Transplant Immunology 33 (2015) 95–102

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

Rat renal transplant model for mixed acute humoral and cellular rejection: Weak correlation of serum cytokines/chemokines with intragraft changes

Anja Lemke [a,d], Mercedes Noriega [b], Anja M. Röske [c], Markus J. Kemper [d], Björn Nashan [c], Christine S. Falk [e,f], Martina Koch [c,*]

[a] Department of Hepatobilary and Transplant Surgery and Pediatric Nephrology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
[b] Department of Pathology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
[c] Department of Hepatobiliary and Transplant Surgery, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
[d] University Childrens Hospital, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
[e] Institute of Transplant Immunology, IFB-Tx, Hannover Medical School, Hannover, Germany
[f] Deutsches Zentrum für Infektionsforschung DZIF, Heidelberg, Germany

ARTICLE INFO

Article history:
Received 30 April 2015
Received in revised form 14 August 2015
Accepted 17 August 2015
Available online 20 August 2015

Keywords:
Mixed acute rejection
MHC disparity
Macrophages
B-cells

ABSTRACT

Background: Acute renal allograft rejection remains a major cause of allograft dysfunction; especially for episodes with mixed humoral and cellular character which can be detrimental for graft survival. We established a rat RT model with exclusive and complete MHC-disparity to investigate pathomechanisms of acute rejection and evaluate serum multiplex assays as a diagnostic tool in this context.
Methods: LEW rats receive congeneic LEW.1W allografts (allo), no immunosuppression. Planned duration of the experiment was 4 weeks (n = 13 allo, n = 3 iso). To study kinetics of rejection, additional animals were sacrificed at day 7 (n = 6 allo and n = 3 iso) and day 21 (n = 3 allo). Serum cytokines and chemokine were longitudinally analyzed with multiplex assays in n = 5 allo and n = 5 controls. Allografts were assessed by histopathology, immunohisto-chemistry and PCR.
Results: Animals develop allograft dysfunction acute humoral rejection with additional cellular components. Donor-specific MHC-antibodies are already detectable at day seven (d7) after RT. Leukocytic graft infiltrates are dominated by macrophages and additionally consist of T-cells, B-cells and NK-cells. Increased intragraft expression of interleukin-2, interferon gamma, tumor necrosis factor alpha as well as B-cell activating factor and its receptor are observed. Of the 24 serum cytokines/chemokines, only CCL2 is significantly different (higher) in allo vs. controls at d7 (p = 0.02).
Conclusions: Correlation of serum chemokines/cytokines with features of humoral and cellular rejection, as reproduced in our LEW.1W to LEW rat renal transplant model, is limited. Macrophages, B-cells and their signaling pathways deserve more attention in genesis and possibly also treatment of acute rejection.

1. Introduction

Although the incidence of rejection after renal transplantation (RT) could successfully be reduced with potent immunosuppressives over the last three decades, sustainable treatment of rejection episodes remains critical for long-term graft function [1,2]. Diagnosis and classification of acute rejection is based on analysis of histological changes, most commonly using the BANFF criteria. These criteria do not include differentiation of leukocytic infiltrates, which can consist of not only T-cells but also B-cells, NK-cells and monocytes/macrophages [3–5]. Great effort is made to replace the gold standard kidney biopsy and develop less invasive diagnostic tools to differentiate rejection from other causes for allograft dysfunction using peripheral blood or urine. For this, a more detailed understanding of underlying pathological mechanisms is essential. Obviously, it is difficult to prospectively study the dynamics of allograft rejection in humans as they usually are on life-long medication modulating the immune system. Rat models of renal transplantation are acknowledged as useful investigational tool in the field of experimental transplantation research [6]. Usually, animal strains differ

Abbreviations: BAFF, B-cell activating factor of the TNF-family; BAFF-R, B-cell activating factor receptor; CCL2, Chemokine (CC motif) ligand 2 or monocyte chemotactic protein 1 (MCP1); CCL5, Chemokine ligand 5 or RANTES; IFN-gamma, Interferon gamma; Il-2, Interleukin 2; LEW, Lewis rat strain; RT, Renal transplantation; TNF-alpha, Tumor necrosis factor alpha.

* Corresponding author at: University Medical Center Hamburg-Eppendorf, Hepatobiliary Surgery and Transplantation, Martinistrasse 52, 20246 Hamburg, Germany.
E-mail addresses: lemke_anja@gmx.de (A. Lemke), m.noriega@uke.de (M. Noriega), anjaroeske@live.com (A.M. Röske), kemper@uke.de (M.J. Kemper), nashan@uke.de (B. Nashan), Falk.Christine@mh-hannover.de (C.S. Falk), martina.koch@uke.de, m.koch@uke.de (M. Koch).

http://dx.doi.org/10.1016/j.trim.2015.08.003
0966-3274/

Table 1
Overview of pathological evaluation of allografts according to the BANFF criteria.

Animal number	Group	t	i	g	ah	v	cg	ci	ct	cv	mm	ptc
LEW 1	d7	1	2	0	0	1	1	1	1	0	1	1
LEW 2	d7	1	1	0	0	0	0	1	1	0	0	1
LEW 3	d7	1	2	1	0	0	0	1	1	0	0	2
LEW 4	d7	1	2	0	0	0	0	1	1	0	0	1
LEW 5	d7	1	1	0	0	0	0	1	1	0	0	1
LEW 6	d7	1	1	0	0	1	0	1	1	0	0	1
LEW 7	d21	2	3	1	0	0	0	1	1	0	0	3
LEW 8	d21	2	2	1	0	0	0	1	1	0	0	2
LEW 9	d21	2	3	1	0	0	0	1	1	0	0	2
LEW 10	d28	1	3	0	0	0	3	3	3	0	1	1
LEW 11	d28	1	3	0	0	0	3	3	3	0	1	2
LEW 12	d28	1	3	2	0	0	1	3	3	0	1	3
LEW 13	d28	3	2	3	0	0	3	2	2	0	1	3

in MHC as well as non-MHC molecules. The rat RT model with congenic Lewis (LEW) rat strains LEW.1W to LEW has exclusive differences in MHC I and II haplotypes. These animals have previously been used to study chronic MHC dependent allograft damage after initial treatment with cyclosporine A [7]. Animals developed impairment of allograft function and signs of mixed cellular and humoral rejection with MHC-antibodies together with interstitial fibrosis and tubular atrophy.

