Cell Transplantation
2017, Vol. 26(8) 1428-1440

DOI: 10.1177/0963689717720295
journals.sagepub.com/home/cll
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Acute Renal Graft-Versus-Host Disease in a Murine Model of Allogeneic Bone Marrow Transplantation

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Abstract

Acute kidney injury (AKI) is a very common complication after allogeneic bone marrow transplantation (BMT) and is associated with a poor prognosis. Generally, the kidneys are assumed to not be no direct targets of graft-versus-host disease (GvHD), and renal impairment is often attributed to several other factors occurring in the early phase after BMT. Our study aimed to prove the existence of renal GvHD in a fully major histocompatibility complex (MHC)-mismatched model of BALB/c mice conditioned and transplanted according to 2 different intensity protocols. Syngeneically transplanted and untreated animals served as controls. Four weeks after transplantation, allogeneic animals developed acute GvHD that was more pronounced in the high-intensity protocol (HIP) group than in the low-intensity protocol (LIP) group. Urea and creatinine as classic serum markers of renal function could not verify renal impairment 4 weeks after BMT. Creatinine levels were even reduced as a result of catabolic metabolism and loss of muscle mass due to acute GvHD. Proteinuria, albuminuria, and urinary *N*-acetyl-beta-D-glucosaminidase (NAG) levels were measured as additional renal markers before and after transplantation. Albuminuria and NAG were only significantly increased after allogeneic transplantation, correlating with disease severity between HIP and LIP animals. Histological investigations of the kidneys showed renal infiltration of T cells and macrophages with endarteriitis, interstitial nephritis, tubulitis, and glomerulitis. T cells consisted of CD4+, CD8+, and FoxP3+ cells. Renal expression analysis of allogeneic animals showed increases in indoleamine-2,3 dioxygenase (IDO), different cytokines (tumor necrosis factor α, interferon-γ, interleukin 1 α [IL-1α], IL-2, IL-6, and IL-10), and adhesion molecules (intercellular adhesion molecule 1 and vascular cell adhesion molecule 1), resembling findings from other tissues in acute GvHD. In summary, our study supports the entity of renal GvHD with histological features suggestive of cell-mediated renal injury. Albuminuria and urinary NAG levels may serve as early markers of renal impairment.

Keywords

renal GvHD, cytokines, IDO, apoptosis, urinary NAG

Introduction

Since its clinical introduction in the early 1960s, allogeneic bone marrow transplantation (BMT) has been increasingly used for the treatment of malignant and nonmalignant diseases. Often, BMT is the only curative treatment option available.[1] Despite the development of novel strategies and the improved treatment of side effects, graft-versus-host disease (GvHD) remains a life-threatening complication, limiting an even broader use of BMT. In general, the skin, liver, and gastrointestinal tract are the main targets in acute GvHD.[2] Although up to 70% of patients develop acute kidney injury (AKI) after allogeneic BMT,[3] the kidneys are not assumed to be a direct target of T cells in acute GvHD.

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Submitted: September 29, 2016. Revised: February 16, 2016. Accepted: February 21, 2016.

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Instead, several other factors are responsible for renal impairment in the early phase after BMT, such as tumor lysis, sepsis, nephrotoxic medications, hepatic veno-occlusive disease, or thrombotic microangiopathy.[3-6] However, GvHD as a severe inflammatory disease may also indirectly cause nephropathy.[7] The exact etiology of renal dysfunction after BMT often remains unclear, because no renal biopsy is taken in most cases. However, AKI is a risk factor for developing chronic kidney disease (CKD) and is associated with increased morbidity and mortality.[8,9] Chronic GvHD of the kidneys has been described in several studies,[10-12] but reports of GvHD resulting in nephrotic syndrome are rare.[13] In contrast, only some studies have indicated renal involvement in acute GvHD.[14,15] A recent study specifically addressing this topic has described cell infiltration suggestive of acute GvHD of the kidneys in a rat model of allogeneic BMT.[16]

In our present study, we used a fully mismatched mouse model of GvHD to further investigate whether kidneys are a target of acute GvHD and to characterize pathophysiological processes involved.

Materials and Methods

Bone Marrow Transplantation

Animal experiments were approved by the institutional animal committee of the University of Regensburg and conducted in accordance with German animal protection laws. The transplantation procedure was conducted as recently described elsewhere.[17,18] In short, female C57BL/6 N (H-2^b) and BALB/c (H-2^d) mice (Charles River, Sulzbach, Germany) were transplanted at an age of 11 to 12 wks. First, BALB/c recipient mice were conditioned with lethal total body irradiation using a linear accelerator (150 cGy/min). A mixture of bone marrow cells and splenocytes from either syngeneic (BALB/c, synTx) or allogeneic (C57BL/6, alloTx) donors was injected through the tail vein. The effect of different transplantation conditions on alterations of the kidneys was tested by diversifying the irradiation dose as well as the count of transplanted bone marrow cells and splenocytes according to 2 different intensity protocols (Table 1). Untreated animals of the same age were used as controls to detect possible effects of irradiation and transplantation procedures ($n = 5$, ctrl).

Clinical GVHD Score, Serum, and Urine Samples

After transplantation, clinical GVHD scores were assessed weekly by a well-established standard scoring system incorporating weight loss, posture (hunching), mobility, fur texture, and skin integrity.[19] Each parameter was graded between 0 and 2 followed by calculation of the cumulative score for each mouse. Blood samples were obtained immediately after euthanasia in week +4. Urine samples were taken the week before transplantation and in the third week after transplantation to measure protein (Pierce protein method with bicinchoninic acid solution (B-9643, Sigma-Aldrich, Munich, Germany) and copper(II) sulfate solution (C-2284, Sigma-Aldrich) and albumin (enzyme-linked immunosorbent assay (ELISA) #ab108792, Abcam, Cambridge, MA, USA) content as well as creatinine (enzymatic kinetic colorimetric method (creatinine peroxidase-antiperoxidase (PAP) test) #LT-CR-0101, Labor+Technik, Berlin, Germany) and N-acetyl-beta-D-glucosaminidase (NAG; ELISA #MBS703771, My BioSource, San Diego, CA, USA) levels. The albumin to creatinine ratio was calculated. Four weeks after transplantation, animals were euthanized, blood samples were taken, and creatinine as well as blood urea nitrogen (enzymatic ultraviolet method (urea UV test) #LT-UR-0010, Labor+Technik, Berlin, Germany) levels were measured to test renal function. After that, the kidneys were prepared for further investigation.

Histology and Immunohistochemistry

For histological analysis, the kidneys were fixed in 20% buffered formalin and embedded in paraffin. Tissue samples were stained with hematoxylin/eosin (HE; Merck, Darmstadt, Germany) and periodic-acid-Schiff (PAS; Roth, Karlsruhe, Germany) for light microscopy. For detection of infiltrating T cells and macrophages, tissues were incubated with CD3 (anti-CD3 #ab16669, Abcam), CD4 (anti-CD4-Aff–Purified #AP20210PU-N, Acris, Herford, Germany), CD8 (anti-CD8 #bs-0648 R, Bioss Antibodies Inc., Woburn, MA, USA), forkhead box P3 (anti-FoxP3 #ab54501, Abcam), and MAC2 (anti-MAC2 #CL8942AP, Cedarlane, Burlington, Ontario, Canada) antibodies. To verify that CD4+, CD8+, and FoxP3+ cells were T cells, we performed a double staining with CD3 (anti-CD3 #ab5690 and anti-FoxP3 #ab54502, Abcam; anti-CD4 #14-9766, eBioscience, San Diego, CA, USA; anti-CD8 #bs-0648 R, Bioss Antibodies Inc., Woburn, MA, USA). Additionally, apoptosis was detected by means of a terminal deoxynucleotidyl transferase deoxyuridine triphosphate nucleotide (dUTP) nick end labeling (TUNEL) assay (TUNEL #G7130, Promega, Mannheim, Germany). In each kidney, the number of infiltrating CD3+ T cells, CD4+ T cells, CD8+ T cells, FoxP3+ T cells, and MAC2+ macrophages as well as apoptotic cells was counted in 20 randomly selected interstitial fields of view at 40× magnification.

