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## **A comprehensive genotype-phenotype interaction of different Toll-like receptor variations in a renal transplant cohort**

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**ABSTRACT**

**BACKGROUND:** To date, the impact of the Toll-like receptor (TLR) system on early and late kidney transplantation outcome such as acute rejection episodes or cardiovascular morbidity and mortality has still not been conclusively elucidated. Genetically determined alterations in TLR expression exhibit a possibility to evaluate their role in transplantation. In this study we sought to determine a comprehensive genotype-phenotype association with early and late allograft outcomes.

**METHODS:** We studied 11 single nucleotide polymorphisms (SNPs) in TLR2, TLR3, TLR4, TLR5, TLR9 and within a co-molecule CD14 in 265 patients receiving their first kidney transplant and associated them with the occurrence of delayed graft function (DGF), acute rejection episodes (ARE) or major adverse cardiovascular events (MACE).

**RESULTS:** ARE were significantly more frequent in patients carrying the TLR3 TT/CT-allele (43.8% vs. 25.8%,  $p=0.001$ ) as were rates of DGF (21.4% vs. 12.0%,  $p=0.030$ ). Furthermore, TLR9 was significantly involved in the occurrence of MACE (TLR9 -1237,  $p=0.03$ ). Interestingly there was no significant effect of any TLR-polymorphism on graft survival or renal function and the incidence of any infection, including cytomegalovirus (CMV) infection.

**CONCLUSION:** Our study in renal transplant recipients suggests the TLR system to be involved in both acute rejection and MACE. Modulation of the TLR system may be promising targets in future therapeutic strategies.

## INTRODUCTION

Kidney transplantation is considered the “gold standard” in the treatment of end stage renal disease. Although improvements in therapeutic regimens with one year patient and graft survival rates > 90% have been achieved over the last years, the incidence of acute rejection remains at 15-25% with its adverse impact on long term graft survival [1]. However, the more intense immunosuppressive regimens lead to more infectious complications and development of malignancy [2]. Besides acute infection episodes, chronic inflammation seems to be associated strongly and independently with accelerated vasculopathy and subsequently cardiovascular disease (CVD) [3].

Along with the adaptive immune system, mainly assumed to be responsible for early transplant complications, the innate immune system, mostly through its key player the Toll-like receptor system (TLR) but also through other members such as NOD2/CARD15 (Nucleotide Oligomerisation Domain-2/Caspase-Recruiting Activating Domain-15) or Mannose-binding protein, play an important role in the development of both acute and chronic allograft dysfunction [4-6]. Furthermore, increasing evidence exists, that innate immunity plays a role in other chronic human diseases, e.g. atherosclerosis, which is thought to be the result of a chronic infectious state by some [7, 8]. In different animal models as well as in humans, a direct role of TLR4 and TLR2 in the progression of atherosclerosis, with expression of different TLRs in atherosclerotic plaques, has been established. Furthermore the adipocyte fatty acid-binding protein, an important player in atherosclerotic plaque development, has been shown to be regulated upon activation of TLRs, especially TLR2, TLR3 and TLR4 [9].

Although TLRs are primarily involved in recognizing exogenous ligands, recent studies have clearly shown that endogenous ligands, e.g. high mobility group box protein-1 (HMGB1), heat shock proteins, heparan sulphate, fibrinogen, exist as well [10]. Importantly, some of these endogenous ligands (e.g. HMGB1, heat shock proteins) are known to be induced by ischemia reperfusion damage and/or up-regulated during transplant rejection [11]. Recent publications suggested that a TLR4 polymorphism, that causes hyporesponsiveness to lipopolysaccharide (LPS), reduced severity and frequency of delayed graft function, and acute lung allograft rejection, improved outcome after lung transplantation, and affected the rate of atherosclerotic events in renal transplant recipients [10, 12-14]. In bone marrow transplantation the presence of a TLR4 mutation was associated with a reduced risk for acute GvHD (graft versus host disease) and a higher risk for gram-negative bacteremia or invasive aspergillosis [15, 16].

Therefore we hypothesized that genetic variations in the TLR-system may affect clinical outcome (e.g. acute rejection, cardiovascular morbidity, infections) of kidney transplantation through different activation pathways of the innate immunity and the respective impacts on infection, rejection or vasculopathy. To analyze such potential influences we determined selected polymorphisms in the TLR2, TLR3, TLR4, TLR5 and TLR9 genes in a cohort of first renal transplant recipients.

## METHODS

### Transplant population

Between 1995 and 2004 >93% of all patients receiving their first renal transplant at the University of Regensburg were included in this study. Our only inclusion criterion was first renal transplant, the exclusion criteria were re- or multi-organ-transplantation or no written informed consent. Since 1999 all patients were prospectively enrolled at the time of transplant, the earlier ones were carefully recruited retrospectively. Demographic and clinical data, as well as outcome parameter did not differ between enrolled and non-enrolled patients in the two time periods. Demographic data for donor and recipient age and sex, HLA-mismatch, panel reactive antibodies, cold ischemia time, immunosuppressive therapy, presence of rejection episodes, laboratory values, clinical examinations and graft survival were extracted from the hospital records. All events were registered prospectively in the patients group enrolled since 1999, events that happened before 1999 were extracted from hospital records. These events were collected highly accurate because >90% of all patients were treated for complications in our center, or treated in close cooperation with our transplant center.

Standard immunosuppressive regimen included a calcineurin inhibitor (cyclosporine A or tacrolimus), a proliferation inhibitor (mycophenolate mofetil or azathioprine) and steroids which did not differ between the different groups. In cases of high immunological risk (e.g. high levels of panel reactive antibodies (PRA)) induction by either polyclonal antithymocyte globulins or monoclonal anti-CD25 antibodies was followed by a standard maintenance immunosuppressive treatment.

The Internal Review Board approved the study and written consent was obtained at the time of enrolment.

