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Implication of Matrix Metalloproteinase 7 and the Noncanonical Wingless-Type Signaling Pathway in a Model of Kidney Allograft Tolerance Induced by the Administration of Anti-Donor Class II Antibodies

Vojislav Jovanovic,* Anne-Sophie Dugast,* Jean-Marie Heslan,* Joanna Ashton-Chess,* Magali Giral,† Nicolas Degauque,* Anne Moreau,† Annaick Pallier,* Elise Chiffoleau,* David Lair,* Claire Usal,* Helga Smit,* Bernard Vanhove,* Jean-Paul Souillou,2*‡ and Sophie Brouard2,3* 

In rats, tolerance to MHC-incompatible renal allografts can be induced by the administration of anti-donor class II Abs on the day of transplantation. In this study we explored the mechanisms involved in the maintenance phase of this tolerance by analyzing intragraft gene expression profiles by microarray in long-term accepted kidneys. Comparison of the gene expression patterns of tolerated to syngeneic kidneys revealed 5,954 differentially expressed genes ($p < 0.05$). Further analysis of this gene set revealed a key role for the wingless-type (WNT) signaling pathway, one of the pivotal pathways involved in cell regulation that has not yet been implicated in transplantation. Several genes within this pathway were significantly up-regulated in the tolerated grafts, particularly matrix metalloproteinase 7 (MMP7; fold change $> 40$). Analysis of several other pathway-related molecules indicated that MMP7 overexpression was the result of the noncanonical WNT signaling pathway. MMP7 expression was restricted to vascular smooth muscle cells and was specific to anti-class II Ab-induced tolerance, as it was undetectable in other models of renal and heart transplant tolerance and chronic rejection induced across the same strain combination. These results suggest a novel role for noncanonical WNT signaling in maintaining kidney transplant tolerance in this model, with MMP7 being a key target. Determining the mechanisms whereby MMP7 contributes to transplant tolerance may help in the development of new strategies to improve long-term graft outcome.

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D espite improvements in renal allograft survival over the last three decades, the half-life of renal allografts has increased only marginally, largely due to late graft injury resulting from drug-related nephrotoxicity and chronic rejection (1–4). Such chronic injury is poorly influenced by the currently used immunosuppressors. Moreover, life-long immunosuppression puts transplant patients at a higher risk of infection and malignancy (5). Thus, inducing donor-specific tolerance, i.e., indefinite survival of a well-functioning graft in an immunocompetent adult host in the absence of immunosuppression, is a major goal in transplantation. A number of tolerance trials are currently taking place, but there are still many challenges to face before tolerance can be achieved routinely in clinic practice (6).

In rodents, a variety of maneuvers can induce donor-specific allograft tolerance, including pretransplantation priming with donor MHC Ags (blood or splenocytes) (2) (1, 2, 7–10), transfected cells expressing donor MHC Ags (11, 12), MHC gene transfer (13, 14), or DNA vaccination (15).

We have previously shown that donor-specific tolerance to MHC-mismatched renal allografts in adult rats can also be induced by the administration of Abs directed against donor MHC class II to recipients on the day of transplantation (16–18). These animals display normal and stable kidney function, and their grafts display normal histology with no signs of chronic rejection (18). Although numerous studies have shown the importance of MHC II signaling in the regulation of APC activity and fate (19), the mechanisms responsible for maintaining this anti-MHC II Ab-induced tolerant state remain to be fully elucidated. In the current study we took advantage of genome-wide transcriptome analysis to further explore these mechanisms. By analyzing intragraft gene expression and networks 100 days after transplantation in tolerated kidneys compared with syngeneic controls, we were able to identify a major involvement of several gene pathways, including that of wingless-type (WNT) signaling, a pathway known to control a variety of processes.

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4 Abbreviations used in this paper: WNT, wingless-type; Axin, axis inhibitor; Dkk3, dickkopf homologue 3; DST, donor-specific blood transfusion; PC, fold change; HPRT, hypoxanthine phosphoribosyltransferase; LEW, Lewis (rat); MMP7, matrix metalloproteinase 7; NFATc1, NFAT cytoplasmic, calcineurin-dependent 1; PKC, protein kinase C; PLC, phospholipase C; TCF7, transcription factor 7; TGF, transforming growth factor.

