Recipient Toll-like receptors contribute to chronic graft dysfunction by both MyD88- and TRIF-dependent signaling

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SUMMARY

Toll-like receptors (TLRs) recognize specific molecular patterns derived from microbial components (exogenous ligands) or stressed cells (endogenous ligands). Stimulation of these receptors leads to a pronounced inflammatory response in a variety of acute animal models. Chronic allograft dysfunction (CAD) was regarded as a candidate disease to test whether TLRs influence chronic fibrosing inflammation. Potential endogenous renal TLR ligands, specifically for TLR2 and TLR4, have now been detected by a significant upregulation of glucose regulated protein (GRP)-94, fibrinogen, heat shock protein (HSP)-60, HSP-70, biglycan (Bgn) and high-mobility group box chromosomal protein 1 (HMGB1) in the acute and chronic transplant setting. In a genetic approach to define the contribution of TLR2 and TLR4, and their adaptor proteins MyD88 and TRIF [Toll/interleukin (IL)-1 receptor domain-containing adaptor-protein inducing interferon β], to CAD, kidney transplantation of TLR wild-type grafts to recipients who were deficient in TLR2, TLR4, TLR2/4, MyD88 and TRIF was performed. TLR and adaptor protein deficiencies significantly improved the excretory function of chronic kidney grafts by between 65% and 290%, and histopathologic signs of chronic allograft damage were significantly ameliorated. T cells, dendritic cells (DCs) and foremost macrophages were reduced in grafts by up to 4.5-fold. The intragraft concentrations of IL-6, IL-10, monocyte chemotactic protein-1 (MCP-1) and IL-12p70 were significantly lower. TLR-, MyD88- and TRIF-deficient recipients showed a significant reduction in fibrosis. α-smooth muscle actin (α-SMA)-positive cells were decreased by up to ninefold, and collagen I and III were reduced by up to twofold. These findings highlight the functional relevance of TLRs and their two major signaling pathways in graft-infiltrating mononuclear cells in the pathophysiology of CAD. A TLR signaling blockade may be a therapeutic option for the prevention of CAD.

INTRODUCTION

Despite major advances in the treatment of acute allograft rejection, chronic allograft dysfunction (CAD) still develops in a majority of kidney transplant recipients (Nankivell et al., 2003). The chronic changes affect vessels, glomeruli, tubules and the interstitium. They are characterized by the histologic features of arteriopathy, glomerulopathy, peritubular capillaropathy, interstitial fibrosis and tubular atrophy (Cornell et al., 2008). This process can start within several weeks after kidney transplantation and is the major cause of organ loss (Nankivell et al., 2003).

Previous research on the mechanisms of graft rejection was mainly focused on T-cell-mediated immune regulation (Cornell et al., 2008). New evidence now hints at an important role of the innate immune system in the development of organ rejection (Goldstein et al., 2003; Palmer et al., 2006; de Groot et al., 2008; Wang et al., 2008). The innate immune system recognizes pathogen-associated molecular patterns by pattern recognition receptors (Medzhitov et al., 1997).

The Toll-like receptor (TLR) family, which includes 13 members in mammals, binds to a variety of microbial products, e.g. lipopolysaccharide (LPS), zymosan and flagellin. Endogenous ligands, such as glucose regulated protein (GRP)-94, fibrinogen, heat shock protein (HSP)-60 and -70, biglycan, high-mobility group box chromosomal protein 1 (HMGB1), heparan sulphate and RNA, have been shown to potently activate TLRs (Matzinger, 2002; Iwasaki and Medzhitov, 2004; Schaefer et al., 2005; Cavassani et al., 2008; Kawai and Akira, 2008). MyD88 is an adaptor protein used by TLRs, with the exception of TLR3, that activates the transcription factors nuclear factor kappa B (NF-κB), activator protein 1 (AP-1) and cAMP response element binding protein (CREB) leading to cytokine and chemokine production (Hazeki et al., 2007; Kawai and Akira, 2008). The TRIF [Toll/interleukin (IL)-1 receptor domain-containing adaptor-protein inducing interferon (IFN) β]-dependent signaling cascade is a MyD88-independent signaling pathway associated with TLR3 and TLR4 that stimulates cytokine production through interferon regulatory factor 3 (IRF3), NF-κB and AP-1 (Kawai and Akira, 2008). Exogenous and endogenous TLR ligands are not only able to trigger the release of inflammatory cytokines [e.g. tumor necrosis factor (TNF)-α, IL-12, IFNs] and chemokines [e.g. IL-8, monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2)], but they also foster the maturation of dendritic cells (DCs) into potent antigen presenting cells (APCs) (Kaisho and Akira, 2001). TLR-activated DCs induce naïve T cells into antigen-specific effector T cells of the T helper (Th)1 and Th2 lineage (Iwasaki and Kelsall, 1999; Rissoan et al., 1999). In B cells,

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antigen-specific antibody responses are boosted by TLRs (Pasare and Medzhitov, 2005). Through these effects, TLR signaling connects innate and acquired immunity.

TLRs are expressed by a variety of hematopoietic cells [DCs, monocytes, T cells, neutrophils, mast cells, natural killer (NK) cells, basophils and eosinophils] and in tubular epithelial cells, podocytes and endothelial cells of the kidney (Akira and Takeda, 2004; Shigeoka et al., 2007; Banas et al., 2008). A deficiency in TLR2 and TLR4 has been reported to protect animals from renal ischemia reperfusion injury (IRI). In a murine acute renal failure model, TLR2-deficient mice showed less secretion of proinflammatory cytokines and chemokines, less recruitment of leukocytes, renal damage and dysfunction (Leemans et al., 2005; Shigeoka et al., 2007). Similar data could be obtained in TLR4- and MyD88-deficient mice (Wu et al., 2007).

The role of TLRs in experimental allogenic transplantation is still controversial. TLR deficiency has been reported to either have no effect or to be beneficial to the allograft. In a mouse skin transplantation model, TLR2 deficiency was associated with slower rejection rates compared with the wild-type controls (Goldstein et al., 2003), whereas TLR4 did not seem to influence rejection (Samstein et al., 2004). Using fully mismatched skin and cardiac allografts, all grafts from MyD88-deficient mice and wild-type controls were rejected within 2 to 3 weeks, however, decreased activation of DCs and diminished Th1 responses could be observed (Tesar et al., 2004). This impaired activation of DCs led to a reduction of naïve T-cell priming (Tesar et al., 2004) and a higher susceptibility of T cells to suppression by regulatory T cells (Walker et al., 2006).

Increased expression of *TLR2* mRNA, but not *TLR4* mRNA, in protocol biopsies of human kidney transplants has been correlated with superior graft function (de Groot et al., 2008). A loss-of-function polymorphism in the *TLR4* gene has been associated with a reduction in inflammatory cytokines and delayed graft function of kidney transplants (Kruger et al., 2009).

In vivo and in vitro experiments have hinted at a role of TLRs in fibrogenic responses, which might be important for the development of graft fibrosis. A deficiency of TLR4, TLR9 and MyD88 decreased markers of liver fibrosis in different toxic and surgical models of experimental hepatic fibrosis (Seki et al., 2007; Watanabe et al., 2007).

