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Toll-Like Receptor 4 Contributes to Small Intestine Allograft Rejection

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Abstract

Background—Although outcomes for small intestine transplantation (SIT) have improved in recent years, allograft rejection rates remain among the highest of solid organ grafts. The high load of commensal bacteria in the small intestine may contribute through activation of the toll-like receptor (TLR) pathway. In this study, we examine the participation of TLR4 in acute allograft rejection in an orthotopic mouse model of SIT.

Methods—Wild-type C57Bl/6 (H-2^b) or TLR4^{−/−} (H-2^b) mice were transplanted with syngeneic (C57Bl/6), allogeneic (BALB/c; H-2^d), or F1 (BALB/c×C57Bl/6; H-2^{d/b}) vascularized, orthotopic small intestine grafts. Graft recipients were killed on days 2 to 6 posttransplant. Serum cytokines were measured by Luminex, and tissue was obtained for histology and quantitative real-time polymerase chain reaction.

Results—BALB/c grafts transplanted into C57Bl/6 recipients exhibited mixed inflammatory infiltrates, destruction of the mucosa, and significant apoptosis. TLR2 and TLR4 transcripts were modestly increased in syngeneic grafts on days 2 and 6 compared with native bowel, whereas TLR2 and TLR4 were significantly increased on days 2 and 6 in allogeneic grafts. Although fully mismatched and F1 grafts were rejected by C57Bl/6 recipients (mean survival time=8.2 and 9.3 days, respectively), graft survival was significantly prolonged in TLR4^{−/−} recipients (mean survival time=10.6 and 14.3 days, respectively). Proinflammatory cytokines were markedly reduced in TLR4^{−/−} graft recipients.

Conclusions—Small intestine graft survival is prolonged in the absence of TLR4, suggesting that gut flora associated with the graft may augment alloimmune responses through TLR4. Thus, the TLR pathway may be a novel therapeutic target for improving SIT allograft survival.

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S.M.K., R.O.C., C.O.E., and O.M.M. participated in the research design; M.W., L.P., and T.I. performed the experiments; S.M.K., R.O.C., and O.M.M. analyzed the data; J.H. and N.K. performed histologic analyses; and S.M.K. and O.M.M. prepared the manuscript.

Keywords

Small intestine transplantation; Toll-like receptors; TLR4

Small intestine transplantation (SIT) is an important therapeutic option for children and adults with end-stage intestinal failure. However, the success of SIT has historically been hampered by high incidences of acute graft rejection (80%–90%) and infection. Improvements in short-term intestinal graft outcome have been reported in the past decade as 1-year graft and patient survival is 74% and 86%, respectively (1). Nevertheless, long-term outcomes remain inferior to other solid organs as 3- and 5-year patient survival is 61% and 47%, respectively, in isolated intestine transplants (2, 3). The development of acute rejection is strongly linked to both graft loss and patient death (3). Because of the high incidence of rejection, and the dire consequences, intensive immunosuppression has been routinely used in SIT patients. Accordingly, these patients are at increased risk of infection. Epstein Barr virus-associated posttransplant lymphomas (posttransplant lymphoproliferative disease) remain a serious, and often fatal, complication in SIT, particularly in pediatric recipients. Moreover, the transfer of bacterial flora associated with the graft may also contribute to an increased risk of posttransplant infectious complications in SIT patients. Thus, a greater understanding of the factors that determine posttransplant rejection and infection could markedly improve SIT outcome.

Toll-like receptors (TLRs) are a family of receptors (TLR1–TLR11) expressed on a variety of cell types including leukocytes that recognize conserved molecular patterns derived from extracellular microbes. Ligation of TLR by these pathogen-associated molecular patterns (PAMPs) derived from surface or intracellular components of microbes activates the innate immune response and can potentiate the adaptive immune response. In the case of TLR4, the known PAMP is the gram-negative bacterial cell wall component lipopolysaccharide, but TLR4 can also recognize viral envelope proteins (4) and a variety of endogenous ligands including heat shock proteins, necrotic or dying cells, and fibrinogen (5). Signaling downstream of TLR4 involves MyD88, a common cytoplasmic adaptor protein used by all TLR, with the exception of TLR3, to propagate the signal transduction cascade involving nuclear factor- κ B and mitogen-activated protein kinases that culminates in the production of inflammatory cytokines including interleukin (IL)-1, IL-6, tumor necrosis factor- α , and IL-12. TLR4, along with TLR3, can also initiate MyD88-independent signaling through the TIR-domain-containing adaptor-inducing interferon-beta (TRIF) adaptor protein that results in production of type 1 interferons (IFNs) through activation of the transcription factor, IFN regulator factor 3.