We established the congenic LEW.1W to LEW model without any immunosuppressive treatment to study dynamics of cellular graft infiltration and cytokine expression in acute graft rejection exclusively caused by MHC incompatibility. Our goal was to evaluate, whether the systemic serum cytokine and chemokine response correlates with histological changes, leukocytic infiltrates or the cytokine/chemokine milieu in the graft.

2. Material and methods

2.1. Animals and kidney transplantation

Animal experiments were approved by the local ethical committee and performed according to local and EU guidelines. Male Lewis rats (LEW, Charles River, Germany) received an allogenic kidney graft (allo group) from weight matched Lewis.1W rats (LEW.1W, Zentrales Tierlabor, Medizinische Hochschule Hannover, Germany). Sharing the same genetic background, donor and recipient differed completely in MHC haplotypes, resulting in MHC class I (RT1.A and RT1.C) as well as MHC class II (RT1.B/D) incompatibilities [7]. LEW to LEW rats served as isogeneic control (iso group). Life sustaining kidney transplantation was performed as previously described [8]. Graft ischemia time was limited to 30 min. The left kidney of the recipient was removed simultaneously during transplantation, whereas the right kidney was excised five to seven days after transplantation. Planned duration of the experiment was 4 weeks (n = 13 allo, n = 3 iso). To enlighten kinetics of rejection and intragraft cytokine expression in our model, a group of animals was sacrificed at day 7 (n = 6 allo and n = 3 iso) and day 21 (n = 3 allo).

2.2. Renal function assays

Body weight and graft function were monitored on alternate days. Serum creatinine (SCr) level was analyzed with Reflovet Plus (Roche Diagnostics, Switzerland; detection limit 0.5 mg/dl). Urine (U)-albumin concentrations (collected after 24 h in metabolic cages every other week) were quantified by competitive ELISA specific for rat albumin (Nephrat II, Exocell Inc., USA).

2.3. Pathology and immunohistochemistry

Morphological studies were performed by light microscopy. Kidney grafts were fixed in buffered formalin. Paraffin-sections were stained with periodic acid-Schiff (PAS) and were evaluated by a pathologist blinded for criteria according to the BANFF classification [9–12].

For immunohistochemistry on frozen sections, single staining techniques were performed as previously described [13]. Briefly, 5 μm sections were blocked, incubated with primary antibody, washed and treated with peroxidase-coupled secondary antibody rat-anti-mouse IgG (Dianova, Germany). Peroxidase activity was visualized with 3-amino-9-ethyl-carbazole. Sections were counterstained with Mayer's hemalaun (Merck, Germany). Graft infiltrating cells within the renal cortex were counted in ten 400-fold high power fields (HPFs; 0.196 mm^2 per HPF) per section by the same independent investigator. The following primary antibodies were used: R73 (TCR constant determinant; BioLegend, Germany), ED1 (CD68, pan-macrophage marker) and 10/78 (CD161, NK cells) (both from Serotec, Germany), Ki-B1R (pan B-cell marker; Dianova, Germany) and 3.4.1 (CD8, BD Biosciences, Germany). BAFF-R (ProSci Inc., USA) was used together with Fast Red staining kit.

2.4. Donor-specific allo-MHC antibodies

MHC antibodies were detected from recipient serum as described before by flow cytometry using donor type lymphocytes as antigen carriers [7] in six allotransplanted animals. LEW lymphocytes served as controls.

In short, after incubation with recipients sera lymphocytes were double stained for rat immunoglobulins and CD4 (mAbw3/25). T-cells become positive for rat IgG if anti-MHC class I antibodies are present in recipient's sera. The difference of the mean fluorescence intensity (MFI) of T-cells from LEW.1W and LEW rats is given as ΔMFI for time point d0 (pretransplant) and d7 (posttransplant).

Samples were analyzed using a FACScanto (BD Bioscience, San Jose, California, USA) and results were evaluated using the FlowJo computer program (Ashland, Orlando, USA).

2.5. Intragraft cytokine mRNA expression

Total RNA from kidneys was isolated and purified using RNeasy mini kit (Qiagen, Germany) or Nucleospin II RNA kit (Macherey-Nagel GmbH & Co. KG, Germany). The quantity and quality of RNA were determined using Infinity M200 (Tecan, Germany). Complementary DNA (cDNA) was synthesized from mRNA using SuperScript® III reverse transcriptase (Invitrogen, Germany) or high capacity cDNA reverse transcription kit (Applied Biosystems/Life Technologies GmbH, Germany). Quantitative real-time PCR (qRT-PCR) was performed in a Thermocycler MX3000P (Stratagene, Germany) using SYBR premix Ex Taq (Lonza, Switzerland) or QuantiFast SYBR green PCR kit for interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) (Quiagen, Germany). Transcriptions were normalized to GAPDH. The mRNA expression level was calculated by the ΔΔCt method in comparison to naive LEW rats (n = 3). Primers for GAPDH (QT00199633), Interleukin 2 (Il2, QT00185360), IFN-γ (QT00184982), TNF-α (QT00178717), and B-cell activating factor (BAFF, QT01596266) were purchased from QuantiTect Primer Assays (Quiagen). Chemokine (C-C motif) ligand 2 (CCL2, PPR06714B) was also purchased from Quiagen. BAFF-receptor primers: 5′-agc tca gtg gag ccc agt tc-3 and 5′-ccg aag gag tcc agc aag agt-3′.

2.6. Serum cytokine expression

Clotted blood samples were centrifuged twice at 3400 rpm for 6 min and the serum was kept at −20 °C until analysis. Bio-Plex Pro Rat Cytokine Assay with 24-Plex Panel (Biorad, Germany) was performed according to instructions by the manufacturer. For each analyte and sample, >50 beads were measured and mean fluorescence intensity (MFI) was used to calculate cytokine concentrations (pg/ml) according to the standard curve ranging from >20.000 to 1 pg/ml using the 5 parameter logistic plot formula.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.0f for Mac OS X (GraphPad Software, USA). D'Agostino & Pearson omnibus normality test used to test for normal distribution. Kruskal–Wallis and Dunns's multiple comparisons test were used to compare multiple groups. Longitudinal serum cytokine data were analyzed using 2-way ANOVA with Bonferroni's multiple comparisons test. Multiplicity adjusted p values were reported.

