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Haptoglobin Enhances Cardiac Transplant Rejection

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Abstract

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 up to 72 hours after transplantation revealed that 22 proteins including haptoglobin, a known anti-oxidant, are significantly upregulated in our grafts. Through the use of haptoglobin deficient mice, we show that 80% of haptoglobin deficient recipients treated with peri-operative administration of the costimulatory blocking agent CTLA4 immunoglobulin exhibited > 100 days survival of full major histocompatibility complex mismatched allografts, whereas all similarly treated wild type recipients rejected their transplants by 21 days post transplantation. We found that haptoglobin modifies the intra-allograft inflammatory milieu by enhancing levels of the inflammatory cytokine IL-6 and the chemokine MIP-2 but impair levels of the immunosuppressive cytokine IL-10. Haptoglobin also enhances dendritic cell graft recruitment and augments anti-donor T cell responses. Moreover, we confirmed that the protein is present in human cardiac allograft specimens undergoing acute graft rejection.

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DISCLOSURES

None.

Conclusions—Our findings provide new insights into the mechanisms of inflammation after cardiac transplantation and suggest that, in contrast to its prior reported anti-oxidant 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.

Keywords

Transplantation; inflammation; rejection; immunosuppression transplantation; immunology

INTRODUCTION

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 intra-graft 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 [8], 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 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 prior to 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 anti-oxidative 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 of the online data supplement.

Mice heart transplant model

C57BL/6J (stock# 000664), BALB/cJ (stock# 000651) mice were purchased from Jackson Laboratories (Bar Habor ME). C57BL/6 haptoglobin deficient ($Hp^{-/-}$), C57BL/6 $MyD88^{-/-}$ (each backcrossed ten times) and relevant wild type littermate controls 2–4 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, Pisa Italy). Use of vertebrate animals was approved by the Yale School of Medicine IACUC.

A murine heterotopic heart transplant model was employed to assess intra-graft inflammatory responses [10] (detailed in the supplemental material). Anesthesia was induced by isofluorane via a pressurized vaporizer and maintained with ketamine (0.1mg/g body weight) and xyalazine (0.1mg/g body weight).

In vitro culture, cytokine measurement and flow cytometry

To determine if cardiac transplantation led to an increase in inflammatory modulators, we procured cardiac lysates pre- and post-transplantation and cultured them with bone marrow-derived dendritic cells (DCs) as previously reported [11]. 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 DC cultures overnight as indicated. Target cytokines and chemokines analytes in the cardiac lysates were measured by ELISA for IL-6, IL-10, MCP-1, (eBioscience), MIP-2, (R&D Systems), HO-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 Milenyi Biotec, Inc., CA) as detailed in the online data supplement. Purified human haptoglobin was obtained from Sigma and found to contain <40pg/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. Anti-donor T cell responses were assessed either by measuring cytokine production by ELISPOT, ELISA or proliferation by thymidine incorporation. Cellular suspensions (i.e., 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 intra-graft 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 [12]. These specimens were paraffin-embedded at the time of routine protocol biopsy after heart transplantation and were de-paraffinized and counterstained with an anti-human haptoglobin antibody (AbCam, Cambridge, MA) or isotype control via immune histochemistry. Slides were read blindly. Use of clinical specimens and accessing patient data from de-identified medical records was 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 (HE) by routine methods. An anti-murine haptoglobin antibody (Proteintech, Chicago, IL) was used to stain for haptoglobin in murine hearts. Murine histological data was read in a blinded fashion.

Statistics

Statistical analysis is detailed in the on line data supplement.

RESULTS

Characterization of early intra-graft inflammation

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

When we measured inflammatory cytokines and chemokines within the transplant 24h 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 two to ten-fold over those in non-transplanted hearts (Online Figure IA), indicating an early phase of intra-graft inflammation after organ implantation.

Characterization of the intra-graft levels of cytokines and chemokines demonstrated similar levels between syngeneic and allogeneic transplants at 24h post transplantation (Figure 1B). 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 4h of cold storage prior to 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 (i.e., 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 6h and 48h after transplantation and then subsided by 72h post 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 peri-operative stressors, but not

to the effects of organ procurement and implantation, were comparable to those in the donor heart prior to implantation (Online Figure IB + C), 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 intra-graft inflammation further after cardiac transplantation, we adapted an in vi assay in which dendritic cells (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 in mice [14–16]. The IL-6 response by DCs was increased after the culture with the lysates obtained from cardiac transplants at 24h and 48 post transplantation but not with the lysates obtained from non-transplanted 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 either RNase, DNase or pronase separately to lysates from syngeneic cardiac grafts at 24h post transplantation in order 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 employed in our experiments effectively digested known RNA and DNA-based DC activators, and the concentration of pronase employed in the assay did not impair DCs to respond to LPS, Online Figures III C–D).