The high load of commensal bacteria associated with the small intestine, combined with the ability of the TLR pathway to augment immune activation, suggests that TLR signaling may play an important role in the increased incidence of acute rejection in small intestine allografts. Here, we investigate the contribution of TLR4 to allograft rejection using a mouse model of orthotopic SIT.

RESULTS

Establishment of an Allogeneic Model of Orthotopic Small Intestine Transplantation

To establish a model of acute rejection in SIT, we performed orthotopic intestinal transplants in syngeneic (C57Bl/6 $\times$ C57Bl/6) and allogeneic (BALB/c $\times$ C57Bl/6) donor-recipient pairs. Allogeneic grafts (n=5) were rejected with a mean survival time (MST) of 8.2 ± 0.8 days, whereas syngeneic grafts survived for more than 60 days (n=4). In a separate group of animals, syngeneic and allogeneic SIT recipients (n=3) were killed on days 2, 4,

and 6 posttransplant, and intestinal tissue was obtained for histopathologic analyses. Syngeneic grafts showed normal histology with no evidence of rejection on days 2, 4, or 6 posttransplant (Fig. 1A, C, and E). In marked contrast, day 2 allografts (Fig. 1B) showed increased cellularity in the villi with activity in the crypts. Day 4 allografts (Fig. 1D) showed increased apoptosis, whereas on day 6, posttransplant allografts (Fig. 1E) had high levels of apoptosis, loss of the mucosa, and a classic mixed inflammatory infiltrate. To eliminate any influence of graft-versus-host disease, we also transplanted small intestine grafts from F1 animals (BALB/c×C57Bl/6) into semiallogeneic C57Bl/6 recipients. F1 grafts survived slightly longer than fully allogeneic grafts (9.3 MST vs. 8.2 MST), but this difference was not significant. Thus, small intestine allografts display the histologic hallmarks of acute graft rejection.

TLR Expression Is Increased in SIT Allografts

To begin to examine the role of TLR signaling in rejection of SIT, we analyzed expression of TLR2 and TLR4 in syngeneic and allogeneic small intestine grafts by quantitative real-time polymerase chain reaction. Syngeneic grafts showed modest up-regulation of TLR2 and TLR4 on posttransplant days 2 and 6 compared with normal, nontransplanted C57Bl/6 mice (Fig. 2A). In contrast, C57Bl/6 recipients of BALB/c allografts had significant up-regulation of both TLR2 and TLR4 on days 2 and 6, although increases in TLR4 were more rapid and notably greater than increases in TLR2 (Fig. 2B). These results indicate that increased TLR expression in the graft is an early feature of SIT and suggest that TLR may participate in small intestine allograft rejection.

Recipient TLR4 Participates in Rejection of SIT

Because SIT grafts contain significant bacterial flora and because TLR4 is markedly up-regulated in SIT allografts, we reasoned that TLR4 could contribute to small intestine allograft rejection. To determine whether TLR4 participates in the allogeneic response in SIT, we transplanted intestines from fully major histocompatibility complex-mismatched BALB/c mice (H-2^d) into TLR4^{-/-} mice (H-2^b). As shown in Figure 3(A), TLR4^{-/-} recipients of BALB/c small intestine had significantly prolonged graft survival compared with wild-type C57Bl/6 recipients (10.6±1.1 days vs. 8.2±0.8 days; *P*=0.02). Allograft survival was also significantly prolonged in TLR4^{-/-} recipients of F1 (BALB/c×C57Bl/6) small intestine grafts compared with wild-type C57Bl/6 recipients (Fig. 3B; MST 14.3 days vs. 9.3 days; *P*=0.008). F1 C57Bl/6 grafts showed a mild increase in crypt apoptosis on days 2 and 4 compatible with mild acute rejection (Fig. 4A and C). By day 6 posttransplant, C57Bl/6 recipients of F1 grafts showed significantly increased apoptosis and increased inflammatory cells within the lamina propria consistent with moderate acute rejection (Fig. 4E). In marked contrast, TLR4^{-/-} recipients of F1 grafts showed no evidence of acute rejection on days 2 and 4 (Fig. 4B and D). However, by day 6, increases in apoptosis and inflammatory infiltrate were consistent with mild to moderate rejection (Fig. 4F). Interestingly, the absence of TLR4 in recipients of fully mismatched BALB/c heterotopic cardiac allografts did not result in prolonged allograft survival compared with survival in wild-type C57Bl/6 recipients (data not shown). Together, these data indicate that recipient TLR4 plays an important role in small intestine allograft rejection.