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such as cell migration, polarity, or differentiation (20). Deregulation of this pathway has been reported in numerous human diseases (20), but to date it has never been implicated in the field of immune tolerance. One of the targets of this pathway, matrix metalloproteinase 7 (MMP7; also known as matrilisin, MAT, MMPM, MPSL1, and PUMP-1), was explored further in tolerated and rejected rat heart allografts. We show that in rodents MMP7 up-regulation is governed by a noncanonical WNT pathway and is specific to the renal transplant tolerance induced by anti-donor class II treatment, where it is located on vascular smooth muscle cells within the graft. These results reveal a novel role for MMP7 in allograft responses. Determining the mechanisms whereby MMP7 contributes to transplant tolerance may help in the development of new strategies to improve long-term graft outcome.

Materials and Methods

Rodent transplant models

Surgical procedures, treatments, and experimental groups. Inbred male adult Lewis (LEW) rats (200–250 g) of the LEW.1A (RT1a) and LEW.1W (RT1u) congenic strains were purchased from Janvier and maintained in an animal facility under standard conditions according to European and institutional guidelines.

Kidney transplantation. The model used was that of kidney allotransplantation from LEW.1W donors to MHC-mismatched LEW.1A recipients receiving i.v. 0.5 ml of anti-LEW.1W class II alloimmune serum prepared as described (17) on the day of transplantation (n = 12). At day 100 posttransplantation, these animals tolerate a skin graft from the same donor and their kidney grafts show no histological signs of chronic humoral rejection (18). Controls included a group of LEW.1A recipients of LEW.1A kidneys (syngeneic transplants; n = 5) and a group of untreated LEW.1A recipients of LEW.1W kidneys (allogenic, untreated; n = 3). Kidney transplantations were performed aseptically and a binephrectomy was performed 7 days after transplantation as previously described (19). Rejection, indicated by the death of the binephrectomized rat, was confirmed by histology. Blood urea and creatinine and urine protein/creatinine ratios were measured throughout the posttransplant period. Blood urea at <8 mmol/L and blood creatinine at <40 mmol/L were considered as normal (18).

Heart transplantation. The model used was that of heart allotransplantation from LEW.1W donors to MHC-mismatched LEW.1A recipients receiving either a donor-specific blood transfusion (DST) 7 and 14 days before transplantation (20) or a 20-day course of the deoxyspergualin analog LF015-095 (21). At day 100 posttransplantation, the grafts of DST-treated animals show clear-cut histological signs of chronic humoral rejection (chronic transplant vasculopathy) (9), whereas those of LF015-095-treated animals do not and are thus tolerated (21, 22). Analyses were performed on DST-treated (n = 3) and LF015-095-treated recipients (n = 3) at day 100 posttransplantation. Syngeneic transplants (LEW.1A donors to LEW.1A recipients) were performed as controls (n = 3). Heterotopic cardiac allografts were performed as previously described (23).

Sample preparation

Organs were harvested at day 100 (tolerant/syngeneic kidney allografts) or 1 day before rejection (acutely rejected kidney allografts). Blood was collected by cardiac puncture into a heparinized syringe and PBMC were collected by cardiac puncture into a heparinized syringe and PBMC were collected by density sedimentation using a Ficoll gradient and then stored in TRIZol reagent (Invitrogen Life Technologies). Sections of the spleen and kidney graft were either snap frozen in liquid nitrogen for future RNA extraction, or embedded in O.C.T. compound (Tissue-Tek; Miles Laboratories) and snap frozen in liquid nitrogen for future immunohistochemistry analysis. Blood cell contamination was avoided by perfusing the organ with PBS. All samples were stored at −70°C until use.