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 non-transplanted cardiac tissue to tissue from syngeneic cardiac transplants at various time points after transplantation via mass spectrometry. By 6h post transplantation, only one protein, NADP transhydrogenase was differentially (down) regulated in cardiac grafts, whereas by 24 to 72h after transplantation, several proteins were differentially down and up regulated within the transplant (Online Figure IV A–B). The entire proteomic screen identified a total of 1318 up and down regulated proteins (Online Table I). Among these proteins, bioinformatic analyses indicated enrichment for biological and molecular processes including immune response, phagocytosis, and cytoskeletal re-organization (Online Tables II–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 24h and 72h after transplantation were calgranulin A, haptoglobin, chitinase-like 3 protein, and vimentin (Online Table V). We had previously employed an unbiased proteomic screen of skin transplants and noted that haptoglobin, a protein with anti-oxidant 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 intra-graft haptoglobin in the syngeneic heart transplant model via an ELISA test (Figure 1A), and found that haptoglobin was highly induced in the serum 3h after cardiac transplantation in mice (Figure 1A). At 24h after transplantation, haptoglobin levels were six-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 intra-graft haptoglobin levels were similar between allografts and syngeneic grafts 1 day after transplantation, by 3 days post transplantation, haptoglobin levels were two-fold higher in allografts than in syngeneic grafts (Figure 1C), although by day 7, post 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 intra-graft 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 wild type (WT) or $Hp^{-/-}$ recipient mice with a WT BALB/c cardiac allograft with or without peri-operative 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 [20]. Without CTLA4 Ig treatment, $Hp^{-/-}$ recipients exhibited a 3 day significant extension of allograft survival compared to 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. Hp expression in the donor allograft did not impact 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 Videos 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 co

stained with some cardiomyocytes, macrophages and exhibited scant perivascular staining (Figure 2D).

Haptoglobin alters the intra-graft 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 proinflammatory cytokines, specifically the production of IL-6 [11]. Therefore, we evaluated how recipient haptoglobin impacted the intra-graft inflammatory milieu after cardiac transplantation and peri-operative treatment with CTLA4 Ig. During the first 3 weeks after transplantation, we noted lower intra-graft levels of IL-6 and MIP-2 in $Hp^{-/-}$ than in WT recipients treated with CTLA4 Ig (Figure 3A–B). 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 heme-oxygenase-1 (HO-1) and IL-10 production [19, 21]. We determined that intra-graft levels of HO-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 intra-graft 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 treated with CTLA4 Ig but not in non-transplanted 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 post 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–B), we measured IL-6 and MIP2 in the supernatants after 12h of cell culture. Compared to cells obtained from non-transplanted WT BALB/c hearts, immune cells enriched from BALB/c allografts from WT recipients produced significantly elevated (i.e., 5–10 fold) levels of both IL-6 and MIP-2 compared to immune cells enriched from $Hp^{-/-}$ recipients (Figure 3E–F). We also noted that there were significantly more immune cells (i.e., four fold higher) in the allografts of WT recipients as compared to $Hp^{-/-}$ 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 non-transplanted hearts (Online Figure X). Immune cells enriched from allografts also produced more IL-10 after culture than cells obtained from non-transplanted

hearts (Online Figure XI). Furthermore, immune cells enriched from the allografts of $Hp^{-/-}$ recipients exhibited somewhat lower levels (i.e., 50% reduced) of IL-10 than immune cells enriched from the allografts of WT recipients (Online Figure XI). ECs, but not fibroblasts, enriched from allografts had higher levels of IL-10 production than cells obtained from non-transplanted 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 intra-graft 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 if there were alterations in the activation phenotype and accumulation of intra-graft DCs (defined as $CD11c^+$ MHC class II^+ cells) in the cardiac allografts of $Hp^{-/-}$ compared to WT recipients treated with CTLA4 Ig at day +7 post transplantation. We found that the upregulation of the costimulatory molecule CD80 was reduced three-fold on the surface of intra-graft DCs in the $Hp^{-/-}$ recipients as compared to WT recipients treated with CTLA4 Ig (Figure 4A–B). 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 post transplantation, we found a two fold lower total number of immune cells within the allografts of $Hp^{-/-}$ recipients than in WT counterparts at day +7 post transplantation (Figure 4D). Analysis at day +7 post transplantation also found reduced numbers of neutrophils and T cells in the allografts of $Hp^{-/-}$ recipients as compared to their WT counterparts (Online Figure XII).

