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Kers, J.

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Triggering Receptor Expressed on Myeloid Cells 1 (TREM-1) appears inessential during experimental renal injury and kidney transplantation

Alessandra Tammaro¹,*
Jesper Kers¹,*
Diba Emal¹
Ingrid Stroo²
Tessa Vermeer¹
Gwendoline Teske¹
Loes Butter¹
Nike Claessen¹
Jeffrey Damman¹
Marc Derive³
Henri G.D. Leuvenink⁴
Harry van Goor⁵

Jan-Luuk Hillebrands⁵
Bouke G. Hepkema⁶
Harold Snieder⁷
Jacob van den Born⁸
Martin H. de Borst⁸
Stephan J.L. Bakker⁸
Gerjan J. Navis⁸
Rutger J. Ploeg⁹
Sandrine Florquin¹,¹⁰
Marc Seelen⁸
Jaklien C. Leemans¹
Mark C. Dessing¹,¹¹

*Authors contributed equally

¹Department of Pathology, Academic Medical Center, Amsterdam (NL)
²Department of Immunopathology, Sanquin Research, Amsterdam (NL)
³Institut National de la Santé et de la Recherche Médicale (INSERM) UMR_S1116, Faculté de Médecine de Nancy, Université de Lorraine, Vandoeuvre-les-Nancy (FR)
⁴Department of Surgery, University Medical Center Groningen, Groningen (NL)
⁵Department of Pathology and Medical Biology, University Medical Center Groningen, Groningen (NL)
⁶Department of Laboratory Medicine, University Medical Center Groningen, Groningen (NL)
⁷Department of Epidemiology, Unit of Genetic Epidemiology & Bioinformatics, University Medical Center Groningen, Groningen (NL)
⁸Department of Internal Medicine, Division of Nephrology, University Medical Center Groningen, Groningen (NL)
⁹Nuffield Department of Surgical Sciences, University of Oxford, Oxford (UK)
¹⁰Department of Pathology, Radboud University Nijmegen Medical Center, Nijmegen (NL)
¹¹Department of Stem Cell Biology and Regenerative Medicine, Keck School of Medicine of the University of Southern California, Los Angeles (USA)

(Submitted)
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Abstract

The innate immune response plays a significant role in the induction of renal ischemia reperfusion injury (IRI), which represents a major cause of delayed graft function (DGF) in patients that received a renal transplant. Triggering receptor expressed on myeloid cells-1 (TREM-1) is an evolutionary conserved pattern recognition receptor expressed on a variety of innate immune cells and its expression is increased in several renal injury models. However, little is known about the function of TREM-1 in murine and human renal injury. Here, we investigated the expression and function of TREM-1 in a murine model of renal IRI using different TREM-1 inhibitors: LP17, LR12 and TREM-1 fusion protein. In human renal transplantation, we analyzed the association of single nucleotide variants in the TREM1 gene, in a cohort comprising 1263 matching donors and recipients with post-transplant outcomes, including DGF. In the murine study, following IR, renal TREM-1 expression increased due to the influx of TREM-1-expressing cells. However, LP17, LR12 and TREM-1 fusion protein did not ameliorate IRI after reperfusion. In the human transplantation cohort, neither the donor nor the recipient TREM1 gene variant p.Thr25Ser associated with DGF, biopsy-proven acute rejection or death-censored graft failure. We conclude that TREM-1 appears inessential in the immune response during experimental renal IRI or graft outcome following renal transplantation.
Introduction

Kidney transplantation is at present the most optimal renal replacement therapy for patients with end-stage renal disease (ESRD). Following transplantation, renal ischemia-reperfusion injury (IRI) is a major cause of delayed graft function (DGF). DGF is associated with an increased risk for acute rejection and subsequently a decreased survival of the allograft (Cooper and Wiseman, 2013; Siedlecki et al., 2011). Innate immunity plays an important role in the mechanism underlying IRI. Following kidney injury, so-called damage-associated molecular patterns (DAMPs) are being released from necrotic cells, which are subsequently recognized by Pattern Recognition Receptors (PRRs) that include Toll-like Receptors (TLRs). Activation of TLRs is known to induce inflammation that deleteriously affects renal function following IRI (Leemans et al., 2014; Pulskens et al., 2010). Over the past decade, an additional family of evolutionary conserved innate immune receptors has been identified and characterized: the triggering receptors expressed on myeloid cells (TREMS) (Arts et al., 2013; Colonna, 2003; Sharif and Knapp, 2008). TREM-1 is the founding member of the TREM receptor family and is mainly expressed on granulocytes (neutrophils) and monocyte/macrophages in mice and men (Bouchon et al., 2000). TREM-1 is an activating receptor, which associates with its adaptor molecule TYRO protein tyrosine kinase-binding protein (TYROBP, also called DAP12) to induce cytokine production (Colonna, 2003; Pulskens et al., 2010; Sharif and Knapp, 2008). Asides from activating its own intracellular signalling pathway, TREM-1 synergizes with diverse TLRs, subsequently leading to an amplified inflammatory responses (Arts et al., 2013; Bouchon et al., 2000; Colonna, 2003; Sharif and Knapp, 2008). Most of the studies addressing the pathogenic role of TREM-1 have been performed in various infectious studies (Hommes et al., 2015; Klesney-Tait et al., 2013). The general concept thus far is that TREM-1 is specifically involved in anti-microbial immune responses (Weber et al., 2014). Recent evidence, however, has also pointed towards a beneficial effect of TREM-1 inhibition during sterile inflammatory responses, like IRI (Boufenzer et al., 2015; Campanholle et al., 2013; Gibot et al., 2008). Murine studies have shown that TREM-1 expression increases upon chronic obstructive nephropathy and renal IRI (Campanholle et al., 2013; Lo et al., 2014; Tammaro et al., 2013). In humans, renal TREM-1 expression has been observed on interstitial cells, most probably myeloid cells, of patients with obstruction-related hydronephrosis (Tammaro et al., 2013). Blockade of the
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TREM-1 signalling pathway by a short inhibitory peptide (LP17 and LR12) reduced tissue injury during mesenteric IR in rats and myocardial infarction, emphasizing the potential therapeutic benefit of TREM-1 inhibition in sterile inflammation (Boufenzer et al., 2015; Gibot et al., 2008). Currently, the treatment of patients with acute kidney injury in the context of DGF is purely supportive, whereas manipulation of innate immunity during necroinflammation might reduce further alloimmune priming, leading to a reduction in rejection. In the current study we investigated whether TREM-1 could be such a potential target during experimental and human renal IRI. We therefore investigated 1) the expression and function of TREM-1 in a mouse model of renal IRI and 2) determined the association between non-synonymous single nucleotide variants in the TREM1 gene and outcomes following renal transplantation, with a particular interest to DGF.