3. Results

3.1. Allotransplanted animals develop acute rejection with humoral and cellular components

At day 7, four of six animals showed signs compatible with humoral rejection with focal glomerular and preglomerular thrombotic micoangiopathy and cortical necrosis. One animal showed signs of cellular rejection (BANFF IIa) in addition. One animal had T-cell mediated vascular rejection BANFF IIa and one animal showed borderline changes together with cortical necrosis. At day 21, all three animals showed signs of mixed acute humoral and cellular (BANFF Ia) rejection. At day 28, all four animals showed glomerular changes as signs of acute (glomerulitis) and beginning chronic humoral rejection (double contours of the basal membranes), accompanied by diffuse tubular damage, which can be understood as secondary Borderline changes (Table 1 and Fig. 2).

Renal function was impaired in all animals at day 7 post-RT, but creatinine values were significantly higher in allo animals compared to iso-controls (2.8 ± 1.1 vs 0.8 ± 0.5 mg/dl, $p = 0.0003$) and returned to normal after the first week in the iso-controls (Fig. 1a). The renal impairment was paralleled by increased excretion of albumin in the allo-group in the second week after RT (26 (9–172) vs. 5 (2–38) mg/dl in controls, $p = 0.007$, which declined again over time). All animals lost weight within the first week after transplantation. Thereafter, allotransplanted animals continued to loose weight whereas iso-controls regained weight (Fig. 1b). 8 of 13 animals (62%) did not reach the end point (day 30) of the experiment (Fig. 1c).

3.2. Leukocytic graft infiltrates do not only consist of T-cells but also B-cells, NK-cells and dominating macrophages

All allografts had significantly more cellular infiltrates than iso-controls (Fig. 3). The number of infiltrating cells was highest at day 21 (TCR + T-cells 76 ± 20 cells per HPF (388 ± 102 per mm^2), CD8 + fraction of T-cells 73 ± 15 (373 ± 77 per mm^2), CD161 + NK-cells 44 ± 10 (225 ± 51 per mm^2), KiB1 + B-cells 59 ± 33 (301 ± 168 per mm^2), CD68 + macrophages 149 ± 29 (760 ± 148 per mm^2)). However, CD68 + and CD161 + cells already were high at day 7, therefore, no statistically significant difference was seen between day 7 and day 21. KiB1 + cells (30 ± 13 (153 ± 66 per mm^2)) and TCR + T-cells (62 ± 19 (316 ± 97 per mm^2)) were not statistically lower at day 28, whereas the numbers of CD8 + T-cells (33 ± 7 (168 ± 36 per mm^2)), as well as CD161 + cells (19 ± 11(97 ± 56 per mm^2)) and ED1 + cells (104 ± 36(531 ± 184 per mm^2)) were significantly lower again.

3.3. All animals developed donor specific antibodies

While no preformed MHC antibodies were present in any of the 6 animals tested, de novo synthesis of circulating allo-MHC antibodies was detectable in all animals already at day 7 after RT. Mean ΔMFI pre-RT was 18 ± 25, increasing to 1130 ± 733 at day 7. Antibodies persisted in all animals tested until end of the experiment. A representative staining of rat IgG on CD4 pos. T-lymphocytes is shown in Fig. 4.

3.4. IFN-γ and BAFF receptor are abundantly expressed in rejected allografts

The pro-inflammatory cytokines IFN-γ and TNF-α were already significantly higher expressed in allografts on day 7 median 271-fold [range 140–405] expression compared to normal LEW-controls (Fig. 5) and 25-fold [2–79], respectively, and even higher at day 21 with a 367-fold [273–422] and 35-fold [19–48] increase. Both were still expressed in animals of d28 group but not statistically different from iso-controls. IL-2 mRNA was highest at day 28 with a 22-fold [7–41] increase. BAFF followed a similar pattern as IL-2 with highest expression at day 28 (17-fold [3–32]). In contrast, BAFF receptor (BAFF-R) expression was highest at day 21 (641-fold [262–1308]) and almost returned to the level of iso-controls again at day 28 (39-fold [30–158] vs. 11-fold [4–160], ns).

CCL2 expression in the grafts was low compared to the other cytokines analyzed. A higher expression was found in the allografts (8-fold [3–13]) compared to the isografts (3-fold [1–4]), but this difference did not reach statistical significance in the multicomparison tests. There was a significant difference between the allo and the iso group looking at day 7 ($p = 0.036$) and day 28 (0.024).

3.5. Systemic cytokine and chemokine milieu: CCL2 correlates with acute rejection

Longitudinal data of serum cytokines and chemokines were available of five allo and five iso control animals. The following rat proteins were analyzed in parallel: EPO, G-CSF, GM-CSF, CXCL1 (GRO-a/KC), IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12p70, IL-13, IL-17A, IL-18, M-CSF, CCL2 (MCP-1), CCL3 (MIP-1α), CCL (MIP-3α), CCL5 (RANTES), TNF-α, and VEGF. Of these, only CCL2 (chemokine ligand 2, monocyte chemotactic protein 1) was significantly different between pre-RTx values and day 7 ($p = 0.0001$) in allotransplanted animals as well as different from day 7 iso-controls ($p = 0.02$) (Fig. 6). M-CSF (Macrophage colony-stimulating factor 1), VEGF (vascular endothelial

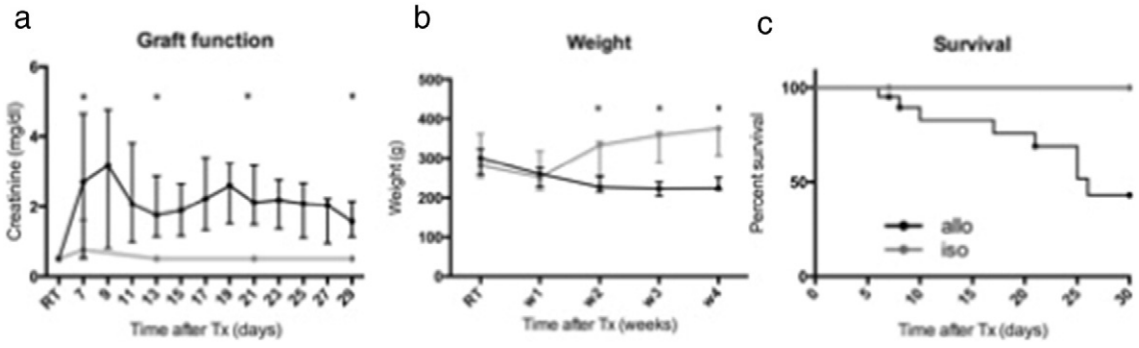

Fig. 1. Graft function (a), weight (b) and survival (c) of LEW.1W to LEW allotransplanted animals (black) and LEW to LEW iso-controls (gray). Significant differences between allo and iso groups are marked with *.

growth factor) and CCL5 (chemokine ligand 5, RANTES) were significantly increased at day 7 in allo-animals, but not statistically different from iso-controls at the same day. No statistically significant differences were observed in any of these three cytokines on day 21. Interestingly, none of the classical pro-inflammatory cytokines displayed a statistically significant change at days 7 or 21.

