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Brief Report **Transplantation**

Immune tolerance to transplanted organs is impaired when the innate immune system is activated in response to the tissue necrosis that occurs during harvesting and implantation procedures. A key molecule in this immune pathway is the intracellular TLR signal adaptor known as myeloid differentiation primary response gene 88 (MyD88). After transplantation, MyD88 induces DC maturation as well as the production of inflammatory mediators, such as IL-6 and TNF-α. However, upstream activators of MyD88 function in response to transplantation have not been identified. Here, we show that haptoglobin, an acute phase protein, is an initiator of this MyD88-dependent inflammatory process in a mouse model of skin transplantation. Necrotic lysates from transplanted skin elicited higher inflammatory responses in DCs than did nontransplanted lysates, suggesting DC-mediated responses are triggered by factors released during transplantation. Analysis of transplanted lysates identified haptoglobin as one of the proteins upregulated during transplantation. Expression of donor haptoglobin enhanced the onset of acute skin transplant rejection, whereas haptoglobin-deficient skin grafts showed delayed acute rejection and antidonor T cell priming in a MyD88-dependent graft rejection model. Thus, our results show that haptoglobin release following skin necrosis contributes to accelerated transplant rejection, with potential implications for the development of localized immunosuppressive therapies.

Haptoglobin activates innate immunity to enhance acute transplant rejection in mice

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Immune tolerance to transplanted organs is impaired when the innate immune system is activated in response to the tissue necrosis that occurs during harvesting and implantation procedures. A key molecule in this immune pathway is the intracellular TLR signal adaptor known as myeloid differentiation primary response gene 88 (MyD88). After transplantation, MyD88 induces DC maturation as well as the production of inflammatory mediators, such as IL-6 and TNF-α. However, upstream activators of MyD88 function in response to transplantation have not been identified. Here, we show that haptoglobin, an acute phase protein, is an initiator of this MyD88-dependent inflammatory process in a mouse model of skin transplantation. Necrotic lysates from transplanted skin elicited higher inflammatory responses in DCs than did nontransplanted lysates, suggesting DC-mediated responses are triggered by factors released during transplantation. Analysis of transplanted lysates identified haptoglobin as one of the proteins upregulated during transplantation. Expression of donor haptoglobin enhanced the onset of acute skin transplant rejection, whereas haptoglobin-deficient skin grafts showed delayed acute rejection and antidonor T cell priming in a MyD88-dependent graft rejection model. Thus, our results show that haptoglobin release following skin necrosis contributes to accelerated transplant rejection, with potential implications for the development of localized immunosuppressive therapies.

Introduction

Activation of the innate immune system enhances alloimmunity and impairs transplant tolerance (1–3). Transplant tolerance is impeded by the production of inflammatory mediators, such as IL-6 and TNF-α, by DCs through activation of the TLR signal adaptor, myeloid differentiation primary response gene 88 (MyD88) (4–6). However, the substances that activate MyD88 in response to tissue necrosis during organ implantation are not known. Identification of these innate immune ligands could allow the development of therapeutic interventions to inhibit allograft rejection within the transplant. Such localized inhibition of innate immunity could maximize the potential of immune suppressive therapies while preserving the innate immune pathways required for protection from infections.

Using DCs from mice with genetic deletions of MyD88, we show that the ability of DCs to mount an inflammatory response to tissue necrosis is MyD88 dependent. In addition, through mass spectrometry–based analyses, we identify haptoglobin as a protein that contributes to the MyD88-dependent inflammatory response to tissue necrosis. These observations are further supported by experiments using haptoglobin-deficient ($hp^{-/-}$) skin grafts, which exhibit a greater than 50-day graft survival time compared with WT grafts in an experimental model in which graft rejection is MyD88 dependent (6).

Results and Discussion

Necrotic lysates from skin induce the release of IL-6 and TNF-α by CD11c$^+$ DCs in a tissue culture assay. Harvesting of organs and subsequent implantation induce reperfusion injuries with resultant tissue necrosis (7). Moreover, the tissue necrosis that occurs in organ transplantation activates innate immunity to initiate the alloimmune response (6). Hence, we first determined whether necrotic skin lysates elicit the production of IL-6 and TNF-α using the innate immune responder cells, CD11c$^+$ DCs, that produce these cytokines in response to skin transplantation (4, 5). When necrotic skin lysates were added to BM-derived CD11c$^+$ DCs in a cell culture assay, the lysates induced the production of IL-6 and TNF-α in a dose-dependent manner (Figure 1A). We also found that lysates harvested from necrotic cardiac tissue under sterile conditions induced IL-6 production, but this response was lower than that induced by necrotic skin lysates (Figure 1B).