TLR4^{-/-} Recipients of Small Intestine Allografts Have Reduced Levels of Proinflammatory Cytokines in the Periphery

TLR4 signaling in dendritic cells (DCs) is known to stimulate production of proinflammatory cytokines that could contribute to allograft rejection (4). To determine the effect of host TLR4 deficiency in SIT recipients, we used Luminex assays to measure serum cytokine levels in TLR4^{-/-} recipients of F1 grafts 5 days after transplantation. Recipients deficient in TLR4 (TLR4^{-/-}) had significantly reduced levels of IL-12, -1, and -6 compared

with C57Bl/6 recipients ($P < 0.05$) and diminished IFN- γ , although this difference was not significant (Fig. 5). These results suggest that decreases in proinflammatory cytokine production could play a role in the prolongation of small intestine survival observed in TLR4 $^{-/-}$ recipients.

DISCUSSION

Improvements in surgical approaches, posttransplant patient management, and innovations in immunosuppression protocols have markedly improved SIT outcomes in the past decade (2). Nevertheless, graft rejection and infection continue to be major causes of morbidity and mortality in SIT recipients (1). We considered the possibility that activation of innate immune pathways by commensal microbiota within the small intestine may play a role in SIT rejection. Here, we tested the contribution of the TLR pathway, a key component of host innate immunity through recognition of microbial products, to SIT acute rejection. In particular, we focused on TLR4, known to detect lipopolysaccharide in gram-negative bacteria, by using TLR4-deficient mice as recipients of orthotopic small intestine transplants. Our findings indicate that the absence of TLR4 in the recipient is sufficient to significantly extend graft survival of SIT allografts. Moreover, posttransplant graft histology was improved in TLR4 $^{-/-}$ recipients compared with wild-type recipients. The mechanistic underpinnings for this observation are likely to be complex, but our data suggest that diminished activation of host DC may be involved because circulating proinflammatory cytokine levels are markedly reduced in TLR4 $^{-/-}$ recipients compared with wild-type recipient and because DCs are an important source of IL-6 and IL-12 after TLR4 activation (4, 6, 7).

The link between TLR signaling and alloimmunity was initially reported by Goldstein et al. (8). These authors showed that the absence of the TLR adaptor protein MyD88 $^{-/-}$ in the donor and the recipient led to acceptance of skin grafts mismatched for the male H-Y antigen. The absence of MyD88 in graft recipients was associated with reduced number of mature DCs in draining lymph nodes and reduced IFN- γ expression. Further, the absence of both MyD88 and TRIF was required to achieve prolonged skin graft survival across complete major histocompatibility complex and minor antigen mismatches (9). These studies do not permit identification of specific TLR that participate in graft rejection, but it is noteworthy that TLR4 can use both the MyD88- and TRIF-dependent pathways of TLR activation. Indeed, few studies have directly examined the role of TLR4 in allograft rejection. TLR4 has been implicated in human chronic cardiac allograft rejection (10) and in rejection of islets transplanted intraportally (11). In the later study, TLR4 deficiency in the donor, but not the host, diminished proinflammatory cytokine production and prolonged graft survival. Goldberg et al. (12) who found that TLR4-deficient islets had prolonged survival in allogeneic recipients reported similar findings. These findings are consistent with the idea that TLR4 signaling impacts on graft survival through effects on ischemia/reperfusion injury of the donor organ or during isolation of cellular grafts.