### Determination of TLR-polymorphisms

Genomic DNA from allograft recipients was isolated from peripheral white blood cells using a standard salting out procedure. The different polymorphisms in the TLR2 (R753Q, rs5743708; R677W, del -196/-174 [17]), TLR3 (F412L, rs3775291; T737S, rs5743318), TLR4 (D299G, rs4986790; T399I, rs4986791), TLR5 (392STOP, rs5744168), TLR9 (P545P, rs352140; -1237T/C rs5743836) and CD14 (-159C/T, rs2569190) genes were analyzed using the ABI Prism 7900HT Sequence Detection System® by Taqman PCR except the TLR2 del -196/-174, using a standard PCR procedure with subsequent detection by electrophoresis on 3% NuSieve gel. Primers and probes were designed using the genomic sequences published in dbSNP ([www.ncbi.nlm.gov](http://www.ncbi.nlm.gov)) or as described in the references cited above.

### Endpoints

#### *Acute rejection and delayed graft function (DGF), graft function and survival*

Acute rejection was determined by allograft biopsy in > 95% of cases or was defined by an increase in creatinine level by 30% or more from baseline, not attributable to other causes, with subsequent return to baseline after treatment with pulse steroids. Episodes of acute rejection were primarily treated with a pulse steroid therapy over 3 days, followed by an ATG (anti-thymocyte globulin) treatment in steroid resistant cases. In our institution we had introduced a protocol biopsy program, performing biopsies on day 14 and at 3 months posttransplant. Borderline rejections were treated like any other acute rejection episode, and therefore regarded as acute rejection episodes in our analysis.

Delayed graft function (DGF) was defined as need for dialysis within the first seven days after transplantation [18].

Graft survival was defined as recipient survival with a functioning renal transplant (i.e. censoring for death).

Glomerular filtration rate was estimated by the abbreviated formula of MDRD, as suggested by the K/DOQI Group [19].

#### *Major adverse cardiovascular events (MACE)*

Myocardial infarction, malignant ventricular arrhythmias, acute cardiac failure or any cardiac intervention (PCI +/- stent or revascularization), cerebral ischemia or intracerebral haemorrhage, peripheral amputation or intervention or death due to one of these events was defined as MACE.

#### *Severe infection*

Severe infection was assumed if bacterial or viral infections required hospitalization. Other infections were assumed (urinary or non-urinary) when antibiotics according to clinical signs were needed.

#### *Analysis of cytomegalovirus (CMV) infection*

CMV-load was routinely measured by PCR. The frequency of measurement varies depending on time after transplantation, from weekly in the first 4 weeks to every month or less thereafter. CMV infection was assumed if CMV-PCR was found positive (cut-off level  $10^2$ - $10^3$  copies per ml). CMV risk was assumed if the donor was CMV IgG positive and the recipient CMV IgG negative. CMV prophylaxis with ganciclovir/valganciclovir was used in patients with a CMV risk constellation, i.e. recipient CMV IgG negative and donor CMV IgG positive, for a three-month period post-transplant.

#### **Statistical analysis**

Results are expressed as mean  $\pm$  SD, unless stated otherwise. Comparisons of continuous variables between groups were performed by non-parametric tests, and of categorical variables by two-sided  $\chi^2$  or Fisher's Exact test where applicable. Survival analysis was performed by the Kaplan Meier method comparing groups using the log-rank test, and by Cox regression modeling. Endpoints were used as described above. Categorical and continuous variables were corrected for potential confounding covariates in a multivariate analysis including all variables with a significance level  $p < 0.01$ .  $P < 0.05$  (two-tailed) was considered as statistically significant. Statistical analysis was performed with the SPSS<sup>®</sup> Version 17.0 software package (SPSS Inc. Chicago, Illinois, USA).

Power-analyses were performed using the Power and Sample Size Calculation Statistical Package (Vanderbilt Medical Center, Nashville, TN). Assuming a type I error rate of 0.05, an odds ratio of 2 for acute rejection episodes or DGF, we calculated a power of  $>80\%$  for TLR2, TLR3 and TLR9, as well as at least  $>60\%$  for the other SNPs with a low prevalence. For MACE, assuming a type I error rate of 0.05, a hazard ratio of 2, an accrual period of 120 months, a follow-up time of 30 months and a median time to MACE of 24 months, we calculated a power of  $>80\%$  for all SNPs studied.

## RESULTS

In terms of basic demographic and clinical data, segregated by gender, no differences were observed (Table 1), which were also not seen for all different genotypes tested (data not shown). For all polymorphisms, our population displays similar allele frequencies corresponding to published data and did not deviate from Hardy-Weinberg equilibrium, except TLR3 T737S and TLR2 R677W, which both were monoallelic, as described also by others in a European population [20, 21].

### **Association with acute rejection or delayed graft function (DGF)**

The overall incidence of acute rejection was 35.8%. For the F412L-TLR3-polymorphism (TT/TC-allele) we found a significantly higher rate of acute rejection (43.8% vs. 25.8%,  $p=0.001$ ). After correction for different confounders these results could be confirmed (Table 2). Furthermore the F412L-TLR3 polymorphism showed a significantly higher rate of DGF (21.4% vs. 12.0%,  $p=0.030$ ) in univariate analysis, which was not significant after adjusting for known risk factors ( $p=0.078$ , Table 2). Every other tested polymorphism showed no significant association or trend for acute rejection rates or occurrence of DGF.

### **Impact on graft and patient outcome**

Despite the significant impact of TLR3 on acute rejection, no effect on graft survival (censored for death with functioning graft), i.e. mainly caused by chronic rejection/ chronic allograft nephropathy, as well as for all-cause mortality was seen for this SNPs in the observational period of 6 years. Likewise no significant effect of any other polymorphism has been observed, however we saw a trend for a better graft survival in the group with mutated TLR4 ( $p=0.078$ ). Besides mortality, cardiovascular morbidity is a major burden after transplantation. The TLR9 -1237TT-allele showed a significantly higher incidence of MACE (log-rank  $p=0.030$ , Figure 1) which was still present after multivariate correction for different risk factors, with a 2.923-fold risk in the presence of the TT-allele ((95% CI 1.012 to 8.441,  $p=0.047$ , Table 3). Another SNP, namely TLR2R753Q showed a trend for a higher rate of MACE ( $p=0.072$ ). All-cause mortality (un-censored graft survival) is not altered by any genetic variation.

### **Effect of genotypes on renal function**

During follow-up we assessed the estimated glomerular filtration rate (eGFR) at different time points, i.e. at 6 and 12 months, 3 and 5 years. For all polymorphisms tested, no association could be found at any time point.