We have assumed that mononuclear cells infiltrating the graft represent a major cell-damaging component in CAD. To assess the potential role of TLR2 and TLR4, and of the adaptor proteins MyD88 and TRIF, in the complex pathophysiology of CAD, we used a genetic dissection approach by transplanting wild-type kidneys from BALB/c mice into mice with a targeted deletion of the *Tlr2/Tlr4*, *Myd88* and *Trif* (also known as *Ticam1*) genes. By using TLR2/4-deficient mice, we investigated the combined contribution of the membrane receptors TLR2 and TLR4 to CAD, as some endogenous ligands activate TLR2 and TLR4 simultaneously (Schaefer et al., 2005). By comparing MyD88-deficient animals with TRIF-deficient animals, we sought to analyze the distinct contributions of MyD88 and TRIF signaling in CAD.

The endogenous TLR ligands biglycan (Bgn), GRP-94, HSP-60, HSP-70, fibrinogen and HMGB1 were found to be significantly upregulated at day 7 and 42 after transplantation. TLR and adaptor protein deficiencies significantly improved the creatinine clearance of kidney grafts and ameliorated histopathological signs of chronic allograft damage. We found stronger protection in the MyD88-deficient animals than in TRIF-deficient animals, indicating a major contribution of MyD88 in mediating immune responses in CAD.

RESULTS
Renal allografts showed increases in endogenous TLR ligands
To determine whether renal allograft rejection induces an upregulation of endogenous TLR ligands in kidney grafts, we determined the expression of GRP-94, fibrinogen, HSP-60, HSP-70, Bgn and HMGB1 at day 7 and 42 after transplantation by immunohistochemistry. At day 7, a significant upregulation of all endogenous ligands was seen in all allografts when compared with untreated control kidneys. This increase in potential endogenous TLR ligands was accentuated at day 42 (Fig. 1A-G; supplementary material Fig. S1).

Table 1. TLR2, TLR4, TLR2/4, MyD88 and TRIF deficiencies improved excretory renal allograft function in chronically rejecting organs

Recipient background		
C57BL/6 mice	**Creatinine clearance, ml/min**	**Improvement in renal function**
Control – C57BL/6 allograft	3.68±0.49	
Tlr2/4⁻/⁻	14.40±2.41***	291.3%
Myd88⁻/⁻	13.27±0.87***	260.6%
Trif⁻/⁻	11.82±1.20***	221.2%
Isograft-C57BL/6	10.59±1.75***	187.8%
C3H/HeN mice		
Control – C3H/HeN allograft	7.14±1.24	
Tlr2⁻/⁻	15.63±2.26**	118.9%
Tlr4⁻/⁻	11.78±1.42*	65.0%
Isograft-C3H/HeN	11.78±2.22*	65.0%

In comparison to the control group of mice, creatinine clearances (ml/min) were preserved in the TLR-deficient mice (*Tlr2/4*⁻/⁻, *Myd88*⁻/⁻, *Trif*⁻/⁻) with a C57BL/6 background at 42 days after murine kidney transplantation. There was no significant difference in the creatinine clearance of TLR-deficient mice when compared with C57BL/6 isograft controls. *Tlr2*⁻/⁻ and *Tlr4*⁻/⁻ animals on a C3H/HeN background showed the same amelioration of kidney graft function and were also comparable to the isotype controls (*n*=6-8). *$P<0.05$, **$P<0.01$, ***$P<0.001$.

TLR2/4, MyD88 and TRIF deficiencies improve excretory renal allograft function in chronically rejecting organs

