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ORIGINAL ARTICLE

Autoimmune responses against renal tissue proteins in long-term surviving allograft recipients

Cheng Fang,[1,2,3]* Caroline Ballet,[1,2,3]* Anne-Sophie Dugast,[1,2,3] Anne Godard,[4] Anne Moreau,[5] Claire Usal,[1,2,3] Helga Smit,[1,2,3] Bernard Vanhove,[1,2,3] Sophie Brouard,[1,2,3] Jean Harb[1,2,3]* and Jean P. Soulillou[1,2,3]*

1 Institut National de la Santé Et de la Recherche Médicale (INSERM) – Université de Nantes, UMR 643, Nantes, France
2 Institut de Transplantation Et de Recherche en Transplantation (ITERT), CHU-Hotel Dieu, Nantes, France
3 Réseau Thématique de Recherche et de Soin (RTRS) Centaure, Nantes, France
4 Institut National de la Santé Et de la Recherche Médicale (INSERM) – Université de Nantes, UMR 892, Nantes, France
5 Pathology Laboratory, CHU-Hotel Dieu, Nantes, France

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Correspondence
Pr. Jean Paul Soulillou, INSERM UMR 643, CHU-Hotel Dieu, 30 Bd. Jean Monnet, 44093 Nantes Cedex 01, France. Tel.: +33 2 40 08 74 10; fax: +33 2 40 08 74 11; e-mail: jean-paul.soulillou@univ-nantes.fr

*All these authors contributed equally to this work.

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Summary

Major histocompatibility complex antigens (MHC) are classical targets of recipient responses to allotransplants. However, the role of an immune response directed against autologous graft tissue determinants is poorly defined. In this study, we investigated (i) whether autologous kidney tissue extract can induce an immune response to autologous kidney proteins in normal rats, and (ii) if a similar autologous response develops in the long-term surviving LEW.1A recipients of an MHC-mismatched LEW.1W kidney ($RT1^u$ to $RT1^a$). LEW.1A rats immunized with allo- or syngeneic soluble kidney extracts developed a T-cell response to self antigens as shown by the frequency of specific IFN-γ-producing T cells from LEW.1A rats in the presence of extracts (ELISPOT). In contrast, they responded only marginally to dominant $RT1^u$ determinants. The ELISPOT against fractions of soluble autologous kidney extracts separated by an FPLC gel-filtration system indicated a preferential response to megalin, a high molecular weight protein that has been shown to be involved in experimental Heymann nephritis. In a model of long-term kidney allograft survival by anti-CD28 administration, recipients also developed humoral but not cellular responses to megalin. Our data suggest that autoimmune processes develop in long-term surviving kidney allograft recipients.

Introduction

Immune-mediated graft injury resulting in chronic rejection is the most frequent cause of long-term allograft loss [1]. Major histocompatibility complex (MHC) antigens have been overwhelmingly established as the major molecular targets of T cells following allotransplantation [2,3]. However, little is known regarding autoimmunity following allotransplantation, the nature of the autologous antigens potentially involved, or the respective frequency of T cells committed to MHC and non-MHC determinants in long-term surviving transplant recipients undergoing chronic rejection.

In humans, the role of an immune response directed against autologous determinants within the graft following transplantation is poorly defined, particularly for T-cell effectors [4]. Despite effective immunosuppression in cardiac transplant recipients, antibodies reacting with vimentin, a major endothelial antigen are an independent predictor of transplant-associated coronary artery disease [3,5]. Vimentin-specific tetramer-binding CD8+T cells have also been detected in the blood [6]. In addition, long-term kidney graft recipients with chronic allograft nephropathy (CAN) and transplant glomerulopathy also develop antibodies against a series of autologous determinants [1]. Histocompatible patients can also develop

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anti-donor antibodies against non-MHC antigens, associated with CAN [7]. For example, antibodies reacting with agrin, a heparan sulfate proteoglycan (HSPG) of the glomerular basement membrane [8], were found in seven out of 16 (44%) patients with transplant glomerulopathy [9].

Recipients of cardiac transplant undergoing chronic rejection develop a response to cardiac myosin in rodent [10–13]. In lung recipients, a response to a collagen V determinant has been also reported [14,15]. Thus, the possibility that graft recipients develop an immune response to autologous determinants and the possibility that allograft rejection could create conditions that rupture tolerance to self-antigens [10] have raised another level of complexity concerning the host response to an allograft, particularly in the understanding of late graft dysfunction.

In this article, we first established that immunization of normal LEW.1A rats against soluble kidney extract, without adjuvant, can elicit a weak but significant T-cell immune response directed against autologous kidney determinants. Using these data as positive control, we then show that immunized normal or long-term LEW.1A recipients of a mismatched kidney graft can also develop antibodies against megalin, a high molecular weight kidney tissue protein that has been shown to be involved in Heymann nephritis disease [16,17]. Furthermore, we show that this response still occurs in a model of induction of long-term kidney graft survival: injection of anti-CD28 antibody [18] with chronic rejection [19]. These results indicate that manoeuvres promoting long-term graft survival do not prevent the breakdown of self-tolerance in kidney allograft recipients. Altogether, these data suggest that autoimmunity occurs in model of long-term surviving grafts and show that further analyses of cellular and humoral responses to tissue antigens are warranted in humans with chronic graft dysfunction.

Materials and methods

Animals

Seven- to eight-week-old male LEW.1W (LEW.1W, haplotype $RT1^u$) and LEW.1A (LEW.1A, haplotype $RT1^a$) congenic rats were obtained from the Centre d'Elevage Janvier, Le Genest-Saint-Isle, France and housed under conditions according to European and Institutional guidelines.