4. Discussion

In our rat RT model LEW.1W to LEW, with exclusive MHC incompatibility animals develop acute allograft rejection with cellular and humoral features, allograft dysfunction and premature graft loss. Cellular infiltrates of the graft are dominated by macrophages and, correspondingly, CCL2 detected from serum by multiplex assay, correlates with the early phase of rejection.

To our knowledge, we here present the first description of the rat RT model LEW.1W to LEW developing mixed acute rejection with cellular rejection (ACR) and findings compatible with acute humoral or antibody-mediated rejection (AMR). The LEW.1W to LEW model with cyclosporine A-based immunosuppression develops chronic rejection with a mixed cellular and humoral picture [7]. Bickerstaff et al. previously described a pre-sensitized mouse renal transplant model with acute humoral rejection of renal allografts associated with concomitant cellular rejection [14]. Our animals develop de novo donor-specific allo-MHC antibodies, detectable already 7 days after transplantation. Over the last years, it has become apparent that ACR and AMR are not always easy to differentiate and can occur in combination, then called mixed acute rejection (MAR). The clinical behavior of mixed lesions is often more aggressive over time [15]. MAR is differentiated from pure AMR, primarily by a predominant lymphocytic infiltrate in the grafts [16]. Already at day 7 post-RT, we could observe signs of rejection (primarily humoral) and significant allograft impairment as well as cellular infiltrates and up-regulated cytokine and cytokine-receptor expression in the graft. One could argue that these features might still be caused by ischemia/reperfusion (IR) injury, which is unlikely as exclusive reason, as it was previously described in rat RT models that, renal function is back to normal at day 7 after IR-injury [17]. We performed nephrectomy of the contralateral native kidneys at day 5 post-RT in our animals, which may have aggravated changes because of hyperperfusion of the graft.

T-cell infiltration and increased IL-2 expression are well-acknowledged features of allograft rejection, which can also be seen in our model. The dominance of macrophages in the cellular infiltrates as observed by us has previously been described in other rat RT models, where not only MHC alleles but also other genes were disparate. These data suggest that macrophages may primarily contribute to cellular but not to humoral mechanisms of acute rejection [18]. High levels of macrophages in rejecting allografts have been associated with severe rejection, and glomerular macrophage infiltration in particular has been shown to be an indicator of poor graft outcome [19]. Our data on dynamics of cellular graft infiltration support the hypothesis that T-cells are necessary to induce a rejection episode, but monocytes/macrophages finally impair graft function by the release of soluble immune modulators like cytokines [5]. For example the pro-inflammatory cytokine TNF-α, which we also found to be upregulated in the allografts, stimulates macrophage function and increases MHC class II antigen expression during rejection [20,21]. Significant intragraft IFN-γ mRNA expression paralleled macrophage infiltration, which is not surprising as IFN-γ signaling via IFN-γ receptors in allografts promotes infiltration and MHC induction but prevents early thrombosis, congestion, and necrosis [22]. Experimental data suggest that depletion of macrophages can improve allograft dysfunction. However, the benefit of such strategies and the implication in human disease is not known so far [23].

There is growing evidence that B-cells play a significant role in allograft rejection [3,24]. Nodular B-cell infiltrates are associated with treatment refractory rejection [25]. In our model B-cell infiltration of rejected allografts is paralleled by significantly increased BAFF-R expression in day 7 and day 21 animals. BAFF-R expression is remarkably low on day 28 although we still detected a relevant number of B-cells. We could confirm by immunohistochemistry that BAFF-R expression was restricted to infiltrating B-cells (data not shown). Our data suggest an increase in BAFF-R expression paralleling maturation of B-cells, as BAFF-R is mainly expressed on mature B-cells [26]. We postulate down-regulation of BAFF-R expression on day 28, possibly in response to increased intragraft BAFF-expression. We previously observed down regulation of BAFF-R on peripheral B-cells in response to increased serum BAFF after RT in pediatric patients [27]. Our data highlight the relevance of the BAFF/BAFF-R axis in allograft rejection.

Infiltrating macrophages and B-cells are not yet reflected in the BANFF-classification although they significantly contribute to pathogenesis of allograft rejection.

Multiplex arrays offer opportunities to examine physiologically relevant panels of cytokines and chemokines in a time and cost-efficient fashion. We used the rat-specific cytokine composition commercially available. In this selection of cytokines and chemokines, only CCL2 (monocyte chemotactic peptide-1, MCP-1) was significantly elevated in our model only in the very early phase of acute rejection. M-CSF, CCL5 and VEGF were significantly higher on day 7 compared to prior transplantation values but did not differ between allo- and iso-controls. CCL2 is a specific and powerful chemoattractant and activating factor for monocytes and macrophages and its expression strictly correlates with monocyte infiltrates and tubulointerstitial damage in acute and chronic nephritides [28]. In the fully allogeneic rat RT model Dark Agouti (DA) to LEW, intragraft gene expression of CCL2 strongly

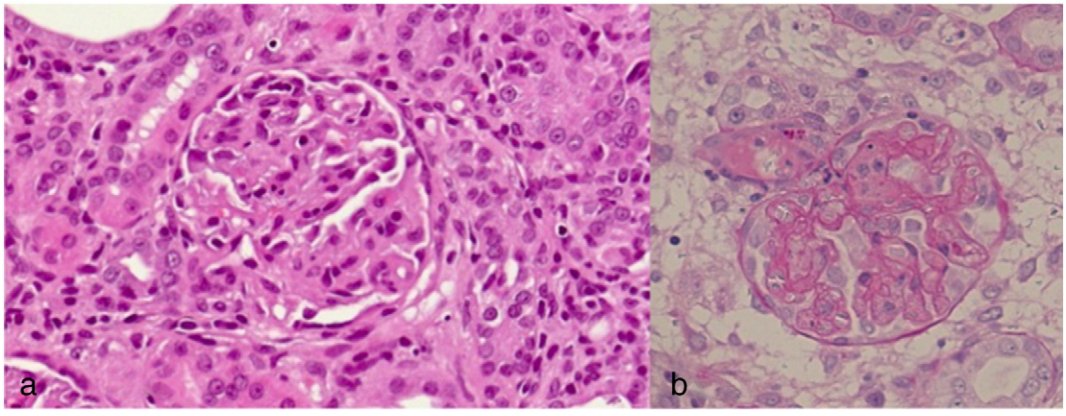

Fig. 2. Histopathological changes and cellular infiltrates in rejected grafts of allotransplanted animals. (a) Glomerular thrombotic microangiopathy (LEW 1, d7). (b) Focal glomerulitis and capillaritis as well as tubulitis, double contours of glomerular basement membrane (LEW 11, d28); both PAS staining, magnification 20×.