Forty-two days after murine kidney transplantation, excretory graft function was found to be preserved in TLR-deficient mice in comparison with the control group of mice. Creatinine clearances were significantly improved in all experimental groups, varying from between 65% and 290%. Isografts showed creatinine clearances that were comparable to the TLR groups and that were significantly
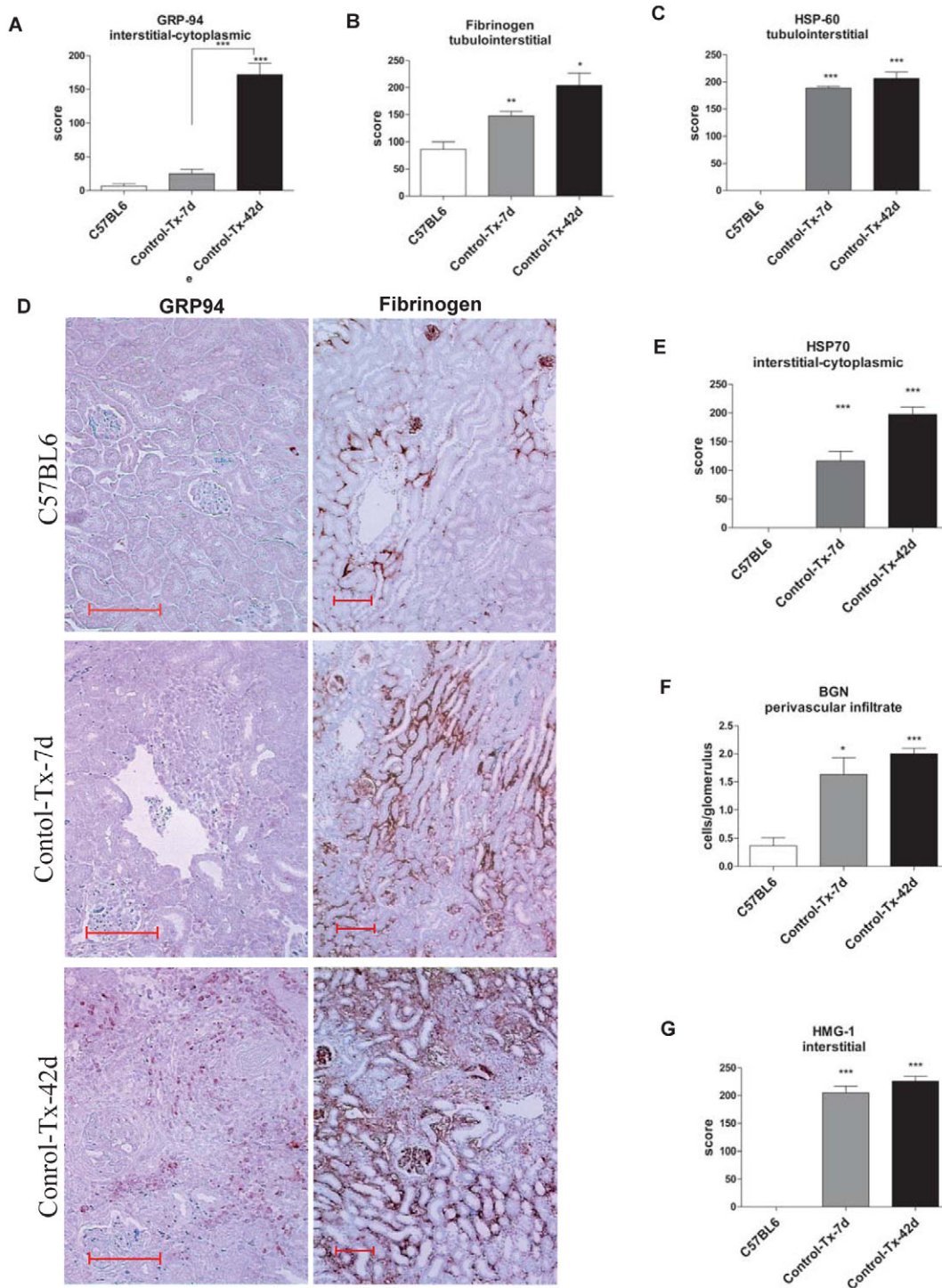
Fig. 1. Endogenous TLR ligands were upregulated in kidney allografts at day 7 and 42 after transplantation. (A-C,E-G) Quantitation showed that GRP-94, fibrinogen, HMGB1, HSP-60, HSP-70 and Bgn are expressed at very low, or undetectable, levels in untreated kidneys and were significantly upregulated at 7 and 42 days post-transplantation (*n*=6-8) (*$P<0.05$, **$P<0.001$, ***$P<0.0001$). (D) Micrographs showing the significant upregulation of GRP-94 and fibrinogen in the interstitial space of kidney allografts at day 7, which was even more pronounced at day 42, in comparison with untreated kidneys. Bars, 200 µm.
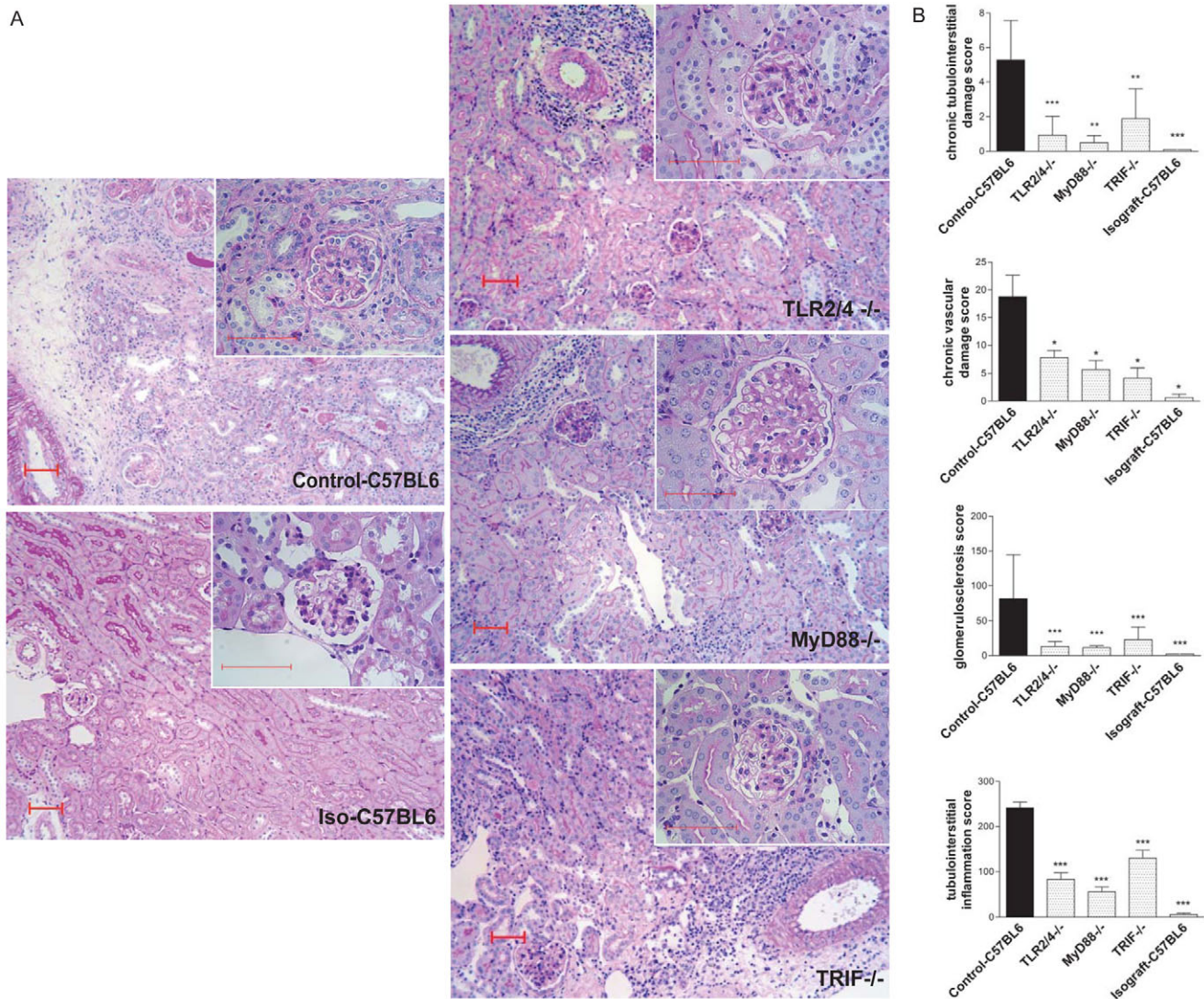
Fig. 2. Effects of TLR deficiency on glomerular and tubulointerstitial damage. (A) Histological evidence of tubulointerstitial damage. Compared with the C57BL/6 transplant controls with a severe mononuclear cell infiltrate, tubular atrophy and interstitial fibrosis, the isotype control grafts and the grafts with a deficient TLR function showed essentially normal renal morphology and only discrete immune cell infiltration. Bars, 200 μm. (B) Quantitation of the tubulointerstitial damage confirmed the histologic findings. Micrographs and quantitation of glomerular damage showed that recipients with deficient TLR function had significantly reduced glomerular damage at day 42 in comparison to wild-type controls that had severe transplant glomerulopathy and focal segmental glomerulosclerosis. The insets in A show magnifications of the glomeruli, demonstrating edematous mesangium with matrix increase with segmentally duplicated membranes and activated endothelium with mononuclear cells. In the isograft control and the TLR and adaptor-deficient groups, the glomeruli had regular cell numbers, normal basement membranes, and no mononuclear cell infiltration (n=6-8) (*P<0.05, **P<0.001, ***P<0.0001).

higher than the C57BL/6 (an increase of 188%) and C3H/HeN (an increase of 65%) control allografts (Table 1).

Histological analysis of renal allograft rejection

Histological evaluation of the control allografts demonstrated a mononuclear cell infiltrate in the glomeruli (transplant glomerulitis) and tubulointerstitium. In addition, a focal infiltration of the subendothelial space could be found in some preglomerular arteries (vascular rejection). These phenomena increased with time. After 7 days, vascular rejection was reduced by TLR blockade; interstitial rejection was lowered in mice with a TLR adapter deficiency (supplementary material Table S1). The most pronounced effect

of TLR inhibition could be seen in the chronic transplant setting 42 days after transplantation. At this time point, a deficiency in TLR2, TLR4, TLR2/4, MyD88 or TRIF resulted in a significant amelioration of glomerular damage, tubulointerstitial inflammation, fibrosis, and atrophy and vascular rejection (Fig. 2). Mice with the combined TLR2/4 deficiency did not show an additive difference in morphology compared with mice with a single TLR2 or TLR4 deficiency (data not shown).

