Bone marrow-derived cells contribute to contractile dysfunction in endotoxic shock

Brian W. Binck, May F. Tsen, Miguel Isla, D. Jean White, Roger A. Schultz, Monte S. Willis, J. Victor Garcia, Jureta W. Horton, and James A. Thomas

Department of Pediatrics, University of Texas Southwestern Medical Center, Dallas, Texas

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Toll-like receptor 4; contractile function; immune cells

The studies presented below tested the hypothesis that TLR4 also mediates myocardial contractile depression following endotoxin exposure. To test this hypothesis, WT and TLR4-deficient hearts were first placed in Langendorff preparations. Unlike hearts from WT mice, TLR4-deficient hearts did not reveal significant contractile dysfunction following LPS administration, as measured by decreased responses in maximal left ventricular pressure, +dP/dt max, and −dP/dt max in ex vivo Langendorff preparations. These findings indicate a requirement for TLR4 in LPS-induced contractile depression. To determine the contribution of bone marrow-derived TLR4 function to LPS-induced myocardial dysfunction, we generated TLR4 chimeras using adoptive transfer between histocompatible mouse strains: either TLR4-deficient mice with TLR4+/+ bone marrow-derived cells or TLR4−/− animals lacking TLR4 in their hematopoietic cells. We then compared the contractile responses of engrafted animals after LPS challenges. Engraftment of TLR4-deficient mice with WT marrow restored sensitivity to the myocardial depressant effects of LPS in TLR4-deficient hearts (P < 0.05). Inactivation of bone marrow-derived TLR4 function, via transplantation of WT mice with TLR4−/− marrow, however, did not protect against the depressant effect of endotoxin. These findings indicate that bone marrow-derived TLR4 activity is sufficient to confer sensitivity to mice lacking TLR4 in all other tissues. However, because inactivation of marrow-derived TLR4 function alone does not protect against endotoxin-triggered contractile dysfunction, TLR4 function in other tissues may also contribute to this response.

Toll-like receptor 4; contractile function; immune cells
TLR4-deficient mice were challenged with LPS, and their contractile responses were assessed using modified ex vivo Langendorff preparations. We also hypothesized that activation of bone marrow-derived TLR4 function would restore myocardial contractile dysfunction in an otherwise endotoxin-insensitive mouse. Using adoptive transfer between histocompatible mouse strains, we generated TLR4 chimeras: either TLR4-deficient mice with TLR4+/+ immune systems or TLR4+/+ animals lacking TLR4 in their hematopoietic cells. We then compared the contractile responses of engrafted animals to LPS challenges.

MATERIALS AND METHODS

Generation of TLR4 chimeric mice. C57BL10/ScSn (TLR4+/+) mice (Jackson Labs, Bar Harbor, ME) and C57BL10/ScSn (TLR4−/−) mice (National Cancer Institute, Bethesda, MD) were used for transplantation experiments as both strains express the H-2b MHC haplotype. Female mice from 6 to 8 wk of age underwent lethal irradiation at day −1. Each group received 500 rad followed 3 h later by an additional 300 rad. On day 0, marrow from femurs and spines of male donor mice were harvested, ground in media, and strained through fine mesh. After red blood cell lysis, the remaining cells were spun, washed, resuspended, and counted. For transplantation, 3 × 10^7 cells/animal were injected into the tail vein of irradiated recipients. Mice were housed in a barrier facility and fed irradiated food and water to prevent infection during engraftment. TLR4−/− mice were challenged with LPS, and their contractile function was assessed using ex vivo modified Langendorff-isolated perfusion. After red blood cell lysis, the remaining cells were spun, washed, resuspended, and counted. For transplantation, 3 × 10^7 cells/animal were injected into the tail vein of irradiated recipients. Mice were housed in a barrier facility and fed irradiated food and water to prevent infection during engraftment. TLR4−/− mice were challenged with LPS, and their contractile function was assessed using ex vivo modified Langendorff-isolated perfusion.