Materials & Methods

Surgical procedure of murine ischemia-reperfusion injury

Pathogen-free 8-to 12 week-old male C57BL/6 wild-type (Wt) were purchased from Charles River Laboratories (Wilmington, MA, United States). The Animal Care and Use Committee of the University of Amsterdam approved all the experiments. Renal IRI was induced as described previously with small alterations (Dessing et al., 2015a). The renal pedicles were clamped for 25 minutes (uni- or bilaterally) through a midline abdominal incision under 2.5% isoflurane-induced anaesthesia. Before and after surgery, mice received a subcutaneous injection of 50 µg/kg buprenorphin (Shering-Plough, Kenilworth, NJ, United States) for analgesic purposes. Sham mice underwent the same procedure without clamping of the renal pedicles. Successful reperfusion was validated by regain of the initial colour of the kidney after removal of the vascular clamp.

TREM-1 inhibitors

We used 3 previously validated approaches to modulate TREM-1 function (Bouchon et al., 2001; Boufenzer et al., 2015; Gibot et al., 2008). LP17 (LQVTDSGLYRCVIYHPP), LR12 (LQEEDTGEYGCV) and LP17/LR12–scramble protein (TDSRCVIGLYHPPLQVY / YQDVELCETGED) were chemically synthesized (Pepsan Systems, Lelystad, The Netherlands) based on the extracellular domain of TREM-1 and TREM-like
transcript 1 (TLT1) respectively. TREM-1 fusion protein (Fc-TREM-1) and control 
IgG were purchased from R&D. Animals were (pre)-treated intraperitoneally with 
endotoxin-free LP17, LR12, Fc-TREM-, and their respective controls dissolved in 
sterile NaCl 0.9% (as described in the Figure legends). Mice were sacrificed 24 
hours after reperfusion.

**Biological parameters related to murine kidney function, damage 
and inflammation**

Murine renal function was determined by measuring plasma creatinine as described 
before (Dessing et al., 2015a). The degree of tubular damage was assessed 
on periodic acid-Schiff after diastase treatment (PASD)-stained tissue sections 
(Dessing et al., 2015a). The PASD score was obtained by a nephropathologist 
(SF) in a blinded fashion on a 5-point scale: 0 = no damage, 1 = 10% necrosis 
of the corticomedullary region, 2 = 10–25%, 3 = 25–50%, 4 = 50–75%, 5 = more 
than 75%. For granulocyte staining, we used FITC-labeled anti-mouse Ly6G mAb; 
(BD Biosciences-Pharminen, Franklin Lakes, NJ, United States) followed by 
appropriate secondary antibodies as described before (Dessing et al., 2015a). 
Ly6G positive cells were counted in 10 high power fields (HPF). Levels of (renal) 
sTREM-1 and keratineocyte-derived cytokine (KC) (all R&D Systems, Minneapolis, 
MN, United States) were measured in snap-frozen and lysed kidney homogenates 
(30 minutes on ice in 300 mM NaCl, 15 mM Tris, 2 mM MgCl₂, 1 mM CaCl₂ and 1% 
Triton X100, pH 7.4 with 100 µg/ml pepstatin A, leupeptin and aprotinin mix) (Lo et 
al., 2014). Protein levels in renal tissue were corrected for total protein level using 
BioRad protein assay (Bio-Rad Laboratories, Veenendaal, The Netherlands) with 
IgG as standard.