### **Effect of genotypes on viral or bacterial infection**

There were no effects with regard to the rate of hospitalization, the onset or recurrence of urinary infection or any other kind of infection, including CMV infection, except for the TLR9 -1237 SNP with a marginally significant difference for more recurrent urinary infection (11.3 % vs. 24.3 %,  $p=0.025$ , data not shown).

## DISCUSSION

Our comprehensive analysis of genetic TLR variations demonstrated a significant association of a polymorphism in the TLR3 gene (F412L) with the incidence of acute rejection and delayed graft function, a known risk factor for acute rejection (Table 2) [22]. Despite finding no association of any polymorphism with graft survival, graft function or all-cause mortality during the 6 year observational period, a significantly higher incidence of MACE in the presence of the TLR9 -1237 TT-allele (log rank  $p=0.030$ ) could be observed.

The impact of the TLR system on graft outcome exhibits different facets. We and others could demonstrate a significant impact of TLRs in ischemia reperfusion injury, a sterile inflammation initiated/maintained by endogenous ligands such as high-mobility group box protein 1, heat shock proteins or biglycan [10, 23, 24]. Besides ischemia reperfusion injury, in normal post-transplant homeostasis ample other ligands accrued, such as oxygen radicals, fibrinogen or part of necrotic cells which are also capable to activate the innate immune system [4]. This leads to the frequently observed chronic inflammatory status post-transplant with potentially deleterious effects, e.g. indicated by slightly elevated CRP levels, itself a stimulator of the innate immune system [3, 25, 26].

TLR3, an intracellular receptor of the innate immune system expressed on dendritic cells and kidney or lung epithelial cells is activated by double- or single-stranded viral RNA (ss-, dsRNA) or mRNA [27, 28]. Stimulation of PBMCs or mesangial cells with Poly(I:C), a synthetic stimulus for TLR3, resulted amongst others in an up-regulation of interferon- $\gamma$  and IP-10 in PBMCs or interleukin-8 and RANTES/CCL5 in mesangial cells [27, 29]. All four cytokines/chemokines are known to be involved in acute rejection after kidney transplantation [30, 31]. In a recent study in chronic hepatitis C and liver disease, TLR3 expression seemed to be positively affected by the presence of the TT-allele [32]. These results may give an explanation for the higher incidence of acute rejection episodes in patients with the TT/TC-allele. However overall graft survival and time to first acute rejection were not significantly altered by the recipient TLR3 F412L polymorphism, suggesting that early well treated acute rejections do not cause a sustained injury to the graft. Our analysis is also supported by a recent publication by Hwang et al. [33], who analyzed 3 polymorphisms within the TLR3 gene, and found a trend for a significant impact of reconstructed haplotypes on the rate of acute rejection.

In the last two decades acute rejection rates declined due to newer and more effective immunosuppressive therapies, however acute rejection still remains one of the major risk factors for poor graft function and development of chronic allograft nephropathy after kidney transplantation [1]. Furthermore intensified immunosuppression to avoid or treat rejection episodes may have deleterious side effects such as drug related nephrotoxicity, induction of cardiovascular damage, malignancies, and increased rates of infections, e.g. polyoma virus nephropathy. Especially major adverse cardiovascular events (MACE) and subsequently fatal cardiovascular events cause up to 50% of all late allograft losses, revealing the important impact on late transplant outcome [25, 26, 34].

Recently Ducloux et al. [12] reported a significant association of TLR4 polymorphisms D299G and T399I with rates of acute rejection and the occurrence of atherosclerotic events in kidney transplant recipients. In a Korean population, Hwang et al. [33] also reported a significant association with acute rejection episodes and TLR4, however, this was a different SNP in the TLR4 gene, rs10759932. In our study, we could not confirm these findings with regard to acute rejection, graft survival or MACE in renal transplant recipients. These controversial findings could be explained by significant different patient

population/characteristics, i.e. we prospectively enrolled >93% of all patients transplanted in our institution within the stated time period, Ducloux et al. enrolled preselected patients with a stable renal function (creatinine <400  $\mu\text{mol/l}$ ) one year after transplantation [12, 30], as well as Hwang et al. could only enroll 63% of all patients transplanted in their enrollment period [33]. These enrolment strategies imply a selection bias against early graft losses, and an over representation of patients with good clinical standings, and good adherence to the transplant center. Furthermore, in the Korean population these two SNPs, D299G and T399I, are not present, and the effect of the examined promoter SNP was not adjusted to different confounders [33]. However, we found a trend for better graft survival in the presence of a mutated TLR4 ( $p=0.078$ ). Although this was not significant, most likely due to the low incidence of the mutated receptor, it is a possible hint for an involvement of TLR4.

The role of TLRs in the pathogenesis of atherosclerosis has increasingly been recognized. Whereas Edfeldt et al. [35] demonstrated the expression of TLR2 and TLR4 in atherosclerotic lesions, Kiechl et al. [8] found a significant association between the two known TLR4 loss-of-function mutations (D299G, T399I) and the thickness of carotid lesions. Other studies could also show the involvement of the TLR signaling pathway, including alterations by genetic variations, in the development of atherosclerosis or cardiac events [36-38]. Therefore we studied the influence of different TLR-polymorphisms on MACE. We found a significant association of the TLR9-polymorphism -1237C/T with (HR 2.923, 95% CI 1.012-8.441), as well as a trend for the TLR2-polymorphism R753Q (Figures 1, 2). The impact of TLR9, a receptor for bacterial and viral unmethylated cytosine-guanosine dinucleotide (CpG)-DNA, on atherosclerosis is currently under debate. Lazarus et al. [39] sequenced the TLR9 gene in different U.S. ethnic groups, and they found several polymorphisms within this receptor. However, only the -1237C/T polymorphism showed a marginally significant association with asthma, but no tested polymorphism was associated with myocardial infarction. A potential explanation for interaction of this receptor and cardiovascular events is the hypothesis of atherosclerosis being a result of chronic inflammation that is accelerated in patients with chronic renal failure and renal transplant recipients. Differences in formation of atherosclerotic plaques due to altered response to endogenous or exogenous ligands might be responsible for different outcomes, a hypothesis that is supported by different experiments which found elevated promoter activity for the TT-allele or potential additional binding sites [40, 41].