Molecular analysis of engraftment with donor cells. Eight weeks posttransplantation, peripheral leukocytes from transplanted mice were genotyped using PCR. Mice were bled by retro-orbital plexus puncture using a small-diameter heparin-coated microhematocrit capillary tube (Allegiance Healthcare, McGaw Park, IL), and genomic DNA was isolated using a commercial kit (Qiagen, Venica, CA). Primers that either flank the 70-kb TLR4 deletion in ScN cells (up 5′-ATA TGC ATG ATC AAC ACA GCA g-3′; down 5′-TCT CTT GTA TCT CAA GCT AC 3′) or anneal to sequences in the WT TLR4 locus deleted in ScN mice (up 5′-GCA AGT TTC TAT CAT TCT C 3′; down 5′-CTT CTA TTT CTA ATT AGT 3′) were used to amplify isolated DNA. Amplification of the WT TLR4 locus yields a 390-bp amplicon, whereas amplification of the region containing the TLR4 deletion yields a 140-bp amplicon. Amplification products were then separated on a 1.2% agarose gel to determine the origin of DNA in the peripheral leukocyte population. Amplification of leukocyte DNA from untransplant TLR4+/+ and TLR4−/− mice was performed as a control.

Cytogenetic analysis of reconstitution. Peripheral blood smears were made from whole blood collected by retro-orbital bleeding and allowed to air dry overnight. Blood smears were fixed in 3:1 methanol/glacial acetic acid for 5 min and allowed to air dry. Fluorescence in situ hybridization (FISH) to detect X and Y chromosomes was performed using whole chromosome paint probes recognizing mouse X and Y chromosomes (Vysis, Downers Grove, IL) according to the manufacturer’s protocols. The X and Y probes were mixed in hybridization buffer and placed on the prepared slide. Slides were then hybridized in a HYBrite hybridization chamber (Vysis) where they were denatured for 5 min at 72°C and then hybridized at 37°C overnight. The slides were washed for 5 min in 0.4 × SSC/0.3% NP-40 (72°C) followed by a 1-min wash in 2 × SSC/0.1% NP-40 (room temperature). Before a coverslip was applied, counterstain solution containing DAPI was added to the slide. Cells were viewed on a fluorescence microscope (Olympus, Tokyo, Japan). For each blood smear, 200 cells were graded on the presence of X (green) and Y (red) chromosomes and reported as XY or XX.

Assessment of systemic TNF-α responses following endotoxin challenge in chimeric mice. Transplanted mice were challenged by intraperitoneal injection with 1 mg/kg LPS (Sigma, St. Louis, MO) and bled through retro-orbital puncture 1 h later. Serum was separated and TNF-α levels were determined using a commercially available kit (R&D Systems, Minneapolis, MN). Groups were compared statistically using two-tailed unpaired t-tests.

Modified Langendorff preparations. Mice challenged with LPS (1 mg/kg ip) and appropriate controls were anticoagulated with heparin sodium and euthanized by cervical dislocation 18 h after injection. Hearts were removed and placed in ice-cold (4°C) Krebs-Henseleit bicarbonate-buffered solution (in mM: NaCl, 147; KCl, 4.7; CaCl2, 2.5; MgSO4, 1.2; NaH2PO4, 1.2; KH2PO4, and 11 glucose). All solutions were prepared each day with deionized water and bubbled with 95% O2-5% CO2 (pH 7.4). All groups were compared statistically using two-tailed unpaired t-tests.

RESULTS

TLR4-deficient animals are protected from LPS-induced contractile dysfunction. To determine whether TLR4 is involved in myocardial depression following endotoxin exposure, we compared the contractile responses of WT and TLR4-deficient mice following the administration of LPS. Animals were given 1 mg/kg LPS intraperitoneally and euthanized 18 h later. The hearts were removed, and contractile function was assessed using ex vivo modified Langendorff-isolated perfusion preparations. As seen in Fig. 1A, hearts from untreated WT animals (n = 8) respond to increases in coronary flow rates with increased responses in maximal LVP (LVPmax), maximal rate of pressure generated (+dP/dtmax), and maximal rate of relaxation (−dP/dtmax). Hearts from LPS-treated WT mice (n = 8) in contrast exhibit significant decreases in systolic performance, as determined by impaired LVPmax generated
and maximal rate of pressure generation (+dP/dt\text{max}) (Fig. 1A). LPS-exposed WT hearts also exhibited significantly impaired diastolic function, with decreased maximal relaxation rates (−dP/dt\text{max}) compared with untreated controls (Fig. 1A).

We then compared contractile function of hearts from untreated and LPS-exposed TLR4-deficient mice. Hearts from untreated TLR4-deficient mice (n = 7) had systolic and diastolic function similar to WT controls (Fig. 1B). Unlike their WT counterparts, hearts from LPS-challenged TLR4-deficient mice (n = 10) did not reveal any significant depression in LVP\text{max}, +dP/dt\text{max}, or −dP/dt\text{max} with increasing coronary flow. These findings indicate that functional TLR4 is required for LPS-induced contractile dysfunction.