MyD88 expression within the allograft alters the intra-graft 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 IL-6 and MIP-2 were significantly reduced three to five fold in $MyD88^{-/-}$ allografts as compared to WT allografts (Figure 5A–B). IL-10 levels were significantly elevated three fold in the $MyD88^{-/-}$ allografts as compared to WT allografts (Figure 5C). These results phenocopy the graft inflammatory alterations observed in $Hp^{-/-}$ recipients of WT allografts (Figure 3A–C).

To determine the cellular targets of haptoglobin within resident heart cells, we isolated immune cells, fibroblasts and ECs from WT and $MyD88^{-/-}$ non-transplanted 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 anti-donor T cell responses without impacting intrinsic T cell function

As prior in vitro studies have indicated that haptoglobin may impact T cell function to nominal antigens or non-specific 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 spleen cells exhibited similar production of the Th1 cytokine, IFN-γ, and similar IL-2 levels as WT T cells (Figure 6A–B). 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 anti-donor 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 2–3 fold reduction in splenic anti-donor T cell IFN-γ and IL-2 responses (Figure 6D–E). 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 appears to amplify intra-graft inflammation (Figure 3A–B) and enhance anti-donor Th1 T cell alloimmunity to impair the graft prolonging effects of costimulatory blockade.

Intra-graft 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 post transplantation were compared to 9 biopsies that exhibited moderate histological evidence of rejection. We found that haptoglobin staining was present in seven out of nine human cardiac transplant specimens that exhibited evidence of moderate allograft rejection but only in two of ten cardiac transplant specimens that were free of acute rejection ($p = 0.02$, Fisher's exact test) (Figure 7A–B). Examination of the seven biopsies that exhibited moderate rejection and haptoglobin staining suggested that haptoglobin was present within some cardiomyocytes (Online Figure XIV) and within some scattered endothelial cells (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 intra-graft 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 anti-oxidant 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 and 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 ischemia reperfusion injury. 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 24h 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 impacts intrinsic T cell function in particular Th1 responses, at least to nominal antigens or non-specific stimulation in vitro [18,25]. Furthermore, haptoglobin increases the cellularity of secondary lymphoid organs in mice [18], which could impact 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 [29], 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 impact intrinsic T cell function during in vitro stimulation with allogeneic spleen cells (Figure 6A–C). 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 employed.

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 believe 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 intra-graft 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

impacts other possible cellular sources of pro and anti-inflammatory molecules with the heart, such as cardiomyoctyes or vascular smooth muscle cells. Regardless of the source of inflammatory mediators, recipient haptoglobin enhances the intra-graft inflammatory milieu and increases anti-donor 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 (i.e., elevating levels of IL-6 and MIP-2 but impairing IL-10 levels) similarly 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 graft to enhance alloimmunuty. Future studies will be required to identify the MyD88 sensing cell within the allograft that is activated by haptoglobin in vivo to enhance intra-graft inflammation after cardiac transplantation.

The cellular recognition mechanisms and pathways by which haptoglobin alters the intra-graft 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 24h and 72h after transplantation (Online Table V). Prior work has shown that calgranulin A suppresses DC priming and slows the tempo of MHC Class II mismatched cardiac allograft rejection in mice [30]. 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 [31], 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 [32]. These other proteins therefore warrant further exploration as they may potentially also contribute to inflammation after heart transplantation.

Aside from the murine data that implicates haptoglobin as an amplifier of inflammation after cardiac transplantation, our study also showed that there is an association between intra-graft 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 three weeks post transplantation in recipients treated with CTLA4 Ig (Figure 3D). Human heart transplant biopsies are not routinely available before one week after transplantation due to patient safety issues. We therefore could not determine if haptoglobin is upregulated within the immediate peri-operative 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 if 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

DCs	dendritic cells
ECs	endothelial cells
Hp	haptoglobin
IRI	ischemia reperfusion injury
MHC	major histocompatibility complex
SEM	Standard error of the mean
Tx	transplantation
WT	wild type

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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 anti-oxidant 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 pro-inflammatory cytokine IL-6, the chemokine MIP-2 but impairs the immune suppressive cytokine, IL-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.