Preparation of soluble kidney extract (SKE)

The kidneys were obtained from LEW.1A and LEW.1W naive rats. After careful washing, kidney tissues were minced finely with scissors in ice-cold PBS. Next, small kidney pieces were homogenized with three volumes of PBS at 4 °C. The homogenate was submitted to three cycles of freeze-thawing and centrifuged at 2000 *g* for 20 min at 4 °C. Insoluble material was further removed from the centrifuged crude extract by ultracentrifugation at 100 000 *g* for 60 min at 4 °C. The final supernatant was then dialysed against PBS (3 × 100 volumes) for 24 h and concentrated by dialysis against 50% sucrose at 4 °C using Spectra/Pro 3 dialysis membranes (Spectrum, Gardena, CA, USA). This solution, referred to as soluble kidney extract (SKE), was filtered through cellulose nitrate membranes with a pore size of 0.2 μm (Poly Labo, Strasbourg, France). The protein concentration was measured using a protein assay reagent kit (Uptima, Montluçon, France) and was about 40 mg/ml. The SKE was stored in 1 ml aliquots at −80 °C until use.

Experimental groups

Group auto-immunization ($n = 4$) consisted of LEW.1A rats immunized by intraperitoneal (i.p.) injection with 1 mg of LEW.1A SKE at 0, 7 and 21 days without complete or incomplete Freund's adjuvant. In group with allo-immunization, LEW.1A rats ($n = 4$) were injected with 1 mg of LEW.1W SKE at 0, 7 and 21 days. At each time point (10, 14, 21, 35 and 70 days), rats were sacrificed for harvesting spleen in each group. The LEW.1A rats injected with 1 mg hen egg lysozyme (HEL; Sigma, MO, USA) or PBS only were sacrificed at 35 days ($n = 3$) and served as positive and negative controls respectively.

Isolation of T cells from spleens

Isolation of T cells was performed as previously described [20]. Briefly, spleen cells were isolated by passing the spleen through a stainless steel mesh. Erythrocytes were depleted by osmotic shock. Mononuclear cells were washed twice in phosphate-buffered saline (PBS) and re-suspended in RPMI 1640 (life Technologies, Grand Island, NY, USA). T cells were then purified using a rat T-cell enrichment column (R&D systems, Lille, France). Purity was checked by flow-cytometry and was typically ≥94%.

Kidney transplantations and induction of long-term survival of MHC-mismatched kidney grafts

Life-sustaining, heterotopic LEW.1W kidney transplantation ($n = 4$) was performed in binephrectomized recipients as previously described [21]. Briefly, the kidney (right side) of the recipient (LEW.1A) was replaced by a LEW.1W donor allograft and a contralateral nephrectomy was performed 7 days later. Recipients were sacrificed at

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day 10 just before rejection to serve as acute rejection. In addition, a group of long-term surviving kidney graft recipients ($n = 3$) was used.: LEW.1A recipients of a LEW.1W kidney allograft received daily intraperitoneal injections of anti-CD28 (JJ319 mouse hybridoma anti-rat CD28, gift from Dr. Thomas huning, Wurzburg, Germany) from the day of transplantation to day 7 post-transplantation (1 mg/day) [18].This antibody induces a transient down-modulation of CD28 expression *in vivo*, without depleting target cells. Without treatment, the kidney grafts were rejected 11 days post-tranplantation. Syngenic transplants (LEW.1A to LEW.1A) served as controls and were not rejected.

ELISPOT

ELISPOT assays were performed as indicted by the manufacturer (BD, San Diego, CA, USA). Briefly, ELISPOT plates were coated with 5 μg/ml anti-rat IFN-γ capture antibody at 4 °C overnight. Then the plates were washed and blocked with RPMI 1640 containing 10% FBS for 2 h at room temperature. Splenocytes of LEW.1A rats were harvested at different times after the first injection of SKE lysates and T cells were purified. To measure T-cell responses, 8×10^5 T cells were incubated in complete RPMI 1640 medium in the absence or presence of Ags (SKE LEW.1A or SKE LEW.1W) (2 μg/well) for 24 h. After washing plates, 2 μg/ml biotinylated anti-rat IFN-γ detection antibody was added, followed by incubation with streptavidin-HRP diluted at 1:100 in PBS/10% FBS. After washing, final substrate solution was added for spot development within 10 min. The reaction was terminated with de-ionized water. The resulting spots were enumerated automatically using an ELISPOT plate reader (AID, StraBberg, Germany). ELISPOT using MHC-derived LEW.1W peptides have been performed as described in [19]. Briefly, after coating the plates with anti-rat IFNγ (5 μg/ml), day 35 T cells from LEW.1A rats immunized with LEW.1W SKE or from LEW.1A recipients rejecting a LEW.1W allograft (day 7), were stimulated for 24 h at 37 °C, 5% CO_2 in an IFN-γ ELISPOT assay with peptides 29 and 37 from donor RT1.D^u (β1 domain) molecules. Detection of spots was then performed as described above.

Gel-filtration of kidney-derived soluble extracts

A Hiload 16/60 Superdex 200 column connected to an AKTA FPLC system (GE Healthcare, Chalfont St Giles, UK) was equilibrated in PBS at room temperature. One millilitre of SKE was loaded onto the column, eluted by PBS at a flow rate of 1 ml/min, and each 1.5 ml fraction was collected. Molecular size markers were eluted by the same method as SKE and the chromatogram pattern was recorded simultaneously. For sensitization experiments, approximately 4% of each FPLC fraction was incubated with rat lymphocytes in ELISPOT assays.

