treatment with CTLA4 Ig (200μg day 0, 2, 4 post transplantation) or from hearts of non-transplanted mice and cultured in vitro for 16h. IL-6 and MIP-2 were measured in the culture supernatant via ELISA. ECs enriched from allografts secreted increased amounts of IL-10 relative to ECs enriched from non-transplanted cardiac tissue. There were no significant differences between the ECs enriched from the allografts of WT and Hp⁻/⁻ recipients. Pooled data from 3 independent experiments, with 2 biological repeats / experiment, *p<0.01 (t-test)
Immune cells from the allografts of Hp⁻/⁻ recipients exhibit reduced IL-10 production as compared to allografts of WT recipients. Immune cells (i.e., CD45⁺ cells) were enriched from allografts (day +21 post transplantation) of recipients treated peri-operatively with CLTA4 Ig (200μg day 0, 2, 4 post transplantation). Immune cells were cultured in vitro for 16h and IL-10 was measured in the culture supernatant via ELISA. Pooled data from 3 independent experiments, with 2 biological repeats / experiment, *p = 0.05 (t-test)
Online Figure XII Defective immune cell recruitment in the allografts of Hp-/- recipients. Hp-/- recipients of cardiac allografts exhibit a reduction in graft numbers of neutrophils (i.e., Ly6G+ cells) and CD3+ T cells at day +7 post transplantation vs. WT recipients. Recipients treated with peri-operative CTLA4 Ig as stated in the methods. N = 3 / group. Recipients received CTLA4 Ig 200μg day 0, 2, 4 post transplantation.
Online Figure XIII Immune cells, fibroblasts and ECs enriched from WT hearts produce IL-6 in response to LPS. Immune cells, fibroblasts and ECs were enriched from non-transplanted hearts of WT mice as detailed in supplementary methods. Cells were stimulated ex vivo with LPS (50ng/ml) and IL-6 was measured in culture supernatant in ELISA after 12h culture.
Online Figure XIV Representative endomyocardial biopsy specimens from patients exhibiting moderate rejection and counter stained with anti-haptoglobin antibody (left panel). Arrow indicates DAB-positive (brown) staining within cardiomyocytes. Isotype negative controls were DAB-negative (right panel). Scale bars = 50 µm
Online Figure XV Working model of action of haptoglobin in amplifying inflammation after cardiac transplantation to impair immune modulation.

Graft inflammation is induced by the initial ischemia reperfusion injury after cardiac transplantation, which in turn leads to the production of haptoglobin in the recipient, most likely from the liver. The haptoglobin from the liver is then released into the circulation and subsequently enters the graft to amplify inflammation, possibly through activating DCs or other immune cells leading to the production of chemokines and cytokines to enhance immune cell recruitment to the allograft. Increased immune cell recruitment increases graft inflammation and in turn enhances DC activation and promotes T cell activation to impair immunoregulatory therapy.
Supplemental Online Tables I-VI and Videos I-II

Online Table I Differentially regulated proteins after cardiac transplantation. C57BL/6 mice received a C57BL/6 cardiac transplant.

Table footnote: At 6h, 24h and 72h after transplantation the donor hearts were procured and lysates were generated. The proteome of the transplant heart lysates were compared to non-transplanted heart lysates (denoted as 0h) by mass spectrometry. At 6h post transplantation, the native heart of the recipient was also procured and the proteome was compared to the non-transplanted heart. N = 3 / group / time point. FDR = false discovery rate.

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**Online Table II** Comparison of enriched GO-biological processes for lysates of cardiac transplants and non-transplanted cardiac lysates.

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<td>-Cell activation ($p=4.8 \times 10^{-3}$)</td>
<td>-Wound healing ($p=5.0 \times 10^{-5}$)</td>
<td>-Electron transport chain ($p = 4.36 \times 10^{-7}$)</td>
</tr>
<tr>
<td></td>
<td>-Actin filament-based process ($p=1.2 \times 10^{-5}$)</td>
<td>-Cytoskeleton organization ($p=1.5 \times 10^{-5}$)</td>
<td>-Inflammatory response ($p=1.5 \times 10^{-4}$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Homotypic cell-cell adhesion ($p=1.5 \times 10^{-8}$)</td>
<td>-Cytoskeleton organization ($p=5.5 \times 10^{-5}$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Localization of cell ($p=4.3 \times 10^{-6}$)</td>
<td>-Immune response ($p=4.1 \times 10^{-4}$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Inflammatory response ($p=5.9 \times 10^{-6}$)</td>
<td>-Acute inflammatory response ($p=3.1 \times 10^{-4}$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-Phagocytosis ($p=3.5 \times 10^{-4}$)</td>
</tr>
</tbody>
</table>

As per Table S1, hearts from C57BL/6 strain mice were implanted into C57BL/6 recipients.
**Online Table III** Comparison of enriched GO molecular function for syngeneic cardiac transplants compared to non-transplanted cardiac tissue.