In our model of SIT, the deficiency in TLR4 was confined to the graft recipient. Thus, we favor the hypothesis that a different mechanism in SIT involving translocation of graft-associated flora may stimulate TLR4-expressing recipient antigen-presenting cells leading to augmented alloimmune activation in wild-type recipients. Infiltration of host-derived cells lacking TLR4, which replace graft-derived immune cells, into the small intestine of TLR4 $^{-/-}$ recipients may also contribute to a reduction in alloactivation. Nevertheless, it would be of interest to test whether TLR4 deficiency in the graft affects survival of small intestine transplants. Certainly, the extremely high microbial load of the small intestine is unique with respect to other solid organ grafts. Underscoring this point is our finding that the absence of TLR4 $^{-/-}$ in C57Bl/6 recipients of BALB/c cardiac allograft recipients did not prolong graft

survival, in agreement with a previous report by Zhai et al. (13). Together, these findings indicate that TLR4 activation may be particularly important in SIT.

Although our studies do not directly examine the participation of commensal bacteria in graft rejection, there is strong evidence that translocation of bacteria in small intestine grafts does occur posttransplant (14–16). Translocation can result in migration of gut flora into extraintestinal sites that can then lead to TLR activation of recipient cells. Thus, in certain settings, endogenous ligands of TLR may be important in initiating inflammation, but in the case of SIT, we favor the possibility that gut flora are a source of PAMP for TLR4.

Previous experimental approaches to prolong SIT survival in experimental models by our group and others have centered on costimulation blockade (17–19), establishment of mixed chimerism with donor bone marrow (18, 19), depletion of CD4 or CD8 T cells (17, 18, 20–22), and disruption of chemokine or cytokine pathways (21–24). Altogether, these studies indicate that both CD4⁺ and CD8⁺ T cells contribute to SIT rejection, that cytokines, in particular IFN- γ and tumor necrosis factor- α , play an important role, and that SIT allografts are more resistant to tolerance induction than other solid organ allografts. So far, no studies have directly examined the contribution of microbial antigens to the immunogenicity of SIT allografts and their impact on graft rejection. Our results demonstrating that the absence of TLR4 alone is sufficient to significantly prolong survival of small intestine allografts suggest that the TLR pathway may be an important new therapeutic target to improve the outcome of SIT.

MATERIALS AND METHODS

Mice

BALB/c (H-2d), C57Bl/6 (H-2b), and F1 (BALB/c $\times$ C57Bl/6) mice and TLR4-deficient mice (C57Bl/10ScNJ, H-2b) were all obtained from Jackson Labs (Bar Harbor, ME).

Small Intestine Transplantation

All animal studies were approved by the Institutional Animal Care and Use Committee. Donor animals were fasted for 24 hr and given water containing trimethoprim/sulfamethoxazole (20 mg/kg/day). The donor operation involved a full laparotomy. The mesenteric vessels were ligated with 8-0 silk suture to isolate the entire jejunum and ileum. The portal vein was separated from the pancreas, and heparin was infused into the inferior vena cava. The graft was then perfused in situ with 0.2- to 0.4-ml cold heparinized Lactated Ringer's solution and removed with a Carrel patch of aorta and stored in Lactated Ringer's solution at 4°C. Recipient animals were fasted 12 hr before surgery. End-to-side anastomoses were performed between the donor portal vein and the recipient inferior vena cava and between the donor aortic patch and the recipient aorta. An orthotopic graft was created by anastomosing both the proximal and distal portions of the graft to proximal and distal locations of the recipient's small bowel after removal of the native small bowel.

Histology

Small intestine allografts were harvested on days 2, 4, and 6 posttransplant. The proximal and distal portions of the graft were fixed in formalin, embedded in paraffin, and stained with hematoxylin-eosin. Tissue was analyzed and scored by two pathologists blinded to the identity of the specimens according to the criteria described in the study by Wang et al. (25).

Quantitative Real-Time Polymerase Chain Reaction

Small intestine graft tissue was harvested and placed in RNeasy lysis reagent (Qiagen, Valencia, CA) and stored at –80°C until RNA isolation. RNA was isolated from

samples using the RNEasy Kit (Qiagen, Valencia, CA) and reverse transcribed using random primers. cDNA samples were run in duplicate using a SYBR Green qPCR Kit (ABI) using GAPDH as a reference gene as described previously (26). A threshold cycle (Ct) was observed in the exponential phase of amplification, and quantification of relative expression levels was performed using standard curves for target genes and the endogenous control. Geometric means were used to calculate the delta delta Ct values, and the relative quantification was expressed as $2^{-\text{delta delta Ct}}$. TLR2 and TLR4 relative expression level were compared with naïve small bowel samples.