Messenger RNA Expression in Kidneys

The expression of indoleamine-2,3 dioxygenase (IDO), different cytokines, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1; Table 2) was quantified by real-time polymerase chain reaction (RT-PCR). In brief, kidneys were collected, and total RNA was extracted (RNeasy kit, Quiagen, Hilden, Germany) according to the manufacturer's instructions. A deoxyribonuclease digestion step was included. The absorbance ratios A260/280 and A260/230 were calculated as measures of RNA purity; for all samples, only ratios >2 were

Table 1. Conditions of the 2 Transplantation Protocols and Numbers of Transplanted Animals.

Protocol	Irradiation Dose [Gy]	BMC [$\times 10^6$]	SC [$\times 10^6$]	alloTx [n]	synTx [n]
Low-intensity protocol Tx	8	2	1	8/7	5/5
High-intensity protocol Tx	9	2.5	2	8/5	5/4

Abbreviations: alloTx, number of allogeneically transplanted and surviving animals after 4 weeks; BMC, transplanted bone marrow cells; Gy, Gray; HIP, high-intensity protocol; LIP, low-intensity protocol; SC, transplanted splenocytes; synTx, number of syngeneically transplanted and surviving animals after 4 weeks.

Table 2. Primer Sequences Used for Gene Expression Analysis.

Gene	Forward Primer	Reverse Primer	Company
IDO	CCGGTCACGAATGTGGAACT	AGCTGCCCGTTCTCAATCAG	Eurofins
eNOS	Mm00435217_m1		Applied Biosystems
iNOS	Mm00440502_m1		Applied Biosystems
TNF-α	AGGCACTCCCCCAAAAGATG	TTTGCTACGACGTGGGCTAC	Eurofins
IFN-γ	CTTCAGCAACAGCAAGGCG	AGCGACTCCTTTTCCGCTTC	Eurofins
IL-1α	Mm00439620_m1		Applied Biosystems
IL-1β	Mm00434228_m1		Applied Biosystems
IL-2	TTTTACTTGCCCAAGCAGGC	GAAAGTCCACCACAGTTGCTG	Eurofins
IL-4	CAAACGTCCTCACAGCAACG	GGCATCGAAAAGCCCGAAAG	Eurofins
IL-6	Mm00446190_m1		Applied Biosystems
IL-10	AAGGGTTACTTGGGTTGCCA	GAAATCGATGACAGCGCCTC	Eurofins
IL-11	Mm00434162_m1		Applied Biosystems
ICAM-1	CACGCTACCTCTGCTCCT	AGGCTTCTCTGGGATGGATG	Eurofins
VCAM-1	TCTTGGGAGCCTCAA	CGTAGTGCTGCAAGTGAG	Eurofins
Beta-actin	Mm04394036_g1		Applied Biosystems

Abbreviations: eNOS, endothelial nitric oxide synthase; iNOS; inducible nitric oxide synthase; IDO, indoleamine-2,3 dioxygenase; IFN-γ, interferon γ; IL, interleukin; ICAM-1, intercellular adhesion molecule 1; TNF-α, tumor necrosis factor α; VCAM-1, vascular cell adhesion molecule 1.

accepted. For first-strand complementary DNA (cDNA) synthesis, 1 µg of total RNA was reverse transcribed with 1U Moloney Murine Leukemia Virus reverse transcriptase, 1 µg random primer, 1 mM deoxynucleotide triphosphate mixture, 1 µL recombinant RNasin ribonuclease inhibitor, and transcription buffer with 5 mM $MgCl_2$ in a final volume of 10 µL (all from Promega, Mannheim, Germany). The reaction mixture was incubated at 37°C for 60 min, followed by heat inactivation of the enzyme at 95°C for 5 min. In parallel, 1 µg of total RNA was processed without reverse transcription to control for contamination with genomic DNA. After cooling on ice for 5 min, the cDNA was stored at −20°C until further use. Real-time polymerase chain reaction was detected using the ABIPrism 7900 TaqMan, and data were analyzed with SDS Version 2.2.2 software (Applied Biosystems, Foster City, CA, USA). Beta-actin served as housekeeping gene for normalization of input amounts. For the relative quantification of target genes, a standard curve method with a cDNA pool from each single target sample was used. Measurements were carried out in triplicate, and their means were calculated for each animal.

Data Analysis

All data are shown as mean ± standard error of the mean (SEM). Differences among the treatment groups were evaluated either by analysis of variance (ANOVA) (Holm-Sidak as post hoc test) or Student's *t* test as appropriate with a software program (SigmaPlot, Systat Software Inc., San Jose, CA, USA). The following groups were compared by ANOVA: ctrl versus alloTx(HIP) versus synTx(HIP), ctrl versus alloTx(LIP) versus synTx(LIP), ctrl versus alloTx(HIP) versus alloTx(LIP), ctrl versus synTx(HIP) versus synTx(LIP). A P value of <0.05 was considered significant.

Results

Body Weight and GvHD Score as Markers of Acute Systemic GvHD

The average body weight before transplantation was about 20 g in each group. By week +4 after allogeneic BMT, the weight of the mice had decreased by 24.6% in the HIP group and by 9.9% in the LIP group. Syngeneic animals in both protocol groups showed only a transient loss of body weight in week +1 (synTx(LIP): −7.9%, synTx(HIP):11.1%) but had fully recovered by week +4 (synTx(LIP): +5.3%, synTx(HIP): +6.9%). In contrast, untreated controls showed a continuous increase in body weight by 16.2% during the animal experiment. These changes were significant in allogeneic animals in comparison with all other groups in week +4 before euthanasia and the subsequent investigations of the kidneys ($P < 0.001$ for alloTx(HIP) vs. ctrl, synTx(LIP)

and synTx(HIP); $P < 0.001$ for alloTx(LIP) vs. ctrl; $P < 0.05$ for alloTx(HIP) vs. alloTx(LIP) and for alloTx(LIP) vs. synTx(LIP) and synTx(HIP)). According to their weight changes, allogeneic animals also showed distinct clinical signs of acute GvHD as indicated by the GvHD score. Four weeks after the allogeneic transplantation, HIP animals reached a GvHD score of 6.0 ± 0.3, which was significantly higher than that in the LIP group with 3.9 ± 0.7 ($P = 0.048$). In contrast, syngeneic animals had no signs of relevant GvHD in week +4 with values of 1.4 ± 0.1 in the LIP group and 2.2 ± 1.1 in the HIP group. These results were already summarized in our recent publication.[18]