TLR2, as mentioned above, plays a currently not fully understood role in the initiation and progression of atherosclerosis, and the TLR2-R753Q polymorphism was described to be associated with hyporesponsiveness to bacterial peptides [42]. Accordingly, Hamann et al. [43] reported this TLR2-R753Q polymorphism as a risk factor for coronary stent restenosis. The role of different pathogens like *Chlamydia pneumoniae*, also a ligand for both, TLR2 and TLR4, in the development of atherosclerosis, is discussed controversially at present time [42]. Nevertheless, decreased response to such pathogens could lead to ameliorated atherosclerotic lesions.

However certain potential limitations have to be addressed, such as the study is not fully prospective. But in addition to the high level of completeness with over 93%, there were also no differences between the enrolled and non-enrolled patients as well as patients within the retro- and prospective part of the study, which in our opinion may compensate for this disadvantage. Our high completeness of enrollment is also helpful to obtain reliable results of genetic interactions in lower patient numbers, which is also supported by the power calculations we made, even though we do not have enough power for the low frequency of some of the SNPs. The fact of our successful nearly complete enrolment and follow-up minimizes the bias of particularly early graft loss or patients' death, but also the loss of follow-up of well functioning grafts while recruiting months after transplantation, and

thereby reduces the potential interactions of cumulating negative or positive alleles. Therefore, and because of the exploratory nature of our study, we did not correct for multiple testing. However, if we correct for multiple testing, our key message (TLR3 influences the rate of acute rejection) does not change after a Bonferroni's correction ( $p=0.03$ ), in contrast to the outcome for MACE which loses its significant association.

In conclusion our study in renal transplant recipients suggests the TLR system to be involved in both acute rejection and cardiovascular morbidity. Modulation of the TLR system may offer new therapeutic strategies, particularly via development of small molecule blockers.

#### **AUTHOR CONTRIBUTION**

Bernd Krüger designed and performed the study, performed and analyzed experiments/data, and wrote the manuscript;

Miriam C. Banas collected samples, performed experiments and wrote the paper;

Andreas Walberer, Carsten A. Böger, Stefan Farkas and Ute Hoffmann collected samples, analyzed and discussed data;

Michael Fischereeder, Bernhard Banas, Bernhard K. Krämer designed the study, collected samples, discussed data, and wrote the manuscript.

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## REFERENCES

1. Wu, O., Levy, A. R., Briggs, A., Lewis, G. and Jardine, A. (2009) Acute rejection and chronic nephropathy: a systematic review of the literature. *Transplantation*. **87**, 1330-1339.
2. Marcén, R. (2009) Immunosuppressive drugs in kidney transplantation: impact on patient survival, and incidence of cardiovascular disease, malignancy and infection. *Drugs*. **69**, 2227-2243.
3. Hansson, G. K. (2005) Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med*. **352**, 1685-1695.
4. Alegre, M. L., Leemans, J., Le Moine, A., Florquin, S., De Wilde, V., Chong, A. and Goldman, M. (2008) The Multiple Facets of Toll-Like Receptors in Transplantation Biology. *Transplantation*. **86**, 1-9.
5. Krüger, B., Schröppel, B. and Murphy, B. T. (2008) Genetic polymorphisms and the fate of the transplanted organ. *Transplant Rev (Orlando)*. **22**, 131-140.
6. Krüger, B., Böger, C. A., Schröppel, B., Obed, A., Hoffmann, U., Murphy, B. T., Fischereder, M., Holler, E., Banas, B. and Krämer, B. K. (2007) Impact of NOD2/CARD15 haplotypes on the outcome after kidney transplantation. *Transpl Int*. **20**, 600-607.
7. Curtiss, L. K. and Tobias, P. S. (2009) Emerging role of Toll-like receptors in atherosclerosis. *J Lipid Res*. **50 Suppl**, S340-345.
8. Kiechl, S., Lorenz, E., Reindl, M., Wiedermann, C. J., Oberhollenzer, F., Bonora, E., Willeit, J. and Schwartz, D. A. (2002) Toll-like receptor 4 polymorphisms and atherogenesis. *N Engl J Med*. **347**, 185-192.
9. Kazemi, M. R., McDonald, C. M., Shigenaga, J. K., Grunfeld, C. and Feingold, K. R. (2005) Adipocyte fatty acid-binding protein expression and lipid accumulation are increased during activation of murine macrophages by toll-like receptor agonists. *Arterioscler Thromb Vasc Biol*. **25**, 1220-1224.
10. Krüger, B., Krick, S., Dhillon, N., Lerner, S. M., Ames, S., Bromberg, J. S., Lin, M., Walsh, L., Vella, J., Fischereder, M., Krämer, B. K., Colvin, R. B., Heeger, P. S., Murphy, B. T. and Schröppel, B. (2009) Donor Toll-like receptor 4 contributes to ischemia and reperfusion injury following human kidney transplantation. *Proc Natl Acad Sci U S A*. **106**, 3390-3395.
11. Huang, Y., Yin, H., Han, J., Huang, B., Xu, J., Zheng, F., Tan, Z., Fang, M., Rui, L., Chen, D., Wang, S., Zheng, X., Wang, C. Y. and Gong, F. (2007) Extracellular hmgbl functions as an innate immune-mediator implicated in murine cardiac allograft acute rejection. *Am J Transplant*. **7**, 799-808.
12. Ducloux, D., Deschamps, M., Yannaraki, M., Ferrand, C., Bamoulid, J., Saas, P., Kazory, A., Chalopin, J. M. and Tiberghien, P. (2005) Relevance of Toll-like receptor-4 polymorphisms in renal transplantation. *Kidney Int*. **67**, 2454-2461.
13. Palmer, S. M., Burch, L. H., Davis, R. D., Herczyk, W. F., Howell, D. N., Reinsmoen, N. L. and Schwartz, D. A. (2003) The role of innate immunity in acute allograft rejection after lung transplantation. *Am J Respir Crit Care Med*. **168**, 628-632.
14. Palmer, S. M., Burch, L. H., Trindade, A. J., Davis, R. D., Herczyk, W. F., Reinsmoen, N. L. and Schwartz, D. A. (2005) Innate immunity influences long-term outcomes after human lung transplant. *Am J Respir Crit Care Med*. **171**, 780-785.
15. Lorenz, E., Schwartz, D. A., Martin, P. J., Gooley, T., Lin, M. T., Chien, J. W., Hansen, J. A. and Clark, J. G. (2001) Association of TLR4 mutations and the risk for acute GVHD after HLA-matched-sibling hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. **7**, 384-387.