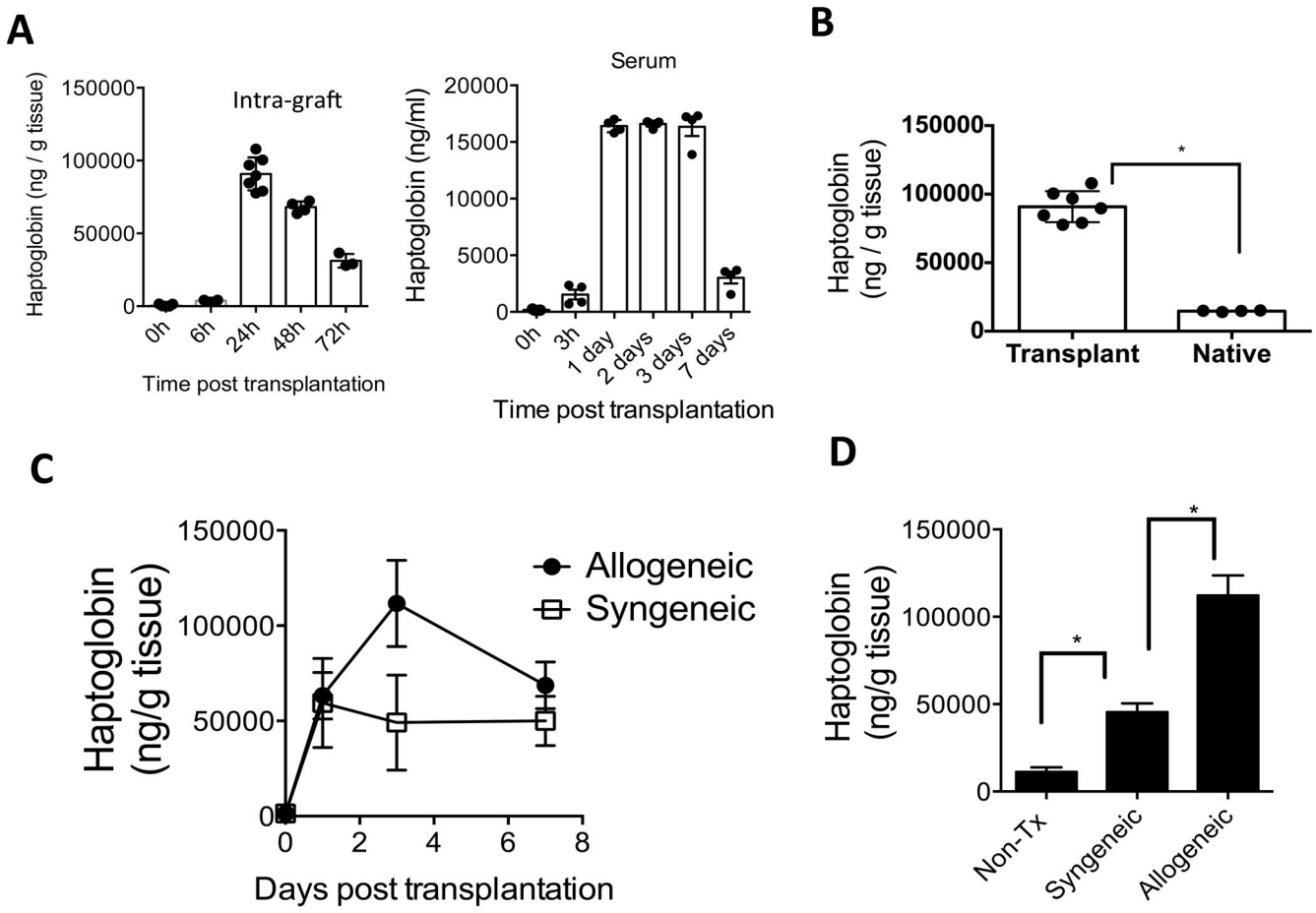

Figure 1. Haptoglobin levels increase within cardiac transplants

A: WT C57BL/6 hearts transplanted into WT C57BL/6 recipients. Haptoglobin levels were measured in the cardiac transplant and in sera. Hearts were exposed to 4h cold storage prior to transplantation. **B:** As per A; intra-graft haptoglobin levels measured in A compared to levels in native heart of the recipient at 24h after transplantation. * $p<0.0001$ (Mann-Whitney) **C:** Intra-graft levels of haptoglobin in syngeneic (i.e., donor and recipient both = C57BL/6) cardiac transplants and cardiac allografts (i.e., donor = C57BL/6 and recipient = BALB/c) during the 1st week after transplantation. At least 4 transplants/time point. **D:** Haptoglobin levels in the liver from non-transplanted mice and mice that received either syngeneic or allogeneic cardiac allografts, day +3 post transplantation. N = 4–6 mice/group. *$p<0.01$(Mann-Whitney)