Parameters of Renal Function After BMT

Figure 1 depicts important markers of renal function in serum and urine. Serum urea levels (Fig. 1A) in each group did not show any significant differences (ctrl: 42.1 ± 2.6 mg/dL; alloTx(LIP): 37.0 ± 2.0 mg/dL; synTX(LIP): 36.9 ± 1.1 mg/dL; alloTx(HIP): 41.5 ± 1.8 mg/dL; synTx(HIP): 38.5 ± 3.9 mg/dL). Serum creatinine (Fig. 1B) was significantly lower in allogeneic animals than in control and syngeneic animals (ctrl: 18.1 ± 0.6 mg/dL; alloTx(LIP): 10.4 ± 1.8 mg/dL; synTX(LIP): 18.8 ± 2.4 mg/dL; alloTx(HIP): 9.7 ± 1.1 mg/dL; synTx(HIP): 14.3 ± 1.9 mg/dL). Urine analysis in the week before (week −1) and 3 weeks after transplantation (week +3) showed a significant increase in albuminuria objectified by the albumin/creatinine ratio (Fig. 1C) after allogeneic transplantation (alloTx(LIP) week −1: 11.6 ± 1.6 mg/g, week +3: 20.1 ± 2.6 mg/g; alloTx(HIP) week −1: 10.4 ± 1.2 mg/g, week +3: 31.1 ± 6.2 mg/g). A comparison of allogeneic animals showed significantly higher albuminuria levels in the HIP group than in the LIP group. In contrast, no significant changes could be observed for control and syngeneic animals between the 2 time points (ctrl week 1: 8.4 ± 0.5 mg/g, week +3: 7.3 ± 1.8 mg/g; synTx(LIP) week −1: 15.2 ± 2.4 mg/g, week +3: 12.5 ± 0.9 mg/g; synTx(HIP) week −1: 9.4 ± 0.5 mg/g, week +3: 10.9 ± 1.7 mg/g). In the LIP group, allogeneic animals had significantly higher albuminuria levels than control and syngeneic animals, and the same results applied to the HIP group. However, total urinary protein levels, also measured as protein/creatinine ratio, showed no changes between week −1 and week +3 (ctrl week −1: 33.8 ± 3.3 g/g, week +3: 36.0 ± 4.8 g/g; alloTx(LIP) week −1: 59.1 ± 6.1 g/g, week +3: 52.7 ± 3.6 g/g; synTx(LIP) week −1: 59.2 ± 4.4 g/g, week +3: 45.5 ± 2.7 g/g; alloTx(HIP) week −1: 43.1 ± 3.7 g/g, week +3: 50.8 ± 10.6 g/g; synTx(HIP) week −1: 35.1 ± 4.9 g/g, week +3: 37.2 ± 4.7 g/g). As a novel marker of renal function, we also assessed urinary NAG levels (Fig. 1D). Similar to albuminuria, NAG was significantly increased after allogeneic transplantation in both the LIP and the HIP groups (alloTx(LIP) week −1: 240.6 ± 2.1 μUI/mL, week +3: 354.1 ± 6.1 μUI/mL; alloTx(HIP) week −1: 330.7 ± 4.1 μUI/mL, week +3: 361.1 ± 5.3 μUI/mL). Apart from albuminuria levels, the difference between allogeneic LIP and HIP animals was not statistically significant. All of the other animals did not show any significant change in urinary NAG levels at the 2 time points (ctrl week −1: 229.8 ± 2.5 μUI/mL, week +3: 333.2 ± 1.8 μUI/mL; synTx(LIP) week −1: 337.1 ± 3.0 μUI/mL, week +3: 339.4 ± 2.4 μUI/mL; synTx(HIP) week −1: 331.03 ± 2.1 μUI/mL, week +3: 341.2 ± 3.3 μUI/mL). Allogeneic animals in the LIP group showed significantly higher NAG levels than control animals, whereas allogeneic animals in the HIP group had significantly higher NAG levels than both control and syngeneic animals.

Histological Alterations in Kidneys after BMT

Figure 2 shows representative histological alterations in kidneys after allogeneic (A to D) compared to syngeneic transplantation (E and F). A pathological infiltration of mononuclear cells into the renal interstitium was only found in allogeneic animals. Immunohistological characterization showed mainly CD3+ T cells (Fig. 3A to D) and MAC2+ macrophages (Fig. 3E and F) that reached from the perivascular tissue into the renal interstitium, infiltrating tubules, and glomeruli. Concomitantly with cellular infiltration, we detected apoptosis of tubular and glomerular cells as illustrated by TUNEL staining (Fig. 3G and H). Such changes were not observed after syngeneic BMT (Figs. 2e and f and 3I and K) or in untreated controls (not shown). Quantification of the infiltrating cells (Fig. 4) showed significantly more CD3+ T -cells in kidneys after allogeneic transplantation than in control and syngeneic animals (ctrl: 1.2 ± 0.1 cells/field; synTx(LIP): 1.2 ± 0.4 cells/field; alloTx(LIP): 10.1 ± 1.2 cells/field; synTx(HIP): 1.4 ± 0.2 cells/field; alloTx(HIP): 11.4 ± 0.9 cells/field). The same applied to MAC2+ macrophages that contained more cells after allogeneic BMT (ctrl: 1.6 ± 0.4 cells/field; synTx(LIP): 2.7 ± 0.1 cells/field; alloTx(LIP): 13.8 ± 1.2 cells/field; synTx(HIP): 2.1 ± 0.3 cells/field; alloTx(HIP): 11.1 ± 1.2 cells/field). Kidneys of all animals after allogeneic transplantation (7 of 7 in the LIP group and 5 of 5 in the HIP group) were affected by CD3+ and MAC2+ cell infiltrates. Double staining revealed CD4+, CD8+, and FoxP3+ cells costaining with CD3, which confirmed that they represent T cells. Their quantification again showed significantly more CD4+ T cells (ctrl: 1.0 ± 0.3 cells/field; synTx(LIP): 1.6 ± 0.2 cells/field; alloTx(LIP): 4.1 ± 0.6 cells/field; synTx(HIP): 2.2 ± 0.5 cells/field; alloTx(HIP): 3.6 ± 0.2 cells/field) and FoxP3+ (ctrl: 0.6 ± 0.1 cells/field; synTx(LIP): 1.1 ± 0.1 cells/field; alloTx(LIP): 2.9 ± 0.1 cells/field; synTx(HIP): 1.0 ± 0.2 cells/field; alloTx(HIP): 2.4 ± 0.1 cells/field) in animals after allogeneic transplantation. Differences in CD8+ T cells were not significant among the different groups (ctrl: 2.5 ± 0.2 cells/field; synTx(LIP): 1.6 ± 0.2 cells/field; alloTx(LIP): 1.7 ± 0.2 cells/field; synTx(HIP): 2.2 ± 0.2 cells/field; alloTx(HIP): 2.1 ± 0.2 cells/field). A significant difference between the 2 allogeneic animal groups was only found for FoxP3+ T cells that were more often detected in LIP animals with less severe disease than in the HIP group. Quantification