Because TLR4-deficient mice do not express receptors in any tissues, the tissue site(s) in which TLR4 function is required is unknown. Many candidate tissues may contribute to myocardial contractile depression. Because macrophage transfer has been demonstrated to restore the lethal effect of LPS in LPS-insensitive mice (7), we chose to examine the contribution of immune cell-derived TLR4 function to cardiac depression following LPS exposure.

Leukocytes from recipient mice exhibit donor genotype. After engrafted animals were bled and DNA was isolated from whole blood, our studies indicate that peripheral leukocytes from recipient mice exhibit the donor genotype 8 wk after transplantation. Genomic DNA isolated from leukocytes was genotyped using PCR primers that either flank the TLR4 deletion or anneal to sequences in the TLR4 gene deleted in TLR4-deficient mouse strain. Figure 2 shows PCR products amplified from peripheral leukocyte DNA from control and engrafted animals. The first four lanes contain products from the amplification of the WT TLR4 locus. Bands are present in the WT control and the TLR4-deficient animal engrafted with WT immune cells, indicating the presence of WT TLR4 in engrafted leukocytes. Reaction products from primers that flank the deleted TLR4 sequence in TLR4-deficient mice are shown in the final four lanes. Bands are present in the TLR4-deficient control, as well as from the WT mouse that has been transplanted with TLR4-deficient marrow, demonstrating the absence of TLR4 in engrafted leukocytes. These findings indicate the presence of donor DNA in the peripheral leukocyte population and suggest extensive engraftment.

Engraftment is extensive in transplanted mice. To determine the extent of engraftment in transplanted animals, peripheral smears of blood were obtained from representative animals from each transplant group and their sex chromosome makeup was determined using FISH with X and Y chromosome-specific probes. Bone marrow and peripheral smears contain >95% male leukocytes 3 mo following transplantation (mean

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**Fig. 1.** Toll-like receptor 4 (TLR4) is required for contractile depression following exposure to endotoxin. Wild-type (WT) and TLR4-deficient mice were challenged with 1 mg/kg LPS. Eighteen hours after endotoxin challenge, contractile dysfunction was assessed using a modified Langendorff-isolated perfusion preparation. A: responses of WT hearts after saline and treatment with LPS; maximal left ventricular (LV) pressure generation (LVP\text{max}, left), maximal rate of pressure generation (+dP/dt\text{max}, middle), and maximal rate of relaxation (−dP/dt\text{max}, right). B: responses of TLR4-deficient hearts following LPS or saline-injected controls. *P < 0.05.
Toxin (1 mg/kg ip) and contractile function curves were generated contractile dysfunction, mice were injected with endo-
marrow-derived immune cells contribute to endotoxin-trig-
generated contractile dysfunction. To determine whether bone

Lane 1 is a DNA ladder. Lanes 2-5 represent reaction products using primers that anneal to sequences in the WT TLR4 locus. Lanes 6-9 represent reaction products using primers that flank the 70-kb TLR4 deletion.

Genomic DNA was isolated from blood of transplanted animals. Primers that either flank the 70-kb ScN TLR4 deletion or anneal to sequences in the WT TLR4 locus deleted in ScN mice were used to amplify isolated DNA. Amplification products were then separated on a 1.2% agarose gel. Amplification of leukocyte DNA from untransplanted TLR4 donors. Mice from different groups were injected with 1 mg/kg LPS, and their serum TNF-α levels (means ± SE) were assayed after 1 h (Fig. 3). Unchallenged WT mice and TLR4-deficient mice had undetectable basal TNF-α levels before endotoxin challenge. After LPS challenge, WT mice have a robust TNF-α response (5,672 ± 2,292 pg/ml), whereas TLR4-deficient animals produce little TNF-α (361 ± 144 pg/ml). In contrast, TLR4-deficient mice that have been engrafted with WT bone marrow exhibit TNF-α responses similar to WT mice (5,897 ± 2,243 pg/ml). WT mice transplanted with TLR4-deficient immune cells tended to have blunted responses to LPS (1,390 ± 967 pg/ml) compared with untransplanted WT controls, but this trend did not meet statistical significance. Additionally, serum TNF-α levels remained significantly higher in this group compared with TLR4-deficient controls.