16. Bochud, P. Y., Chien, J. W., Marr, K. A., Leisenring, W. M., Upton, A., Janer, M., Rodrigues, S. D., Li, S., Hansen, J. A., Zhao, L. P., Aderem, A. and Boeckh, M. (2008) Toll-like receptor 4 polymorphisms and aspergillosis in stem-cell transplantation. *N Engl J Med.* **359**, 1766-1777.
17. Noguchi, E., Nishimura, F., Fukai, H., Kim, J., Ichikawa, K., Shibasaki, M. and Arinami, T. (2004) An association study of asthma and total serum immunoglobulin E levels for Toll-like receptor polymorphisms in a Japanese population. *Clin Exp Allergy.* **34**, 177-183.
18. Perico, N., Cattaneo, D., Sayegh, M. H. and Remuzzi, G. (2004) Delayed graft function in kidney transplantation. *Lancet.* **364**, 1814-1827.
19. National Kidney Foundation (2002) K/DOQI clinical practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Am J Kidney Dis.* **39**, S1-266.
20. Schröder, N. W., Hermann, C., Hamann, L., Göbel, U. B., Hartung, T. and Schumann, R. R. (2003) High frequency of polymorphism Arg753Gln of the Toll-like receptor-2 gene detected by a novel allele-specific PCR. *J Mol Med.* **81**, 368-372.
21. Misch, E. A. and Hawn, T. R. (2008) Toll-like receptor polymorphisms and susceptibility to human disease. *Clin Sci (Lond).* **114**, 347-360.
22. Shoskes, D. A. and Cecka, J. M. (1998) Deleterious effects of delayed graft function in cadaveric renal transplant recipients independent of acute rejection. *Transplantation.* **66**, 1697-1701.
23. Leemans, J. C., Stokman, G., Claessen, N., Rouschop, K. M., Teske, G. J., Kirschning, C. J., Akira, S., van der Poll, T., Weening, J. J. and Florquin, S. (2005) Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. *J Clin Invest.* **115**, 2894-2903.
24. Wu, H., Chen, G., Wyburn, K. R., Yin, J., Bertolino, P., Eris, J. M., Alexander, S. I., Sharland, A. F. and Chadban, S. J. (2007) TLR4 activation mediates kidney ischemia/reperfusion injury. *J Clin Invest.* **117**, 2847-2859.
25. Abedini, S., Holme, I., Marz, W., Weihrauch, G., Fellstrom, B., Jardine, A., Cole, E., Maes, B., Neumayer, H. H., Gronhagen-Riska, C., Ambuhl, P. and Holdaas, H. (2009) Inflammation in renal transplantation. *Clin J Am Soc Nephrol.* **4**, 1246-1254.
26. Krüger, B., Walberer, A., Debler, J., Böger, C. A., Farkas, S., Reinhold, S. W., Obed, A., Schlitt, H. J., Fischereeder, M., Banas, B. and Krämer, B. K. (2010) Is inflammation prior to renal transplantation predictive for cardiovascular and renal outcomes? *Atherosclerosis.* **210**, 637-642.
27. Allam, R., Lichtnekert, J., Moll, A. G., Taubitz, A., Vielhauer, V. and Anders, H. J. (2009) Viral RNA and DNA trigger common antiviral responses in mesangial cells. *J Am Soc Nephrol.* **20**, 1986-1996.
28. Anders, H. J., Banas, B. and Schlöndorff, D. (2004) Signaling danger: toll-like receptors and their potential roles in kidney disease. *J Am Soc Nephrol.* **15**, 854-867.
29. Re, F. and Strominger, J. L. (2004) IL-10 released by concomitant TLR2 stimulation blocks the induction of a subset of Th1 cytokines that are specifically induced by TLR4 or TLR3 in human dendritic cells. *J Immunol.* **173**, 7548-7555.
30. Krüger, B., Böger, C. A., Obed, A., Farkas, S., Hoffmann, U., Banas, B., Fischereeder, M. and Krämer, B. K. (2007) RANTES/CCL5 polymorphisms as a risk factor for recurrent acute rejection. *Clin Transplant.* **21**, 385-390.
31. Tatapudi, R. R., Muthukumar, T., Dadhania, D., Ding, R., Li, B., Sharma, V. K., Lozada-Pastorio, E., Seetharamu, N., Hartono, C., Serur, D., Seshan, S. V., Kapur, S., Hancock, W. W. and Suthanthiran, M. (2004) Noninvasive detection of renal allograft