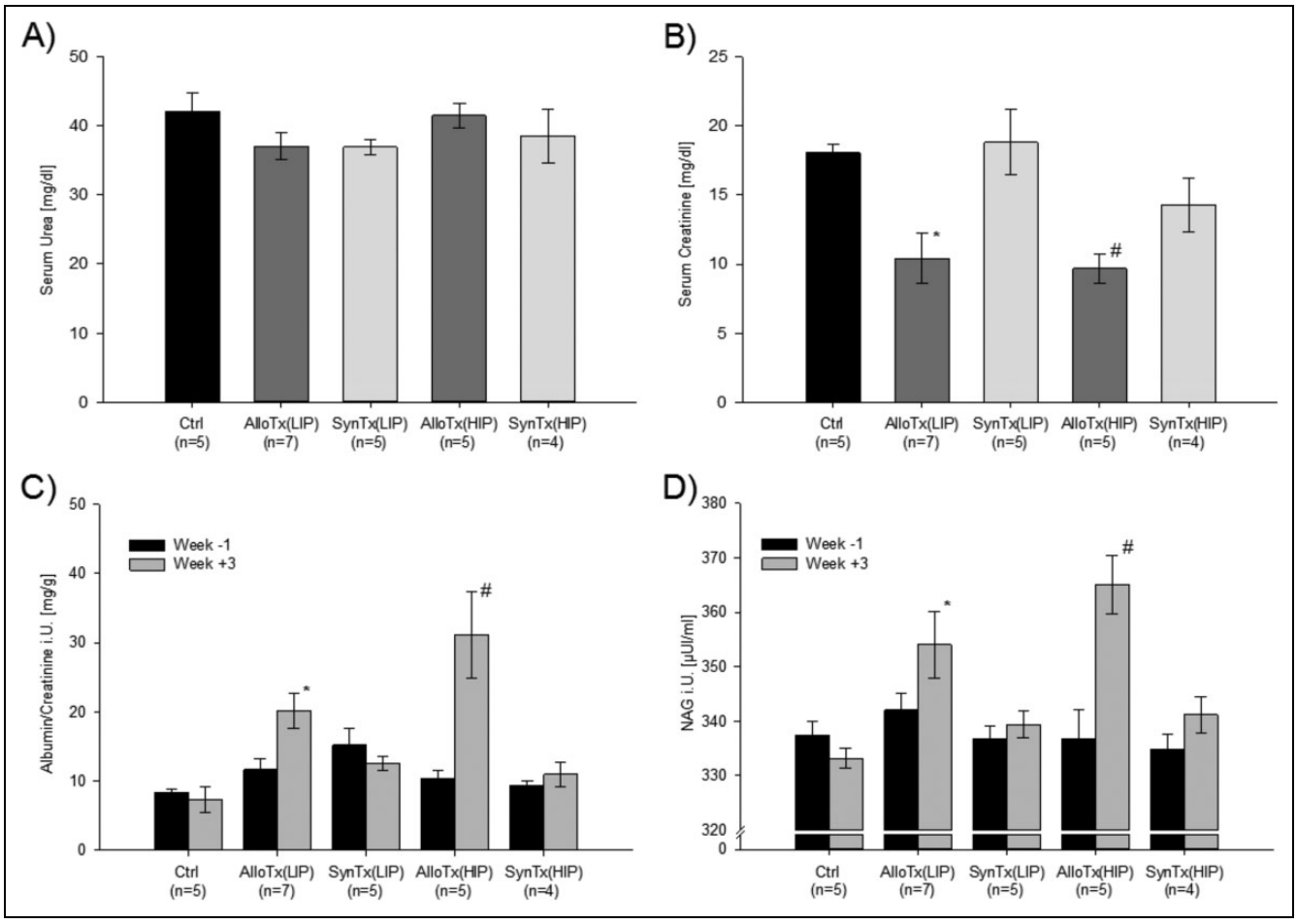

Figure 1. Serum and urine markers of renal function in acute GvHD. (A) Serum levels of urea. Serum urea levels do not significantly differ among untreated controls, allogeneic, or syngeneic animals. One-way analysis of variance (ANOVA): $P = 0.17$ for ctrl versus alloTx(LIP) versus synTx(LIP); one-way ANOVA: $P = 0.64$ for ctrl versus alloTx(HIP) versus synTx (HIP).(B) Serum levels of creatinine. After allogeneic transplantation, creatinine levels were significantly decreased in both protocol groups. Creatinine levels were unaffected by syngeneic transplantation. One-way ANOVA ($P = 0.015$): $^{*}P < 0.05$ versus ctrl and synTx(LIP), one-way ANOVA ($P = 0.002$): $^{\#}P < 0.01$ versus ctrl, $P < 0.05$ versus synTx(HIP).(C) Urinary albumin/creatinine ratio as a marker of albuminuria in week −1 and week +3. Only allogeneic animals showed a significant increase in albuminuria after transplantation. HIP allogeneic animals had even significantly higher levels than LIP allogeneic animals. Within the LIP group, allogeneic animals had the significantly highest albuminuria levels and the same finding applied to the HIP group. *t* test: $^{*}P = 0.02$ versus alloTx(LIP) week −1. One-way ANOVA ($P = 0.002$) in week +3: $^{*}P < 0.01$ versus ctrl, $P < 0.05$ versus synTx(LIP). *t* test: $^{\#}P < 0.001$ versus alloTx(HIP) week −1. One-way ANOVA ($P = 0.002$) in week +3: $^{\#}P < 0.01$ versus ctrl and synTx(HIP). One-way ANOVA ($P = 0.002$) in week +3: $^{*}P < 0.05$ versus ctrl, $^{\#}P < 0.01$ versus ctrl, $P < 0.05$ versus alloTx(LIP).(D) Urinary levels of *N*-acetyl-beta-D-glucosaminidase (NAG) in week −1 and week +3. After allogeneic transplantation, urine NAG levels significantly increased, but control and syngeneic animals did not show any change in NAG levels. HIP allogeneic animals tend to have higher NAG levels than LIP allogeneic animals. Inside the LIP group, allogeneic animals have higher NAG levels than controls and only slightly higher NAG levels than syngeneic animals. In the HIP group, allogeneic animals had significantly higher NAG levels than control and syngeneic animals. *t* test: $^{*}P = 0.048$ versus alloTx(LIP) week −1. One-way ANOVA ($P = 0.011$) in week +3: $^{*}P < 0.05$ versus ctrl, $P = 0.06$ versus synTx(LIP). *t* test $^{\#}P < 0.001$ versus alloTx(HIP) week −1. One-way ANOVA ($P < 0.001$) in week +3: $^{\#}P < 0.001$ versus ctrl, $P < 0.05$ versus synTx(HIP). One-way ANOVA ($P = 0.001$) in week +3: $^{*}P < 0.05$ versus ctrl, $P = 0.13$ versus alloTx(LIP), $^{\#}P = 0.001$ versus ctrl. alloTx, allogeneically transplanted animals; ANOVA, analysis of variance; ctrl, control group; GvHD, graft versus host disease; HIP; high-intensity protocol; LIP, low-intensity protocol; NAG, *N*-acetyl-beta-D-glucosaminidase; synTx, syngeneically transplanted animals.

of TUNEL+ cells showed a significant increase in apoptotic cells in allogeneic animals of both protocol groups (ctrl: 1.5 ± 0.7 cells/field; synTx(LIP): 5.5 ± 1.5 cells/field; alloTx(LIP): 18.3 ± 3.1 cells/field; synTx(HIP): 6.3 ± 1.9 cells/field; alloTx(HIP): 18.7.1 ± 3.0 cells/field). Such TUNEL+ cells were found in 5 of 7 allogeneic LIP and 5 of 5 allogeneic HIP animals.

Cytokine, IDO, and Adhesion Molecule Expression in Kidneys after BMT

Table 3 lists the renal expression at the mRNA level of all analyzed cytokines for the different treatment groups. IDO, tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), interleukin-1α (IL-1α), IL-2, IL-6, and IL-10 were all