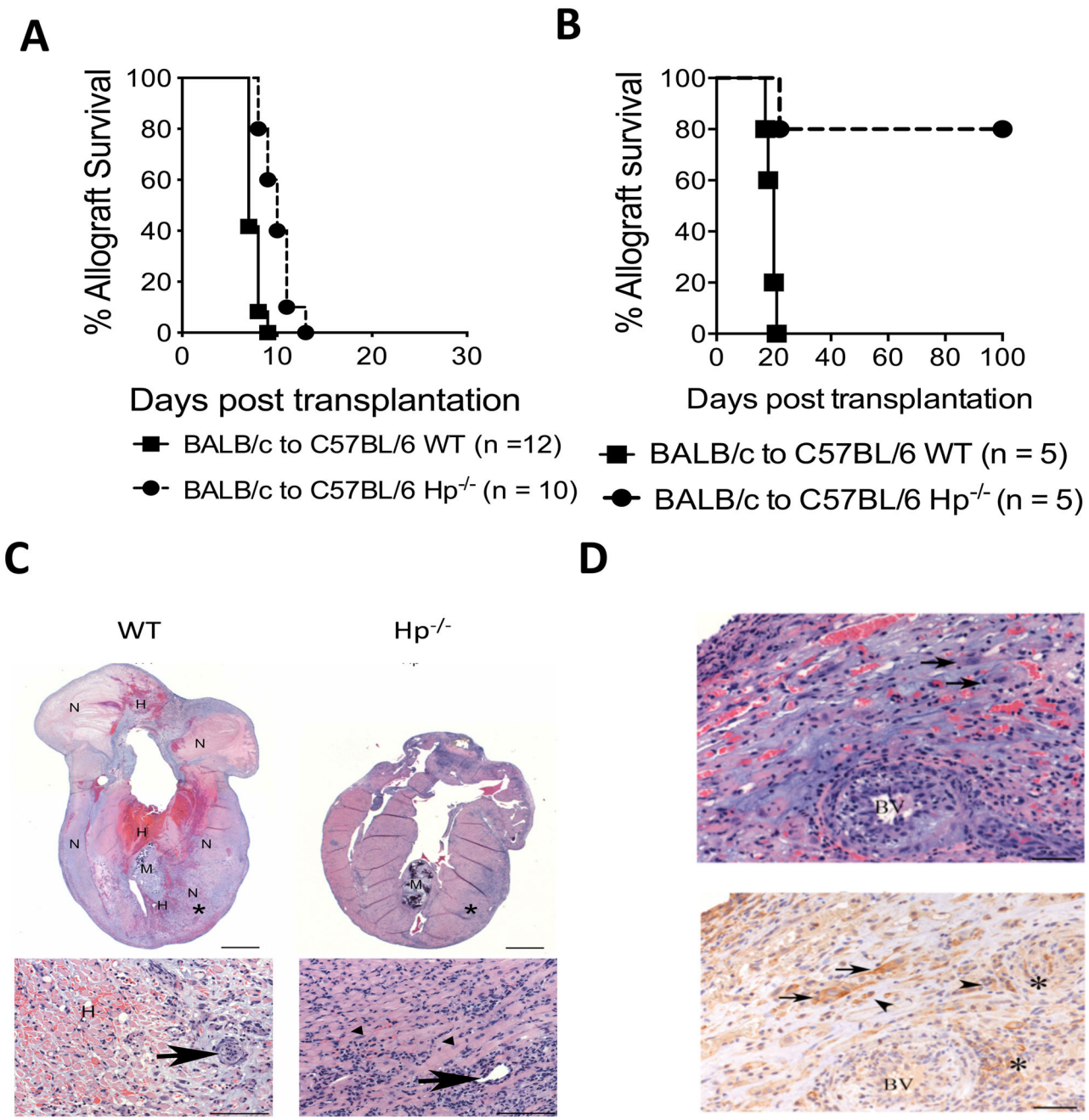

Figure 2. Haptoglobin affects the tempo of cardiac allograft rejection in mice

A: WT BALB/c cardiac transplants implanted into WT C57BL/6 recipients or C57BL/6 $Hp^{-/-}$ recipients. Rejection time was monitored. P = 0.003 between groups (Log Rank). **B:** Recipients were treated with peri-operative CTLA4 Ig (200μg day 0, 2, 4 post transplantation). P = 0.002 between groups (Log Rank). **C:** Histological assessment of WT BALB/c allografts at day +21 post transplantation in either WT C57BL/6 or C57BL/6 $Hp^{-/-}$ recipients treated with CTLA4 Ig per Figure 2B. Allografts transplanted into WT recipients are diffusely swollen and necrotic (markers as N), with large areas of hemorrhage (H), and thrombosis of myocardial blood vessels (arrow). $Hp^{-/-}$ recipients exhibited diminished

hemorrhage within the allograft and patent myocardial vessels (arrow) with retention of myocardiocyte nuclei and sarcoplasm (arrow heads). M = mineralization. Higher power images from the areas marked (*) appear in lower panels. Upper panel scale bars = 1000 μm, lower panel scale bars = 100 μm. **D:** WT BALB/c cardiac transplants were implanted into WT C57BL/6 recipients treated with peri-operative