RNA extraction and reverse transcription

Total RNA from rat kidney grafts and PBMC was prepared using the TRIZol (Invitrogen Life Technologies) extraction method. RNA quantity and quality were determined using a NanoDrop spectrophotometer and an Agilent 2100 bioanalyzer. Ten micrograms of total RNA was set aside for microarray analyses (see below). The remaining RNA was treated with Dnase (Roche) to remove genomic DNA and reverse transcribed into first strand cDNA using poly(dT) oligonucleotide and Moloney murine leukemia virus reverse transcriptase (Invitrogen Life Technologies).

Microarray experiments

RNA preparation, amplification, and hybridization. Ten micrograms of total RNA was cleaned up using RNeasy columns (Qiagen). RNA quantity and quality were determined using a NanoDrop spectrophotometer and an Agilent 2100 bioanalyzer. A rRNA 28S/18S ratio of 1.0 ± 0.1 was considered as acceptable for RNA amplification. The Applied Biosystems rat genome survey microarray (part no. 4337467) used contained 26,857 60-mer oligonucleotide probes representing 27,088 individual rat genes. An additional 3400 control spots were present on the chip to cover various steps in the hybridization process. Digoxigenin-UTP labeled cRNA was generated and amplified from 0.5 μg of total RNA from each sample using an Applied Biosystems NanoAmp chemiluminescent reverse transcriptase-in vitro transcription (RT-IVT) labeling kit (part no. 4365715). Array hybridization was performed for 16 h at 55°C. Chemiluminescence detection, image acquisition, and analysis were performed using the Applied Biosystems chemiluminescence detection kit (part no. 436875D), analyzer (part no. 4338036) and version 1.1 analyzer software (part no. 4338391) according to the manufacturer’s protocol.

Microarray analysis. Microarray raw data were analyzed by the R language and environment for statistical computing and graphics. Genes were identified using the Panther Protein Classification System Probe ID database. Raw data were analyzed according to p value and fold change (FC). FC corresponds to the ratio of the signal intensity of the gene in one treatment group to the signal intensity of the gene in syngeneic rats. FC was calculated for genes that fulfilled the criterion of significance at p < 0.05 (Fig. 1A), and the genes were then processed using software available on www.pantherdb.org. Genes were classified according to their signaling pathway, molecular function, or biological process. For each category, the 20 up-regulated and down-regulated genes were chosen and matched whenever they belonged to the same group (Tables I–III).

Real-time quantitative PCR

Real-time quantitative PCR was performed in an Applied Biosystems GenAmp 7700 sequence detection system using SYBR-Green PCR core reagents as previously described (18) or TaqMan gene expression assays according to the manufacturer’s instructions (Applied Biosystems). Rat primer and probe sets were either designed (rat transcription factor (TCF) GCTGAACAGTACATTGCTCAT (forward) and 5′-GGACACCAGTGCACACCAGAAAG (reverse); rat β-actin 1 and 2, ACTATGGCCACAGACGACGACAT (forward) and CCCTCTCCCTGACGAGACTGTC (reverse); rat hypoxanthine phosphoribosyltransferase (HPRT), CTTTG TGAACAGTACAGCC (forward) and CGCTGTACGACACAACTG (reverse); and NFAT cytoplasmic, calcineurin-dependent 1, GGAGATGGAAGCGAAAACTGA, NFATc1 (forward) and TAAGTCTCCGGCTACCTGTGC (reverse)) or purchased from Applied Biosystems (Dkk3, Rn00593415_m1; Axin inhibitor 2, Rn00577441_m1; cadherin 13, Rn00594145_m1; HPRT, Rn01527838; g1; and Mmp7, Rn00563467_m1). HPRT was used as an endogenous control gene to normalize the varying starting amounts of RNA. Relative expression between a given sample and a control sample, used for all experiments, was calculated with the 2−ΔΔCt threshold cycle method (ABI PRISM 7900 user bulletin 2:11-24 (1997); PE Applied Biosystems). All samples were analyzed in duplicate. Expression of genes of interest was compared between tolerated animals and syngeneic controls.