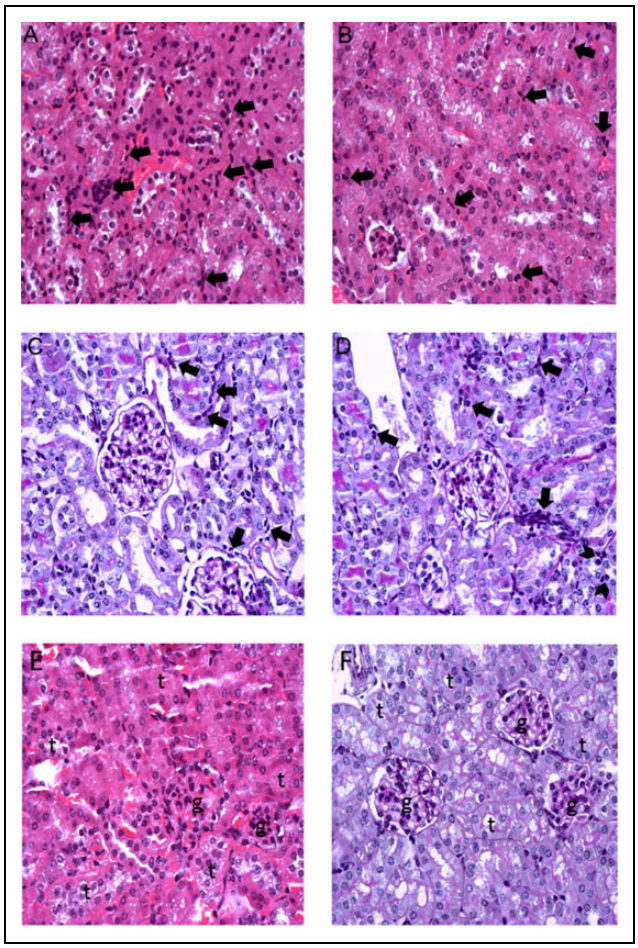

Figure 2. Histological alterations of kidneys in acute GvHD HE (A,B), and PAS (C,D) stainings of kidneys after allogeneic BMT. Mononuclear cell infiltrate was present in the renal interstitium (arrows in A-D). Some cells had also infiltrated the tubules (arrowheads in D). In syngeneic animals (E and F), no pathological infiltration of mononuclear cells was detected ("g" indicates glomeruli, "t" indicates tubules in E and F). (A-F magnification 40×). BMT, bone marrow transplantation; GvHD, graft versus host disease; HE, hematoxylin/eosin; PAS, periodic-acid Schiff.

upregulated at the mRNA level after allogeneic transplantation compared to untreated and syngeneic transplanted animals. Expression of TNF-α, IFN-γ, IL-6, and IL-10 was significantly higher in the HIP group than in the LIP group after allogeneic BMT, reflecting the clinical severity of acute GvHD in both groups. Interleukin-1 β (IL-1β) and IL-11 were the only cytokines with a similar expression level in all treatment groups. The expression of IL-4 was highest in untreated control animals and significantly lower after syngeneic and allogeneic BMT. A comparison of IL-4 expression between transplanted animals only showed higher levels after allogeneic but not after syngeneic BMT. However, the difference was only statistically significant for LIP animals, whereas only a trend was detectable in HIP animals ($P = 0.11$ for alloTx(HIP) vs. synTx(HIP)). The expression of the 2 adhesion molecules ICAM-1 and VCAM 1 was significantly elevated after allogeneic BMT. VCAM-1 also showed higher levels for syngeneic animals than for controls.

Discussion

Prophylaxis and treatment of acute GvHD after allogeneic BMT remain the main cornerstone in modern hematology and oncology. Although different organ systems are known to be affected by this life-threatening complication,[2] renal failure after BMT is attributed to several factors other than GvHD as stated by Hingorani.[7] A recent experimental study by Higo et al. has indicated that kidneys may also be a direct target of T cells in allogeneic BMT rats.[16] In our study, we used a fully mismatched mouse model of acute GvHD to further address this topic. Mice were transplanted according to 2 different intensity protocols with varying irradiation doses and numbers of transplanted splenocytes and bone marrow cells. As published previously, this type of transplantation caused acute GvHD in allogeneic animals that was clinically more severe in the HIP group than in the LIP group as indicated by the GvHD score and animal body weight. Syngeneic animals and untreated controls did not develop GvHD.[18]

In our model, we first aimed to assess alterations in renal function by different serum and urinary parameters. Serum urea levels did not differ among the different groups (Fig. 1A). Elevated serum urea levels as an indicator of renal injury and catabolic metabolism may have been expected, but serum urea is also affected by fluid homeostasis and liver function, which may explain our results. Serum creatinine levels (Fig. 1B) that are typically elevated in renal failure were even decreased in acute GvHD. Since creatinine is mainly influenced by body muscle content, we suggest that the observed decrease was caused by the loss of muscle mass due to catabolic metabolism in the context of acute GvHD. This hypothesis is also supported by the significant decrease in body weight after allogeneic BMT. Because these classic serum parameters of renal function did not seem to be practical for measuring renal injury in our animal model of GvHD, we also assessed proteinuria, albuminuria, and urinary NAG levels. We did not find any difference in proteinuria but a significant increase in albuminuria (Fig. 1C) between week −1 and week +3 in allogeneic animals. The amount of albuminuria even correlated with the clinical severity of GvHD between the LIP and the HIP groups, indicating glomerular damage after allogeneic BMT. The same results were yielded for urinary NAG (Fig. 1D) as a marker of tubular injury. Since renal injury in GvHD may be induced either by direct T-cell-mediated damage or indirectly by the severe inflammatory state in systemic GvHD,[7,20] albuminuria, and elevated NAG levels alone are not sufficient to diagnose renal GvHD.

Therefore, we conducted histological examinations of the kidneys that showed infiltration of mononuclear cells, causing endarteritis (Fig. 3A), interstitial nephritis (Fig. 3A and B, tubulitis (Fig. 3A to C), and glomerulitis (Fig. 3D and F).