To determine whether these effects could be caused by radiation received during transplantation, we also performed syngeneic transplants, both with WT and TLR4-deficient mice. TLR4-deficient mice transplanted with TLR4-deficient marrow do not produce significant TNF-α following treatment with LPS (451 ± 34 pg/ml). This suggests that irradiation itself does not confer sensitivity to LPS. Interestingly, WT mice transplanted with WT bone marrow had a more dramatic response to LPS than untransplanted WT mice (11,383 ± 3,591 pg/ml), suggesting that regulation of the TNF-α response may not be fully reconstituted following irradiation and transplantation.

Bone marrow-derived cells contribute to endotoxin-trig-
gerated using modified Langendorff preparations. Endotoxin-challenged hearts from TLR4-deficient mice do not exhibit significant contractile depression following LPS exposure. However, hearts from endotoxin-challenged TLR4-deficient mice that have undergone bone marrow transplantation with WT marrow (n = 5; Fig. 4A) exhibit depressed contractility. They show significant decreases in LVPmax as well as +dP/dtmax and −dP/dtmax in response to increasing coronary flow rates (P < 0.05). Similar depressions in contractility indexes were also seen in response to increasing extracellular calcium concentrations (data not shown), another inotropic stimulus.

To ensure that the contractile dysfunction observed was not due to irradiation and transplantation itself, contractile studies were performed on hearts from syngeneic TLR4-deficient transplants (data not shown). These mice did not reveal any significant depression of contractility.

Inactivation of bone marrow-derived TLR4 function does not protect against LPS-induced contractile dysfunction. Although bone marrow-derived TLR4 function is sufficient to confer sensitivity to the cardiodepressant effects of endotoxin, it is unknown whether TLR4 inactivation in the same cells confers a cardioprotective effect in otherwise WT mice. To answer this question, cardiac contractile responses of endotoxin-exposed WT mice with and without marrow-derived TLR4 function were compared (Fig. 4B). WT mice (n = 8) and WT mice transplanted with TLR4-deficient bone marrow (n = 9) were injected with LPS (1 mg/kg ip) and contractile function was studied as before. Both groups exhibit depressed contractile function, compared with untreated WT controls, with decreased responses in LVPmax, +dP/dtmax, and −dP/dtmax, indicating that inactivation of marrow-derived TLR4 function does not protect against LPS-induced contractile dysfunction (P < 0.05).

DISCUSSION

Results from the above studies confirm that TLR4 function plays a key role in endotoxin-induced myocardial contractile

![Fig. 2. Peripheral leukocyte DNA from engrafted animals has donor genotype. Genomic DNA was isolated from blood of transplanted animals. Primers that either flank the 70-kb ScN TLR4 deletion or anneal to sequences in the WT TLR4 locus deleted in ScN mice were used to amplify isolated DNA. Amplification products were then separated on a 1.2% agarose gel. Amplification of leukocyte DNA from untransplanted TLR4+/+ and TLR4−/− mice was performed as a control. Lane 1 is a DNA ladder. Lanes 2-5 represent reaction products using primers that anneal to sequences in the WT TLR4 locus. Lanes 6-9 represent reaction products using primers that flank the 70-kb TLR4 deletion.](http://ajpheart.physiology.org/)

![Fig. 3. Transplant recipients exhibit TNF-α responses to LPS similar to donors. Mice from different groups were injected with 1 mg/kg LPS, and their serum TNF-α concentrations (means ± SE) were assayed after 1 h. *P < 0.05 compared with TLR4−/−, +P < 0.05 compared with WT.](http://ajpheart.physiology.org/)
dysfunction. Previous work has demonstrated that TLR4 signaling is critical to the innate immune response to infection, and recent studies, using TLR4 mutants, have suggested that TLR4 function may be important in cardiac contractile dysfunction seen following LPS exposure (13). Our ex vivo studies, performed in TLR4 null mice, build on this concept. In this model, TLR4 function is absolutely required for LPS-induced myocardial depression. The fact that hearts from mice deficient in the receptor show no significant differences in their contractile responses following endotoxin challenge supports this notion.

Although studies with TLR4-deficient mice suggest that TLR4 function is important in endotoxin-mediated contractile depression, they do not delineate in which tissue compartments TLR4 signaling is required. Previous work demonstrates that the adoptive transfer of WT macrophages is sufficient to restore the lethal effects of LPS in endotoxin-insensitive mice (7), prompting our hypothesis that bone marrow-derived immune cells may contribute to contractile depression.