**Flow cytometry**

Peripheral blood was drawn via a cardiac puncture and red blood cells were lysed. 
(Erythrocyte lysis solution: 160mM NH₄Cl, 10mM KHCO₃ and 0.1mM EDTA, pH 
7.4). Cell suspensions were stained with rat anti-mouse TREM-1 (R&D). Leukocyte 
morphology was determined based on forward and side scatter. Stained Cells were 
fixed in PBS containing 2% paraformaldehyde (PFA) and later acquired on FACS 
Canto II (BD Biosciences). Data analysis was performed using FlowJo v10 (FlowJo 
LLC, Ashland, OR, United States).
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mRNA purification and reverse transcriptase PCR
Total RNA was isolated from 10-15 renal frozen tissue slides (20μM) using Trizol reagent (Invitrogen, Carlsbad, CA, United States) according to manufacturer’s protocol. mRNA samples were quantified by spectrophotometry (NanoDrop, Thermo Scientific, Wilmington, DE, United States) and converted to cDNA using oligo-dT. Mouse Tbp and Trem1 mRNA expression was analyzed by reverse transcriptase PCR with SYBR green PCR master mix on an LC480 lightcycler (Hoffmann-La Roche, Basel, Switzerland). Gene expression of Trem1 was divided by the value obtained from the housekeeping gene (Tbp). Relative expression was analyzed using LinRegPCR v12.4. Primer sequences are Trem1 forward: 5’-GGTCTCCATCTTTATTACA, reverse: 5’-AAACCAGGCTCTTGTGAGA; Tbp forward: 5’-GGGAATCATGGACCAGAACA, reverse: 5’-GATGGGAATTCCAGGAGTCA.

TREM-1 in situ hybridization
In situ hybridization was performed on frozen kidney tissue sections as described before (Liu et al., 2014) with minor adjustments. mTrem1 primers were designed using NCBI primer design tool. A DNA template was generated by nested PCR incorporation of T7 RNA polymerase promoters using mTrem1 primers (forward: 5’-CGTGTGTGTCAGGTAACG and reverse 5’-taatacgactatagggAGGAGAACCCGAG). Riboprobe synthesis from template PCR was checked for specificity with cDNA from Wt and Trem1/3 knockout mice. Kidneys were perfused with ice-cold PBS, fixed in 4% PFA for several hours at room temperature and placed in 30% sucrose overnight at 4°C. The next day, the tissues was placed in O.C.T. compound (Tissue-Tek, Alphen aan den Rijn, The Netherlands) and kept frozen in -80ºC until needed. The frozen kidney tissue sections (5 µm) were let to acclimatize at room temperature, washed 3 times with PBS and fixed overnight in 4% PFA at 4°C. The tissue sections were permeabilized with proteinase K (10 μg/ml) for 20 minutes, followed by fixation in 4% PFA and next acetylated (0.375% acetic anhydride). The sections were hybridized overnight at 68°C with 0.5 μg/ml of riboprobe in hybridization buffer (50% formamide, 5× SSC, 1% SDS, 50 μg/ml yeast tRNA, 50 μg/ml heparin). After the first post hybridization wash, sections were treated with 2 μg/ml RNase for 15 minutes at 37°C. For riboprobe detection, sections were pre-treated with blocking buffer.
(20% heat inactivated sheep serum, 2% blocking reagent; Hoffmann-La Roche no 11096176001) for 1 hour and additionally incubated with anti-DIG-AP antibody (1:4,000, Hoffmann-La Roche no 11093274910) at 4°C overnight. The next day, a chromogenic substrate (BM Purple; Hoffmann-La Roche) was used to visualize the signal. Once a sufficient staining intensity was reached, sections were fixed in 4% PFA and mounted with Glycergel (Dako, Heverlee, Belgium). The stained sections were imaged on an Eclipse 90i (Nikon Instruments, Amsterdam, The Netherlands).

Renal transplant study population

We included samples from the Renal Genetics Transplantation (REGaTTA) cohort collected from the University Medical Center Groningen, Groningen, The Netherlands (Dessing et al., 2015b). Matched donors and recipients from 1430 transplantations were assessed for eligibility. Patients with more than two re-transplantations, simultaneous kidney/pancreas- or kidney/liver transplantations, unavailability of DNA for genotyping, technical problems or patients that were lost to follow-up, were excluded. The final cohort on which statistical analyses was performed comprised of 1263 donor-recipient pairs. A detailed flow diagram is shown in Supplementary Figure 1. The medical ethics committee of the University Medical Center Groningen approved the study under file no METc 2014/077 and written informed consent was acquired from all living transplant donors. By Dutch jurisdiction, deceased donors provide informed consent upon registration of their donation status. No living donors from a vulnerable population were used. The study was conducted according to the Declarations of Helsinki and Istanbul.

DNA isolation, quality control and TREM1 variant selection

Peripheral blood mononuclear cells were used to acquire donor and recipient DNA. DNA samples were validated for DNA concentrations using absorbance at 260 nm with a spectrophotometer (ND-1000, NanoDrop) and purity was assessed by 260/280 and 260/230 absorbance ratios. In case of impurity of the sample, repeated isolation attempts were conducted. Based on a 1000Genomes minor allele frequency (MAF) of >1%, 2 non-synonymous SNVs in the TREM1 gene were selected: rs2234237 (p.Thr25Ser) and rs2234245 (p.Phe214Leu). For the two SNVs, no functional consequence has been described in the literature to date.
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Genotyping of the SNVs was performed using the Illumina VeraCode GoldenGate Assay kit (Illumina, San Diego, CA, United States) according to the manufacturer’s instructions. Genotype clustering and calling were performed using BeadStudio Data Analysis Software (Illumina).