ELISA

Megalin was detected by enzyme-linked immunosorbent assay. Briefly, fractions were coated at 100 μl/well overnight at 4 °C on 96-well enzyme-linked immunosorbent assay plates. Plates were blocked using 2% BSA in PBS. Sheep anti-megalin antibodies (kindly supplied by Dr. Pierre Verroust, Paris, France) were added to the plate in duplicate at 100 μl/well and incubated for 2 h at 37 °C. The plates were developed using peroxidase-conjugated anti-sheep IgG antibodies. To detect the presence of anti-megalin in rat sera, a capture ELISA method using a sheep anti-megalin antibody was performed. Wells were coated with 50 μl of sheep anti-megalin antibodies diluted at 1/500, and kept at 4 °C overnight. After blocking with PBS + 2% BSA, 50 μg of LEW.1A fraction 6 was added for 2 h at 37 °C. After washing, 100 μl rat sera diluted at 1/50 was added for 2 h at 37 °C. After rewashing, 50 μl of peroxidase-conjugated donkey anti-rat IgG antibodies diluted to 1/500 were added (Jackson Immunoresearch, PA, USA). Following 2-h incubation at 37 °C, ABTS substrate solution was added. Absorbance was read at 405 nm using an ELISA reader (Dynatech Laboratories, VA, USA).

RNA extraction and cDNA synthesis

Total RNA was extracted using Trizol reagents (Invitrogen, Carlsbad, CA, USA). A constant amount of 3 μg RNA was reverse-transcribed into cDNA at 42 °C for 60 min in a reaction mixture containing of 25 μg/ml of pdT24, 10 μg/ml random primers, 0.5 mM of each dNTP, 10 mM DTT, 2 U/μl RNase inhibitor, 1 × first-strand buffer and 10 U/μl MMLV reverse transcriptase. The cDNA synthesis reaction was brought to a final volume of 50 μl. Complementary DNA was stored at −20 °C until used for real-time quantitative PCR.

Quantitative reverse-transcriptase polymerase chain reaction

Direct quantification of PCR products was monitored by measuring the increase in fluorescence caused by the binding of the dye labeller SYBR Green to double-stranded DNA. The level of fluorescence, monitored by the ABI PRISM 7700 Sequence Detection Application program (Applied Biosystems, Foster City, CA, USA), is directly proportional to the level of PCR product. Briefly,

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10 microlitres of 1/10-diluted cDNA sample were amplified in 25 μl of SYBR Green PCR Core reagent (Applied Biosystems, Warrington, UK) with 0.6 U of AmpliTaq Gold polymerase, 0.25 U of Amperase uracyl-H-glycosylase, 200 nM of each dNTP, 300 nM of each primer and 3 mM of $MgCl_2$, in 1 × Power SYBR Green PCR Buffer. The transcripts were identified with IFN-γ, IL-2, IL-4, IL-10 and IL-13 primers (MWG-Biotech AG, Ebersberg, Germany). Amplifications were performed in an ABI PRISM 7700. Each amplification was performed using a TaqMan (Perkin-Elmer, Wellesley, MA, USA) in duplicate wells under the following conditions: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s, then 60 °C for 1 min. All results were normalized to HPRT.

Detection of IgG deposit in kidney graft

Renal tissues were processed for routine histological analysis and direct immunofluorescence according to standard techniques. Three-micrometer-thick cryostat sections were incubated at room temperature for 30 min with a Fluorescein (FITC)-conjugated affinipure $F(ab')_2$ fragment goat anti-rat IgG, Fcγ fragment-specific, diluted to 1/100 (Jackson Immunoresearch, Baltimore, PA, USA). Sections were then examined by fluorescence microscope.

Statistics

Statistical analysis was performed with the GraphPad Prism Software version 4.00 for windows (GraphPad Software, San Diego, CA, USA). The differences between two groups were analysed by the Mann-Whitney test. For more than two groups, data were analysed using the Kruskal-Wallis test followed by a Dunn's *post hoc* test. All statistical tests were two-sided. For all experiments, probability of error (*P*-values) was calculated. Values of $P < 0.05$ were regarded as significant.

Results

Immunization of naive LEW.1A rats with allogeneic or autologous SKEs generates specific IFN-γ-secreting T cells

We first tested whether autologous extract could trigger a response without adjuvant, in order to avoid strong artificial co-stimulation. The addition of SKE extracts to ELISPOT test performed with T cells obtained from LEW.1A rats after immunization with autologous SKE led to an increase ($P < 0.05$) in IFN-γ-producing cell frequency in comparison to medium alone (Fig. 1a). The frequency peaked on day 35 (24.5 ± 1.8, 23.5 ± 1.2 vs. 14.5 ± 3.0). Similarly, in LEW.1A rats immunized with allogeneic SKE, the basal level of IFN-γ-producing cells significantly