CTLA4 Ig (200μg day 0, 2, 4 post transplantation). At day +21 post transplantation allografts were obtained and H and E (top panel) and immune histochemistry (bottom panel) for haptoglobin was performed. Haptoglobin positive cells (brown) include scattered cardiomyocytes (arrows), macrophages (arrow heads) and scattered perivascular cells (*). Scale bar = 50 μm, BV = blood vessel. Isotype control staining was negative, data not shown.

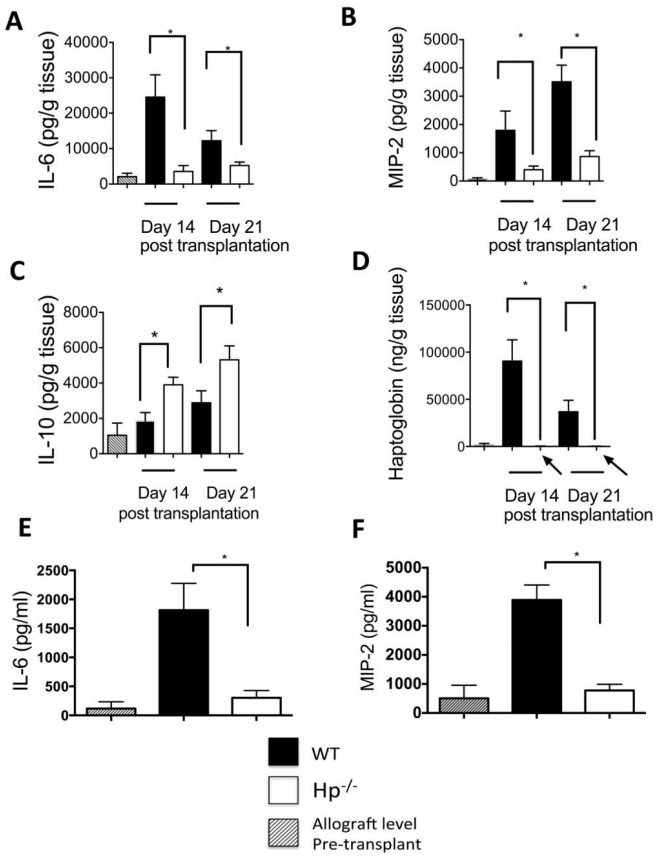

Figure 3. Recipient haptoglobin alters the intra-graft inflammatory environment after cardiac transplantation and treatment with peri-operative CTLA4 Ig

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 IL-6 (A), MIP-2 (B), IL-10 (C) and haptoglobin (D) were measured via ELISA. *p<0.01 (t-test). Arrows in 3D indicate the $Hp^{-/-}$ groups. Figures represent pooled data from three independent experiments. Error bars = SEM, N = 6–9 mice/time point**. E–F**: Immune cells (i.e., $CD45^{+}$ cells) were enriched from hearts of recipients at day +21 post transplantation and treatment with CTLA4 Ig and cultured for 12h. IL-6 and MIP-2 were measured in the culture supernatant. Pooled data from 3 independent experiments, n = 2/experiment, *p<0.01 (t-test)