Immunophenotyping of the graft infiltrations

To analyze the immune phenotype of the inflammatory infiltrate in the kidney grafts, we quantified T cells (CD4+, CD8+),
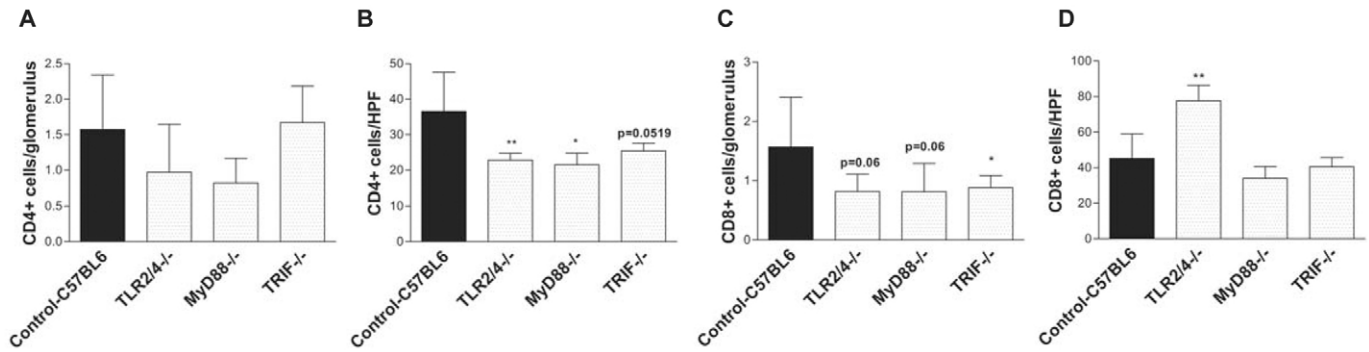
Fig. 3. Immunophenotyping of graft infiltrations in TLR-deficient recipients at day 42 after engraftment. (A) Glomerular CD4+ cells tended to be reduced in the *Tlr2/4$^{-/-}$* and *Myd88$^{-/-}$* mice. (B) Tubulointerstitial CD4+ cell numbers were significantly lower in the grafts of *Tlr2/4$^{-/-}$* and *Myd88$^{-/-}$* recipients. Recipients with a deficiency in TRIF function tended to have a decrease in the number of tubulointerstitial CD4+ cells ($P=0.05$). HPF=high-power field. (C) The numbers of glomerular CD8+ cells were significantly lower in the *Trif$^{-/-}$* group and tended to decrease in *Myd88$^{-/-}$* ($P=0.06$) and *Tlr2/4$^{-/-}$* ($P=0.06$) recipients. (D) In contrast to the glomerular compartment, the numbers of CD8+ T cells were not significantly changed in the tubulointerstitial space ($n=6$-8) (*$P<0.05$, **$P<0.001$, ***$P<0.0001$).

macrophages (F4/80+) and DCs [CD11c+, major histocompatibility complex (MHC) II+] by immunohistochemistry and flow cytometry.

The number of glomerular CD4+ cells was significantly decreased in TLR4 mutant recipients (by 3.5-fold) and tended to be reduced in the TLR2/4 and MyD88 groups (Fig. 3A). The number of tubulointerstitial CD4+ cells was significantly lower in the grafts of TLR2/4- ($P<0.01$) and MyD88-deficient ($P<0.05$) recipients. Recipients with a TRIF deficiency tended to show a non-significant decrease in the number of tubulointerstitial CD4+ cells ($P=0.052$). By contrast, a deficiency in TLR2 or TLR4 did not change the numbers of tubulointerstitial cytotoxic T cells. The glomerular CD8+ cells were significantly lower in the TRIF group ($P<0.05$) and tended to decrease in MyD88- ($P=0.06$) and TLR2/4- ($P=0.06$) deficient recipients (Fig. 3C,D).

Glomerular F4/80+ cells were significantly decreased in all TLR-related deficient allografts: in TLR2-, TLR4- and TLR2/4-deficient mice the numbers of cells were reduced by approximately twofold, fivefold and 12-fold, respectively, and in MyD88- and TRIF-deficient mice there were reductions of approximately tenfold and sixfold, respectively. Immunohistochemistry for tubulointerstitial F4/80+ cells, which detected macrophages and DCs, showed a significant reduction in cell numbers in all of the experimental groups when compared with control mice. The number of F4/80+ cells in the transplanted deficient mice was not significantly different to the very low count in the isograft group (Fig. 4A).

Staining for arginase-1-positive cells, which are induced by the Th2 cytokines IL-4 and IL-13 and are considered to be a marker for alternative activation of macrophages in the mouse, revealed higher numbers in all experimental groups, with the most pronounced increase observed in TRIF-deficient recipients. By contrast, the number of cells that were positive for YM-1 – a heparin binding lectin of the chitinase family of proteins, which is also associated with alternative activation and is detected in macrophages, DCs and granulocytes – was unchanged or even reduced (Fig. 4B).

The number of glomerular CD11c+ cells was significantly lower in TLR2/4-, MyD88- and TRIF-deficient groups than in allograft

controls. In order to specifically detect DCs, flow cytometry was performed on the allograft-derived leukocytes that were isolated from the TLR2/4-deficient group. The number of CD11c and MHC II double positive cells was significantly decreased ($P<0.05$) (Fig. 4C). Furthermore, the levels of MHC II and CD11c were significantly reduced (data not shown), arguing for a lower activation state of intragraft DCs in TLR-deficient recipients. As further evidence of allograft protection by TLR deficiency, we found reduced numbers of Ki67+ proliferating cells, the majority of which were assumed to be leukocytes, in the glomerula and interstitium (Fig. 5A,B).

Kidney transplants in TLR2/4-, MyD88- and TRIF-deficient mice generally showed a decreased concentration of the cytokines that are classically associated with inflammation, such as MCP-1, IL-12p70, IL-6 and IL-10 ($P<0.001$) (Fig. 6).

**TLR deficiency improves markers of renal fibrosis**
Graft fibrosis, one of the major characteristics of chronic allograft damage, was analyzed 42 days after transplantation in the tubulointerstitial compartment by immunohistochemical staining for collagen I/III and α-smooth muscle actin (α-SMA) cells. The staining for myofibroblasts (α-SMA+ cells), which are the major source of interstitial collagen, showed that there were significantly lower numbers of α-SMA+ cells in all gene-deficient groups (Fig. 7A).

Collagen deposition was markedly increased in the interstitial space of control kidney allografts. In contrast to this finding, all experimental groups showed a significant decrease in collagen I and III staining in the tubulointerstitium (Fig. 7B).