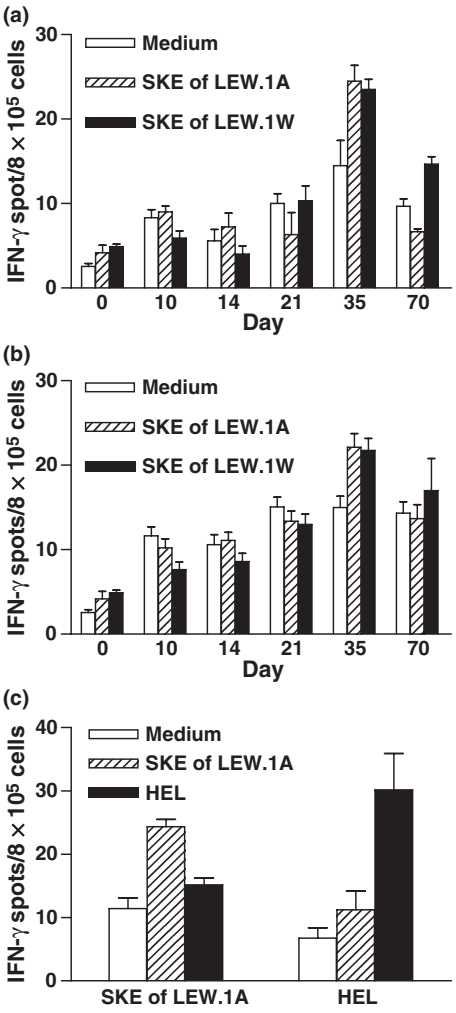

Figure 1 Frequency of specific IFN-γ-secreting T cells of LEW.1A rats following SKE immunization. At different time points after the first injection of SKE into LEW.1A rats, splenocytes were harvested and T cells were tested in an ELISPOT assay. (a) LEW.1A rats were injected i.p. with autologous SKE. Addition of SKE from LEW.1A or LEW.1W to the ELISPOT further increased IFN-γ-producing cell frequencies on day 35 ($P < 0.05$, $n = 4$). (b) LEW.1A rats were injected i.p. with LEW.1W SKE. The frequency peaked 35 days following immunization ($P < 0.05$). Addition of SKE from LEW.1W or LEW.1A to the ELISPOT further increased IFN-γ-producing cell frequencies (versus medium) on day 35 ($P < 0.05$, $n = 4$). (c) On day 35, HEL (control Ag) significantly increased IFN-γ-producing cell frequency in LEW.1A rats immunized with HEL ($P < 0.05$, $n = 3$) but not in those immunized with LEW.1A SKE, and vice versa. Legends on the panel indicate reagents added to the ELISPOT assay.

increased ($P < 0.05$) from day 10 following immunization and peaked on day 35 (Fig. 1b). Addition of SKE from LEW.1W or LEW.1A to the ELISPOT further increased the frequency of IFN-γ-producing cells on day 35 (Fig. 1b) ($P < 0.05$; 22.1 ± 1.6, 21.8 ± 1.4 vs. 15.0 ± 1.3). In contrast, no response to SKE was observed in control rats

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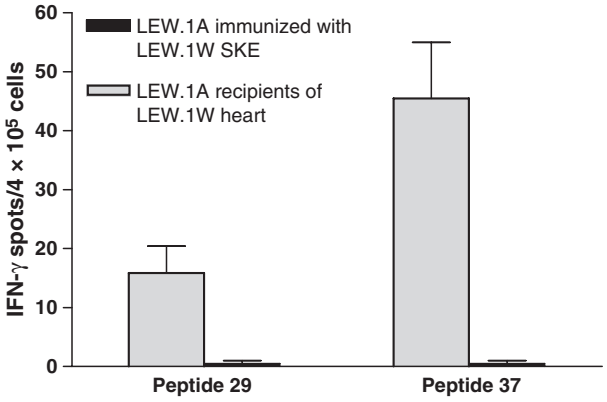

Figure 2 LEW.1A immunized rats do not respond to RT1^u dominant peptides. T cells from LEW.1A rats obtained 35 days after LEW.1W SKE immunization ($n = 2$) or from LEW.1A recipients rejecting a LEW.1W allograft obtained 7 days after transplantation ($n = 6$), were stimulated in an IFN-γ ELISPOT assay with peptides 29 and 37 from donor RT1D^u (β1 domain) molecules, which elicit a strong IFN-γ response in unmodified LEW.1A recipients of LEW.1W heart allografts. None of the LEW.1A immunized with LEW.1W extract responded to such peptides.

35 days after immunization with HEL and no response to HEL was observed in LEW.1A immunized with SKE. Finally HEL did not increase the frequency when added to the ELISPOT assay of immunized LEW.1A recipients (Fig. 1c). These results show that the response to SKE was specific as no response to HEL was observed. The assay did not detect a significant response in naive rats. Specific responses to immunodominant peptides from LEW.1W MHC [19] were then studied in LEW.1A rats immunized with LEW.1W SKE. LEW.1A rats immunized against LEW.1W SKE did not respond to dominant MHC (RT1^u) peptides (Fig. 2). As a positive control, we showed that similar peptides elicit a strong response in LEW.1A recipients of a LEW.1W heart [19]. Altogether, these data demonstrate a significant cellular response induced by repeated immunization of normal rats, with both autologous and allogeneic SKEs in the absence of adjuvant. In contrast, no response to MHC antigens was detected, suggesting that the majority of the response detected in this model was directed against autologous determinants.

IFN-γ and IL-10 cytokine mRNA increasingly accumulates in LEW.1A spleens following immunization

Significant changes in accumulation of cytokine mRNA were also observed on day 35 in LEW.1A rat T cells following immunization with LEW.1W or LEW.1A SKE. In the allo- and auto-immunized groups, IFN-γ mRNA increased gradually and peaked on day 35 (11.14 ± 4.27-fold) (Fig. 3a) confirming the ELISPOT data. IL-10 transcript accumulation also increased on day 14 and peaked on day 35 in both the allo- (6.81 ± 3.15-fold) and auto- (4.76 ± 1.56-fold) immunized groups (Fig. 3b). No accumulation was observed, however, for IL-2, IL-4 and IL-13 transcripts (not shown). Moreover, no change in IFN-γ, IL-2, IL-4, IL-10 or IL-13 mRNA levels (relative expression <1.50) was observed in the spleens of LEW.1A rats injected with PBS (data not shown).