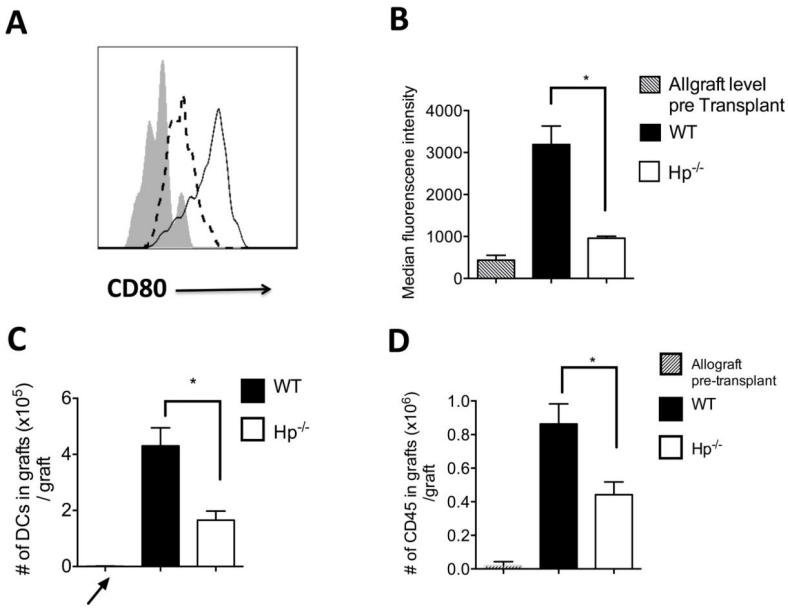

Figure 4. Recipient enhances accumulation of mature DCs after cardiac transplantation and treatment with peri-operative CTLA4 Ig

A: Representative flow cytometric plot showing surface expression of CD80 on $CD11c^+$ MHC class II^+ cells within the graft at day + 7 post transplantation in WT and $Hp^{-/-}$ mice. Grey shadow shows expression on $CD11c^+$ MHC class II^+ cells in the allograft prior to implantation. **B:** Median fluorescent intensity (relative units) of CD80 expression on $CD11c^+$ MHC class II^+ cells in the groups shown in A. *$p<0.01$ (t-test). **C–D:** Enumeration of DCs and immune cells within the allografts of WT and $Hp^{-/-}$ recipients treated with CTLA4 Ig at day +7 post transplantation. Pooled data from 2 independent experiments with n =3/experiment, *$p<0.01$ (t-test). Arrow in 4C indicate pre transplant group.

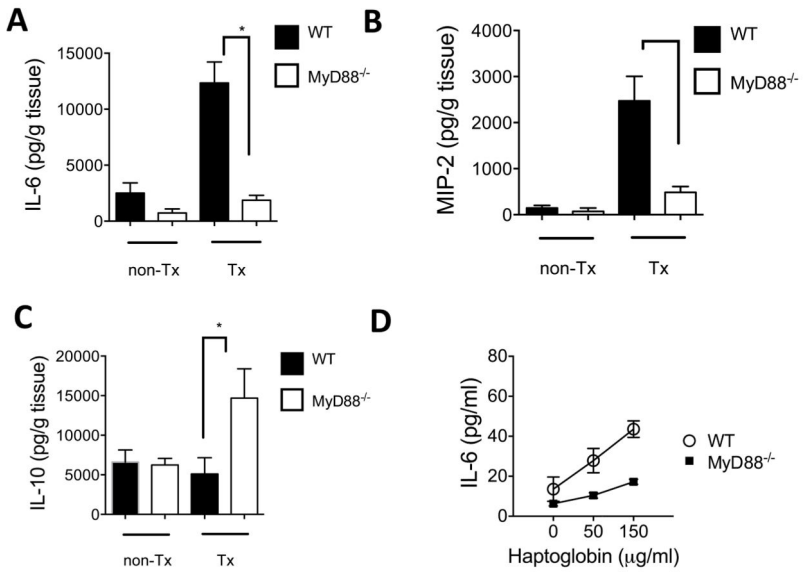

Figure 5. MyD88 expression within allografts modifies intra-graft inflammation after cardiac transplantation and treatment with CTLA4 Ig

A–C: C57BL/6 WT or MyD88$^{-/-}$ hearts were transplanted into WT BALB/c recipients that were treated with CTLA4 Ig 200μg day 0, 2, 4 post transplantation. At day +14 post transplantation, allografts were obtained and intra-graft IL-6 (A), MIP-2 **(B)**, and IL-10 **(C)** were measured via ELISA. *p<0.01 (t-test). Tx = transplanted. **D**: Immune cells (i.e., CD45^{+} cells) were enriched from non-transplanted WT or MyD88$^{-/-}$ C57BL/6 hearts and stimulated *ex vivo* with the indicated dose of haptoglobin. IL-6 was measured in the media via ELISA. ECs and fibroblasts did not produce IL-6 above control levels in response to haptoglobin (data not shown).