Renal transplantation study outcomes
The primary outcome used in this study was DGF, defined as the requirement for dialysis within the first week after transplantation (patients with primary non-function of the graft (Damman et al., 2011), were excluded, since these cases most probably represent arterial and venous thrombosis instead of IRI). Secondary outcomes were time-to-first episode of biopsy-proven acute rejection (BPAR) and death-censored graft survival (defined as the need for indefinite dialysis or re-transplantation).

Statistical analyses
In the murine studies, differences between groups were analysed using non-parametric Mann-Whitney (2 groups) or Kruskal-Wallis tests (>2 groups) using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego CA, United States). Results are expressed as mean ± standard error of the mean. Values of P<0.05 were considered significant. For the human renal transplantation study, deviation from Hardy-Weinberg equilibria were tested with PLINK version 1.07 for
Mac OS X 10.11 (S. Purcell, http://pngu.mgh.harvard.edu/purcell/plink) (Purcell et al., 2007). Differences in allele frequencies between donors and recipients were tested by logistic regression analyses in a genetic additive model construction. Results were adjusted for age and donor-recipient relatedness with the DFAM algorithm. Minor allele frequencies between the HapMap-CEU (a population from Utah with Northern and Western European ancestry) that was genotyped in the context of the 1000Genomes study were compared by Fisher exact tests or \( \chi^2 \) tests where appropriate. The association between \( TREM1 \) single nucleotide variants and DGF were tested with logistic regression models with stratification for donor type. The association with (BPAR) and death-censored graft failure was tested with Cox proportional hazard models. An insufficient amount of patients had a homozygous recessive genotype and we therefore only calculated odds and hazard ratios for the heterozygous genotype. Two-sided P-values below 0.05 after Bonferroni correction were considered significant. Data were analysed with the R computing environment (www.r-project.org) with use of the packages gdata v2.17.0, stats v3.2.1, rms v4.3-1. RStudio v0.99.467 and R v3.2.1 for Mac OS X 10.11 were used.

**Results**

**Renal ischemic injury leads to increased TREM-1 expression**

The S3 segment of the proximal tubules located in the cortico-medullary area is the most sensitive to ischemic injury, due to the high metabolic need (Bonventre and Yang, 2011). Moreover, the interstitial cells surrounding the ischemic tubules are rich in granulocytes that accumulate in the kidney as early as 30 minutes after reperfusion. Since TREM-1 is expressed on the plasma membrane of granulocytes, we determined renal TREM-1 expression 24 hours after renal IRI. Using in situ hybridization, we localized \( Trem1 \) transcript in kidney tissue sections from Wt mice one day after IRI. Sham tissues were used as control. \( Trem1 \) RNA-positive interstitial cells were detected in the S3 segments of the kidney after IR and absent in sham kidney (Figure 1A). \( Trem1/3 \) double KO kidney tissues served as negative controls (Supplementary Figure 2). We measured renal \( Trem1 \) transcript expression moreover by RT-PCR (Figure 1B) and observed a robust increased expression in IRI kidneys compared to sham tissues, which was confirmed on the protein level (Figure 1C).
Following IR, inflammatory cell numbers arise into the circulation to then migrate to the site of tissue injury. By FACS analysis, we detected increased numbers of circulating granulocytes (Figure 2A), but not monocytes (Figure 2B), in mice after IRI. This suggests that renal *Trem1* mRNA-expressing cells are most likely infiltrating granulocytes. We then checked the surface expression of TREM-1 receptor in circulating granulocytes and monocyte from sham and IRI mice. Renal IRI leads to down-regulation of TREM-1 from the plasma membrane of circulating monocytes, but not granulocytes (Figure 2C). Membrane TREM-1 can be shed by matrix metalloproteinases (Gómez-Piña et al., 2012) and released as soluble protein. 

![Figure 1](image1.png)

**Figure 1 | TREM-1 is increased in the kidney after IR.** *Trem1* transcript expression visualized by using in situ hybridization (ISH) in sham and Wt mice 1 day after bilateral IR. Original magnification 10x; inset 60x (A). Intrarenal *Trem1* transcript and protein expression detected by RT-PCR (n=6-7/group) (B) and ELISA (n=6-7/group) (C). Statistics analyzed using the Mann-Whitney test. Values are means ±SEM. *P<0.05 vs sham.

![Supplementary Figure 2](image2.png)

**Supplementary Figure 2 | Trem1 transcript is absent in Trem1/3 double KO mice.** *Trem1* mRNA expression by in situ hybridization performed on renal frozen sections from sham (A) and IR (B) (day 1) Trem1/3 double KO mice.
indeed found increased plasma sTREM-1 levels after renal IRI (Figure 2D). Thus, this suggest that receptor cleavage from monocytes is the source of circulating soluble TREM-1 protein.