Immunohistochemistry

 Cryostat sections of 5 μm were cut, air-dried, and fixed in acetone for 10 min. Sections were then either labeled by a two-step immunofluorescence technique using rabbit anti-rat MMP7 (Calbiochem) as the primary Ab and donkey anti-rabbit FITC (Jackson ImmunoResearch Laboratories) as the secondary Ab or by a three-step indirect immunoperoxidase technique as described (24) with the same primary and secondary Abs. To confirm MMP7 expression by smooth muscle cells, we performed double staining using a two-step immunofluorescence technique using mouse anti-rat smooth muscle actin and rabbit anti-smooth muscle actin as the primary Abs. Anti-rabbit FITC (Jackson ImmunoResearch Laboratories) and goat anti-mouse Alexa Fluor 568 (Invitrogen Life Technologies) were used as secondary Abs.
Statistical analysis

The nonparametric Mann-Whitney and Kruskal-Wallis tests were used to compare two or three or more groups, respectively. Differences were defined as statistically significant as follows: *, \( p < 0.05 \); **, \( p < 0.01 \); and ***, \( p < 0.001 \).

Results

Differential gene expression patterns in tolerated vs syngeneic kidney grafts 100 days after transplantation

We have previously shown that a single administration of anti-donor MHC class II alloimmune serum on the day of transplantation results in the indefinite survival of MHC-mismatched kidney grafts. The kidney grafts of tolerated animals display normal function and have no histological evidence of chronic rejection (18). In this study the gene expression patterns of three tolerated and three syngeneic kidney allografts were analyzed using pangenomic Applied Biosystems genome survey microarrays (part no. 4337467). Of the \( \sim 27,000 \) genes present on the microarray, 26,857 genes were found to be expressed and were subsequently analyzed by R software. Of these 26,857 expressed genes, 5,954 were found to be significantly expressed differentially between the two biological situations (\( p < 0.05 \); Fig. 1). Analysis of these 5,954 genes using the Probe ID database from the Panther protein classification system (www.pantherdb.org) enabled their organization according to signaling pathways.

### Table I. Organization of differentially expressed genes according to signaling pathways

<table>
<thead>
<tr>
<th>Signaling Pathway</th>
<th>No. of Up-Regulated Genes</th>
<th>No. of Down-Regulated Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation mediated by chemokine and cytokine signaling pathway</td>
<td>76</td>
<td>24</td>
</tr>
<tr>
<td>Integrin signaling pathway</td>
<td>57</td>
<td>21</td>
</tr>
<tr>
<td>WNT signaling pathway</td>
<td>45</td>
<td>26</td>
</tr>
<tr>
<td>Platelet-derived growth factor signaling pathway</td>
<td>42</td>
<td>22</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>38</td>
<td>18</td>
</tr>
<tr>
<td>T cell activation</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Epidermal growth factor receptor signaling pathway</td>
<td>34</td>
<td>16</td>
</tr>
<tr>
<td>Apoptosis signaling pathway</td>
<td>31</td>
<td>15</td>
</tr>
<tr>
<td>Interleukin signaling pathway</td>
<td>31</td>
<td>13</td>
</tr>
<tr>
<td>Huntington disease</td>
<td>29</td>
<td>15</td>
</tr>
<tr>
<td>Heterotrimeric G protein signaling pathway/G(<em>{\alpha_i}) and G(</em>{\alpha_s})-mediated pathway</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>Heterotrimeric G protein signaling pathway/G(<em>{\alpha_i}) and G(</em>{\alpha_s})-mediated pathway</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>TLR signaling pathway</td>
<td>24</td>
<td>12</td>
</tr>
<tr>
<td>PI3K pathway</td>
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<td>12</td>
</tr>
<tr>
<td>B cell activation</td>
<td>23</td>
<td>12</td>
</tr>
<tr>
<td>Fibroblast growth factor signaling pathway</td>
<td>22</td>
<td>16</td>
</tr>
<tr>
<td>Cytoskeletal regulation by Rho GTPase</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Parkinson disease</td>
<td>21</td>
<td>11</td>
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<tr>
<td>Ras pathway</td>
<td>18</td>
<td>11</td>
</tr>
<tr>
<td>Endothelin signaling pathway</td>
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<td>11</td>
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<tr>
<td>Nicotinic acetylcholine receptor signaling pathway</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Ubiquitin-proteasome pathway</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Adrenaline and noradrenaline biosynthesis</td>
<td>13</td>
<td>13</td>
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<tr>
<td>Caderhin signaling pathway</td>
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<tr>
<td>SHT2-type receptor-mediated signaling pathway</td>
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<td>De novo purine biosynthesis</td>
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<td>Alzheimer disease-presenilin pathway</td>
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<tr>
<td>Tricarboxylic acid cycle</td>
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</table>
signaling pathways (Table I), molecular function (Table II), and biological process (Table III). According to this analysis, the most significantly up- and down-regulated genes in tolerance belonged to the categories of the inflammation chemokine- and cytokine-mediated signaling pathway, integrin signaling, the WNT signaling pathway, platelet-derived growth factor signaling, and angiogenesis, as well as epidermal growth factor receptor, IL, and apoptosis signaling (Table I). Certain major categories consisted of genes that were up-regulated only, e.g., T cell activation and G-protein, TLR, and B cell signaling (Table I). The majority of the up-and down-regulated genes belonged to the molecular function categories of nucleic acid binding, receptors, transcription factors, and regulatory molecules, whereas again certain functional categories were up-regulated only, e.g., defense/immunity proteins, signaling molecules, and ribosomal proteins (Table II). The highest-ranking biological processes were signal transduction, protein/nucleic acid metabolism, and immunity/defense (Table III), suggesting that tolerance is correlated with an active intragraft immune response (18).