Bone marrow-transplanted animals provide a means of examining the contribution of a single tissue compartment to TLR4 function. Our results indicate that after irradiation and bone marrow transplantation, recipients are extensively engrafted by 3 mo. Peripheral leukocytes possess donor DNA, and FISH studies document the predominance of male donor cells in female recipients. Moreover, TLR4-deficient mice, normally unresponsive to LPS, produce serum TNF-α levels similar to WT controls when engrafted with TLR4-competent immune cells. Conversely, WT mice transplanted with TLR4-deficient marrow have blunted TNF-α responses when challenged with LPS. Together, these observations indicate that bone marrow-derived TLR4 function contributes to the serum TNF-α response following exposure to endotoxin. TLR4-deficient mice that have been transplanted with TLR4-deficient marrow, however, do not exhibit significant TNF-α responses, indicating that irradiation alone is not sufficient to confer sensitivity to LPS. Why WT mice transplanted with WT marrow have an exaggerated TNF-α response is unknown but may indicate that regulatory control of the TNF-α response may take longer to reestablish following engraftment or may be lost following irradiation and adoptive transfer.

A central finding from these studies is that, in this model, bone marrow-derived immune cells confer sensitivity to contractile dysfunction following endotoxin exposure. TLR4-deficient mice that have been engrafted with WT (TLR4+/+) immune cells have statistically significant contractile dysfunction on stimulation with LPS, suggesting that immune cells may contribute, at least in part, to the contractile dysfunction seen in endotoxic shock. Our findings indicate that activation of bone marrow-derived TLR4 function is sufficient to confer sensitivity to LPS-induced contractile dysfunction in an otherwise TLR4-deficient animal. Although our findings help identify a possible contributing compartment, they do not preclude the involvement of other tissues, nor do they conclusively
identify the degree to which bone marrow-derived TLR4 function is involved. Our results could be due to TLR4 signaling in a single cell population or, conversely, the result of multiple bone marrow-derived cell types, including traditionally “non-immune” cells such as endothelial stem cells.

It is interesting that, in this model, inactivation of bone marrow-derived TLR4 function does not protect hearts from contractile dysfunction following endotoxin challenge. WT mice engrafted with TLR4-deficient marrow display contractile curves that are statistically indistinguishable from WT mice. This observation suggests that other tissues, including perhaps the heart itself, contribute to the contractile depression seen in endotoxic shock. Increased TLR4 mRNA levels have been documented in patients with enteroviral-associated dilated cardiomyopathy (19). Also, others have demonstrated increased TLR4 mRNA levels in the myocardium of patients with non-infectious heart failure (3), supporting the notion that myocardial TLR4 signaling may participate in contractile dysfunction. TLR4 upregulation in heart failure in uninfected patients raises interesting questions as to the nature of TLR4 signaling in injury. Contractile dysfunction, and perhaps injury, may represent a cumulative effect of TLR4 function in multiple tissues or the result of tissue damage in a single compartment. Tissues traditionally thought of as nonimmune may act as relays for myocardial depression through TLR4 signaling. Myocardial depression may therefore represent a cumulative aggregate response to a variety of inputs, both from the innate immune system as well as nonimmune tissue. If true, Toll/IL-1 signaling may have a role in nonimmune physiology and hint at a broader function of the pathway. Although clearly important in the recognition and elimination of pathogen, the pathway may serve a more generic role in sensing injury. Currently, our understanding of myocardial injury is somewhat limited, due to the complexity of multiple pathways involved in cardiac function. For example, both LPS and ischemia-reperfusion injury are known to promote apoptosis in cardiac myocytes, and various ion channels have been implicated in preconditioning from ischemia-reperfusion injury. The complex interplay between these protein pathways remains the focus of much research, but if indeed Toll/IL-1 signaling is involved in more than simple pathogen recognition, one might expect it to play a part in multiple forms of injury. Previous studies documented a role for the Toll/IL-1 signaling pathway in thermal injury (21), and the pathway has been implicated in ischemia-reperfusion injury as well (15). Additional studies demonstrated that the Toll/IL-1 pathway can be activated by endogenous signals such as heat shock proteins (14, 22–25), degradation products of hyaluronan (20), and pulmonary surfactant proteins (8). If indeed the Toll/IL-1 signaling pathway senses a range of inflammatory disease, it might also be a target of therapeutic potential in the treatment of cardiac disease.

GRANTS

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REFERENCES