**Administration of TREM-1 inhibitors does not lead to renoprotection in renal IRI**

In order to determine the functional role of TREM-1, we tested if blocking the ligand-receptor binding could prevent renal IRI. Wt mice were treated with different types and doses of compounds with known modulatory functions and underwent unilateral or bilateral clamping of the renal arteries. Previous studies have shown that TREM-1 fusion protein by binding to endogenous TREM-1 ligands, successfully reduced pathology in several models of infection (Bouchon et al., 2001). Thus we investigated whether TREM-1 fusion protein could affect renal pathology in a sterile inflammatory model. We administrated Fc-TREM-1 fusion protein 1 hour prior to induction of renal IRI. Mice pre-treated with different doses of fusion protein or matching isotype control did not display any change in renal damage (PASD score) and inflammation (renal KC and Ly6G-positive cells) induced by unilateral IRI (Figure 3A-C).

Next, we investigated the effect of a recently described soluble peptide, LR12, which has been proposed as another, more potent synthetic TREM-1 inhibitor (Derive et al., 2013). Due to the short half-life of LR12 ([Derive et al., 2014] and personal...
communication with M. Derive], mice were treated every 8 hours for 24 hours starting 2 hours before surgery. Sham operated mice received the same treatment to exclude any detrimental effect due to repeated peptides injections. Consistent with the previous results related to Fc-TREM-1 experiments, LR12 treatment did not lead to any change in terms of renal damage or inflammation following unilateral IRI, compared to mice receiving scramble protein injections (Figure 3D-F).
Lastly, we investigated the effect of LP17 peptide, a well-known synthetic TREM-1 protein that was proposed by others as a therapeutic tool to dampen TREM-1 induced inflammatory responses in a variety of inflammatory diseases (Gibot et al., 2006a, 2008; Schiechl et al., 2013). Using different doses of LP17, again our results were consistent with previous ones. Indeed, LP17-treated mice displayed similar renal damage (PASD score) and inflammation (intrarenal KC protein and Ly6G positive cells/HPF) compared to mice with a control treatment. Because the potential therapeutic significance of this type of treatment has been shown especially in sterile inflammation (Gibot et al., 2008; Schiechl et al., 2013), we subjected Wt mice to a more severe, bilateral renal IRI surgery with prior treatment with LP17 or control peptide (200ug) and sacrificed the mice 24 hours after reperfusion. Sham mice received the same treatments without clamping. In the bilateral model, LP17 treated mice did not display any significant difference in renal function parameters (urea and creatinine) compared to control peptide-treated mice. Renal damage (as indicated by PASD score) and inflammation (renal granulocyte influx) displayed similar patterns between control and LP17 treated mice (Supplemental Figure 3). Taken together, our experiments do not suggest a beneficial role for modulation of TREM-1 function during the course of sterile renal injury.

Supplementary Figure 3 | Contribution of LP17 pretreatment in bilateral renal IRI experiments. Plasma Urea (A) and creatinine (B), renal necrosis (C) and granulocyte influx (D) in Wt mice pre-treated with 100ug LP17/control protein in the bilateral ischemia model. Sham mice injected with scramble protein/LP17 are used as control (n=10/group).
### Table 1 | Donor, recipient and transplant characteristics

| Variable | Study cohort N=1263 |
|----------|---------------------|
| **Donor characteristics** | |
| Age (mean years ± SE) | 44.4 ± 14.4 |
| Male N (%) | 645 (51%) |
| Donor type, N (%) | |
| Living donor | 282 (22%) |
| Deceased donor (DBD+DCD) | 989 (79%) |
| Donor cause of death N (%) | |
| CVA | 549 (43%) |
| Trauma | 305 (24%) |
| Other | 135 (11%) |
| Unknown | 282 (22%) |
| **Recipient characteristics** | |
| Age (mean years ± SE) | 47.9 ± 13.4 |
| Male N (%) | 739 (58%) |
| Primary kidney disease N (%) | |
| Glomerulonephritis | 271 (21%) |
| Adult polycystic kidney disease | 167 (13%) |
| Renovascular disease | 124 (10%) |
| IgA nephropathy | 98 (8%) |
| Chronic pyelonephritis | 148 (12%) |
| Diabetic nephropathy | 51 (4%) |
| End-stage renal disease not otherwise specified | 168 (13%) |
| Other | 244 (19%) |
| Initial immunosuppression N (%) | |
| Corticosteroids | 1201 (95%) |
| Cyclosporine A | 1065 (85%) |
| Tacrolimus | 97 (8%) |
| Azathioprine | 72 (6%) |
| Mycophenolic acid | 907 (71%) |
| mTOR inhibitor | 38 (3%) |
| ATG induction | 103 (8%) |
| Anti-CD3 moab induction | 19 (2%) |
| Interleukin-2 RA induction | 199 (16%) |
| Transplant number N (%) | |
| First | 1142 (90%) |
| Second | 128 (10%) |
| **Transplant characteristics** | |
| Cold ischemia time (mean hr ± SE) | |
| Living donors | 2.7 ± 1.9 |
| Deceased donors | 20.7 ± 6.5 |
| HLA no. of 0 mismatches N (%) | 241/1050 (23%) |
| Cause of graft loss N=212 (%) | |
| Rejection | 132 (62%) |
| Technical problems | 37 (17%) |
| Primary recurrent disease | 16 (8%) |
| Primary non-viable | 12 (6%) |
| Infection | 3 (1%) |
| Other | 12 (6%) |

DBD, deceased brain death; DCD, deceased cardiac death; CVA, cerebrovascular accident; ATG, anti-thymocyte globulin; moab, monoclonal antibody; RA, receptor antagonist.
Study characteristics and distribution of the TREM1 single nucleotide variants in donors and recipients

In human renal transplantation, we investigated single nucleotide variants (SNVs) in the TREM1 gene in the context of human renal transplantation, with DGF in particular. The flow diagram of the included and excluded patients is shown in Supplementary Fig. 1. The cohort characteristics are shown in Table 1.