The IL-6 response of CD11c$^+$ cells was not due to contaminating bacterial components such as LPS. The lysate LPS concentration (<10 pg/ml) was the same as the baseline amount in the control medium, and this concentration of LPS did not induce an inflammatory response (Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI58344DS1). Moreover, treating the skin with betadine and ethanol solution to reduce the number of contaminating bacteria did not affect DCs' IL-6 production (Supplemental Figure 1B); neither did the use of plasmocin to inhibit *Mycoplasma* bacteria contamination in necrotic skin lysates (Supplemental Figure 1C).

The production of IL-6 and TNF-α by DCs in response to necrotic skin cell lysates is MyD88 dependent, but independent of TLR2 and TLR4. DCs upregulate costimulatory molecules (e.g., CD40 and CD86) and produce IL-6 and TNF-α in response to a MyD88-dependent pathway after skin transplantation (4–6). Lower MyD88 expression correlates with operational tolerance of kidney transplants in humans (8), and MyD88 signaling induces transplant rejection in

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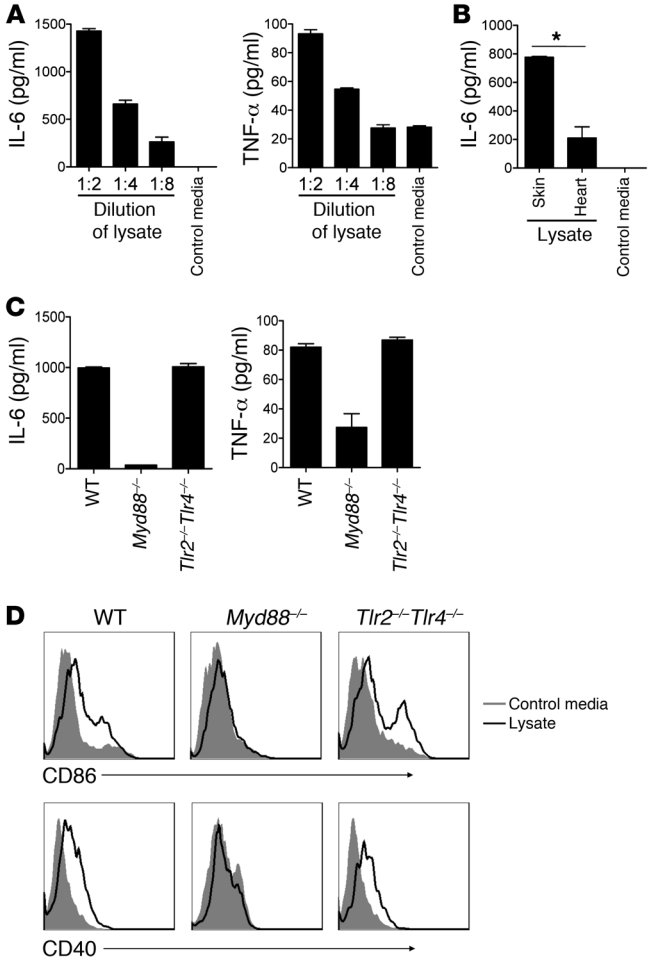

Figure 1
Lysates from nontransplanted necrotic skin induce MyD88-dependent production of IL-6 and TNF-α by DCs. (**A**) Lysates from necrotic skin were cultured with DCs. IL-6 and TNF-α concentrations were determined using ELISA. Lysates alone did not contain cytokines (data not shown). (**B**) Lysates from necrotic skin or from necrotic cardiac tissue were cultured with DCs. and IL-6 was measured by ELISA. *$P = 0.01$, *t* test. (**C** and **D**) Necrotic skin lysates were cultured with WT, *Myd88*$^{-/-}$, and *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ DCs. IL-6 and TNF-α were determined using ELISA. $P < 0.05$ for *Myd88*$^{-/-}$ DCs versus *WT* or *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ (*t* test). In the flow cytometric analysis, cells were gated on the CD11c^{+} pool, and expression of CD40 and CD86 was assessed after culture with control media or necrotic skin lysates. In **A**–**D**, results are representative of 1 experiment repeated independently 4 times with consistent results. Error bars represent SEM. Assays in each experiment run in triplicate.