DISCUSSION
TLR2/4 and the adaptor proteins MyD88 and TRIF, expressed by infiltrating leukocytes, have now been found to be central mediators of chronic rejection in a murine allogeneic renal transplant model in which a wild-type BALB/c kidney graft was transplanted into TLR- or adaptor protein-deficient mice. This model allowed a genetic dissection of the contribution of TLRs and their respective adaptor proteins to the development of CAD. The improvement
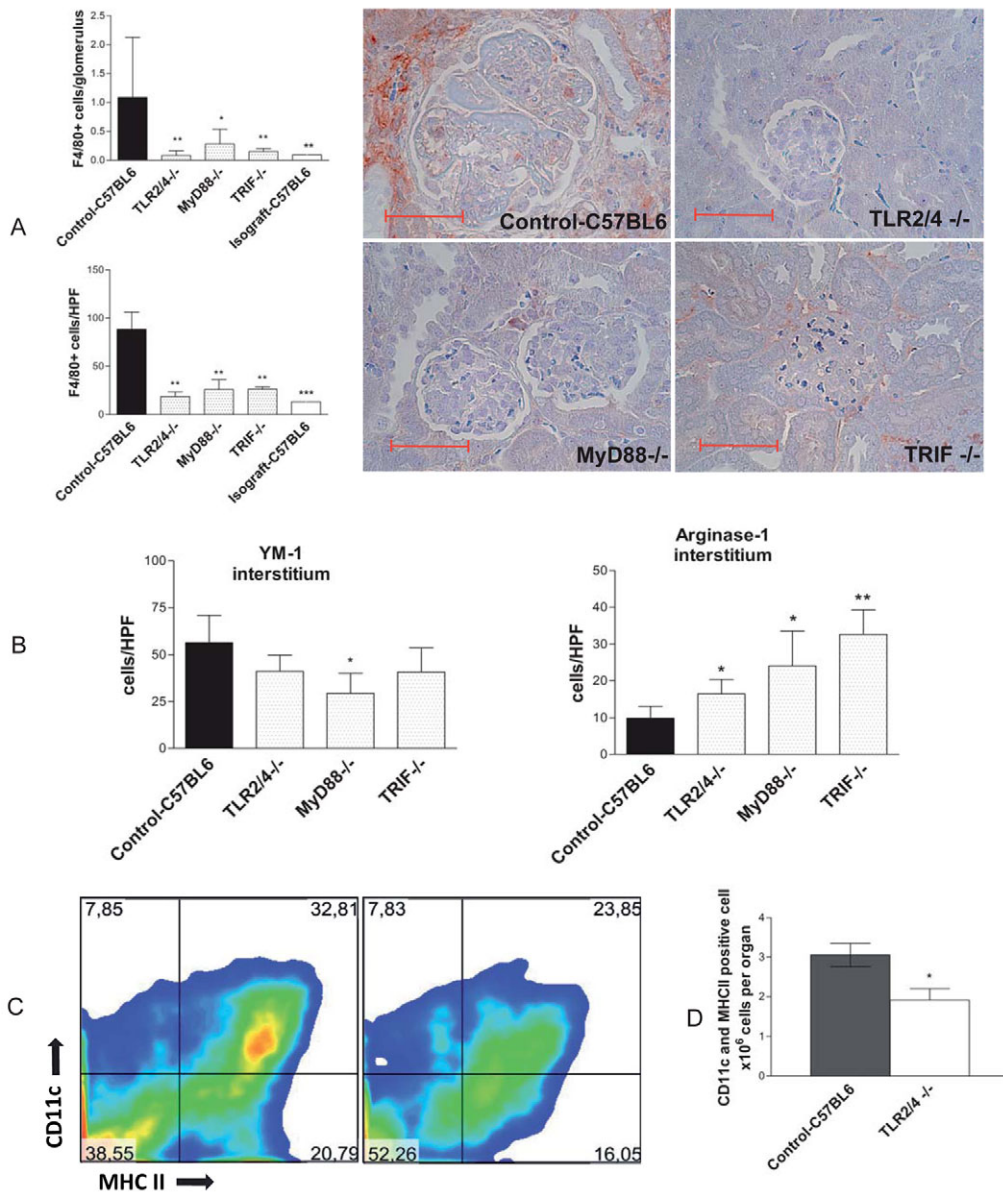
Fig. 4. Deficiencies in TLR function reduce the numbers of monocytes/macrophages and DCs in kidney allografts after 42 days. (A) In kidney grafts, glomerular and tubulointerstitial macrophages, which are identified by F4/80 expression, are significantly reduced in all experimental groups. Bars, 200 μm. (B) Quantitation of staining for arginase-1+ cells revealed a higher number of cells in all experimental groups; this was most pronounced in $Trif^{-/-}$ recipients. By contrast, the YM-1+ cell count was unchanged or even reduced. (C) Freshly isolated renal DCs from control mice (left) and the TLR2/4-deficient group (right) were stained with the monoclonal antibodies anti-MHC II FITC and anti-CD11c PE Cy5.5 and analyzed by flow cytometry, which showed decreased numbers of CD11c+ and MHCI II+ cells. (D) There was a significant downregulation of the maturation markers CD11c and MHC II in total kidney lysates of $Tlr2/4^{-/-}$ animals (n=6-8) (*P<0.05, ** P<0.001, ***P<0.0001).

in chronic allograft dysfunction by the TLR blockade seemed to be a result of the continuous activation of the TLR signaling pathway during chronic allograft rejection and the effects of TLRs on several mononuclear cells of the innate and adaptive immune systems. The persistent expression of potential endogenous ligands of TLRs in the transplant is in agreement with a recently published study showing an upregulation of HMGB1 in non-living donor kidneys (Kruger et al., 2009). The authors of that study postulate that IRI is the main factor responsible for continued ligand, antigen and cytokine release, but that postulate is unlikely because, after

42 days, control isotransplantations showed high creatinine clearances, normal kidney histopathology and low inflammatory cytokine levels when compared with allografts.
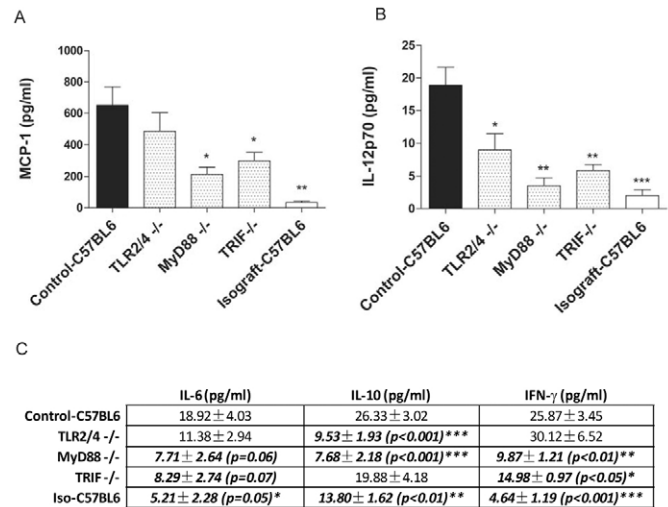
The data from 7 days after transplantation indicated that TLR activation influences acute rejection, a process that is driven mainly by T-cell-mediated events. However, the main effects were seen in the chronic transplants. The results of these experiments were different from the previously described skin and heart transplantation models. TLR-deficient skin grafts transplanted into a TLR-deficient or wild-type background were
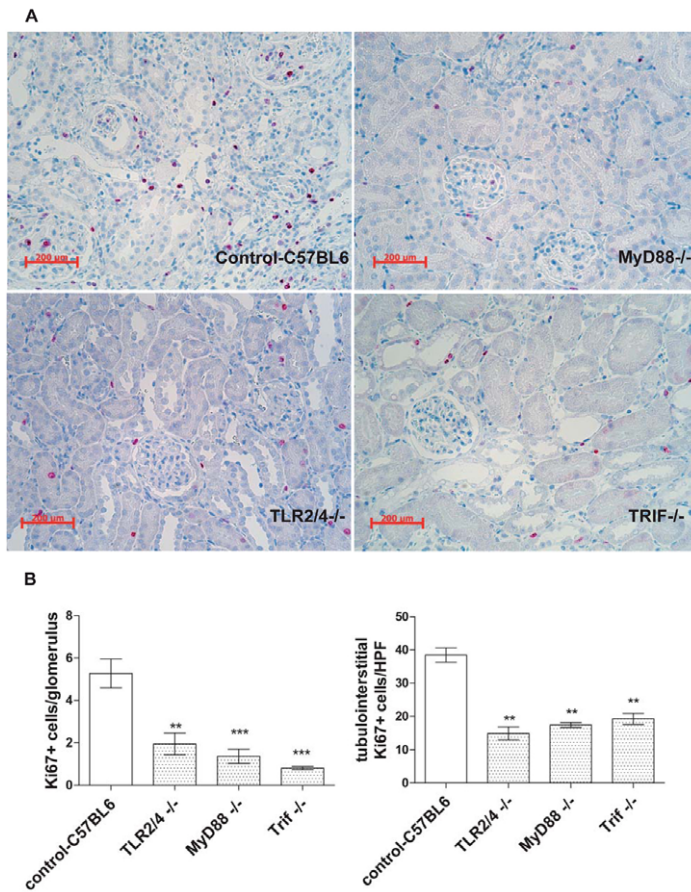
Fig. 5. TLR deficiencies reduced the numbers of Ki67+ cells in kidney allografts after 42 days. (A) Micrographs and (B) quantitation showing that glomerular and tubulointerstitial proliferating cells, identified by Ki67 expression (red), were significantly reduced in kidney grafts in *Tlr2/4$^{-/-}$*, *Myd88$^{-/-}$* and *Trif$^{-/-}$* recipients (*n*=6-8) (*P<0.05, **P<0.001, ***P<0.0001). Bars, 200 μm.