The minor allele frequencies of the two non-synonymous SNVs in the TREM1 gene for donors and recipients were respectively 0.078 and 0.085 for p.Thr25Ser, 0.001 and 0.000 for p.Phe214Leu without statistical differences between donors and recipients (All P > 0.8, corrected for age and donor-recipient relatedness, Table 2). Donors and recipients significantly deviated from Hardy-Weinberg equilibrium (P = 0.003 and P = 0.002 for donors and recipients, Bonferroni corrected), but both distributions were not different from the frequency distribution that was found in the HapMap-CEU population of the 1000Genomes sequencing data (donors and recipients: P = 1).

TREM1 single nucleotide variants do not associate with renal transplant outcomes

DGF occurred in 411/1263 patients (33%), of which 60 resulted in primary non-failure and were therefore excluded from analyses (351/1203 of DGF remaining, 29%). The majority (97%) of patients who experienced DGF was recipient of a deceased donor. For the p.Phe214Leu variant we acquired insufficiently high MAFs for statistical analyses. In the full cohort, the TREM1 p.Thr25Ser variant was not significantly

Table 2 | Donor and recipient genotype distributions of TREM1 single nucleotide variants

| HGVS (rs number) | A/a | 1000 Genomes MAF | Donor | Recipient | p 2 |
|------------------|-----|------------------|-------|-----------|-----|
|                  |     |                  | A/A   | A/a       |     |
|                  |     |                  | a/a   | MAF       |     |
| p.Thr25Ser (rs22342937) | A/t | 0.080 3 | 0.845 | 0.155 | 0.000 | 0.078 | 0.836 | 0.159 | 0.005 | 0.085 | 0.86 |
| p.Phe214Leu (rs2234245) | G/c | 0.028 4 | 0.999 | 0.001 | 0.000 | 0.000 | 1.000 | 0.000 | 0.000 | 0.000 | 1    |

1 Donor and recipient genotype are displayed as homozygous dominant (A/A), heterozygous (A/a) or homozygous recessive (a/a). MAF, minor allele frequency. 2 P-value for logistic regression between donor and recipient allele frequencies in a genetic additive model, adjusted for age and donor-recipient relatedness (DFAM algorithm). 3 HapMap-CEU population. 4 Overall 1000Genomes population.
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Table 3 | Association of TREM1 single nucleotide variant p.Thr25Ser with renal outcomes

| Cohort         | Donor                              | Recipient                             |
|----------------|------------------------------------|---------------------------------------|
|                | $\text{OR}_{\text{heterozygosity}}$ ($95\% \text{ CI}$) | $\text{P-value}^1$ | $\text{OR}_{\text{heterozygosity}}$ ($95\% \text{ CI}$) | $\text{P-value}^1$ |
| **Delayed graft function** |                                   |                                       |                                   |                   |
| Full           | 1.06 (0.77 - 1.52)                 | 1                                     | 0.91 (0.64 - 1.27)                | 1                  |
| DBD            | 1.07 (0.68 - 1.65)                 | 1                                     | 1.03 (0.66 - 1.59)                | 1                  |
| DCD            | 0.77 (0.31 - 2.11)                 | 1                                     | 0.69 (0.28 - 1.90)                | 1                  |
| **Biopsy-proven acute rejection** |                                   |                                       |                                   |                   |
| Full           | 0.72 (0.54 - 0.96)                 | 0.15                                  | 0.81 (0.62 - 1.07)                | 0.84               |
| DBD            | 0.70 (0.49 - 1.00)                 | 0.29                                  | 0.86 (0.62 - 1.20)                | 1                  |
| DCD            | 1.10 (0.56 - 2.16)                 | 1                                     | 1.04 (0.53 - 2.04)                | 1                  |
| **Death-censored graft failure** |                                   |                                       |                                   |                   |
| Full           | 0.75 (0.50 - 1.13)                 | 1                                     | 0.87 (0.59 - 1.27)                | 1                  |
| DBD            | 0.89 (0.57 - 1.41)                 | 1                                     | 0.84 (0.53 - 1.34)                | 1                  |
| DCD            | 0.50 (0.18 - 1.39)                 | 1                                     | 1.18 (0.52 - 2.64)                | 1                  |

$^1$ After Bonferroni correction. $\text{OR}_{\text{heterozygosity}}$, odds ratio (homozygous recessive were not sufficiently represented for statistical analyses); CI, confidence interval; DBD, deceased brain death donor; DCD, deceased cardiac death donor.

associated with DGF in donors ($\text{OR}_{\text{heterozygosity}} = 1.08, 95\% \text{ CI} = 0.77 - 1.52, P = 1$ after Bonferroni correction) and recipients ($\text{OR}_{\text{heterozygosity}} = 0.91, 95\% \text{ CI} = 0.64 - 1.27, P = 1$). When we stratified patients for donation type (DBD or DCD), similar results were obtained (Table 3).