**Signaling pathways distinguish tolerated from syngeneic kidneys: the WNT pathway and its downstream molecular target MMP7**

The two highest ranking pathways were those of inflammation and integrin signaling, whose roles in transplantation are already well known. The third highest ranking pathway, however, has to our knowledge never been described in transplantation. This WNT signaling pathway implicated 45 up-regulated and 26 down-regulated genes, of which three molecules were expressed at least 10-fold more in tolerated vs syngeneic kidneys: MMP7 (FC > 40), TCF7 (T cell specific; FC > 15), and dickkopf homologue 3 (DKK3; FC > 12). DKK3 and TCF7 as measured by quantitative PCR. Results are expressed as mean ± SD of the 2−ΔΔCt (threshold cycle) values for each group of samples. *, p < 0.05; **, p < 0.01.

**FIGURE 2.** Differential expression of MMP7 and other MMP family members in rat kidney allografts. A, Fold change in expression of MMP2, MMP7, MMP12, MMP16, MMP19, and MMP23 in tolerant (n = 3) vs syngeneic (n = 3) kidney allografts 100 days posttransplantation according to microarray. B, MMP7 mRNA accumulation in naive, syngeneic (day 100), tolerant (day 100), and acutely rejecting (day 11) rat kidney allografts as measured by quantitative PCR. Results are expressed as mean ± SD of the 2−ΔΔCt (threshold cycle) values for each group of samples. *, p < 0.05; **, p < 0.01.
confirmed by quantitative PCR performed on a further 12 tolerated kidneys (>100 days posttransplantation) and five syngeneic grafts (>100 days posttransplantation), as well as three naive kidneys that expressed no MMP7 and three acutely rejected kidneys at day 11 ± 1 posttransplantation that expressed MMP7 only moderately (Fig. 2B).

Involvement of the noncanonical WNT signaling pathway in the kidney grafts of tolerated recipients

We next set out to understand the mechanisms governing MMP7 expression in our model. Previous studies have shown that MMP7 expression can be controlled by the canonical WNT signaling pathway (26, 27) (illustrated in Fig. 3, A and B). Briefly, in the absence of WNT or if the WNT pathway is inhibited by DKK, β-catenin becomes phosphorylated by a destruction complex and is degraded by the proteasome. When WNT is present it binds to the Frizzled receptor and the coreceptors, preventing β-catenin degradation. β-Catenin then migrates to the nucleus, where it binds TCFs to activate target genes.