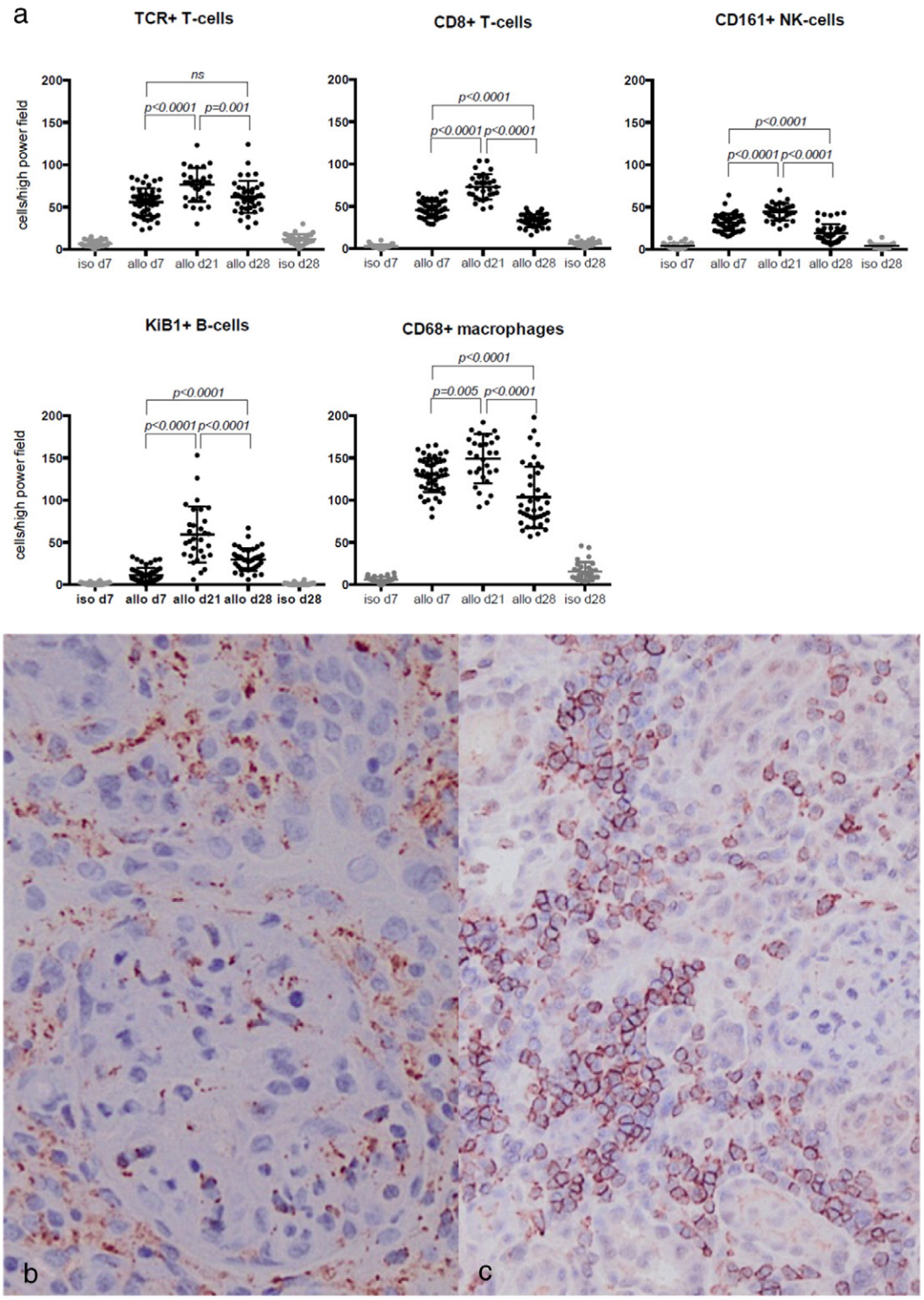

Fig. 3. Characterization of cellular graft infiltrates using immunohistochemical staining. (a) Significantly more cells were seen in allografts compared to iso-controls at any time point. (b) Intracellular staining of CD68 (ED1) in macrophages (LEW 7) and (c) KiB1-R staining on B-cells at day 21 post-allotransplantation (LEW 9), magnification 200×.

increases 3–4 days after RT and returns to control levels at day 6. However, no data on serum concentrations have been presented [29]. Our data on intragraft CCL2 are in accordance with data published by Grau et al. M-CSF is the principal factor for survival, proliferation and differentiation of monocytes and macrophages. This growth factor also has a role in cytokine production, cytotoxicity and phagocytosis [30]. An increased expression of M-CSF paralleling macrophage infiltration has been described in rejected allografts in the DA to LEW rat RT model [31]. It remains unresolved whether serum levels of M-CSF can really differentiate acute rejection from other causes of allograft impairment, e.g. cyclosporine A toxicity or IR injury in humans [32]. VEGF is an endothelial-specific growth factor that promotes endothelial cell proliferation, differentiation and survival. Increased mRNA expression of VEGF was seen in rat allografts in the DA to Wistar Furth model, mainly in chronic rejection [33], where it may be important in inflammation and development of fibrosis. Peng et al. suggested that VEGF in serum and urine might be a new non-invasive approach to supplement a protocol biopsy for detection of subclinical rejection [34]. We are