<table>
<thead>
<tr>
<th></th>
<th>6h post transplantation</th>
<th>24h post transplantation</th>
<th>72h post transplantation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Down-regulated proteins</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-NADH dehydrogenase activity (p=6.5 x10^{-3})</td>
<td></td>
<td>-Calcium-dependent phospholipid binding (p=5.3 x10^{-3})</td>
<td>-Microtubule binding (p=2.3 x10^{-3})</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Calcium-dependent protein binding (p=8.8 x10^{-3})</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Structure-specific DNA binding (p=1.7 x10^{-2})</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Structural constituent of muscle (p=3.0 x10^{-2})</td>
<td></td>
</tr>
<tr>
<td><strong>Up-regulated proteins</strong></td>
<td></td>
<td>-Actin binding (p=1.3 x10^{-4})</td>
<td>-Heme binding (p=1.9 x10^{-4})</td>
</tr>
<tr>
<td>-Cytoskeletal protein binding (p=3.1 x10^{-3})</td>
<td></td>
<td>-Integrin binding (p=3.5 x10^{-4})</td>
<td>-Succinate dehydrogenase activity (p=1.0 x10^{-3})</td>
</tr>
<tr>
<td>-Amino acid binding (p=3.0 x10^{-3})</td>
<td></td>
<td></td>
<td>-NADH dehydrogenase activity (p=3.5 x10^{-3})</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-Structural constituent of cytoskeleton (p=4.6 x10^{-3})</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-Electron carrier activity (p=5.0 x10^{-3})</td>
</tr>
</tbody>
</table>
**Online Table IV** Comparison of enriched GO-cellular components for syngeneic cardiac transplants compared to non-transplanted cardiac tissue.

<table>
<thead>
<tr>
<th></th>
<th>6h post transplantation</th>
<th>24h post transplantation</th>
<th>72h post transplantation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Down-regulated proteins</strong></td>
<td>-Mitochondrial inner membrane ($p=2.1 \times 10^{-3}$)</td>
<td>-Mitochondrial matrix ($p=4.0 \times 10^{-6}$)</td>
<td>-Troponin complex ($p=7.9 \times 10^{-4}$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-Myofilament ($p=4.1 \times 10^{-3}$)</td>
<td>-Mitochondrial matrix ($p=1.3 \times 10^{-3}$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sarcomere ($p=4.6 \times 10^{-3}$)</td>
<td>-Sarcomere ($p=7.1 \times 10^{-3}$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>-Microtubule ($p=2.9 \times 10^{-2}$)</td>
</tr>
<tr>
<td><strong>Up-regulated proteins</strong></td>
<td>-Cytoplasmic vesicle ($p=2.0 \times 10^{-4}$)</td>
<td>-Vesicle ($p=8.8 \times 10^{-4}$)</td>
<td>-Mitochondrial inner membrane ($p=7.6 \times 10^{-12}$)</td>
</tr>
<tr>
<td></td>
<td>-Cytoskeleton ($p=6.1 \times 10^{-3}$)</td>
<td>-Cytoskeleton ($p=9.1 \times 10^{-6}$)</td>
<td>-Endocytic vesicle lumen ($p=2.0 \times 10^{-3}$)</td>
</tr>
<tr>
<td></td>
<td>- Extracellular exosome ($p=5.1 \times 10^{-3}$)</td>
<td>-Ruffle membrane ($p=6.3 \times 10^{-4}$)</td>
<td>- Extracellular space ($p=1.3 \times 10^{-2}$)</td>
</tr>
</tbody>
</table>
**Online Table V** Upregulated Intra-graft Proteins after Cardiac Transplantation