FIGURE 3. Regulation of MMP7 by the canonical WNT signaling pathway. A. The inhibited canonical WNT signaling pathway. In the absence of WNT, β-catenin associates with a multiprotein destruction complex consisting of the tumor-suppressor gene products Axin and adenomatous polyposis coli (APC) and the serine/threonine kinases casein kinase 1 (CK1) and glycogen-synthase kinase 3β (GSK3β). β-Catenin is phosphorylated by CK1 and GSK3β, leading to its recognition by β-transducin-repeat-containing protein (β-TRCP), its ubiquitylation, and its subsequent degradation by the proteasome. Members of the TCF/lymphocyte enhancer-binding factor (LEF) family are blocked in the nucleus by the repressors and are inactive. DKK3 can also take part in the inhibition of the WNT canonical signaling pathway. B. The activated canonical WNT signaling pathway. WNT proteins bind their receptors, Frizzled proteins, in addition to a coreceptor low-density lipoprotein receptor related protein (LRP) 5 or LRP6, resulting in the inactivation of GSK3β by Disheveled. β-Catenin then migrates to the nucleus, where it binds TCFs to activate target genes. C. Expression of various molecules implicated in the canonical WNT pathway in tolerant and syngeneic kidneys 100 days posttransplantation according to microarray. Results are expressed as fold change. D. Expression of various molecules implicated in the canonical WNT pathway in tolerant and syngeneic kidneys 100 days posttransplantation according to quantitative PCR. Results are expressed as means ± SD of 2^(-ΔΔCt) (threshold cycle) values. *, p < 0.05; **, p < 0.01.
kidneys according to both microarray (Fig. 3C) and quantitative PCR (Fig. 3D). Moreover, β-catenin, a key molecule in regulating MMP7 expression, was undetectable. The fact that the level of the WNT pathway inhibitors was increased as well as that of MMP7 indicates the involvement of the noncanonical WNT signaling pathway.

**Regulation of MMP7 by the noncanonical WNT signaling pathway in tolerated kidneys**

In addition to the canonical WNT pathway, there is also a so-called noncanonical pathway (illustrated in Fig. 4A). This pathway is activated by ligand binding to the Frizzled receptor, leading to the release of intracellular calcium possibly via G proteins. Activation of this pathway in our model of tolerance is coherent with the fact that G protein signaling pathways were one of the major pathways up-regulated in tolerated kidneys, accounting for 52 genes (Table I). This pathway involves the activation of phospholipase C (PLC) and protein kinase C (PKC). Elevated Ca2+ can activate the phosphatase calcineurin, which leads to dephosphorylation of the transcription factor NFATc1 and its accumulation in the nucleus (28, 29). Most components of the noncanonical WNT signaling pathway were detected by the microarray, including WNT6 (FC > 3), Frizzled6 (FC > 2), Frizzled2 (FC > 2.5), and NFATc1 (FC > 3) (Fig. 4B). Up-regulation of one of the key molecules in the noncanonical WNT signaling pathway, NFATc1 (which enters the nucleus and activates c-myc; Ref. 30), was additionally confirmed by quantitative PCR (Fig. 4C). Thus, altogether our data suggest that the high MMP7 expression (FC > 40) in tolerated kidney grafts is the result of noncanonical WNT signaling.

**Regulation of MMP7 in the tolerated kidneys and hearts in the same strain combination following different protocols of tolerance induction**

To determine whether MMP7 up-regulation was tissue specific, we next compared MMP7 expression in kidney grafts and spleens from tolerant animals and syngeneic controls. As shown in Fig. 5A, MMP7 transcripts were only detected in tolerated kidney grafts, being very weak in the other compartments (spleen and blood). Kinetic studies also showed that MMP7 expression in tolerant kidneys was time dependent, with a high level of transcript as soon as day 25 after transplantation (24.8- ± 8.7-fold tolerant vs syngenic) (data not shown). We then asked whether MMP7 up-regulation was specific to tolerated kidneys or could it also be relevant to
tolerated kidneys. We therefore compared MMP7 expression in tolerated kidneys to that observed in long-surviving heart grafts (day 100 posttransplantation) transplanted in the same allogeneic strain combination (LEW.1W to LEW.1A). To induce heart allograft tolerance, we used different protocols: DST 7 and 14 days before transplantation (grafts showing clear histological signs of chronic rejection) (9) or a 20-day course of the deoxyspergualin analog LF015-095 (grafts showing no signs of rejection; i.e., tolerated) (21). The results clearly show an absence of MMP7 expression in heart grafts from both DST-treated (chronic rejection) and LF015-095-treated (tolerated) recipients at this time (Fig. 5B), suggesting that MMP7 expression is specific to kidney graft tolerance induced by the anti-donor MHC class II protocol.