There were 5 renal transplant recipients that were homozygous recessive for the TREM1 variant p.Thr25Ser. None of these patients developed DGF, but due to the small amount of patients, we did not perform statistical analysis on this group separately. Supplementary Table 1 shows the characteristics of these 5 patients.

On follow-up, 430/1263 (34%) of recipients encountered a first episode of biopsy-proven acute rejection (BPAR). The median (interquartile range) time to rejection was 51 months (1 - 105 months). Neither donor nor recipient TREM1 p.Thr25Ser variants associated with the cumulative incidence of BPAR in donors ($\text{HR}_{\text{heterozygosity}} = 0.72, 95\% \text{ CI} = 0.54 - 0.96, P = 0.15$ and $\text{HR}_{\text{heterozygosity}} = 0.81, 95\% \text{ CI} = 0.62 - 1.07, P = 0.84$, respectively). Similar results were acquired after stratification for donor
type (Table 3). Death-censored graft failure occurred in 215/1263 (17%) at a median (interquartile range) of 5.5 years (2.9 - 8.7 years) after transplantation. Corroborating on the results obtained for DGF and BPAR, neither donor nor recipient TREM1 p.Thr25Ser associated with the cumulative incidence of death-censored graft failure in donors (HR$_{\text{heterozygosity}}$ = 0.75, 95% CI = 0.50 - 1.13, P = 1 and HR$_{\text{heterozygosity}}$ = 0.87, 95% CI = 0.59 - 1.27, P = 1, respectively), again with similar results after stratification for donor type (Table 3). In conclusion, we did not acquire (sufficient) evidence for a potential beneficial or detrimental effect of single nucleotide variants in the TREM1 gene for post-transplant renal outcomes, including delayed graft function.

**Discussion**

Infiltrating inflammatory cells, especially granulocytes, contributes to the development of acute kidney injury following IRI, which increases the risk of delayed graft function (Bonventre and Yang, 2011). TREM-1 is an innate immune receptor expressed on the plasma membrane of granulocytes and monocytes. Increasing evidence suggest that TREM-1 plays a pivotal role in modulating the innate immune response in (non) sterile acute and chronic inflammation (Boufenzer et al., 2015; Hommes et al., 2015; Klesney-Tait et al., 2013; Wu et al., 2012). TREM-1 induced amplification of the inflammatory response and hence collateral tissue damage, occurs through synergism with Toll like receptors (TLRs) (Arts et al., 2013). The contribution of TLRs in sterile renal injury has been broadly investigated in mice and human (Dessing et al., 2015b; Pulskens et al., 2010). However, the role of TREM-1 herein is still unclear. Interestingly, blockade of TREM-1 activation by synthetic analogues successfully reduced pathology in mesenteric ischemia reperfusion and
myocardial infarction (Boufenzer et al., 2015; Gibot et al., 2008). In the current study we showed that synthetic TREM-1 inhibition in murine renal IRI does not appear to be a therapeutic option to prevent injury and/or improve function. Consistent with the results of the experimental study, we found no association of the p.Thr25Ser heterozygous variant with renal outcome following kidney transplantation.

Our findings demonstrated that renal IRI induced a significant increase in *Trem1* transcripts in renal tissue. This up-regulation was in line with the increased number of circulating granulocytes, detected by FACS analysis 24 hours after IRI. Granulocytes are well-known to express TREM-1 and to accumulate surrounding the S3 segment of the proximal tubuli, the part of the nephron that is most susceptible to ischemic damage (Dessing et al., 2015a). We speculate that in our model interstitial *Trem1*-expressing cells detected by ISH are most probably infiltrating granulocytes. Following renal IRI, monocytes migrate from the bone marrow into the blood to infiltrate the inflamed kidney tissue and differentiate into macrophages and dendritic cells. Monocytes are known to express high levels of TREM-1 on their plasma membrane, which can be proteolytically cleaved by matrix metalloproteinases (MMPs), resulting in soluble TREM-1 protein release (Gómez-Piña et al., 2012). No differences in the number of circulating monocytes were detected between sham and IRI mice. However, we observed a down-regulation of TREM-1 surface expression on circulating monocytes, which mirrored a significant increase in soluble protein concentration in the circulation.

The increased circulating sTREM-1 protein concentration following IRI is in line with other studies showing that the soluble protein can be detected in plasma, gastric secretions, bronchioalveolar lavage fluid and urine during infection and inflammation (Gibot et al., 2004; Su et al., 2011; Tzivras et al., 2006). Functionally, the soluble protein acts as a counter regulatory molecule by scavenging available TREM-1 ligand thus preventing further amplification of the inflammatory signal. Nevertheless in renal IRI the significance of increased sTREM-1 concentration appears to be dispensable in modulating the inflammatory response. Several studies have shown that treatment with synthetic analogues of TREM-1 protein or recombinant fusion protein represents a therapeutic strategy to contain excessive and unregulated inflammation resulting in decreased organ damage and mortality. The TREM-1 inhibitory peptide LP17 was
TREM-1 in experimental and human kidney injury