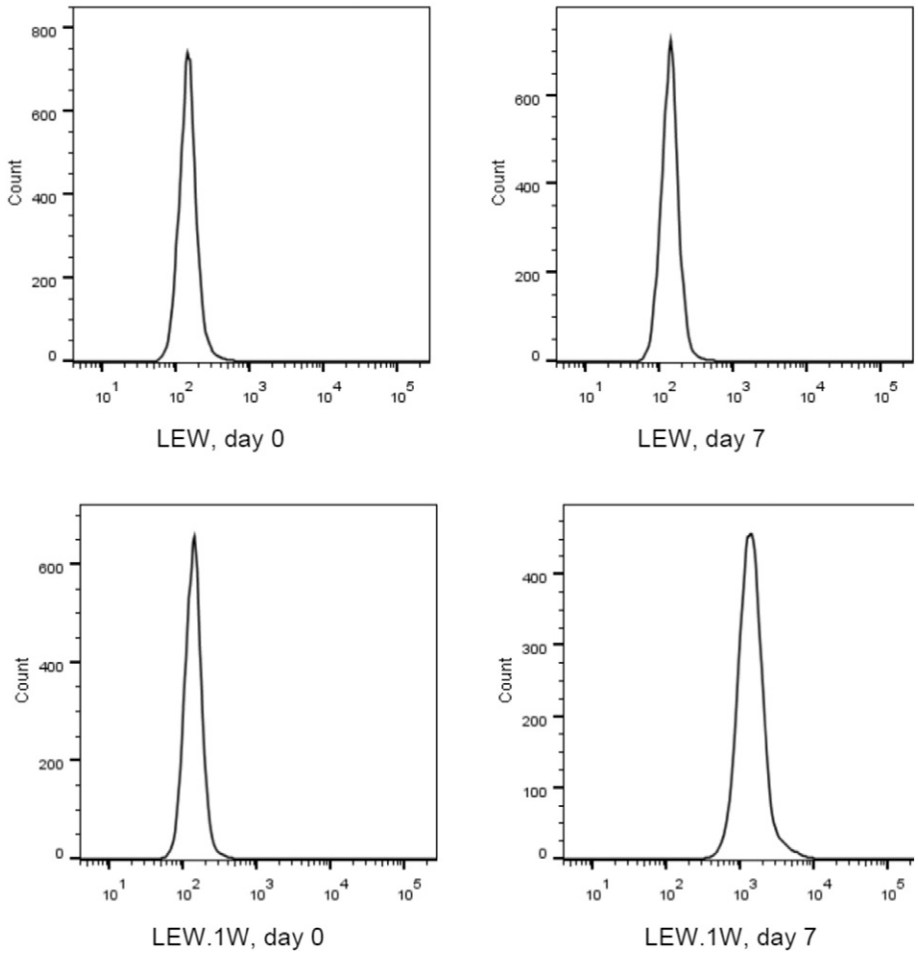

Fig. 4. Detection of donor specific MHC class I antibodies by staining recipient (LEW, upper panel) and donor (LEW.1W, lower panel) type T-lymphocytes with anti-rat IgG after incubation with sera of a representative animal pre-transplant (day 0) and 7 days post-transplant (day 7). No antibody detection on recipient cells pre- and post-transplantation (upper panel, control), but clear positivity for donor specific antibodies on day 7 post transplantation (lower panel).

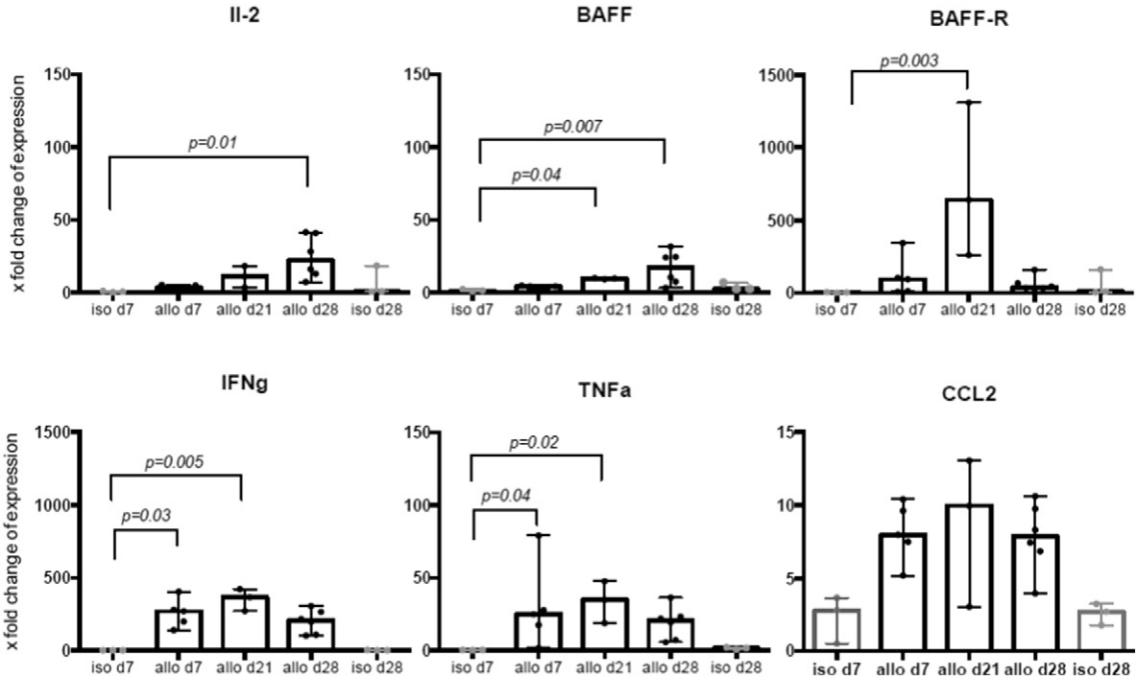

Fig. 5. Expression of intragraft mRNA of Interleukin 2 (IL-2), B-cell activating factor (BAFF), interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α) and BAFF-receptor (BAFF-R) at different time points after RT compared to d7 iso-controls.