<table>
<thead>
<tr>
<th>Protein</th>
<th>Log2 fold change 24h post transplantation</th>
<th>Log2 fold change 72h post transplantation</th>
<th>GO Molecular Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) Calgranulin A (SA109)</td>
<td>5.2</td>
<td>4.6</td>
<td>Ant-oxidant activity, Innate immune response, chemotaxis</td>
</tr>
<tr>
<td>2) Haptoglobin (HPT)</td>
<td>3.9</td>
<td>3.1</td>
<td>Anti-oxidant activity, hemoglobin binding, immune system response, host defense</td>
</tr>
<tr>
<td>3) Cathelin-related antimicrobial peptide precursor (Cramp)</td>
<td>3.9</td>
<td>-</td>
<td>Anti-microbial peptide</td>
</tr>
<tr>
<td>4) Lipocalin (NGAL)</td>
<td>3.4</td>
<td>-</td>
<td>Iron binding, transporter activity</td>
</tr>
<tr>
<td>5) Chitinase Like protein (CH3L3)</td>
<td>2.6</td>
<td>2.2</td>
<td>Carbohydrate and chitin binding</td>
</tr>
<tr>
<td>6) Thrombospondin 1 (TSP1)</td>
<td>2.5</td>
<td>-</td>
<td>Cell adhesion</td>
</tr>
<tr>
<td>7) Talin (TLN1)</td>
<td>1.7</td>
<td>-</td>
<td>Cell adhesion, actin cytoskeleton</td>
</tr>
<tr>
<td>8) Vimentin (VIME)</td>
<td>1.5</td>
<td>1.4</td>
<td>Protein binding, structural molecule activity</td>
</tr>
<tr>
<td>9) Filamin A alpha (FLNA)</td>
<td>1.8</td>
<td>-</td>
<td>Actin and protein binding</td>
</tr>
<tr>
<td>10) Protein canopy homolog 2 (CNPY2)</td>
<td>1.9</td>
<td>-</td>
<td>Protein binding</td>
</tr>
<tr>
<td>11) Coactosin like protein (Cotl1)</td>
<td>2</td>
<td>-</td>
<td>Actin, protein and enzyme binding</td>
</tr>
<tr>
<td>12) Protein canopy homolog 2 (CNPY2)</td>
<td>1.9</td>
<td>-</td>
<td>Negative regulation of gene expression</td>
</tr>
<tr>
<td>13) Ribosomal protein 32 (RL32)</td>
<td>1.9</td>
<td>-</td>
<td>Structural component of ribosome</td>
</tr>
<tr>
<td>14) Tropomysin alpha 4 chain (TPM4)</td>
<td>1.6</td>
<td>-</td>
<td>Metal ion binding</td>
</tr>
<tr>
<td>15) inter alpha-trypsin inhibitor, heavy chain 4 isoform 3 precursor (ITIH4)</td>
<td>1.5</td>
<td>-</td>
<td>Protein binding</td>
</tr>
<tr>
<td>16) Tubulin beta-4B chain (TBB4B)</td>
<td>1.3</td>
<td>-</td>
<td>GTP binding, structural constituent of cytoskeleton</td>
</tr>
<tr>
<td>17) Tubulin alpha 1C chain (TBA1C)</td>
<td>1.3</td>
<td>-</td>
<td>GTP binding, structural constituent of cytoskeleton</td>
</tr>
<tr>
<td>18) adenylate cyclase-</td>
<td>1.6</td>
<td>-</td>
<td>Protein binding</td>
</tr>
</tbody>
</table>
Proteins were identified by mass spectrometry of cardiac tissue before, 24h and 72h after transplantation. All proteins listed were significantly upregulated (p<0.01 after adjustment to control for a false discovery rate of <5% per statistical methods in online supplement). Label in parenthesis refers to protein label in Figure S4. The entire proteomic screen of proteins is shown in Supplemental Table 1.
**Online Table VI** Comparison of clinical characteristics of patients with and without evidence of acute moderate cellular rejection on endomyocardial biopsy.

<table>
<thead>
<tr>
<th></th>
<th>Moderate rejection, n = 9</th>
<th>No rejection, n = 10</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>47 +/- 5</td>
<td>48.9 +/- 4</td>
<td>0.7</td>
</tr>
<tr>
<td>% Female</td>
<td>29%</td>
<td>40%</td>
<td>0.6</td>
</tr>
<tr>
<td>Time post transplant (days)</td>
<td>166 +/- 51</td>
<td>490 +/- 153</td>
<td>0.07</td>
</tr>
<tr>
<td>% with CMV status mismatch between donor and recipient</td>
<td>11%</td>
<td>63%</td>
<td>0.05</td>
</tr>
<tr>
<td>No. of immune suppressants</td>
<td>3 +/- 0.1</td>
<td>3.1 +/- 0.1</td>
<td>0.55</td>
</tr>
<tr>
<td>% taking steroid taper</td>
<td>88%</td>
<td>50%</td>
<td>0.09</td>
</tr>
<tr>
<td>% taking steroid pulse</td>
<td>22%</td>
<td>0%</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Of the 7 biopsies that stained positive for haptoglobin and also exhibited evidence of moderate acute allograft rejection, the duration of steroid use at the time of endomyocardial biopsy ranged from 1 to 422 days.
Online Videos I-II
Video of WT BALB/c heart transplanted into a WT C57BL/6 recipient (movie 1), and a WT BALB/c heart transplanted into a C57BL/6 Hp⁻/⁻ recipient (movie 2). In the WT recipient the heart is not beating, compatible with a rejected allograft. In contrast, in the Hp⁻/⁻ recipient the heart is still beating. Recipients were treated with peri-operative CTLA4 Ig and transplants were harvested at day +21 post transplantation.
Files uploaded separately.