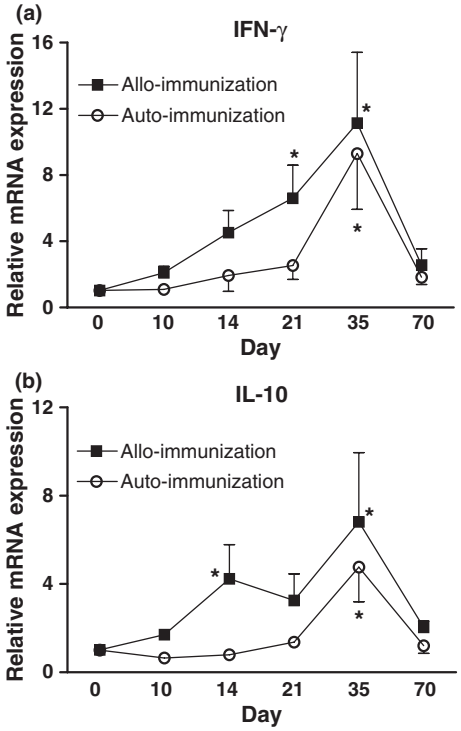

Figure 3 Kinetics of IFN-γ and IL-10 cytokine mRNA accumulation in the spleen of immunized LEW.1A rats. After injection of SKE from LEW.1W (allo) or LEW.1A (auto), IFN-γ mRNA [Panel (a), $*P < 0.05$, $n = 4$] and IL-10 mRNA levels [Panel (b), $*P < 0.05$, $n = 4$] increased gradually and peaked on day 35.

The IFN-γ response is mostly directed against a 600 kDa fraction of SKEs

To tentatively assess the antigenic moieties of the kidney extracts, SKEs from LEW.1W and LEW.1A were separated by FPLC. The FPLC profiles of LEW.1W and LEW.1A SKEs are shown in Fig. 4. Each FPLC fraction was used to stimulate T cells harvested from LEW.1A rats in ELISPOT assays 35 days (peak of the response) after immunization with LEW.1A (Fig. 4b) or LEW.1W SKE (Fig. 4a and c). As shown in Fig. 4a, addition of FPLC fraction 6 from LEW.1W SKE significantly enhanced the production of IFN-γ ($P < 0.05$). FPLC fraction 6 from LEW.1A SKE similarly activated T cells of rats immunized with

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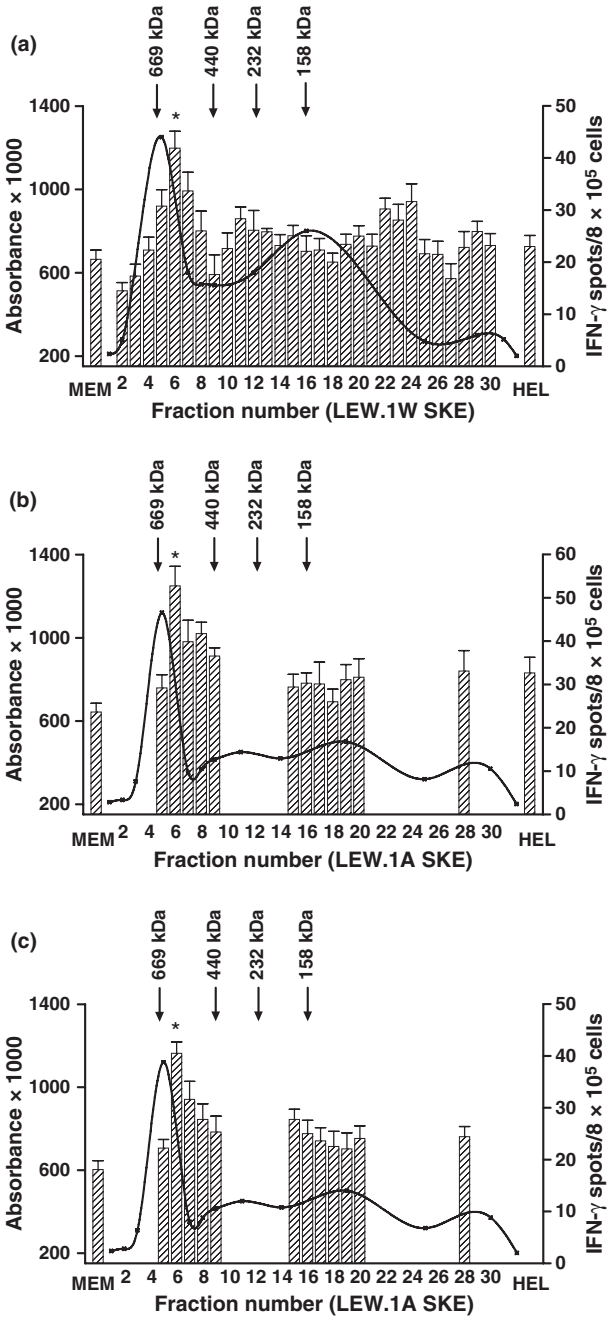

Figure 4 FPLC profiles of LEW.1W SKE and effect of FPLC fractions on the IFN-γ ELISPOT assays of immunized LEW.1A rats. (a) FPLC profiles of LEW.1W SKE and effect on T cells of LEW.1A rats immunized with LEW.1W SKE at day 35. (b) FPLC profiles of LEW.1A SKE and effect on T cells of LEW.1A rats immunized with LEW.1A SKE at day 35. (c) FPLC profiles of LEW.1A SKE and effect on T cells of LEW.1A rats immunized with SKE from LEW.1W at day 35. Only fraction 6 elicited a significant increase in IFN-γ-secreting cells (*$P < 0.05$, $n = 3$).

autologous or allogeneic SKE (Fig. 4b and c) ($P < 0.05$). Fraction 6 contained proteins with a molecular weight of 600 kDa. One protein that falls within this molecular