an experimental skin transplant model (6). Given these findings, we determined whether necrotic skin cell lysates can also induce MyD88-mediated signaling by comparing the cytokine production responses of *Myd88*$^{-/-}$ CD11c^{+} DCs with those of WT DCs. We found that production of these cytokines was 4- to 10-fold lower in *Myd88*$^{-/-}$ CD11c^{+} DCs than in WT CD11c^{+} DCs cultured with necrotic skin lysates (Figure 1C). However, DCs that were deficient in both TLR2 and TLR4 (*Tlr2*$^{-/-}$*Tlr4*$^{-/-}$ DCs), which are TLRs that respond to several microbial substances, including LPS, exhibited IL-6 and TNF-α responses similar to those of WT cells (Figure 1C). Upregulation of CD40 and CD86 was also MyD88 dependent, but TLR2 and TLR4 independent (Figure 1D). Overall, these results indicate that necrotic lysates activate inflammatory responses in DCs via MyD88.

Necrotic lysates from transplanted skin induce greater IL-6 and TNF-α responses than lysates from nontransplanted skin. To determine whether necrotic lysates from skin transplants induce a higher inflammatory response than lysates from nontransplanted skin, we transplanted skin onto recipient mice and then obtained lysates from necrotic skin at several time points after transplantation. We found that up to 7 days after transplantation, lysates of C57BL/6 skin transplants (after transplantation to either syngeneic or allogeneic BALB/c recipients) induced higher IL-6 and TNF-α responses by CD11c^{+} DCs than lysates of nontransplanted skin (Figure 2, A and B). By day 11 after transplantation, the inflammatory response of DCs induced by necrotic allogeneic transplants persisted, whereas the response induced by the syngeneic transplants had begun to diminish (Figure 2, A and B). These results suggest that the process of transplantation induces the release of factors that trigger DC-mediated innate immune responses.

Haptoglobin is released after skin transplantation and induces MyD88-dependent cytokines by CD11c^{+} DCs. To determine whether protein factors in the necrotic lysates contribute to the observed cytokine responses, we denatured proteins in necrotic skin lysates by heating lysates above 65°C. This resulted in abrogation of IL-6 release by CD11c^{+} DCs (Supplemental Figure 2A). We next digested proteins from skin lysates with pronase and found that this digestion reduced the inflammatory response of DCs 3-fold (Supplemental Figure 2B). These results indicate that proteins within necrotic lysates contribute to the DC-mediated inflammatory response.

To identify the corresponding protein components, we compared the protein profiles of nontransplanted necrotic skin lysates with those of either syngeneic or allogeneic skin lysates at day 7 after transplantation using 2D differential gel electrophoresis (DIGE). Several proteins were upregulated more than 2.5-fold in transplanted skin compared with nontransplanted skin; MALDI TOF/TOF analysis identified one of these as haptoglobin (Figure 2C, Supplemental Figure 3, and Supplemental Table 1), an acute phase protein that modulates inflammatory responses (9).

At day 3 after transplantation, the concentration of haptoglobin in skin lysates increased from 211 ± 45 ng/ml (before transplant) to 1688 ± 503 ng/ml for syngeneic grafts and to 2177 ± 345 for allogeneic grafts (Figure 2D). By day 11 after transplantation, the haptoglobin concentration had begun to decline in the lysates from syngeneic grafts, but continued to increase in the lysates from allogeneic grafts (Figure 2D). Additionally, there was a positive correlation between haptoglobin concentrations within the skin lysates and the IL-6 and TNF-α produced by DCs after culture with lysates (Figure 2E). Hence, the process of transplantation induced the release of haptoglobin, which correlated with the inflammatory responses of DCs.