|	IL-6 (pg/ml)	IL-10 (pg/ml)	IFN-γ (pg/ml)
Control-C57BL6	18.92 ± 4.03	26.33 ± 3.02	25.87 ± 3.45
TLR2/4 -/-	11.38 ± 2.94	9.53 ± 1.93 (p<0.001)***	30.12 ± 6.52
MyD88 -/-	7.71 ± 2.64 (p=0.06)	7.68 ± 2.18 (p<0.001)***	9.87 ± 1.21 (p<0.01)**
TRIF -/-	8.29 ± 2.74 (p=0.07)	19.88 ± 4.18	14.98 ± 0.97 (p<0.05)*
Iso-C57BL6	5.21 ± 2.28 (p=0.05)*	13.80 ± 1.62 (p<0.01)**	4.64 ± 1.19 (p<0.001)***

Fig. 6. Expression of cytokine secretion 42 days after renal transplantation. (A) The MCP-1 and (B) IL-12p70 concentrations in transplanted tissue from TLR- and TLR adaptor-deficient recipients were significantly reduced. (C) Overview of the IL-6, IL-10 and IFN-γ concentrations from lysed kidneys, showing reduced concentrations in most experimental groups compared with grafts from wild-type recipients (*n*=5-8) (*P<0.05, **P<0.001, ***P<0.0001).

used to simultaneously test the contribution of TLRs in mononuclear cells of the recipient, as well as in cells of the donor graft. Importantly, the skin model was an acute rejection model, whereas the kidney transplantation model used here has focused on the chronic rejection processes that limit graft survival in the clinical setting.

To our surprise, not only TLR2/4- and MyD88-deficient animals, but also TRIF-deficient animals, were protected from CAD. In most of the damage scores, we found a trend towards less inhibition of CAD in the TRIF-deficient group compared with MyD88-deficient recipients. These results are, in part, analogous to previous reports describing that TRIF-deficient animals were able to reject minor histocompatibility Y (HY)-incompatible skin transplants, whereas MyD88-deficient animals were unable to reject these grafts (Goldstein et al., 2003; Goldstein, 2006; McKay et al., 2006). As MyD88 is downstream of the TLRs (except TLR3), and is therefore potentially initiated by several ligand-TLR interactions, it might induce a more sustained synthesis of inflammatory cytokines than TRIF, which is a downstream signaling adaptor only for TLR3 and TLR4.

Similiar to CAD in human kidney grafts, graft damage in the chronic kidney transplantation model that was used in this study seemed to be driven mainly by DCs and macrophages. By contrast, the acute rejection models of skin and heart transplantation are dependent mainly on T-cell function, because depletion of these cells prevented rejection (Zhai et al., 2006). In addition to the lower total numbers of DCs, the intragraft DCs showed a less mature phenotype, which has been observed previously in the draining lymph nodes of animals that have received skin transplants, in conjunction with a lower number of anti-graft-reactive T cells and diminished Th1 immunity (Goldstein et al., 2003). TLRs can activate DCs to induce naïve T cells into differentiating into antigen-specific effector T cells (Rissoan et al., 1999).

CD8-positive T-cell numbers were not significantly changed in grafts of MyD88- and TRIF-deficient animals. This is consistent with the finding in human kidney grafts demonstrating that changes in creatinine clearance in rejecting organs do not correlate with T-cell infiltration, but rather monocyte/macrophage infiltration. The lower IL-12p70 synthesis in the allografts of deficient mice argues for reduced activation of T cells (Girlanda et al., 2008).

An upregulation of arginase-1-positive cells in the kidney grafts of TLR-deficient recipients was observed. Arginase-1 competes with nitric oxide (NO) synthesis for their common substrate, arginine, thereby reducing NO levels in the tissue. Recent data show that TLR stimulation of macrophages induces NO secretion (El Kasmi et al., 2008). The increase in arginase-1-positive cells in the grafts of all TLR- and TLR adaptor-deficient mice might thus have eliminated the potential cytotoxic effects of high NO concentrations. In addition, arginase-1 has been described as a marker for alternative activation of macrophages, potentially indicating a less robust inflammatory response in these innate cells.
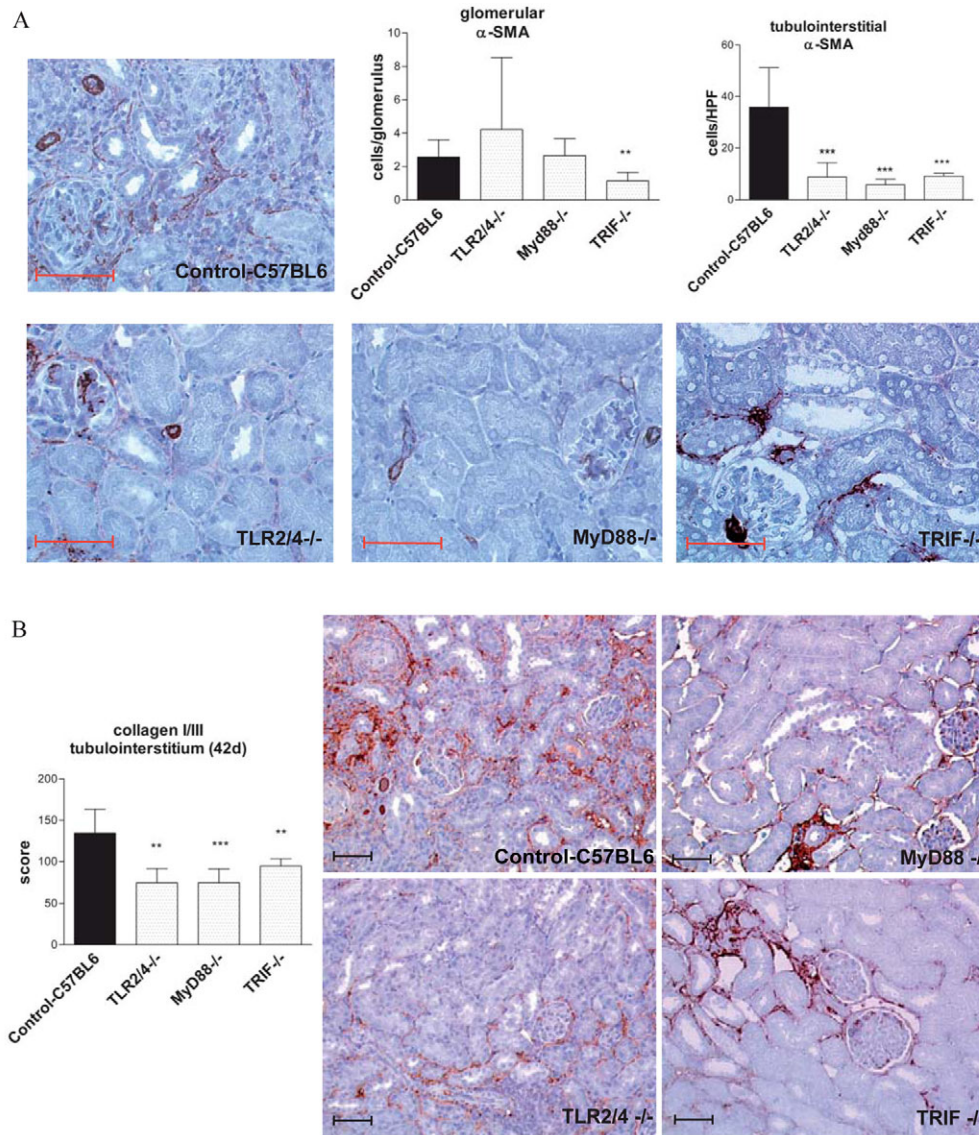
Fig. 7. Graft fibrosis was analyzed 42 days after transplantation in the tubulointerstitial and glomerular compartment by immunohistochemical staining for α-SMA and collagen I/III. (A) The number of myofibroblasts, identified by expression of α-SMA (red), in kidney grafts was significantly reduced in the tubulointerstitium of all experimental groups. In the glomerular compartment, a significant reduction in α-SMA staining could only be found in grafts from *Trif*^{-/-} recipients. (B) Collagen I/III expression (red) in the glomerular and tubulointerstitial compartment were significantly decreased in all experimental groups (*n*=6-8) (*$P<0.05$, **$P<0.001$, ***$P<0.0001$). Bars, 200 μm.