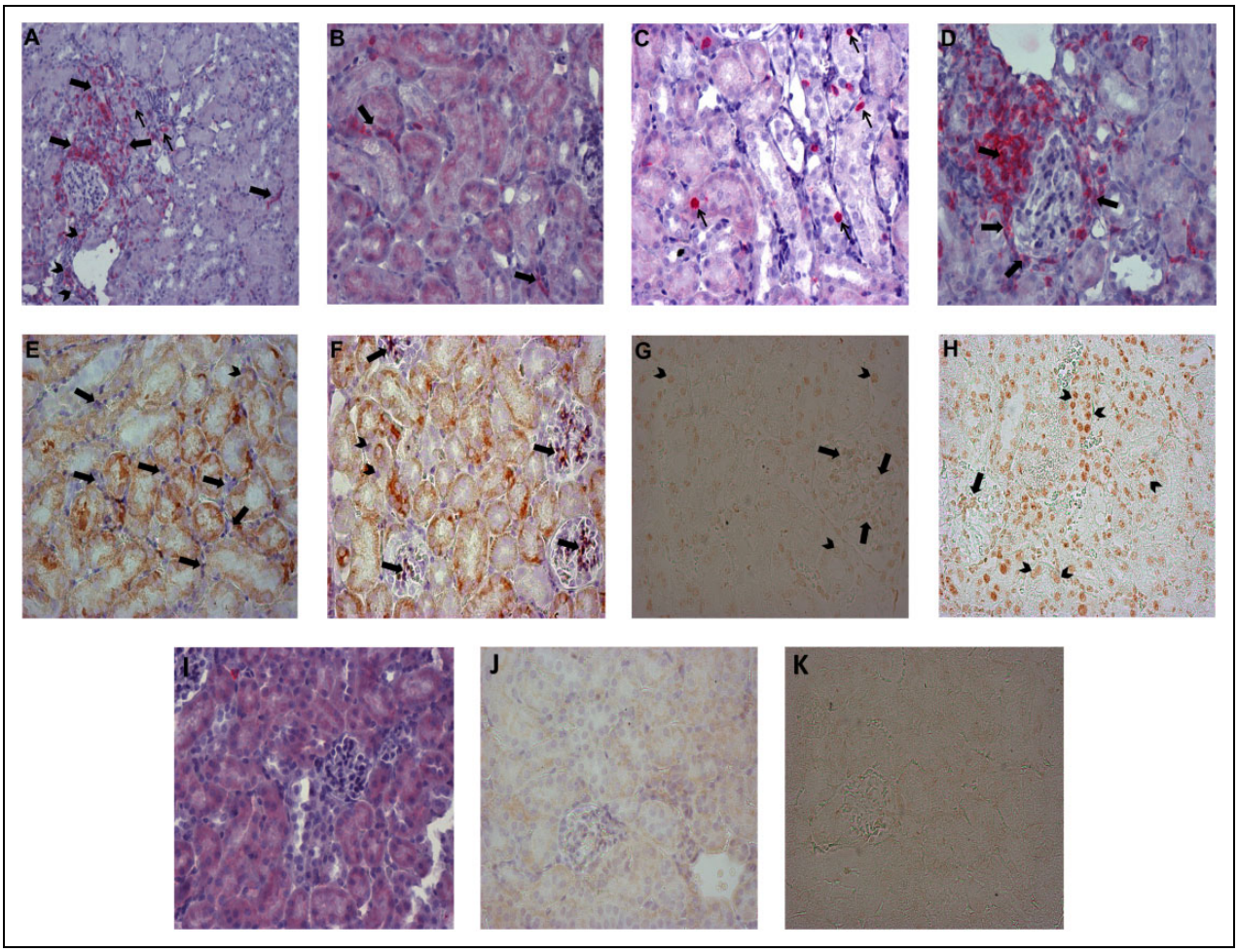

Figure 3. Immunhistological characterization of renal cell infiltrates and apoptosis in acute GvHD. Immunohistological staining for CD3 (A-D), MAC2 (E, F), and TUNEL(G, H) in kidneys after allogeneic BMT. CD3+ T cells were present around the vessels (endarteriitis, arrowheads in A), migrated into the interstitium (interstitial nephritis, arrows in A and B) and infiltrated tubules (tubulitis, thin arrows in A and C) and glomeruli (glomerulitis, arrows in D). MAC2+ macrophages were found in the interstitium (arrows in E) and infiltrate both tubules (arrowheads in E and F) and glomeruli (arrows in F). This infiltration was accompanied by apoptosis of tubular (arrowheads in G and H) and glomerular (arrows in G and H) cells. In contrast, after syngeneic BMT, no pathological infiltration of CD3+ T cells (I) and MAC2+ macrophages (J) as well as apoptosis was observed (K). (A magnification 20×, B-K magnification 40×). BMT, bone marrow transplantation; GvHD, graft versus host disease; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

Concomitantly to cell infiltrations, we could prove apoptosis of tubular and glomerular cells (Fig. 3G and H) that was in line with the above-mentioned level of albuminuria as a marker of glomerular and elevated urinary NAG levels indicating tubular injury. The cellular infiltrate consisted of CD3+ T cells, CD4+ T cells, CD8+ T cells, FoxP3+ T cells, and MAC2+ macrophages (Fig. 4). After allogeneic BMT, the number of CD3+ T cells, CD4+ T cells, FoxP3+ T cells, and MAC2+ macrophages was significantly increased compared to that of syngeneic and control animals. These findings reflect the pathophysiology of GvHD, in which donor T cells get activated by antigen-presenting cells (APCs) and proliferate, differentiate, and finally attack host tissues.[2,21] Both CD4+ and CD8+ cytotoxic T cells are important effectors against major histocompatibility complexes (MHCs) II and I[22] and fundamental for the development of acute GvHD. Thereby, the alloantigen composition of the host tissue is the main determinant for which the T-cell subpopulation gets activated.[21] Actually, it is not clear why in our fully MHC-mismatched mouse model only CD4+ and not CD8+ T cells were significantly increased. This needs to be further investigated, since in the rat model of Higo et al.[16] CD8+ T cells also infiltrated the kidneys in acute GvHD. FoxP3+ T cells have a regulatory effect on the activation and proliferation of T cells that are known to be able to preserve graft-versus-tumor activity and reduce GvHD after BMT.[23-25] A study on GvHD skin biopsies has described an association of the increase in FoxP3+ T cells with lower disease severity.[26] Our data on kidneys are in line with these results, since FoxP3+ T cells were elevated in GvHD and even more in LIP animals with less severe disease than in the HIP group. However, the role of