Haptoglobin activates MyD88 in CD11c^{+} DCs. Purified human haptoglobin induced a lower IL-6 and TNF-α production in *Myd88*$^{-/-}$ CD11c^{+} DCs than in WT DCs (Figure 3, A and B). In addition, haptoglobin-activated WT CD11c^{+} DCs exhibited enhanced priming of allogeneic T cells relative to PBS-treated WT DCs, whereas haptoglobin-activated *Myd88*$^{-/-}$ CD11c^{+} DCs failed to augment T cell priming relative to PBS-treated *Myd88*$^{-/-}$ DCs (Figure 3C). Moreover, nontransplanted skin lysates from *hp*$^{-/-}$ mice failed to elicit a robust

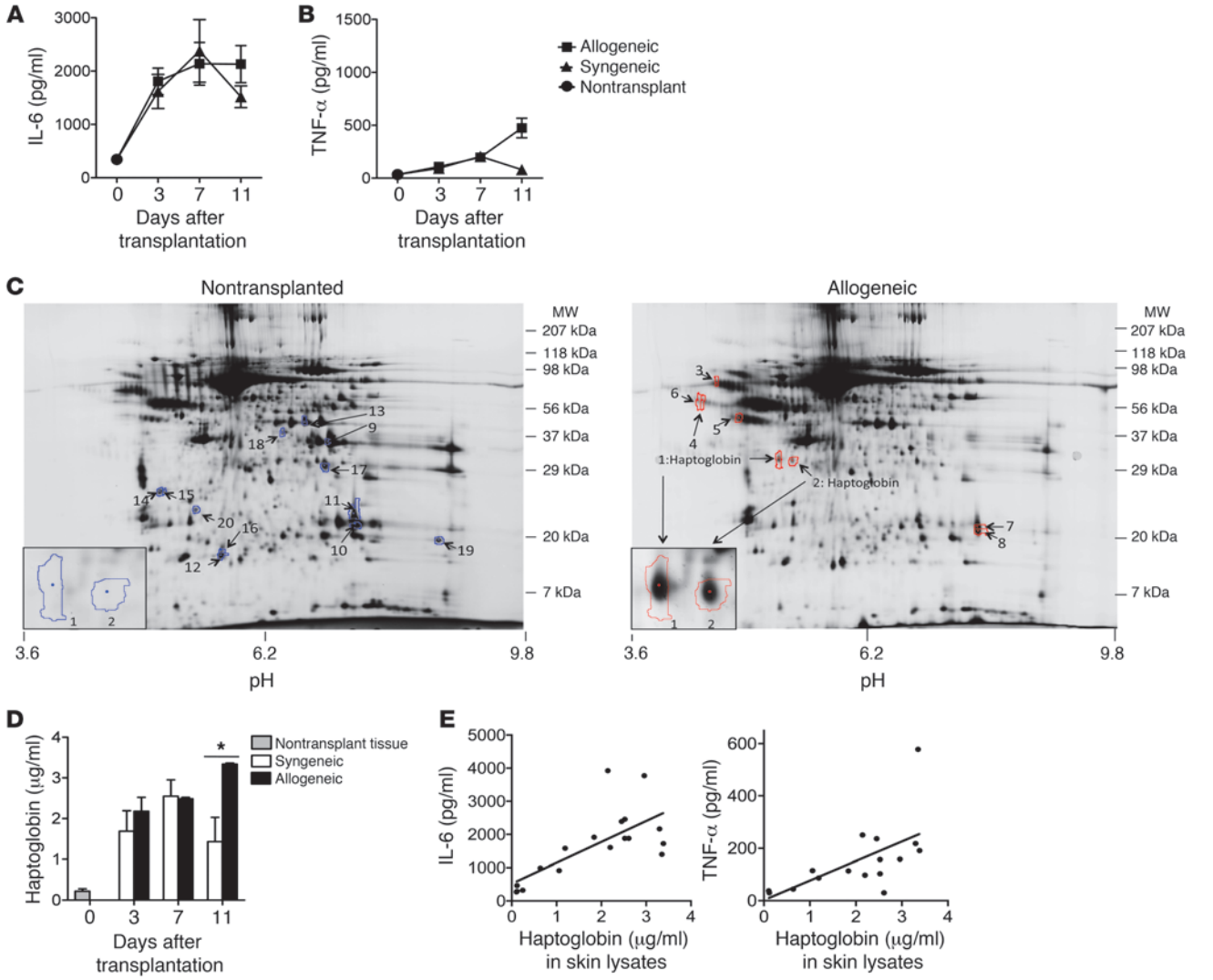

Figure 2

Haptoglobin is released after skin transplantation and positively correlates with inflammatory responses of DCs. (**A** and **B**) Skin from C57BL/6 mice was transplanted to C57BL/6 and BALB/c recipients. Lysates from the grafts, harvested at indicated time points, were cultured with DCs and cytokines measured (ELISA). $n = 6$ recipient mice/time point. The difference between TNF-α values induced by syngeneic versus allogeneic grafts at day 11 after transplantation reached statistical significance. $P = 0.001$, t test. (**C**) 2D-DIGE of lysates obtained from nontransplanted and allogeneic skin transplants (day 7 after transplantation) and labeled with cy2 and cy5 N-Hydroxysuccinimide ester dyes, respectively. MALDI TOF/TOF identified spots 1 and 2 as haptoglobin (inset zoom of spots shown in each gel). Spots outlined in blue (downregulated, allogeneic versus nontransplanted)/red (upregulated, allogeneic versus nontransplanted) were defined by DeCyder software. Identified proteins that exhibited at least a 2.5-fold change are numbered and listed in Supplemental Table 1. (**D**) Haptoglobin levels within lysates of syngeneic and allogeneic transplants (ELISA). $n = 6$ skin grafts/time point. 