- inflammation by measurements of mRNA for IP-10 and CXCR3 in urine. *Kidney Int.* **65**, 2390-2397.
32. Askar, E., Bregadze, R., Mertens, J., Schweyer, S., Rosenberger, A., Ramadori, G. and Mihm, S. (2009) TLR3 gene polymorphisms and liver disease manifestations in chronic hepatitis C. *J Med Virol.* **81**, 1204-1211.
  33. Hwang, Y. H., Ro, H., Choi, I., Kim, H., Oh, K. H., Hwang, J. I., Park, M. H., Kim, S., Yang, J. and Ahn, C. (2009) Impact of polymorphisms of TLR4/CD14 and TLR3 on acute rejection in kidney transplantation. *Transplantation.* **88**, 699-705.
  34. Krämer, B. K., Böger, C., Krüger, B., Marienhagen, J., Pietrzyk, M., Obed, A., Paczek, L., Mack, M. and Banas, B. (2005) Cardiovascular risk estimates and risk factors in renal transplant recipients. *Transplant Proc.* **37**, 1868-1870.
  35. Edfeldt, K., Swedenborg, J., Hansson, G. K. and Yan, Z. Q. (2002) Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. *Circulation.* **105**, 1158-1161.
  36. Edfeldt, K., Bennet, A. M., Eriksson, P., Frostegard, J., Wiman, B., Hamsten, A., Hansson, G. K., de Faire, U. and Yan, Z. Q. (2004) Association of hypo-responsive toll-like receptor 4 variants with risk of myocardial infarction. *Eur Heart J.* **25**, 1447-1453.
  37. Hernesniemi, J. A., Raitakari, O. T., Kahonen, M., Juonala, M., Hutri-Kahonen, N., Marniemi, J., Viikari, J. and Lehtimäki, T. (2008) Toll-like receptor 4 gene (Asp299Gly) polymorphism associates with carotid artery elasticity. The cardiovascular risk in young Finns study. *Atherosclerosis.* **198**, 152-159.
  38. Hommels, M. J., Kroon, A. A., Netea, M. G., de Leeuw, P. W., Bruggeman, C. A., Leiner, T., Koster, D. A., Stalenhoef, A. F. and van der Ven, A. J. (2007) The Asp299Gly Toll-like receptor 4 polymorphism in advanced aortic atherosclerosis. *Neth J Med.* **65**, 203-207.
  39. Lazarus, R., Klimecki, W. T., Raby, B. A., Vercelli, D., Palmer, L. J., Kwiatkowski, D. J., Silverman, E. K., Martinez, F. and Weiss, S. T. (2003) Single-nucleotide polymorphisms in the Toll-like receptor 9 gene (TLR9): frequencies, pairwise linkage disequilibrium, and haplotypes in three U.S. ethnic groups and exploratory case-control disease association studies. *Genomics.* **81**, 85-91.
  40. Novak, N., Yu, C. F., Bussmann, C., Maintz, L., Peng, W. M., Hart, J., Hagemann, T., Diaz-Lacava, A., Baurecht, H. J., Klopp, N., Wagenpfeil, S., Behrendt, H., Bieber, T., Ring, J., Illig, T. and Weidinger, S. (2007) Putative association of a TLR9 promoter polymorphism with atopic eczema. *Allergy.* **62**, 766-772.
  41. Hamann, L., Glaeser, C., Hamprecht, A., Gross, M., Gomma, A. and Schumann, R. R. (2006) Toll-like receptor (TLR)-9 promoter polymorphisms and atherosclerosis. *Clin Chim Acta.* **364**, 303-307.
  42. Schröder, N. W. and Schumann, R. R. (2005) Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. *Lancet Infect Dis.* **5**, 156-164.
  43. Hamann, L., Gomma, A., Schröder, N. W., Stamme, C., Glaeser, C., Schulz, S., Gross, M., Anker, S. D., Fox, K. and Schumann, R. R. (2005) A frequent toll-like receptor (TLR)-2 polymorphism is a risk factor for coronary restenosis. *J Mol Med.* **83**, 478-485.

## Figure Legends

### Figure 1

Kaplan-Meier estimate of onset of cardiovascular event (MACE) in renal transplant recipients and numbers at risk with respect to TLR9 -1237C/T genotype. The presence of the TLR9 -1237 TT-Allele (solid line) is associated with significantly higher rates of cardiovascular events (log rank  $p=0.030$ ).

### Figure 2

Kaplan-Meier estimate of onset of cardiovascular event (MACE) in renal transplant recipients and numbers at risk with respect to TLR2-R753Q genotype. Heterozygosity of the TLR2-R753Q allele (dotted line) is associated with a trend for higher rates of cardiovascular events (log rank  $p=0.064$ ).

**Table 1:** Demographic and clinical data

	<b>N=265</b>
<b>Recipient age [yrs]</b>	50.0 ± 13.6
<b>Living donor</b>	64 (24.2%)
<b>Donor age [yrs]</b>	49.0 ± 16.0
<b>CIT [hrs]</b>	12.1 ± 7.8
<b>HLA-MM [n]</b>	
<b>0-1</b>	71 (26.8%)
<b>2-4</b>	158 (59.6%)
<b>5-6</b>	36 (13.6%)
<b>PRA [n]</b>	
<b>0-10%</b>	255 (96.2%)
<b>11-20%</b>	0
<b>&gt; 20%</b>	10 (3.8%)
<b>Body mass index (kg/m<sup>2</sup>)</b>	25.6 ± 4.3
<b>MACE (prior Tx) [n]</b>	60 (22.6%)
<b>DM (prior Tx) [n]</b>	41 (15.5%)
<b>Hyperchol (prior Tx) [n]</b>	136 (51.3%)
<b>AH (prior Tx) [n]</b>	253 (95.5%)
<b>Duration of dialysis [yrs]</b>	4.0 ± 2.7
<b>Preemptive Tx [n]</b>	17 (6.4%)
<b>Underlying renal disease</b>	
<b>diabetic nephropathy</b>	29 (10.9%)
<b>WG</b>	3 (1.1%)
<b>SLE</b>	3 (1.1%)
<b>IgA-Nephritis</b>	19 (7.2%)
<b>ADPKD</b>	27 (10.2%)
<b>chronic GN</b>	106 (40.0%)
<b>others</b>	78 (29.4%)
<b>Induction therapy [n]</b>	9 (3.4 %)
<b>DGF [n]</b>	45 (17.0%)
<b>ARE [n]</b>	95 (35.8%)
<b>CMV risk constellation [n]</b>	61 (23.0%)
<b>CMV infection [n]</b>	105 (39.6%)
<b>eGFR at 6 months [ml/min]</b>	46.8 ± 20.3
<b>eGFR at 12 months [ml/min]</b>	46.1 ± 20.2
<b>eGFR at 3 years [ml/min]</b>	45.2 ± 19.1
<b>eGFR at 5 years [ml/min]</b>	42.9 ± 19.0

CIT: cold ischemia time, HLA-MM: HLA mismatch, PRA: panel reactive antibodies, MACE: major adverse cardiovascular event, DM: diabetes mellitus, Hyperchol: hypercholesterolemia, AH: arterial hypertension. WG: Wegener granulomatosis, SLE: systemic lupus erythematosus, ADPKD: autosomal dominant polycystic kidney disease, chronic GN: chronic glomerulonephritis, DGF: delayed graft function, ARE: acute rejection episodes, GFR: estimated glomerular filtration rate.