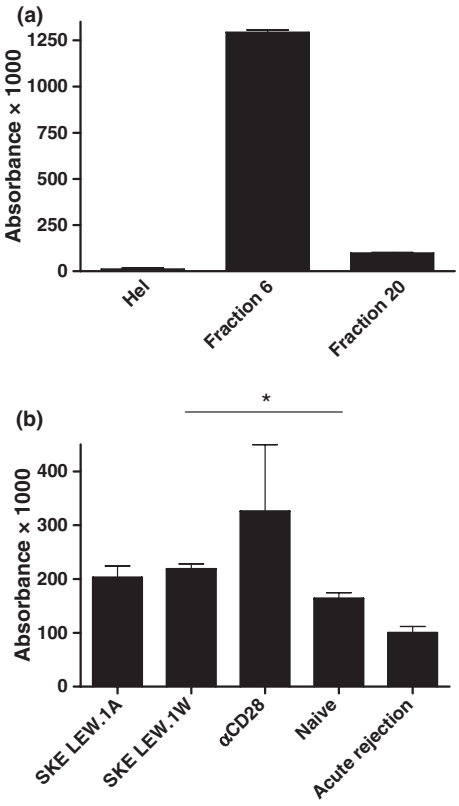

Figure 5 (a) Detection of megalin in SKE fractions by ELISA: All FPLC fractions were coated on 96-well plates. The antibody against megalin was then added. Only fraction 6 of 600 kDa reacted with anti-megalin antibody ($P < 0.01$). Other FPLC fractions and HEL were negative. (b) Detection of anti-megalin antibodies in rat sera: Sera from LEW.1A rats immunized with SKE from LEW.1A ($n = 4$) or LEW.1W ($n = 4$), and long surviving LEW.1A rats recipients of LEW.1W kidneys (100 days) following induction with anti-CD28 treatment ($n = 4$) developed antibodies against megalin (*$P < 0.05$). In contrast, sera from naive rats or recipients with acute rejection (10 days) ($n = 3$), did not show significant reaction with megalin.

weight range is a kidney tissue protein known as megalin. When associated with adjuvant, megalin can induce the production of antibodies in immunized rats and subsequently cause Heymann Nephritis (HN).

Fast pressure liquid chromatography (FPLC) fractions of LEW.1A SKE were screened for the presence of megalin by ELISA with anti-megalin antibodies. Megalin was only detectable in fraction 6 ($P < 0.01$) (Fig. 5a).

A humoral response to Megalin is detected in long-term surviving LEW.1A recipients of LEW.1W kidney grafts

Anti-CD28 antibodies can lead to long-term survival [18,19]. To detect antibodies against megalin in the sera of these animals, we used a capture ELISA where plates were coated with anti-megalin antibodies and LEW.1A

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fraction 6 was added. Fig. 5b shows that sera from LEW.1A rats immunized with SKE from LEW.1A or LEW.1W, and LEW.1A rat recipients of LEW.1W kidney allografts (100 days) preconditioned with anti-CD28 treatment contained antibodies against megalin. In contrast, sera from naive rats or rats undergoing acute rejection (10 days post-transplant) did not show an anti-megalin antibody response.

A cellular response to SKE is absent in long-term surviving kidney allograft recipients

To test the cellular response to SKE and SKE FPLC fractions after kidney transplantation, spleen T cells from LEW.1A recipients of LEW.1W kidneys were cultured in the presence of FPLC fraction 6 from LEW.1A SKE. The presence of a specific cellular response was measured by IFN-γ spots elicited by fraction 6 minus spots obtained with medium alone. No significant cellular response was detected in any of the tested animal groups (untreated or anti-CD28-treated kidney recipients). The values obtained in LEW.1A rats immunized with LEW.1A or LEW.1W SKEs are shown as positive controls. Collectively, these data suggest that allografts, in contrast to repeated kidney extract injection, failed to induce a significant ELISPOT response or that induction treatment to induce long-term graft survival inhibited the anti-autologous determinant response. These data also suggest that despite IFN-γ-producing T cells being nondetectable in the long-term surviving recipients, the animals still mount a significant humoral response to megalin.

Long-term kidney function and histology

Long-term anti-CD28 preconditioned LEW.1A rat recipients did not present a significant increase in creatinine as compared with syngeneic controls (51 ± 12.6 μmol/l; $n = 5$, vs. 34 ± 0.1 μmol/l; $n = 3$). A detailed histological analysis of the grafts from these animals has been described elsewhere [18] as only a mild grade I nonspecific chronic nephropathy and minor foci of interstitial fibrosis, or an infiltrate and slight tubular atrophy.

Anti-megalin antibodies have been shown to deposit in kidneys during experimental Heymans nephritis [22]. Thus, long-term kidney grafts of LEW.1A recipients conditioned with anti-CD28 (as well as ungrafted LEW.1A rats immunized with LEW.1W SKE) were studied for Ig deposits by immuno-fluorescence. No Ig deposits were observed.

Discussion

In this report, we show that in the LEW.1W to LEW.1A combination, long-term surviving allograft recipients conditioned with anti-CD28 antibodies [18] develop a humoral response against autologous kidney determinants. The antibodies recognized a 600 kDa protein in FPLC fractions of soluble kidney extract, confirmed as megalin by ELISA.