1 skin graft/recipient mouse, $^*P < 0.01$, t test. (**E**) Haptoglobin concentrations found within skin lysates exhibit a positive correlation with IL-6/TNF-α production by DCs after DCs are cultured with lysates. $P < 0.01$ and $r = 0.7$ (correlation value) for both cytokines. Pooled data acquired from 3 independent experiments. **A**, **B**, and **D** are representative of 1 experiment repeated independently twice with consistent results. All assays run in triplicate. Error bars represent SEM.

IL-6 response in DCs, in contrast with WT skin (Figure 3D). The reduced response was higher than that for DCs cultured in control medium, suggesting that other protein factors may also contribute to this signaling pathway. These results indicate that haptoglobin activates MyD88 in CD11c$^+$ DCs and contributes to the inflammatory response of DCs induced by necrotic skin lysates.

Donor expression of haptoglobin accelerates acute transplant rejection in a MyD88-dependent transplant model. To determine the impact of haptoglobin on transplant responses, we employed a skin transplant model in which rejection and antidonor Th1 T cell responses are MyD88 dependent (6). Specifically, skin from WT or $hp^{-/-}$ mice was transplanted from male donors, which are HY antigen positive, onto WT female recipients, which are HY antigen negative. Haptoglobin is synthesized within the skin (10–12), and before transplantation, we determined that lysates from WT (including nonlymphoid and lymphoid subpopulations), but not $hp^{-/-}$, male skin contained haptoglobin (Supplemental Figure 4, A and B). We also found that $hp^{-/-}$ skin contained CD11c$^+$ DCs that were