**Table 2:** Uni- and multivariate logistic regression analysis for different risk factors of acute rejection and delayed graft function including TLR3 F412L (TT/TC) genotype.

	acute rejection				delayed graft function			
	univariate		multivariate		univariate		multivariate	
	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)	p-value	OR (95% CI)
<b>TLR3 F412L (ref. CC-allele)</b>	<b>0.001</b>	<b>2.352 (1.395-3.965)</b>	<b>0.003</b>	<b>2.237 (1.304-3.836)</b>	<b>0.030</b>	<b>2.143 (1.02-3.923)</b>	0.078	1.940 (0.928-4.057)
<b>AR (ref. no)</b>					<b>0.021</b>	<b>2.149 (1.123-4.112)</b>	0.222	1.567 (0.762-3.220)
<b>DGF (ref. no)</b>	<b>0.021</b>	<b>2.149 (1.123-4.112)</b>	0.097	1.795 (0.899-3.583)				
<b>Recipient age (yrs)</b>	0.474	1.007 (0.988-1.026)			<b>0.006</b>	<b>1.038 (1.011-1.065)</b>	0.159	1.024 (0.991-1.059)
<b>Gender (ref. male)</b>	0.959	1.014 (0.594-1.732)			0.788	1.100 (0.551-2.194)		
<b>Type of Tx (ref. Living donor)</b>	0.141	1.587 (0.858-2.934)			<b>0.006</b>	<b>5.371 (1.605-17.974)</b>	0.472	1.783 (0.369-8.610)
<b>CIT (hrs)</b>	0.120	1.026 (0.993-1.060)			0.065	1.040 (0.998-1.083)	0.212	1.044 (0.976-1.117)
<b>HLA-MM (number)</b>	<b>0.002</b>	<b>1.271 (1.093-1.477)</b>	0.528	1.053 (0.898-1.235)	<b>0.034</b>	<b>1.226 (1.016-1.479)</b>	0.095	1.237 (0.964-1.588)
<b>PRA (%)</b>	<b>0.063</b>	<b>1.149 (0.992-1.330)</b>	0.101	1.022 (0.996-1.050)	0.647	0.991 (0.954-1.029)		
<b>Donor age [yrs]</b>	<b>0.003</b>	<b>1.025 (1.008-1.043)</b>	<b>0.011</b>	<b>1.024 (1.005-1.042)</b>	<b>0.020</b>	<b>1.026 (1.004-1.048)</b>	0.241	1.015 (0.990-1.040)
<b>Duration of dialysis [yrs]</b>	0.724	1.001 (0.994-1.009)			<b>0.006</b>	<b>1.014 (1.004-1.024)</b>	0.070	1.011 (0.999-1.023)
<b>Induction therapy (ref. no)</b>	<b>0.873</b>	<b>1.122 (0.274-4.592)</b>			<b>0.672</b>	<b>0.707 (0.142-3.518)</b>		

OR: hazard ratio, CI: confidence interval, DGF: delayed graft function, CIT: cold ischemia time, HLA-MM: HLA mismatch, PRA: panel reactive antibodies.

**Table 3:** Cox-proportional-hazard analysis to assess the effect of TLR2-R753Q or TLR9 -1237 C/T on MACE within the first 6 years after transplantation.

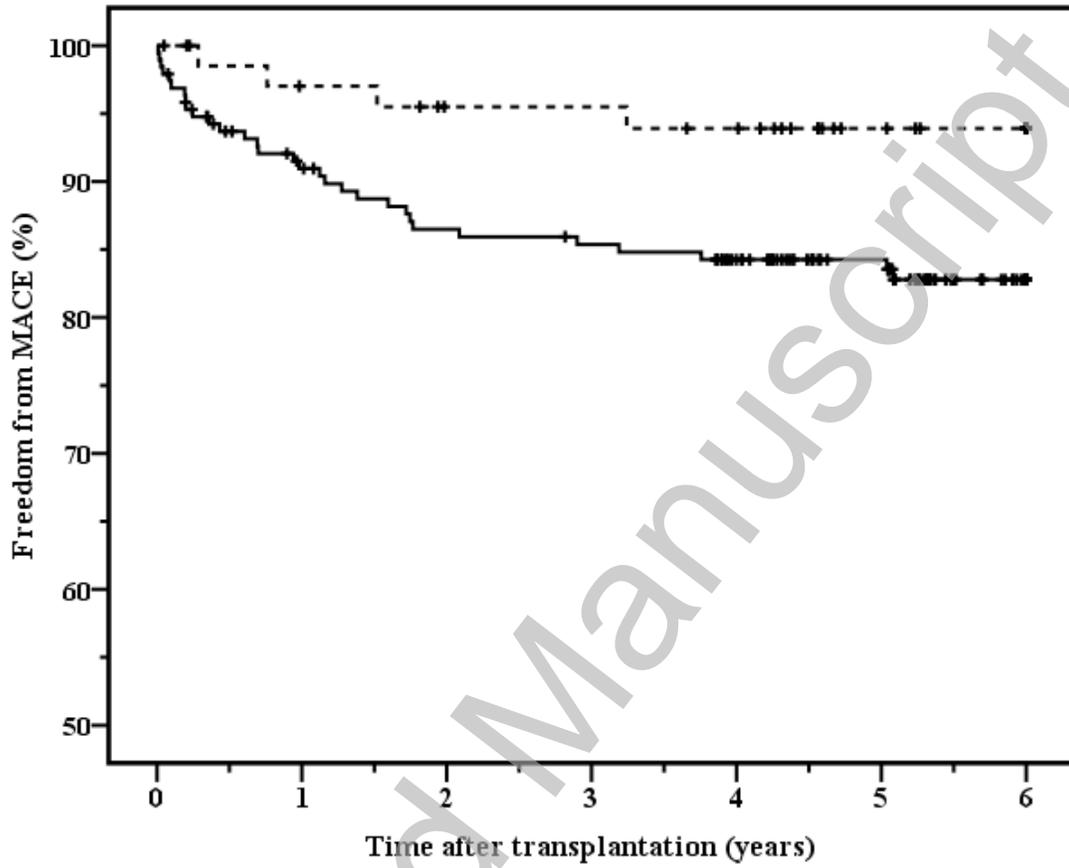
	univariate		multivariate TLR9		multivariate TLR2	
	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)
TLR2 (ref. GA-allele)	<b>0.072</b>	<b>2.378 (0.925-6.118)</b>			0.110	2.267 (0.830-6.192)
TLR9 (ref. TT/CT-allele)	<b>0.039</b>	<b>2.996 (1.058-8.489)</b>	<b>0.047</b>	<b>2.923 (1.012-8.441)</b>		
MACE prior Tx (ref. no)	<b>&lt;0.001</b>	<b>5.007 (2.589-9.685)</b>	<b>0.033</b>	<b>2.335 (1.069-5.097)</b>	<b>0.014</b>	<b>2.609 (1.215-5.603)</b>
DM (ref. no)	<b>0.002</b>	<b>22.995 (1.496-5.997)</b>	0.921	1.041 (0.469-2.312)	0.960	1.021 (0.453-2.302)
AH (ref. no)	0.356	21.785 (0.028-15164)				
Smoking (ref. no)	<b>0.001</b>	<b>2.884 (1.500-5.545)</b>	<b>&lt;0.001</b>	<b>4.119 (1.932-8.781)</b>	<b>&lt;0.001</b>	<b>4.170 (1.989-8.745)</b>
Recipient age [yrs]	<b>0.001</b>	<b>1.051 (1.021-1.081)</b>	<b>0.013</b>	<b>1.049 (1.010-1.088)</b>	<b>0.022</b>	<b>1.043 (1.006-1.081)</b>
Donor age [yrs]	0.269	1.012 (0.991-1.034)				
Duration of dialysis [yrs]	0.578	1.003 (0.993-1.013)				
Gender (ref: male)	<b>0.006</b>	<b>4.302 (1.521-12.169)</b>	<b>0.035</b>	<b>3.226 (1.084-9.603)</b>	<b>0.043</b>	<b>3.120 (1.038-9.379)</b>
Donor type (ref. living)	0.110	2.160 (0.840-5.557)				
AR (ref. no)	<b>0.036</b>	<b>2.012 (1.046-3.870)</b>	0.339	1.427 (0.689-2.957)	0.477	1.302 (0.629-2.688)
DGF (ref. no)	<b>0.007</b>	<b>2.678 (1.316-5.452)</b>	0.281	1.526 (0.708-3.288)	0.213	1.616 (0.759-3.445)
CIT [hrs]	0.383	1.018 (0.977-1.061)				
PRA [%]	0.472	0.931 (0.766-1.131)				
HLA-MM (number)	0.763	1.029 (0.852-1.243)				
Induction therapy (ref. no)	<b>0.007</b>	<b>4.217 (1.490-11.936)</b>	<b>0.028</b>	<b>3.719 (1.155-11.977)</b>	<b>0.011</b>	<b>4.597 (1.429-14.789)</b>