shown to successfully reduce the inflammatory responses in the context of sepsis, pneumonia, mesenteric IR induced injury, hemorrhagic shock, colitis, autoimmune arthritis and acute pancreatitis (Gibot et al., 2006a, 2006b, 2008, 2009; Kamei et al., 2010; Murakami et al., 2009; Schenk et al., 2007; Wiersinga et al., 2007). In addition, TREM-1 fusion protein and LR12 have been used to reduce disease severity in sepsis and in models of acute myocardial infarction (Bouchon et al., 2001; Boufenzer et al., 2015; Chen et al., 2008a; Derive et al., 2013, 2014; Wong-Baeza et al., 2006). These previous studies and the significantly elevated TREM-1 expression during renal IRI suggest a potential benefit for TREM-1 interventions to reduce renal IRI. In this study, we relied on the administration of commercial available TREM-1 synthetic inhibitors. Contrary to previous studies addressing the pathogenic role of TREM-1, our results showed that intervention of TREM-1 function by LP17, LR12 or TREM-1 fusion protein does not prevent renal IR-induced injury.

Supporting our results, Campanholle et al. in a recent study have shown that administration of TREM-1 fusion protein following renal IRI did not prevent renal damage. However, the auteurs aimed to answer a different research question: unravel the therapeutic potential of TREM-1 in macrophage activation and fibrosis. It is important to highlight that these two studies focus on different stages of renal inflammation. Granulocytes and macrophages play different roles in the pathophysiology of renal IRI and have a different temporal distribution (Stroo et al., 2010). Our study aimed to block granulocyte- and monocyte-induced inflammation since TREM-1 is mostly expressed on these cells. Thus, our results are not fully comparable with those of Camphanolle et al, but provide more evidence that blocking TREM-1 signaling at early stage of renal IRI does not represent a suitable therapeutic target (Camphanolle et al., 2013).

Thus far it appears that TREM-1 blocking agents report controversial findings in sterile inflammatory disorders. Although we observed TREM-1 up-regulation following renal IRI, this functional study showed that TREM-1 does not play a pivotal role in granulocyte- and monocyte-induced renal inflammation. We speculate that divergent results obtained by TREM-1 inhibitors might be dependent on affinity ligand availability. In our model of sterile renal injury, damage associated molecular patterns (DAMPs), which are released by damage to parenchymal cells to activate
innate immune cells, might have higher affinity for TLRs compared to TREM-1. In the presence of a low abundance or affinity of TREM-1 ligand, possibly, TLR signaling activation is dominant, thus the inflammatory response in renal IRI is TREM-1 independent or of low significance. However, it cannot be excluded that the ligands through redundancy could activate the inflammatory cells through alternative receptors, for instance TREM-3, which most likely can compensate for TREM-1 absence because of close gene locations. In other models of sterile inflammation where TREM-1 inhibition had a beneficial effect, the amplification of the inflammatory response might have been dependent on TREM-1 signaling, possibly activated by different tissue ligands. In sterile ischemic injury, renal parenchyma-associated TLR2 and TLR4, rather than inflammatory cell-associated TLRs, contribute to renal dysfunction (Leemans et al., 2005, 2014; Wu et al., 2012).

Over the past years, soluble TREM-1 has gained interest as biomarker of several disease settings including renal dysfunction (Essa and Elzorkany, 2015; Hirayama et al., 2011; Jung et al., 2011). Since then, also genetic studies that aimed at finding an association between variants in the \textit{TREM1} gene and outcomes in various clinical settings were conducted, however with conflicting (or disease-specific) results. SNVs in the \textit{TREM1} gene were shown to associate with intestinal Behcet’s disease, pneumonia in burn patients, risk for coronary artery disease but not with inflammatory bowel diseases or infective endocarditis (Chen et al., 2008b; Golovkin et al., 2014, 2015; Su et al., 2012). There is no consensus on the association of TREM1 variants with (the outcome after) sepsis with Chen et al. (Chen et al., 2008b) indicating no association in a Chinese population comprising 175 patients with sepsis and Su et al. describing a significant correlation with the incidence of sepsis in 80 Chinese patients (Su et al., 2012). Interestingly, Su et al. did not describe an association between the p.Thr25Ser variant of \textit{TREM1} with the (dynamic) concentrations of soluble TREM-1 (Su et al., 2012), which questions whether there truly is a biochemical consequence of this non-synonymous variant. To the best of our knowledge, the current study is the first on \textit{TREM1} genetic variants in renal transplant patients. The lack of association between the \textit{TREM1} p.Thr25Ser heterozygous variant and renal outcomes with DGF in particular is in line with the lack of beneficial potential in the preclinical mouse studies of IRI. However, even
though this was a large study, we can not exclude that patients with homozygosity for the p.Thr25Ser variant are in fact protected from IRI after transplantation (N = 5, Supplementary Table 1).

From these studies we conclude that 1) TREM-1 increases during experimental renal ischemia-reperfusion injury due to \textit{Trem1} gene transcription by infiltrating leukocytes, 2) various interventions aiming at modulating the function of TREM-1 did not prevent renal damage, leukocyte influx or dysfunction and 3) genetic variants in the \textit{TREM1} gene do not associate with the development of delayed graft function, biopsy-proven acute rejection or subsequent graft failure. Taken together, these experiments question TREM-1 as a potential target of therapy in these particular disease settings.