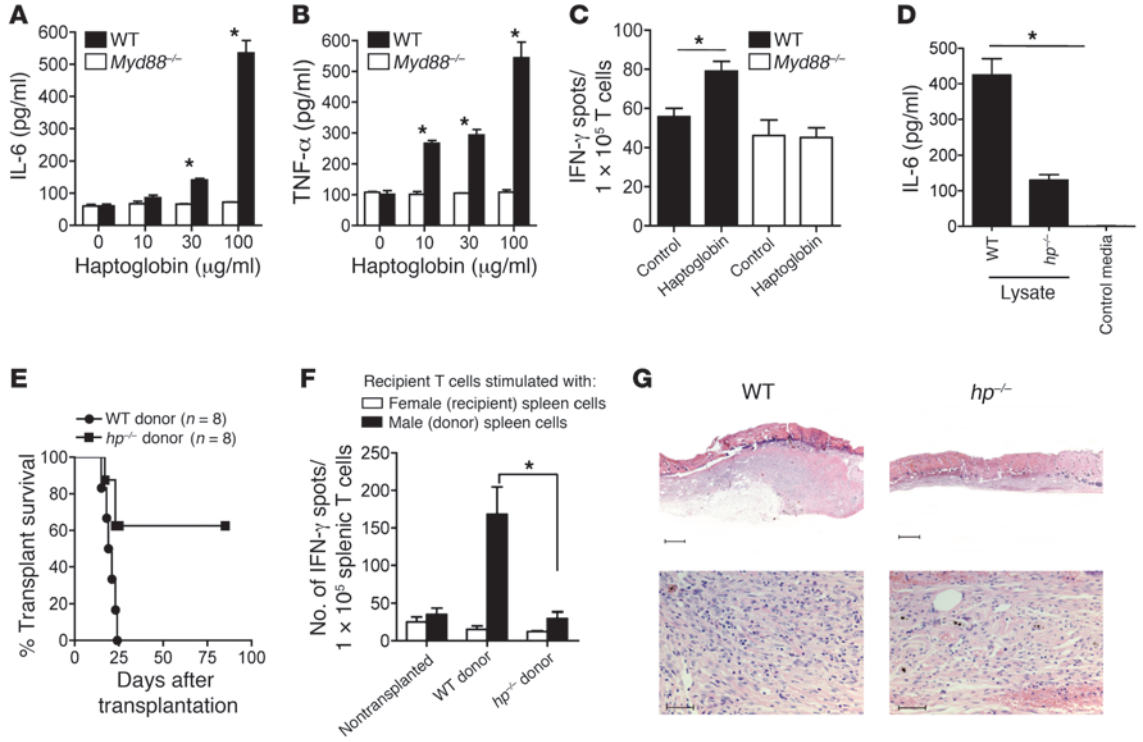

Figure 3

Haptoglobin activates DCs via MyD88, and donor haptoglobin increases the tempo of acute skin transplant rejection. (**A** and **B**) Human haptoglobin was added to WT and *Myd88*$^{-/-}$ DCs and IL-6 and TNF-α measured (ELISA). *$P < 0.01$, *t* test. (**C**) C57BL/6 WT and *Myd88*$^{-/-}$ DCs were haptoglobin (100 μg/ml) or PBS treated, then transferred i.p. to BALB/c recipients ($n = 3$/group). At 21 days after transfer, recipient splenic T cells were stimulated overnight with C57BL/6 spleen cells and IFN-γ recorded (ELISPOT). *$P < 0.001$, *t* test. (**D**) IL-6 production by DCs (ELISA) after culture in necrotic lysates from *haptoglobin*$^{-/-}$ (*hp*$^{-/-}$) or WT nontransplanted skin. *$P = 0.001$, *t* test. (**E**) *hp*$^{-/-}$ or WT male skin was transplanted onto WT female recipients and graft survival measured. Survival times between the 2 groups were different. $P < 0.01$, log rank. (**F**) Antidonor splenic T cell responses were measured after ex vivo stimulation with donor or recipient cells (ELISPOT) at day 21 after transplantation. All recipients were WT. *$P < 0.0004$, *t* test. (**G**) Histology of WT transplants (day 21 after transplantation) shows epidermal and dermal necrosis with abundant scale crust (upper panel, ×4; scale bar: 0.25 mm), with numerous inflammatory cells (lower panel, ×40; scale bar: 0.06 mm). *hp*$^{-/-}$ grafts exhibit less necrosis and inflammation. Representative samples, $n = 3$ mice/group. Data in **A**–**D** and **F** are representative of 1 experiment that was repeated independently once (**C** and **F**) or twice (**A**, **B**, and **D**) with consistent results. $n = 3$/group/experiment. Cell culture assays run in triplicate. Error bars represent SEM.

similar in number to DCs harvested from WT skin and exhibited a comparable expression of costimulatory molecules (Supplemental Figure 4, C and D). Additionally, lymphocytes from *hp*$^{-/-}$ skin primed allogeneic T cells similarly to lymphocytes from WT skin (Supplemental Figure 4E), indicating that haptoglobin deficiency does not indirectly alter skin immunogenicity.

Our study also found that recipients of *hp*$^{-/-}$ grafts showed significantly longer transplant survival than recipients of WT skin (Figure 3E). Furthermore, recipients of *hp*$^{-/-}$ grafts showed lower antidonor splenic T cell IFN-γ responses than recipients of WT skin transplants (Figure 3F). Haptoglobin-deficient grafts also exhibited less necrosis and inflammation than WT grafts (Figure 3G). These results indicate that haptoglobin is a significant contributor to transplant rejection in a MyD88-dependent transplant model.

Our study shows that haptoglobin is an innate immune ligand that is released after skin necrosis. Genetic deletion of haptoglobin in skin transplants significantly delays acute graft rejection and antidonor T cell priming in an experimental model in which graft rejection is MyD88 dependent. Future studies will be required to determine whether haptoglobin promotes alloimmunity via production of IL-6 and TNF-α, cytokines that synergize to induce skin graft rejection (4). Sterile injury induces inflammation via a MyD88-dependent pathway of the IL-1 receptor (13). It would also be interesting to determine whether haptoglobin activates this pathway.