However, YM-1, a chitinase-like secretory lectin that is usually associated with alternative activation of macrophages, was not altered in TLR-deficient mice compared with isograft controls. This set of macrophage markers probably reflects the microenvironment-dependent, highly plastic functional state of monocytes/macrophages (Raes et al., 2002).

The intragraft cytokine data revealed part of the mechanisms of the beneficial effects of TLR inhibition. In comparison to the isograft controls, MCP-1 showed the strongest increase of all the cytokines in the transplanted kidneys and showed a highly significant reduction in the knockout mice. As MCP-1 has been associated with the induction of fibrosis, this finding could be one of the antifibrotic actions of the TLR blockade (Kitagawa et al., 2004; Chow et al., 2006).

IL-12p70, which is secreted by DCs and mediates Th1 responses (Quesniaux, 1992), was reduced strongly by TLR inhibition. Transplantation studies have shown its important role in allograft rejection by promoting cytotoxic T-cell activities in the early post-transplantation phase (Goriely and Goldman, 2007).

In addition to MCP-1 and IL-12p70, an overall prominent decrease in inflammatory cytokine response has now been determined in kidneys that have been transplanted into MyD88- and TRIF-deficient mice. As we have observed a stronger cytokine depletion in MyD88- or TRIF-deficient mice than in TLR2/4-deficient mice, one may surmise a possible involvement of the other TLRs in the chronic transplant setting (e.g. TLR3 for TRIF; TLR7 and 9 for the MyD88 pathway). Despite the fact that the

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cellular origin of the cytokines has not been ascertained owing to the lack of sensitivity and specificity of labeling in immunohistologic sections, our findings have now demonstrated that both MyD88 and TRIF represent important inducers of inflammatory cytokines and mediators of inflammation. Evidence has been published for a supplementary interaction between MyD88 and TRIF in the induction of target genes (Fuentes et al., 1995; Lloyd et al., 1997).

TLR-deficient recipients showed a marked decrease in fibrosis in chronically rejecting transplants. Proliferation and collagen synthesis of myofibroblasts are usually controlled by a variety of cytokines and chemokines [e.g. MCP-1, transforming growth factor (TGF)-β] that are released by inflammatory cells, renal epithelia, and by fibroblasts themselves, and were suppressed by TLR inhibition (Vielhauer et al., 2001; Tesch, 2008). In addition, myofibroblasts in the kidney graft might be recruited from different sources: resident fibroblasts and fibroblasts from circulating bone marrow precursors, a population that is generally considered to be small (Picard et al., 2008; Hewitson, 2009). TLR signaling may have a direct role in fibroblast activation and collagen production (Seki et al., 2007). The amelioration of fibrosis in TLR-deficient recipients could therefore be explained by lower numbers of macrophages, DCs and primed T cells, leading to reduced cytokine release and stimulation of myofibroblasts, and reduced activity of infiltrating TLR-deficient fibroblasts.

The major goal of clinical transplantation of solid organs is the long-term acceptance of grafts with minimal immunosuppression. A significant beneficial effect of the blockade of TLR signaling in kidney transplantation has now been shown and is clearly independent of protection from initial IRI. In this study, the continued interaction between the adaptive and innate immune system through the TLRs and their adaptors seemed to mediate chronic rejection processes and CAD. As antagonists to TLR are currently in clinical trials, the clinical significance of our experimental results may be tested (Hoffman et al., 2005; Bennett-Guerrero et al., 2007; Rossignol et al., 2008).

METHODS
Experimental animals
Male BALB/c, C57BL/6, C3H/HeN (wild-type TLR4) and C3H/HeJ mice were obtained from Charles River Laboratories GmbH (Sulzfeld, Germany). C3H/HeJ mice are TLR4 mutants that are hyposensitive to LPS signaling. The Lps^d allele of C3H/HeJ mice corresponds to a missense mutation in the third exon of the $Tlr4$ gene, with a substitution of proline with histidine at position 712 of the polypeptide chain (Poltorak et al., 1998). The TLR2- and TLR2/4-deficient mice were obtained from Prof. Kirschning (Institute of Medical Microbiology, Immunology and Hygiene, Technical University of Munich, Germany), the MyD88-deficient mice were obtained from Oriental BioService, Inc. (Minamiyamashiro Laboratory, Japan) and TRIF-deficient mice were obtained from Dr Anders (Medical Policlinic, University of Munich, Germany). The TLR2-deficient mice and the TLR4-mutant mice were on a C3H/HeN background, whereas the TLR2/4-, MyD88- and TRIF-deficient mice were on a C57BL/6 background. The TLR2- and TLR2/4-deficient mice were backcrossed at the Technical University of Munich for at least six generations, the MyD88-deficient animals were backcrossed at

Oriental BioService, Inc. for at least ten generations, and the TRIF-deficient mice were backcrossed at the University of Munich for at least six generations. All mice were housed in a specific pathogen-free animal facility at the German Cancer Research Center (DKFZ). Mouse maintenance and experiments were performed according to German laws on animal protection.

Renal transplantation
Mice (male, 8-10 weeks old, weighing 20-25 grams) were anesthetized intraperitoneally with tribromoethanol (avertin). Kidneys from BALB/c mice were transplanted into recipient mice that were TLR2, TLR4, TLR2/4, MyD88 or TRIF deficient. In addition, BALB/c kidneys were transplanted into wild-type control mice (C3H/HeN and C57BL/6 mice) ($n \geq 6$/group). For the isograft transplants, only the kidneys from C57BL/6 or C3H/HeN mice were transplanted into recipients of the same strain. Renal transplantation was performed as described previously (Zhang et al., 1995). Briefly, the abdomen of the donor was opened through a midline incision and the left kidney, with its vessels attached to a segment of the aorta and the renal vein, along with the ureter, was removed en bloc. The donor aorta and inferior vena cava were then anastomosed end-to-side to the recipient abdominal aorta and inferior vena cava, respectively, below the level of the native renal vessels. The native left kidney was removed before revascularization. The donor and recipient ureter were anastomosed, end-to-end, at the ureteropelvic junction. The native right kidney was removed after grafting. Mice were sacrificed on day 7 and 42 post-transplantation.