In order to induce a positive autoimmune response in our experimental kidney transplantation model in LEW.1A recipients immunized without Freund's adjuvant, we first analysed the immunogenicity of autologous and allogeneic soluble kidney extracts in normal LEW.1A rats. Repeated injections of SKE elicited a weak but significant increase in the frequency of T-cell-producing IFN-γ, as measured by ELISPOT. In addition, there was a parallel significant accumulation of IL-10 transcripts, peaking on day 35 postimmunization. The pattern of the other cytokines does not suggest a TH1 or TH2 bias (IL-2 absent but IFNγ present and IL-10 present. IL-4 is usually not transcribed in the LEW.1A strain [23]. Moreover, roughly similar responses were obtained following auto- or allo SKE immunization. Because SKE could trigger a significant cellular response without Freund's adjuvant, we avoided this unspecific agent in all subsequent experiments. This autologous response also provided the positive control required to assess a potential autologous response in LEW.1A kidney graft recipients. Injection with PBS or HEL did not increase the SKE-specific ELISPOT response. Unexpected findings were that the cellular response of LEW.1A rats to allogeneic SKE was not preferentially directed against LEW.1W ($RT1^u$) dominant peptides [19] in the IFN-γ ELISPOT assay. The response to autologous determinants which occurred in the congeneic combination sharing the Lewis background, suggests that autoreactivity is at least as vigorous as alloreactivity in this model.

In naive (ungrafted) LEW.1A rats, the response following immunization with SKE increased progressively until day 35 and was only moderately increased by the further addition of SKE in the ELISPOT test ($P < 0.05$ on day 35). However, a high concentration of SKE in the ELISPOT assay tended to strongly inhibit proliferation of rat lymphocytes *in vitro*, despite preliminary experiments to optimize the test and to determine the less toxic SKE concentration. Even at this concentration the SKE still exhibited some direct effect on PBL in culture, suggesting that caution is required when interpreting the magnitude of the response (which may be underestimated) in the ELISPOT assay performed in the presence of SKE.

Fast pressure liquid chromatography (FPLC)-separated soluble kidney extracts tested on T cells collected at day 35 (at the peak of the response) in the IFN-γ ELISPOT assay showed that the FPLC fraction 6 containing megalin

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(600 kDa) was responsible for most of the response against both allo- and autologous SKE. Megalin has been identified as a major immunogen in Heymann Nephritis, an experimental autoimmune disease in rats [17,22,24].

We have already shown that long-term surviving LEW.1A recipients of LEW.1W hearts develop antibodies directed against both MHC class-1 and -2 $RT1^u$ determinant [19]. We show here that an anti-SKE humoral response was also detected in long-term surviving LEW.1A recipients. Although undetectable during acute rejection or in syngeneic graft recipients, auto antibodies against FPLC fraction 6 were found to be significantly increased in long-term recipients of kidney allografts, contrasting with an undetectable cellular response in the ELISPOT assay. The anti-CD28 induction protocol may have decreased the magnitude of the cellular response in long-term graft recipients because the production of IgG antibodies requires T-cell help. However, some level of T-cell priming against autologous determinant probably occurs. Thus, our data support the concept that an autoimmune response can also be identified in recipients that have been preconditioned for long-term allograft acceptance. Our data are in agreement with previous reports showing that megalin is a major kidney autoantigen in the rat. However, without adjuvant, repeated injections were not sufficient to trigger a kidney autoimmune disease [25]. A specific cellular response against megalin was, however, not analysed because of a lack of purified or recombinant antigen.

Despite this life-sustaining kidney graft model enabling long-term survival, the graft histology showed an increased cell infiltrate compatible with an ongoing chronic rejection. Vigorous autologous responses have already been shown to occur in another animal model where autoreactive CD4+ T cells mediated rejection of a second isogenic [26] skin graft.

Post-transplantation de novo autoimmunity has been associated with hepatitis [27–30], transplant-associated coronary artery disease [5,6] and transplant glomerulopathy [3,9] in the context of chronic rejection. Several tissue proteins recognized by graft recipient antibodies have been characterized, including Vimentin [3,5,6,31,32], Agrin [9], cardiac myosin [10,13], and Type V collagen [14,15] in humans as well as in animal models.

However, despite our data showing an autoimmunity, this was not associated with specific lesions, particularly no glomerulopathy occurred in this model. Collectively, these data are more consistent with the concept that autologous antibodies are poorly pathogenic, particularly when recipients have been preconditioned for the induction of long-term graft survival. These data pave the way for further investigations into autoimmune responses in chronic rejection.

Authorship

CF, CB, A-SD, AG, AM, CU and HS: performed research. BV and SB: contributed important ideas during discussions. JH and JPS: designed research and wrote the paper.

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References

1. Ballet C, Giral M, Ashton-Chess J, Renaudin K, Brouard S, Soulillou JP. Chronic rejection of human kidney allografts. *Expert Rev Clin Immunol* 2006; **2**: 392.
2. Krieger NR, Becker BN, Heisey DM, *et al.* Chronic allograft nephropathy uniformly affects recipients of cadaveric, nonidentical living-related, and living-unrelated grafts. *Transplantation* 2003; **75**: 1677.
3. Carter V, Shenton BK, Jaques B, *et al.* Vimentin antibodies: a non-HLA antibody as a potential risk factor in renal transplantation. *Transplant Proc* 2005; **37**: 654.
4. Joosten SA, van Kooten C, Sijpkens YW, de Fijter JW, Paul LC. The pathobiology of chronic allograft nephropathy: immune-mediated damage and accelerated aging. *Kidney Int* 2004; **65**: 1556.
5. Jurcevic S, Ainsworth ME, Pomerance A, *et al.* Antivimentin antibodies are an independent predictor of transplant-associated coronary artery disease after cardiac transplantation. *Transplantation* 2001; **71**: 886.
6. Barber LD, Whitelegg A, Madrigal JA, Banner NR, Rose ML. Detection of vimentin-specific autoreactive CD8+ T cells in cardiac transplant patients. *Transplantation* 2004; **77**: 1604.
7. Opelz G. Non-HLA transplantation immunity revealed by lymphocytotoxic antibodies. *Lancet* 2005; **365**: 1570.
8. Groffen AJ, Ruegg MA, Dijkman H, *et al.* Agrin is a major heparan sulfate proteoglycan in the human glomerular basement membrane. *J Histochem Cytochem* 1998; **46**: 19.
9. Joosten SA, Sijpkens YW, van Ham V, *et al.* Antibody response against the glomerular basement membrane protein agrin in patients with transplant glomerulopathy. *Am J Transplant* 2005; **5**: 383.
10. Rolls HK, Kishimoto K, Dong VM, *et al.* T-cell response to cardiac myosin persists in the absence of an alloimmune response in recipients with chronic cardiac allograft rejection. *Transplantation* 2002; **74**: 1053.