As MyD88 is dispensable for rejection of other immunogenic grafts (14), but critical to impairing transplant tolerance induced by costimulatory blockade (5), it will be important to determine in the future whether innate immune activation by haptoglobin impairs transplant tolerance. Although our study identifies haptoglobin as an innate ligand that mediates graft rejection, it is possible that other innate ligands could also contribute to transplant rejection. Furthermore, polymorphisms in the human haptoglobin gene are common, and differential expressions of the 3 major phenotypes influence the development of chronic rejection in cardiac transplant recipients (15). It will thus be interesting to determine whether haptoglobin gene polymorphisms influence the clinical course of autoimmune diseases and operational tolerance of organ transplants in humans.

Methods

Mice, skin transplantation, and graft histology. C57BL/6 and BALB/c mice were purchased from Jackson Laboratories. C57BL/6 *Myd88*$^{-/-}$, *Tlr2*$^{-/-}$*Tlr4*$^{-/-}$, and

hp$^{-/-}$ mice were used as previously reported (4, 16). Skin transplantation was performed and transplant rejection recorded as previously described (4). For histological assessment of transplants, 5-micron sections of formalin-fixed, paraffin-embedded skin were stained with H&E.

Tissue harvest and lysate preparation. Generation of necrotic tissue was based on a prior study of tumor cell necrosis and adapted to mouse skin (17). After euthanasia, mice were shaved and then treated with betadine and 70% alcohol solution. Fat and muscle were removed, and the skin was cut into 0.1-g pieces, which were placed in 1 ml of RPMI 1640 containing 5% FCS, penicillin (100 U/ml), and streptomycin (100 μg/ml). Homogenized tissue was frozen at –80°C and thawed at 37°C for 4 cycles to induce cellular fragmentation (17). Supernatants were prepared by spinning necrotic cell lysates (5×10^5 cells/ml) at 390 *g* for 10 minutes and passing the suspension through 0.45-μm filters (Millipore). For all comparisons between experimental groups, skin tissue of equal weight was used. For cardiac tissue, hearts were obtained in a sterile fashion and then rendered necrotic using the same procedure described for skin. For comparisons between skin and heart, tissues of equal weight were used.

BM-derived DC culture, determination of cytokine concentration, and reagents. BM-derived CD11c$^+$ DCs were prepared as previously described (4). DCs (1×10^6 plated per well) were cultured for 16 hours with lysates or haptoglobin; then IL-6 and TNF-α were determined using ELISA (eBioscience). Concentration of haptoglobin in necrotic lysates was also determined via ELISA (Immunology Consultants Laboratory). LPS concentrations were measured by e-Toxate assay (Sigma-Aldrich). Human haptoglobin (purity = 98%–100%; Sigma-Aldrich) was added to BM-derived CD11c$^+$ DCs culture up to a dose of 100 μg/ml containing less than 40 pg/ml LPS.

Antidonor T cell responses. Splenic T cells were enriched using magnetic negative selection (Stem Cell) at day 21 after transplantation or after i.p. injection of 1×10^6 PBS-treated or haptoglobin-treated (100 μg/ml for 6 hours) BM-derived CD11c$^+$ DCs. T cells (1×10^5 cells/well) were cultured with irradiated (5×10^5 cells/well) donor or recipient spleen cells, and ELISPOT was used to measure T cell responses, as previously described (5). For antidonor responses induced by BM-derived CD11c$^+$ DCs, results are reported after background T cell responses induced by ex vivo culture with recipient spleen cells were subtracted.

Flow cytometry. Flow cytometry staining of DCs was performed as previously described (5), after 16 hours of culture, using fluorescent anti-CD40/86 mAbs or isotype controls (eBioscience or BD Biosciences). Data were acquired using a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Treestar).

Statistics. Comparison of means was analyzed using 2-tailed Student's *t* test. Pearson's correlation was performed to analyze the association between haptoglobin levels and cytokine concentrations. Transplant survival between groups was calculated using the log-rank method. $P < 0.05$ was considered significant. All error bars represent SEM. Statistical analysis was performed using GraphPad Prism Software.

Study approval. All procedures were approved by the Yale University IACUC and followed the guidelines of the NIH Guide for the Care and Use of Laboratory Animals.

See Supplemental Methods for description of 2D DIGE, MALDI TOF/TOF, skin cell preparation, and protein digestion of lysates.

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