Determination of creatinine and urea levels in the plasma and urine
For the measurements of creatinine clearance, mice were kept in metabolic cages for 24 hours before sacrifice, with free access to water, but food was withheld. Blood samples were obtained from the hearts of anesthetized mice at day 42 post-transplantation. After centrifugation for 10 minutes at 5000 g/minute, the plasma was collected and stored at −20°C until needed for determinations. The concentrations of creatinine and urea in the plasma and urine were determined with a Hitachi 911 autoanalyzer (Roche, Mannheim, Germany).

Histopathology
Light microscopy was performed on 3 μm sections stained with periodic acid-Schiff (PAS). Kidneys were evaluated for evidence of acute and chronic vascular, glomerular and tubulointerstitial damage, and scored as described previously (Kiss et al., 2003; Adams et al., 2005).

Immunohistochemistry
Immunohistochemical staining was performed on 3 μm sections of frozen or zinc-fixed paraffin-embedded tissue, using rat anti-mouse monoclonal antibodies against CD4 and CD8 (both from BD Biosciences Pharmingen, San Diego, USA) and F4/80 (Serotec, Oxford, UK), and using a mouse anti-arginase-1 monoclonal antibody (BD Biosciences Pharmingen). The rabbit polyclonal antibody directed against the Ym1 protein was a kind gift of Dr Shioko Kimura. To identify myofibroblasts and changes in the extracellular matrix, a mouse anti-α-SMA (Sigma, Missouri, USA) and a rabbit anti-mouse

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collagen I/III antibody (Biogenesis, Poole, UK) were used. Proliferating cells were stained with Ki67 (Dianova, Germany). The expression of TLR ligands was studied by using rabbit anti-mouse polyclonal antibodies against fibrinogen (Glostrup, Denmark), GRP-94 (Santa Cruz, CA, USA) and HMGB1 (BD Pharmingen), and goat anti-mouse antibodies against HSP-60 and HSP-70 (Santa Cruz). Bgn was stained using MAY-01, a chicken anti-rat Bgn antiserum, as described previously (Schaefer et al., 1998).

Glomerular-positive cells were counted in at least 50 glomerular cross sections and given as the mean per glomerular section; interstitial-positive cells were counted in 20 high-power fields (HPFs) ($\times$40 magnification) of the cortex and outer medulla, and recorded as the mean per HPF.

The tubulointerstitial scores for collagen staining and TLR ligands (except for Bgn staining, which was evaluated as stated above) were calculated using the following method (Adams et al., 2005; Bedke et al., 2007), where the extent of the staining was evaluated as: 0, no staining detectable; 1, faint staining; 2, moderate staining; and 3, intense staining. A degree-specific staining index was defined as the percentage of fields with the respective degree of staining in at least ten HPFs of the cortex and outer medulla. The staining score was calculated as the sum of the specific staining indices, whereby the index of the fields with degree 1 was multiplied by 1, the index of the fields with degree 2 was multiplied by 2, and the index of the fields with degree 3 was multiplied by 3. Controls omitting the first or the second antibody for each section gave negative results. All morphological evaluations were performed by a blinded observer (E.K.) and were controlled randomly by a pathologist (H.-J.G.).

Flow cytometry of renal mononuclear cells and cytometric bead array for the measurement of renal cytokines

Explanted kidneys were weighed and mechanically disrupted in six-well plates with 5 mls of a digestion solution consisting of RPMI 1640 with 10 mM HEPES, 0.1% bovine serum albumin (BSA), 0.5 mg/ml collagenase IA and 4.5 kU/ml DNAse I (all Sigma, Schnelldorf, Germany), and incubated at 37°C with 5% CO_2 for 15 minutes. Kidneys were then mechanically disrupted once more and incubated for a further 15 minutes. After repetitive pipetting, the kidney homogenates were sieved through a 100 µm filter (BD, Heidelberg, Germany) and washed with fluorescence-activated cell sorter (FACS) buffer (PBS containing 0.1% BSA). Suspension aliquots were incubated with anti-CD16/32 (Fcγ RIII/II) antibody (BD) followed by staining with monoclonal antibodies: anti-MHCII (M5/114.15.2, eBioscience, CA, USA) and anti-CD11c (N418, eBioscience). Samples were analyzed on a FACSCalibur flow cytometer (BD) after gating on lymphocytes in a forward- and side-scatter plot.

For the measurement of cytokines in mouse kidneys, frozen tissue was lysed in a buffer containing 10 mM HEPES (Roth, Karlsruhe, Germany) in PBS with 0.5% Triton X-100 (Fluka, Steinheim, Germany) and a protease inhibitor cocktail (Roche, Mannheim, Germany), sonicated, and centrifuged at 10,000 ***g*** for 10 minutes. Supernatants were assessed for total protein concentration and measured for IL-6, IL-10, MCP-1, IFN-γ and IL-12p70 by FACSCalibur using BD cytometric bead array (CBA) Flex-Set bead assays (BD). Sample files were analyzed by FCAP Array 1.0.1 software.

TRANSLATIONAL IMPACT

Clinical issue
Kidney transplantation is the treatment of choice for end-stage renal disease, but carries a risk of transplant rejection and death. The rates of late graft loss and subsequent patient mortality are poor, with rejection of up to 50% of donor allografts. The major problem limiting long-term allograft survival is chronic allograft dysfunction (CAD), characterized by scarring of the transplant, with progressive loss of renal function. There is currently no treatment for CAD and its pathophysiology remains poorly defined. Although the contribution of the adaptive immune system to transplant rejection has been known for many years, increasing attention is now focusing on the innate immune system, the body's first line of defence against invading microorganisms, in which an inflammatory response is mounted following activation of pattern recognition receptors (PRRs). In addition to their role in infection, PRRs sense damaged or non-self tissue, and hence may react against grafts. Here, using a mouse renal transplant model, a specific subset of PRRs, the Toll-like receptors (TLRs), is shown to be involved in CAD.

Results
By monitoring the upregulation of renal TLR-binding molecules, the authors show that the innate immune system is activated in both acute and chronic situations in kidney grafts. To determine whether loss of the TLR pathway might result in better graft outcome, mice lacking both TLR2 and TLR4, or either of the key TLR adaptor proteins MyD88 and TRIF, were given kidneys from wild-type animals. In all cases, chronic graft damage developed to a significantly lower extent, and scarring and atrophy of the transplant kidney were largely prevented. The beneficial effect of inhibition of the TLR pathway was mediated by both the adapter proteins MyD88 and TRIF.

Implications and future directions
These findings show that the innate immune system, and specifically the TLR signaling pathway, is involved in CAD. Since conventional immunosuppression, which inhibits adaptive immunity (B- and T-cell activity), does not prevent CAD, the innate immune system represents an important new target for pharmacological intervention. Currently, TLR antagonists are being evaluated as inhibitors of acute inflammation in clinical trials. It would be of great interest to initiate further trials to determine whether blockade of TLRs could be used to prevent CAD in transplant patients.

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Statistical analysis
Statistical analyses were performed with GraphPad Prism 5 software. All quantitative data were analyzed with the unpaired, two-tailed Student's *t*-test. All error bars indicate standard error of the mean (S.E.M.). Values of $P<0.05$ were considered statistically significant.

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