**MMP7 expression by tolerated kidney vascular smooth muscle cells**

Localization of MMP7 was next assessed by the immunostaining of syngeneic vs tolerated kidneys using immunoperoxidase (Fig. 6, A and B) or immunofluorescence (Fig. 6C) techniques. Within the tolerated kidney allografts, MMP7 protein was found to be expressed primarily by the smooth muscle cells of blood vessels (Fig. 6, B and C). This was confirmed using double staining for MMP7 and anti-actin as a marker for the smooth muscle cells. Photos of red fluorescence (α-actin staining; Fig. 6D) and green fluorescence (MMP7 staining; Fig. 6E) were superimposed (Fig. 6F) and orange fluorescence indicated that MMP7 was expressed by the smooth muscle cells.

**Discussion**

In this study, we further explored the mechanisms involved in the maintenance phase of tolerance to kidney allografts induced by the administration of anti-donor class II serum to rat recipients of renal allografts on the day of transplantation (18). By analyzing intra-graft gene expression profiles of tolerated compared with syngeneic kidneys 100 days posttransplantation using microarray technology, we show that the maintenance phase of tolerance brings into play multiple gene pathways and biological processes. In particular, our data reveal a potentially important role for a signaling pathway that has not previously been described in transplantation, that of WNT signaling, as well as for one of its key targets, MMP7 (25), which was up-regulated 40-fold in tolerated kidneys.

WNT signaling, which is initiated by binding members of the WNT family of glycoproteins to their respective receptors (also known as Frizzled proteins), is known to be involved in the regulation of cell growth and differentiation (27). Two main WNT signaling pathways exist, the so-called canonical and the less classical noncanonical pathways. The canonical pathway, when activated, results in a complex signaling cascade ultimately leading to β-catenin-mediated gene transcription. In this pathway’s resting state, β-catenin is degraded by the proteasome. The noncanonical pathway is a Ca\(^{2+}\)-dependant pathway thought to act through G protein-coupled receptors. Activation of this pathway results in gene transcription via the transcription factor NFATc1. Although the precise and relative roles of both pathways in mammals are largely unknown, deregulation of WNT signaling has been reported in a number of human pathologies, from cancer to cardiovascular disease (31–33). To date, its role in transplantation is unknown. However, our findings of an up-regulation of the potential WNT inhibitor DKK3 (34) as well as the up-regulation of other inhibitory molecules acting downstream within the canonical pathway such as Axin2 (35), together with the absence of β-catenin, suggest that this pathway was not responsible for MMP7 up-regulation in the tolerated kidneys. In contrast, the overexpression of components of the noncanonical pathway such as Frizzled 6, Frizzled 2, and NFATc1 in tolerated kidneys indicates that MMP7 expression was driven by this alternative pathway. These results thus imply a role for noncanonical WNT signaling in maintaining...
kidney transplant tolerance in this model, with MMP7 being a key target.