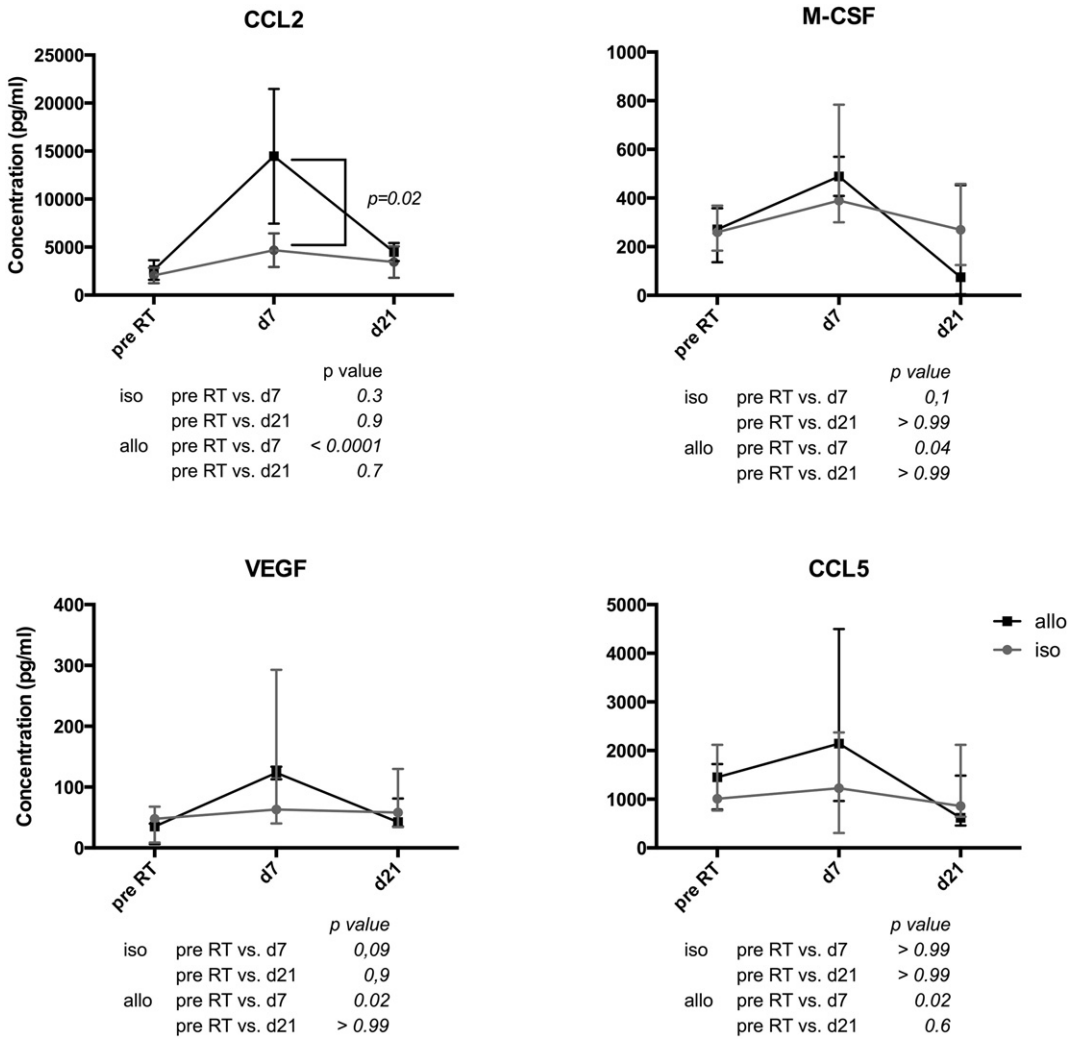

Fig. 6. Longitudinal analysis of serum cytokines and chemokines using multiplex analyses. Only cytokines with significant differences compared to pre-RT values are shown. CCL2 (chemokine ligand 2), M-CSF (macrophage colony-stimulating factor 1), VEGF (vascular endothelial growth factor) and CCL5 (chemokine ligand 5, RANTES).

aware of limitations of our analyses since we do not present a parallel data set of intragraft/serum cytokines (e.g. MCP-1 and BAFF in serum), for instance.

Of the other tested cytokines, several had previously been investigated after renal allograft rejection, for example T-cell cytokines like IL-2, IL-10, IL-4, and IFN-γ. They could not predict early acute rejection in renal transplantation in man [35]. We assume that the increased serum levels of CCL2 in our d7 animals reflect increased intragraft expression and macrophage activity during early phase of rejection. Nevertheless, it seems unlikely that serum cytokine- or chemokine measurement will be able to replace allograft biopsy in diagnosis of rejection; even though, they can contribute to a more detailed understanding of underlying mechanisms.

5. Conclusion

When using multiplex assays for serum quantification of cytokines and chemokines, one has to acknowledge that reflection of intragraft changes is limited. However, CCL2 might be a valuable serum marker in the early phase of rejection. Our rat RT model with exclusive MHC incompatibility reproducing features of humoral and cellular rejection is a valuable tool for further investigations of pathological mechanisms and treatment approaches in mixed acute rejection.

Author contributions

A.L. and M.K. participated in research design, performance of the research, data analysis and writing of the paper.

M.N. and C.F. participated in performance of the research, data analysis and writing of the paper.

A. M. R. participated in research design, performance of the research.

M.J.K. and B.N. participated in writing of the paper.

Acknowledgments

We thank our technicians Claudia Goßler and Sabrina Christiansen, Kerstin Daemen and Jana Keil for the excellent support.

Part of this work was sponsored by Novartis Pharma, Nürnberg, Germany (to M.K.) and by the IFB-Tx (BMBF 01EO1302) and the DFG SFB738 (project B3/B8), and DZIF (TTU-IICH 7.803) to C.S.F.

References

[1] G. Opelz, B. Dohler, Influence of time of rejection on long-term graft survival in renal transplantation, Transplantation 85 (5) (2008) 661.

[2] C. Lefaucheur, D. Nochy, G.S. Hill, et al., Determinants of poor graft outcome in patients with antibody-mediated acute rejection, Am. J. Transplant. 7 (4) (2007) 832.

[3] F. Heller, M.T. Lindenmeyer, C.D. Cohen, et al., The contribution of B cells to renal interstitial inflammation, Am. J. Pathol. 170 (2) (2007) 457.