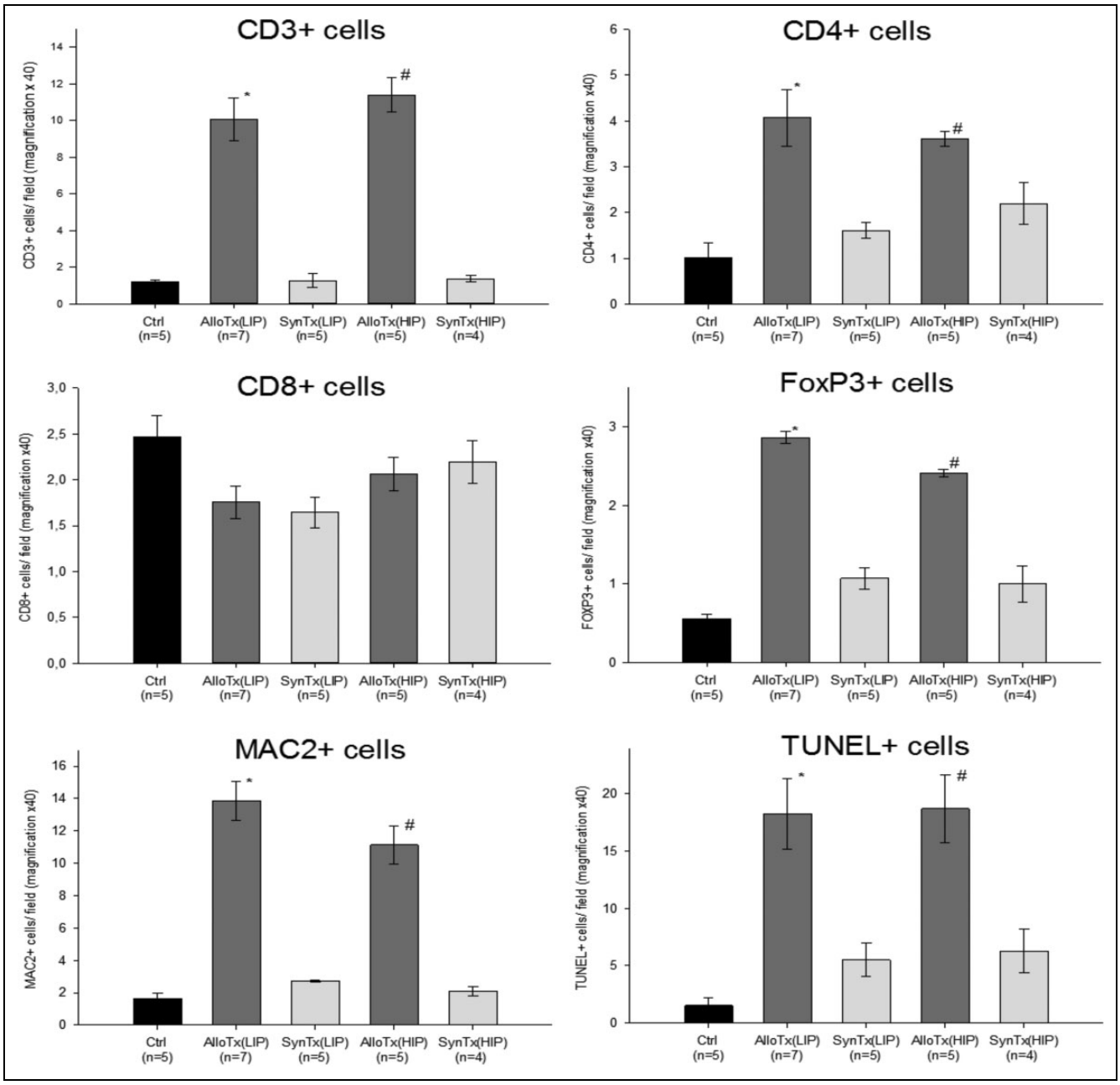

Figure 4. Quantification of infiltrating cell subpopulations and apoptotic cells in kidneys in acute GvHD. Immunohistological quantification of CD3+ T cells, CD4+ T cells, CD8+ T cells, FoxP3+ T cells, MAC2+ macrophages, and TUNEL+ apoptotic cells in kidneys. Four weeks after allogeneic BMT, CD3+ T cells and MAC2+ macrophages infiltrated the kidneys. T cells consist of CD4+, CD8+, and FoxP3+ cells. The numbers of CD3+ (one-way ANOVA ($P < 0.001$): *$P < 0.001$ versus ctrl and synTx(LIP), #$P < 0.001$ versus ctrl and synTx(HIP)), CD4+ (one-way ANOVA ($P < 0.005$): *$P < 0.05$ versus ctrl and synTx(LIP); one-way ANOVA ($P = 0.004$): #$P < 0.05$ versus ctrl and synTx(HIP)) and FoxP3+ T cells (one-way ANOVA ($P < 0.001$): *$P < 0.001$ versus ctrl and synTx(LIP), #$P < 0.001$ versus ctrl and synTx(HIP)) as well as MAC2+ macrophages (one-way ANOVA ($P < 0.001$): *$P < 0.001$ versus ctrl and synTx(LIP), #$P < 0.001$ versus ctrl and synTx(HIP)) per 40× magnification field were significantly higher in allogeneic mice than in untreated controls and syngeneic animals. FoxP3+ T cells showed a significant difference between allogeneic animals with more cells in the LIP than in the HIP group (one-way ANOVA ($P < 0.001$): #$P < 0.001$ vs. ctrl and allo(LIP)). All other infiltrating cell subpopulations did not show any difference between the 2 allogeneic groups. CD8+ T cells did not differ among all groups. Cell infiltration is accompanied by a significant increase in TUNEL+ apoptotic cells (one-way ANOVA ($P < 0.001$): *$P < 0.001$ versus ctrl, $P < 0.01$ versus synTx(LIP); one-way ANOVA ($P = 0.002$): #$P < 0.01$ versus ctrl, $P < 0.05$ versus synTx(HIP)). ANOVA, analysis of variance; alloTx, allogeneically transplanted animals; BMT, bone marrow transplantation; ctrl, control group; GvHD, graft versus host disease; LIP, low-intensity protocol; HIP, high-intensity protocol; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; synTx, syngeneically transplanted animals.

Table 3. Relative Expression of IDO, Cytokines, and Adhesion Molecules in Kidneys.

Gene	Ctrl	AlloTx(LIP)	SynTx(LIP)	AlloTx(HIP)	SynTx(HIP)
IDO	1.24 ± 0.03	1.68 ± 0.16*	1.04 ± 0.16	1.96 ± 0.20#	0.97 ± 0.08
TNF-α	0.43 ± 0.04	0.81 ± 0.08*	0.41 ± 0.02	1.35 ± 0.20#	0.53 ± 0.09
IFN-γ	0.15 ± .02	1.13 ± 0.17*	0.11 ± 0.03	2.05 ± 0.29#	0.11 ± 0.01
IL-1α	0.32 ± 0.04	1.91 ± 0.51*	0.44 ± 0.04	2.43 ± 0.52#	0.53 ± 0.11
IL-1β	1.07 ± 0.04	1.15 ± 0.13	1.23 ± 0.07	1.08 ± 0.21	1.33 ± 0.10
IL-2	0.46 ± 0.05	2.91 ± 0.43*	0.30 ± 0.01	2.58 ± 0.44#	0.31 ± 0.06
IL-4	11.25 ± 1.43†	5.19 ± 1.41*	1.27 ± 0.49	3.34 ± 0.75	0.75 ± 0.35
IL-6	0.33 ± 0.05	1.13 ± 0.12*	0.48 ± 0.08	1.69 ± 0.23#	0.82 ± 0.03
IL-10	0.06 ± 0.01	1.21 ± 0.32*	0.04 ± 0.02	2.27 ± 0.45#	0.07 ± 0.04
IL-11	0.73 ± 0.02	1.04 ± 0.08*	0.85 ± 0.09	1.12 ± 0.21	0.89 ± 0.02
ICAM-1	0.68 ± 0.06	1.05 ± 0.10*	0.57 ± 0.05	1.20 ± 0.14#	0.74 ± 0.01
VCAM-1	0.31 ± 0.02	0.98 ± 0.08*	0.57 ± 0.11†	1.10 ± 0.16#	0.63 ± 0.04π

Abbreviations: ANOVA, analysis of variance; AlloTx, number of allogeneically transplanted and surviving animals after 4 weeks; IDO, indoleamine-2,3 dioxygenase; HIP, high-intensity protocol; IFN-γ, interferonγ; IL, interleukin, ICAM-1, intercellular adhesion molecule 1; LIP, low-intensity protocol; SynTx, number of syngeneically transplanted and surviving animals after 4 weeks; TNF-α, tumor necrosis factor α; VCAM-1: vascular cell adhesion molecule 1.
IDO: one-way ANOVA ($P = 0.021$): *$P < 0.05$ versus synTx(LIP); one-way ANOVA ($P = 0.002$): #$P < 0.01$ versus synTx(HIP), $P < 0.05$ versus ctrl. TNFα: one-way ANOVA ($P = 0.001$): *$P < 0.01$ versus ctrl and synTx(LIP); one-way ANOVA ($P < 0.001$): #$P < 0.01$ versus ctrl and synTx(HIP); one-way ANOVA ($P < 0.001$): *$P < 0.05$ versus alloTx(LIP). IFN-γ: One-way ANOVA ($P < 0.001$): *$P < 0.001$ versus ctrl and synTx(LIP); one-way ANOVA ($P < 0.001$): #$P < 0.001$ versus ctrl and synTx(HIP); one-way ANOVA ($P < 0.001$): #$P < 0.01$ versus alloTx(LIP). IL-1α: one-way ANOVA ($P = 0.004$): *$P < 0.01$ versus ctrl and synTx(LIP); one-way ANOVA ($P = 0.003$): #$P < 0.01$ versus ctrl, $P < 0.05$ versus synTx(HIP). IL-2: one-way ANOVA ($P < 0.001$): *$P < 0.001$ versus ctrl and synTx(LIP); one-way ANOVA ($P < 0.001$): #$P < 0.001$ versus ctrl and synTx(HIP). IL-4: one-way ANOVA ($P < 0.001$): *$P < 0.05$ versus ctrl and synTx(LIP), †$P < 0.001$ versus synTx(LIP); one-way ANOVA ($P < 0.001$): †$P < 0.001$ versus alloTx(HIP) and synTx(HIP). IL-6: one-way ANOVA ($P < 0.001$): *$P < 0.001$ versus ctrl and synTx(LIP); one-way ANOVA ($P < 0.001$): #$P < 0.001$ versus ctrl, $P < 0.05$ versus synTx(HIP); one-way ANOVA ($P < 0.001$): #$P < 0.05$ versus alloTx(LIP). IL-10: one-way ANOVA ($P = 0.003$): *$P < 0.01$ versus ctrl and synTx(LIP); one-way ANOVA ($P < 0.001$): #$P < 0.001$ versus ctrl and synTx(HIP); one-way ANOVA ($P = 0.001$): #$P < 0.05$ versus alloTx(LIP). IL-11: one-way ANOVA ($P = 0.023$): *$P < 0.05$ versus ctrl. ICAM-1: one-way ANOVA ($P = 0.002$): *$P < 0.05$ versus ctrl, $P < 0.01$ versus synTx(LIP); one-way ANOVA ($P = 0.004$): #$P < 0.01$ versus ctrl, $P < 0.05$ versus synTx(HIP). VCAM-1: one-way ANOVA ($P < 0.001$): *$P < 0.001$ versus ctrl, $P < 0.01$ versus synTx(LIP), †$P < 0.05$ versus ctrl; one-way ANOVA ($P < 0.001$): #$P < 0.001$ versus ctrl, $P < 0.05$ versus synTx(HIP), π $P < 0.05$ versus ctrl.