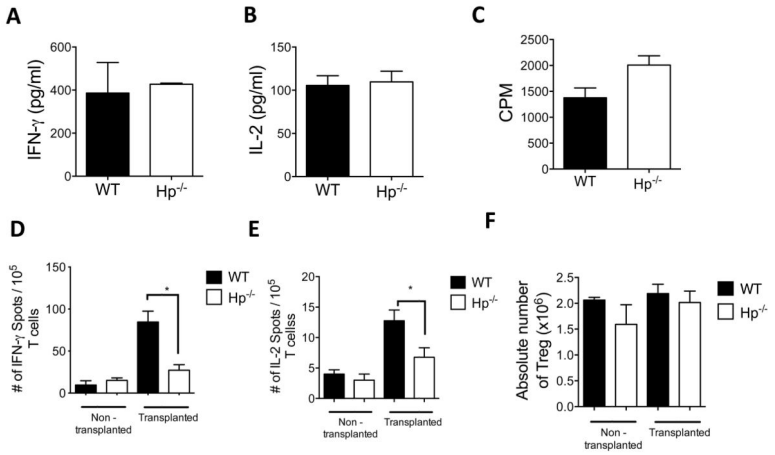

Figure 6. Recipient haptoglobin enhances anti-donor T cell responses after cardiac transplantation and perioperative treatment with CTLA4 Ig

A–B. Purified WT or $Hp^{-/-}$ T cells were stimulated with irradiated donor BALB/c spleen cells *in vitro* and IFN-γ (A) + IL-2 (B) were measured by ELISPOT. T cells stimulated with syngeneic spleen cells did not induce a response (data not shown). **C:** As in A–B but cellular proliferation of T cells measured by thymidine incorporation. **D–E:** Splenic anti-donor IFN-γ (D) IL-2 (E) T cell responses from either WT or $Hp^{-/-}$ recipients before transplantation or at day +21 after cardiac transplantation and treatment with CTLA4 Ig were measured via ELISPOT. Tx = transplantation, *p<0.01 (t-test). **F:** As per D–E but $CD4^+CD25^+FoxP3^+$ cells were enumerated in spleens of mice after relevant staining and flow cytometric analysis.

Figures represent pooled data from two experiments. N = 3 mice/experiment. Error bars = SEM

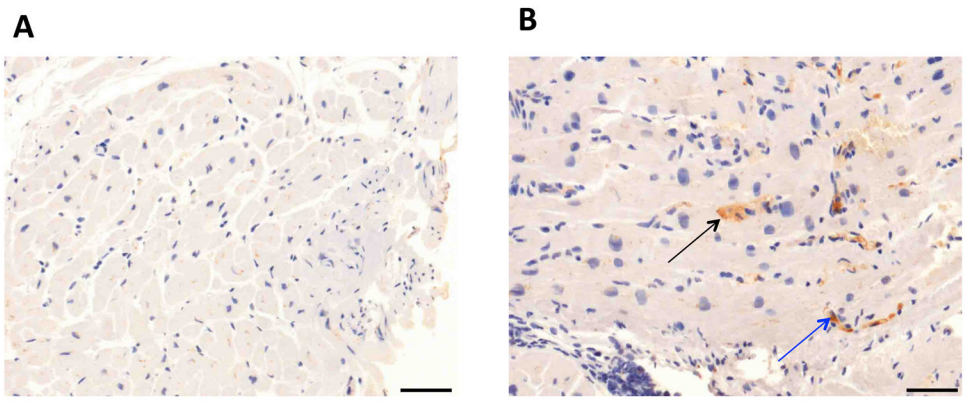

Figure 7. Intra-graft haptoglobin associates with acute allograft rejection in humans

Human heart transplant specimen with no cellular rejection and no staining for Hp (A), and a specimen with evidence of rejection and staining for Hp (black arrow) (B) Blue arrow indicates circumferential staining consistent with endothelial cells. Scale bar = 50 μm