HR: hazard ratio, CI: confidence interval, MACE: major adverse cardiovascular event, DM: diabetes mellitus, AH: arterial hypertension, CIT: cold ischemia time, AR: acute rejection, DGF: delayed graft function, HLA-MM: HLA mismatch. .

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Figure 1

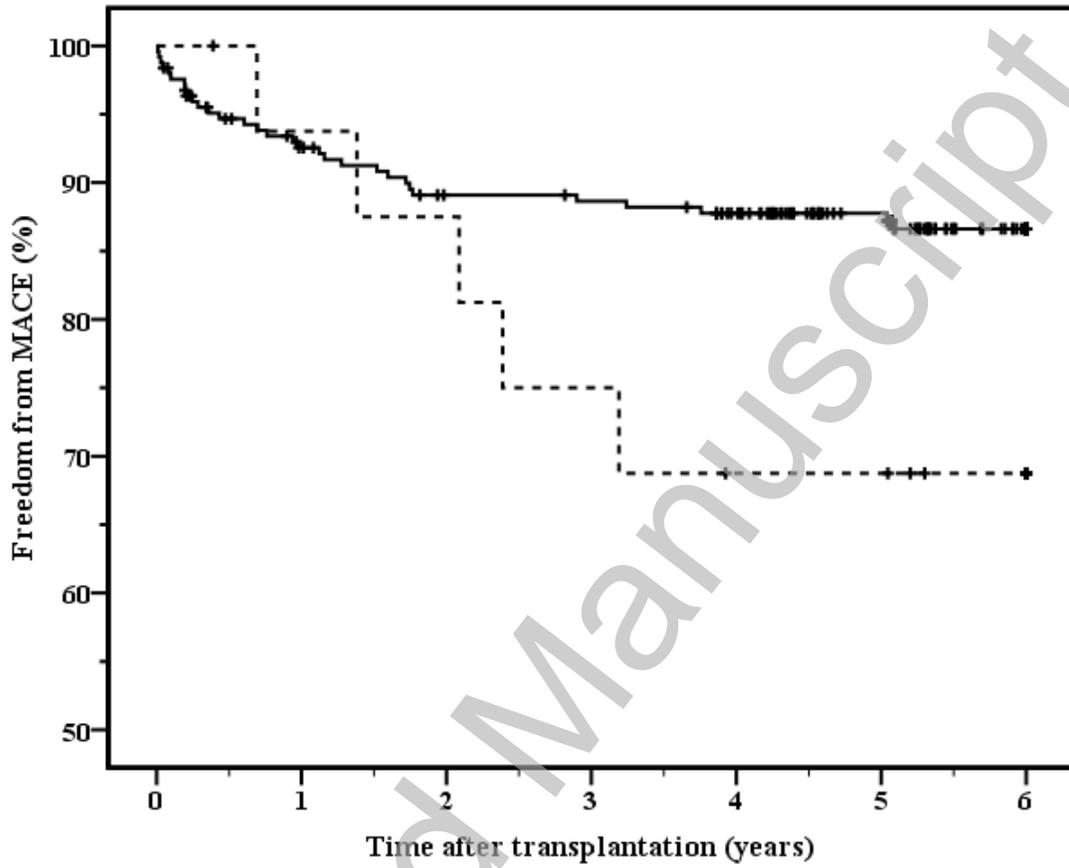


patients at risk

years	0	1	2	3	4	5	6
TT	192	165	155	152	140	119	75
TC/CC	70	64	60	60	58	48	45

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Figure 2



patients at risk

years	0	1	2	3	4	5	6
GG	247	216	203	201	190	158	114
GA	17	15	14	12	10	10	7

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