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11. Fedoseyeva EV, Zhang F, Orr PL, Levin D, Buncke HJ, Benichou G. De novo autoimmunity to cardiac myosin after heart transplantation and its contribution to the rejection process. *J Immunol* 1999; **162**: 6836.
12. Fedoseyeva EV, Kishimoto K, Rolls HK, *et al.* Modulation of tissue-specific immune response to cardiac myosin can prolong survival of allogeneic heart transplants. *J Immunol* 2002; **169**: 1168.
13. Tanaka M, Zwierzchoniewska M, Mokhtari GK, *et al.* Progression of alloresponse and tissue-specific immunity during graft coronary artery disease. *Am J Transplant* 2005; **5**: 1286.
14. Haque MA, Mizobuchi T, Yasufuku K, *et al.* Evidence for immune responses to a self-antigen in lung transplantation: role of type V collagen-specific T cells in the pathogenesis of lung allograft rejection. *J Immunol* 2002; **169**: 1542.
15. Yoshida S, Haque A, Mizobuchi T, *et al.* Anti-type V collagen lymphocytes that express IL-17 and IL-23 induce rejection pathology in fresh and well-healed lung transplants. *Am J Transplant* 2006; **6**: 724.
16. Motoyoshi Y, Matsusaka T, Saito A, *et al.* Megalin contributes to the early injury of proximal tubule cells during nonselective proteinuria. *Kidney Int* 2008; **74**: 1262.
17. Makker SP, Singh AK. Characterization of the antigen (gp600) of Heymann nephritis. *Lab Invest* 1984; **50**: 287.
18. Haspot F, Seveno C, Dugast AS, *et al.* Anti-CD28 antibody-induced kidney allograft tolerance related to tryptophan degradation and TCR class II B7 regulatory cells. *Am J Transplant* 2005; **5**: 2339.
19. Ballet C, Renaudin K, Degauque N, *et al.* Indirect CD4+ TH1 response, antidonor antibodies and diffuse C4d graft deposits in long-term recipients conditioned by donor antigens priming. *Am J Transplant* 2009; **9**: 697.
20. Lair D, Degauque N, Miqueu P, *et al.* Functional compartmentalization following induction of long-term graft survival with pregraft donor-specific transfusion. *Am J Transplant* 2007; **7**: 538.
21. Soulillou JP, Carpenter CB, d'Apice AJ, Strom TB. The role of nonclassical Fc receptor-associated, Ag-B antigens (Ia) in rat allograft enhancement. *J Exp Med* 1976; **143**: 405.
22. Oleinikov AV, Feliz BJ, Makker SP. A small N-terminal 60-kD fragment of gp600 (megalin), the major autoantigen of active Heymann nephritis, can induce a full-blown disease. *J Am Soc Nephrol* 2000; **11**: 57.
23. Brouard S, Blancho G, Moreau A, Heslan JM, Cuturi MC, Soulillou JP. Long-term survival of hamster-to-rat cardiac xenografts in the absence of a Th2 shift. *Transplantation* 1998; **65**: 1555.
24. Heymann W, Hackel DB, Harwood S, Wilson SG, Hunter JL. Production of nephrotic syndrome in rats by Freund's adjuvants and rat kidney suspensions. *Proc Soc Exp Biol Med* 1959; **100**: 660.
25. Mahesh B, Leong HS, McCormack A, Sarathchandra P, Holder A, Rose ML. Autoantibodies to vimentin cause accelerated rejection of cardiac allografts. *Am J Pathol* 2007; **170**: 1415.
26. Valujskikh A, Fedoseyeva E, Benichou G, Heeger PS. Development of autoimmunity after skin graft rejection via an indirect alloresponse. *Transplantation* 2002; **73**: 1130.
27. Brandsaeter B, Schrumpf E, Bentdal O, *et al.* Recurrent primary sclerosing cholangitis after liver transplantation: a magnetic resonance cholangiography study with analyses of predictive factors. *Liver Transpl* 2005; **11**: 1361.
28. Kerkar N, Hadzic N, Davies ET, *et al.* De-novo autoimmune hepatitis after liver transplantation. *Lancet* 1998; **351**: 409.
29. Hernandez HM, Kovarik P, Whitington PF, Alonso EM. Autoimmune hepatitis as a late complication of liver transplantation. *J Pediatr Gastroenterol Nutr* 2001; **32**: 131.
30. Miyagawa-Hayashino A, Haga H, Egawa H, *et al.* Outcome and risk factors of de novo autoimmune hepatitis in living-donor liver transplantation. *Transplantation* 2004; **78**: 128.
31. Jonker M, Danskine A, Haanstra K, *et al.* The autoimmune response to vimentin after renal transplantation in nonhuman primates is immunosuppression dependent. *Transplantation* 2005; **80**: 385.
32. Azimzadeh AM, Pfeiffer S, Wu GS, *et al.* Humoral immunity to vimentin is associated with cardiac allograft injury in nonhuman primates. *Am J Transplant* 2005; **5**: 2349.

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