T-regulatory cells (Tregs) and their interplay with IDO, which suppresses the activation of conventional T cells and promotes the function of Tregs,[27] for the severity of renal GvHD needs to be further clarified. In summary, these findings are suggestive of cell-mediated renal injury as could be expected in GvHD.

In the pathophysiology of acute GvHD, not only cellular factors such as T cells and macrophages are fundamental but different cytokines also trigger and coordinate the attack of T cells against the host tissue.[2,21,28] To the best of our knowledge, this is the first study to extensively analyze renal cytokine expression in the context of GvHD including different proinflammatory type 1 T helper (Th1)- and anti-inflammatory Th2-cytokines.[28,29] TNF-α, IFN-γ, IL-1α, IL-2, IL-6, and IL-10 were upregulated (Table 3). Higher expression levels of TNF-α, IFN-γ, IL-6, and IL-10 in allogeneic HIP animals were associated with more severe GvHD compared to allogeneic LIP animals. The increase in proinflammatory cytokines, such as TNF-α, IL-1, and IL-6 termed "danger signals," was well in line with the actual understanding of GvHD.[29,30] These cytokines are produced during the first step of GvHD as a consequence of damage to the host tissue. In a second step, the cytokines generate a highly inflammatory environment and promote the activation of T cells by APCs. T cells proliferate, differentiate, and produce large amounts of Th1-cytokines such as IFN-γ and IL-2. IFN-γ has many different functions in GvHD and may augment or reduce the clinical course of the disease.[2,21] This function also applies to IL-2, which may enhance the severity and mortality of GvHD[30] and is also important for the expansion of Tregs in chronic GvHD.[31,32] In contrast, similar to Th2-cytokines, IL-10 is generally considered anti-inflammatory.[33] After allogeneic BMT, the effect of IL-10 appears to be dose-dependent. In low doses, IL-10 seems to be protective, but high doses are detrimental to the course of GvHD.[34-37] This fact matches our results, because HIP animals with more severe disease showed even higher renal expression levels of IL-10 than the LIP group. IL-11 is also known to have anti-inflammatory effects.[38,39] Administration of IL-11 after allogeneic BMT protected against acute GvHD and maintained the graft-versus-leukemia effect in a mouse model.[40,41] Yet, a study on humans had to be terminated because of increased mortality after IL-11 application.[42] Our study could show similar expression levels in all groups and no significant differences between allogeneic and syngeneic animals. IL-4 also belongs to Th2-cytokines. Elevated IL-4 levels have been described in acute GvHD,[43,44] and the beneficial effect of natural killer cells on GvHD seems to be IL-4 dependent.[45-48] We found renal IL-4 expression to be significantly elevated in allogeneic LIP animals compared to syngeneic animals. Allogeneic HIP animals only showed a trend toward higher expression. Interestingly, the highest expression levels could be detected in untreated control animals, which would suggest that IL-4

expression could be decreased by irradiation. This finding would be of interest, since irradiation normally favors the development of regulatory natural killer cells accompanied by elevated IL-4 levels,[49] and in our recent study, IL-4 expression was not changed in mesenteric arteries of the same animals.[18] Whether this unexpected finding may be specific for kidneys and whether it represents an effect of antecedent radiation or other mechanisms need further clarification.

Furthermore, we also quantified the expression of IDO. IDO is known to be induced in inflammation and plays an important role in immunomodulation and tumorigenesis.[50,51] IDO is activated by IFN-γ,[52,53] lipopolysaccharides,[54] TNF-α,[55] IL-6, and IL-1β.[50] Its immunosuppressive effect results from inhibited T-cell proliferation and the ability to activate mature Tregs and to convert naive T cells to Tregs.[50] IDO activation is suggested to ameliorate GvHD severity and mortality.[56-58] High expression of IDO in intestinal mucosal mononuclear cells and low expression in endothelial cells were associated with favorable outcome in intestinal GvHD.[59] In our study, we could find higher IDO expression levels after allogeneic BMT in both HIP and LIP animals.

Adhesion molecules play an important role in effector cell migration and in trafficking into target organs in GvHD.[60] They are upregulated in the inflammatory milieu mainly mediated by IL-1, TNF-α, and IFN-γ.[2,61] Our results were in line with these findings because of the increased expression of ICAM-1 and VCAM-1 after allogeneic BMT. Additionally, VCAM-1 showed significantly higher expression in syngeneic than in control animals, suggesting that VCAM-1 expression is also upregulated by irradiation itself. Upregulation of different adhesion molecules has been described for radiation-induced normal tissue injury[62] and especially for VCAM-1 in the case of radiation-induced lung inflammation.[63]

Regarding our renal cytokine, IDO, and adhesion molecule expression analysis in acute GvHD, there are some limitations to admit. First, data in the literature often conflict with regard to the expression of different cytokines in acute GvHD. Often, levels of circulating cytokines are examined. However, we present renal tissue expression in the current study, and the 2 are not necessarily comparable. Second, since we determined mRNA expression in whole kidney samples, we cannot determine which cell populations, specifically infiltrating T cells or cells of renal parenchyma, are the major sources of cytokine production. Third, we only present data on mRNA expression and therefore know nothing about the protein expression. Finally, it has to be stated that the relative differences are often only small. Taking these limitations into account, our expression data are difficult to interpret and further studies regarding the source of cytokine production and determination of circulating and protein levels are needed.

In summary, our current study affirms some of the results from Higo et al.[16] in our mouse model with different GvHD severities and also adds some interesting new findings, which may help to further understand renal GvHD. Our histological investigations verified considerable renal infiltration by T cells and macrophages, causing endarteritis, interstitial nephritis, tubulitis, and glomerulitis. Additionally, with the TUNEL staining, we succeeded in correlating the observed cell infiltrates with tissue damage and apoptosis of tubular and glomerular cells. We confirmed elevated urinary NAG levels as marker of tubular damage and are now the first to describe albuminuria due to glomerular injury in renal GvHD. Furthermore, we extended data regarding the renal cytokine milieu with an extensive expression analysis of cytokines and adhesion molecules, which resembled findings from other tissues in GvHD. Of importance, with the quantification of FoxP3+ T cells and the IDO expression, we identified 2 new potential regulators of GvHD. However, their exact role in the pathogenesis and severity of renal GvHD warrants further study.

In conclusion, our current study further supports the hypothesis of the existence of a renal form of GvHD characterized by cell-mediated renal injury. Albuminuria and the elevation of urinary NAG may be early markers for renal impairment in acute GvHD. The exact pathophysiological mechanisms, in particular the exact targets of T-cell attack in the kidneys, the role of regulatory T cells, and the tryptophan metabolism need to be further elucidated to develop potential prophylactic and therapeutic interventions.

Acknowledgment

We thank Gabriela Pietrzyk for their excellent technical assistance.

Ethical Approval

Animal experiments were approved by the institutional animal committee of the University of Regensburg.

Statement of Human and Animal Rights

Animal experiments were conducted in accordance with German animal protection laws.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: The study was supported by a grant of the "Regensburger Forschungsförderung in der Medizin" (ReForM A) to Peter M. Schmid.

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