Luminex Assay for Cytokines

Serum cytokines were quantitated by Luminex using the mouse 26 plex format (Affymetrix, Santa Clara, CA). Samples are acquired on the Luminex MAP200 instrument, with collection criteria set for 100 beads per analyte (2000 beads total). Data are analyzed using MasterPlex software (Hitachi Software Engineering America Ltd., MiraiBio Group, South San Francisco, CA), and both median fluorescence intensity and calculated concentration values are reported for each analyte.

Statistical Analysis

Statistical analysis was performed using Student's *t* test with *P* less than or equal to 0.05 considered to be significant. Graft survival was compared using Kaplan-Meier and the log-rank test with GraphPad Prism 5.0 (version 5b) (Graph Pad, La Jolla, CA) software. *P* values less than or equal to 0.05 were considered significant.

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FIGURE 1.

Histology of small intestine isografts and allografts on days 2, 4, and 6 posttransplant. Syngeneic (C57Bl/6 $\times$ C57Bl/6, n=9) and allogeneic (BALB/c $\times$ C57Bl/6) (n=9) orthotopic small intestine transplants were performed, and three mice from each group were killed on days 2, 4, and 6 posttransplant. Tissue from the distal small intestine was fixed in formalin, embedded in paraffin, and analyzed by hematoxylin-eosin staining. Representative syngeneic grafts (*top*) showed normal histology on days 2 (A), 4 (C), and 6 (E) posttransplant, whereas representative allogeneic grafts (*bottom*) showed increased cellularity in the villi on day 2 (B); shrinking, blunting of the villi, and apoptosis on day 4 (D); and a classic inflammatory infiltrate and extensive apoptosis on day 6 (F).

FIGURE 2.

Expression of TLR2 and TLR4 in small intestine (A) isografts and (B) allografts. RNA was isolated from two to three small intestine grafts on day 2 and 4 posttransplant, and cDNA was prepared. Quantitative real-time polymerase chain reaction was performed, and data are expressed as a fold expression relative to normal native bowel.

FIGURE 3.

Toll-like receptor (TLR4)-deficient graft recipients show prolonged survival of orthotopic small intestine grafts. (A) Full major histocompatibility complex mismatch, BALB/c (H-2^d)

C57BL/6 (H-2^b) (n=5) and BALB/c TLR4^{-/-} (H-2^b) (n=5) or (B) partially mismatched, C57BL/6×BALB/c C57BL/6 (n=4) or TLR4^{-/-} (n=4), orthotopic small intestine transplants were performed. Mean survival time is (A) 8.2 days versus 10.6 days ($P=0.02$) and (B) 9.3 days versus 14.3 days ($P=0.009$). WT, wild type.

FIGURE 4.

Histology of small intestine allografts in C57Bl/6 and toll-like receptor (TLR) 4^{-/-} recipients on days 2, 4, and 6 posttransplant. F1 (C57Bl/6×BALB/c) grafts were transplanted into C57Bl/6 (n=9) or TLR4^{-/-} (n=9) mice, and three mice from each group were killed on days 2, 4, and 6 posttransplant. Tissue from the distal small intestine was fixed in formalin, embedded in paraffin, and analyzed by hematoxylin-eosin staining. Representative wild-type recipients (*top*) showed evidence of mild acute rejection on days 2 (A) and 4 (C) with moderate acute rejection evident on day 6 (E) posttransplant. Representative TLR4^{-/-} recipients of F1 grafts (*bottom*) showed no evidence of rejection on day 2 (B) or day 4 (D) and mild-moderate rejection on day 6 (F).

FIGURE 5.

Proinflammatory cytokines are reduced in the blood of toll-like receptor (TLR) 4^{-/-} recipients of orthotopic small intestine grafts. Serum was obtained from C57Bl/6 recipients of BALB/c small intestine grafts (n=2) and TLR4^{-/-} recipients (TLR4^{-/-}) recipients of BALB/c grafts (n=2) on day 4 posttransplant. Cytokines were analyzed by Luminex as described in *Materials and Methods*. BALB/c versus TLR4^{-/-} interleukin (IL)-12p70 ($P=0.004$), IL-6 ($P=0.02$), and IL-1 ($P=0.004$) were significantly different, whereas interferon (IFN)- γ was not statistically significant ($P=0.2$).