MMP7 is the smallest matrix metalloproteinase (36), a family of enzymes classically described as being responsible for the turnover and degradation of connective tissue proteins (37, 38) and for the turnover, degradation, catabolism, and destruction of the extracellular matrix (39, 40). The time-dependent up-regulation of MMP7 transcripts in tolerated kidneys (no expression on day 0 and increasing expression on day 25 to day 100 after transplantation compared with syngeneic or acutely rejected grafts) suggests that this molecule may play a role in maintaining tolerance. Interestingly, this role appears to be specific to the anti-donor class II model of kidney transplantation, as no up-regulation of MMP7 was observed in other models of kidney transplant tolerance in the same strain combination (anti-CD28 tolerance induction protocol (41); data not shown) or in models of heart tolerance or chronic rejection (LF015-095 and DST protocols) (9, 21, 42). We have already shown in the laboratory that different mechanisms may operate following different tolerance induction protocols, even in the same genetic LEW.1W to LEW.1A background (8). Indeed, whereas the anti-MHC II Ab-induced tolerant state can be successfully transferred to naive recipients by both T cells and CD103+ cells (18), transfer failed in the same kidney allograft model when tolerance was induced using anti-CD28 Abs (41). In the heart allograft model, Degauque et al. showed that in donor specific transfusion-induced tolerance, spleen CD25+ T cells from tolerant rats were unable to transfer tolerance into naive rats (8), whereas Chiolfela et al. provided evidence that 20-day treatment with LF015-0195, a deoxyspergualin analog, induced spleen CD4+CD25+ cells able to transfer tolerance (43). Finally, in the same heart allograft model when recipients were treated with CD40lg, only the adaptively transferred CD8+CD45RClow subset resulted in donor-specific long-term survival, whereas CD8+CD45RChigh T cells from naive animals did not (44). All of these examples show that, in the same allograft model, different protocols lead to specific and distinct mechanisms of tolerance induction and maintenance.

The mechanisms whereby MMP7 contributes to tolerance maintenance require further investigation. One possibility is that MMP7 helps to maintain tolerance through protective effects, because MMPs have been shown to contribute to tissue repair (45). Another possibility is that MMP7 acts by regulating cellular transmigration to the graft. Indeed, MMPs can act on nonmatrix proteins such as cytokines and chemokines to potentiate their activity (46). As such, MMPs have been shown to regulate inflammatory cell influx by determining the bioavailability of chemokines (46, 47). Thus, MMP7-deficient mice are more sensitive to acute lung injury after the administration of a chemotactic peptide (48). Although the tolerated grafts display a larger infiltrate than their syngeneic counterparts (18), MMP7 may serve to regulate the type of cells entering the graft, perhaps favoring a “friendly” infiltrate as previously suggested (18). Along these lines, MMP7 has been shown to direct cell migration rather than just movement per se (48). Interestingly, the regulation of cell transmigration has been reported to be not only due to the MMP7 protease but also to other proteases such as MMP2, MMP12, and the ADAM17 (a disintegrin and metalloproteinase 17) molecule (49), which were up-regulated (>11-fold, >8-fold, and >2.8-fold, respectively) in the tolerated kidney grafts vs syngeneic controls.

Another hypothesis is that MMP7 cleaves particular substrates, releasing molecules with tolerogenic activity. Along these lines, MMP7-deficient mice have been reported to reject a skin allograft significantly earlier than wild-type mice, possibly through its cleavage of FasL and TNF-α (50). Perhaps even more relevant to our findings, however, is the fact that MMP7 has been described as one of the most potent MMPs in its ability to degrade the ubiquitous proteoglycan decorin, thereby releasing transforming growth factor (TGF-)β1 (51), a cytokine long known for its immunomodulatory capacities (52, 53) and its involvement in transplant tolerance (54). Supporting this hypothesis, in the same LEW.1W to LEW.1A strain combination we have previously shown that TGF-β1 is significantly increased in rat heart allografts with prolongation of graft survival in the first days following transplantation (17). TGF-β1 was also up-regulated in this present study in the tolerated rat kidney allografts >100 days posttransplantation (data not shown). Furthermore, in the same model of tolerance induction to mismatched kidney grafts, anti-TGF-β mAbs break the tolerance process (17). Finally, it is important to note that MMP7 has already been detected in smooth muscle cells in the medium and in the stenotic region in patients with supravalvular aortic stenosis and Williams Beuren syndrome and that remodeling of the stenotic part of the aorta has been shown to be an adaptive mechanism to preserve a correct function of the aorta (55).

To conclude, we report for the first time, a role for noncanonical WNT signaling in the state of kidney allograft tolerance induced by administration of anti-donor class II Abs in rats. We also report on a new potential role for MMP7, a target of this pathway, in maintaining tolerance in this model. Determining the underlying mechanisms of this pathway and MMP7 may help in the design of strategies to improve long-term graft outcome.

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References