[4] A. Kroemer, K. Edtinger, X.C. Li, The innate natural killer cells in transplant rejection and tolerance induction, Curr. Opin. Organ Transplant. 13 (4) (2008) 339.
[5] R. Girlanda, D.E. Kleiner, Z. Duan, et al., Monocyte infiltration and kidney allograft dysfunction during acute rejection, Am. J. Transplant. 8 (3) (2008) 600.
[6] B. Shrestha, J. Haylor, Experimental rat models of chronic allograft nephropathy: a review, Int. J. Nephrol. Renov. Dis. 7 (2014) 315.
[7] D. Poehnert, V. Broecker, M. Mengel, B. Nashan, M. Koch, Induction of chronic renal allograft dysfunction in a rat model with complete and exclusive MHC incompatibility, Transpl. Immunol. 22 (3–4) (2010) 137.
[8] M. Koch, S.A. Joosten, M. Mengel, C. van Kooten, L.C. Paul, B. Nashan, Adoptive transfer of primed CD4+ T-lymphocytes induces pattern of chronic allograft nephropathy in a nude rat model, Transplantation 79 (7) (2005) 753.
[9] L.C. Racusen, K. Solez, R.B. Colvin, et al., The BANFF 97 working classification of renal allograft pathology, Kidney Int. 55 (2) (1999) 713.
[10] K. Solez, R.B. Colvin, L.C. Racusen, et al., BANFF 07 classification of renal allograft pathology: updates and future directions, Am. J. Transplant. 8 (4) (2008) 753.
[11] M. Mengel, B. Sis, M. Haas, et al., BANFF 2011 meeting report: new concepts in antibody-mediated rejection, Am. J. Transplant. 12 (3) (2012) 563.
[12] M. Haas, B. Sis, L.C. Racusen, et al., BANFF 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions, Am. J. Transplant. 14 (2) (2014) 272.
[13] C. Doege, M. Koch, A. Heratizadeh, P. Sotonyi, M. Mengel, B. Nashan, Chronic allograft nephropathy in athymic nude rats after adoptive transfer of primed T lymphocytes, Transpl. Int. 18 (8) (2005) 981.
[14] A. Bickerstaff, R. Pelletier, J.J. Wang, et al., An experimental model of acute humoral rejection of renal allografts associated with concomitant cellular rejection, A. J. Pathol. 173 (2) (2008) 347.
[15] Z. Al-Aly, V. Reddivari, A. Moiz, et al., Renal allograft biopsies in the era of C4d staining: the need for change in the BANFF classification system, Transpl. Int. 21 (3) (2008) 268.
[16] M.D. Stegall, M.F. Chedid, L.D. Cornell, The role of complement in antibody-mediated rejection in kidney transplantation, Nat. Rev. Nephrol. 8 (11) (2012) 670.
[17] Y. Lin, P.T. Manning, J. Jia, et al., CD47 blockade reduces ischemia-reperfusion injury and improves outcomes in a rat kidney transplant model, Transplantation 98 (4) (2014) 394.
[18] F.Y. Ma, N. Woodman, W.R. Mulley, J. Kanellis, D.J. Nikolic-Paterson, Macrophages contribute to cellular but not humoral mechanisms of acute rejection in rat renal allografts, Transplantation 96 (11) (2013) 949.
[19] A.B. Magil, Monocytes/macrophages in renal allograft rejection, Transplant. Rev. 23 (4) (2009) 199.
[20] S. Heidenreich, D. Lang, M. Tepel, K.H. Rahn, Monocyte activation for enhanced tumour necrosis factor-alpha and interleukin 6 production during chronic renal allograft rejection, Transpl. Immunol. 2 (1) (1994) 35.
[21] P.J. McLaughlin, A. Aikawa, H.M. Davies, et al., Evaluation of sequential plasma and urinary tumor necrosis factor alpha levels in renal allograft recipients, Transplantation 51 (6) (1991) 1225.
[22] P.F. Halloran, M. Afrouzian, V. Ramassar, et al., Interferon-gamma acts directly on rejecting renal allografts to prevent graft necrosis, Am. J. Pathol. 158 (1) (2001) 215.
[23] A. Bajwa, G.R. Kinsey, M.D. Okusa, Immune mechanisms and novel pharmacological therapies of acute kidney injury, Curr. Drug Targets 10 (12) (2009) 1196.
[24] V. Zarkhin, G. Chalasani, M.M. Sarwal, The yin and yang of B cells in graft rejection and tolerance, Transplant. Rev. 24 (2) (2010) 67.
[25] A. Lehnhardt, M. Mengel, L. Pape, J.H. Ehrich, G. Offner, J. Strehlau, Nodular B-cell aggregates associated with treatment refractory renal transplant rejection resolved by rituximab, Am. J. Transplant. 6 (4) (2006) 847.
[26] E. Castigli, S.A. Wilson, S. Scott, et al., TACI and BAFF-R mediate isotype switching in B cells, J. Exp. Med. 201 (1) (2005) 35.
[27] A. Lehnhardt, F. Dunst, M. van Husen, et al., Elevated serum levels of B-cell activating factor in pediatric renal transplant patients, Pediatr. Nephrol. 27 (8) (2012) 1389.
[28] G. Grandaliano, L. Gesualdo, E. Ranieri, et al., Monocyte chemotactic peptide-1 expression in acute and chronic human nephritides: a pathogenetic role in interstitial monocytes recruitment, J. Am. Soc. Nephrol. 7 (6) (1996) 906.
[29] V. Grau, D. Gemsa, B. Steiniger, H. Garn, Chemokine expression during acute rejection of rat kidneys, Scand. J. Immunol. 51 (5) (2000) 435.
[30] P. Fixe, V. Praloran, M-CSF: haematopoietic growth factor or inflammatory cytokine? Cytokine 10 (1) (1998) 32.
[31] Y. Le Meur, M.D. Jose, W. Mu, R.C. Atkins, S.J. Chadban, Macrophage colony-stimulating factor expression and macrophage accumulation in renal allograft rejection, Transplantation 73 (8) (2002) 1318.
[32] Y. Le Meur, V. Leprivey-Lorgeot, S. Mons, et al., Serum levels of macrophage-colony stimulating factor (M-CSF): a marker of kidney allograft rejection, Nephrol. Dial. Transplant. 19 (7) (2004) 1862.
[33] S.E. Rintala, J. Savikko, J.M. Rintala, E. von Willebrand, Vascular endothelial growth factor (VEGF) ligand and receptor induction in rat renal allograft rejection, Transplant. Proc. 38 (10) (2006) 3236.
[34] W. Peng, J. Chen, Y. Jiang, Z. Shou, Y. Chen, H. Wang, Prediction of subclinical renal allograft rejection by vascular endothelial growth factor in serum and urine, J. Nephrol. 21 (4) (2008) 535.
[35] A. Ghafari, K. Makhdoomi, P. Ahmadpour, A.T. Afshari, S.S. Lak, L. Fakhri, Serum T-lymphocyte cytokines cannot predict early acute rejection in renal transplantation, Transplant. Proc. 39 (4) (2007) 958.