KIDNEY TRANSPLANTATION AND INNATE IMMUNITY

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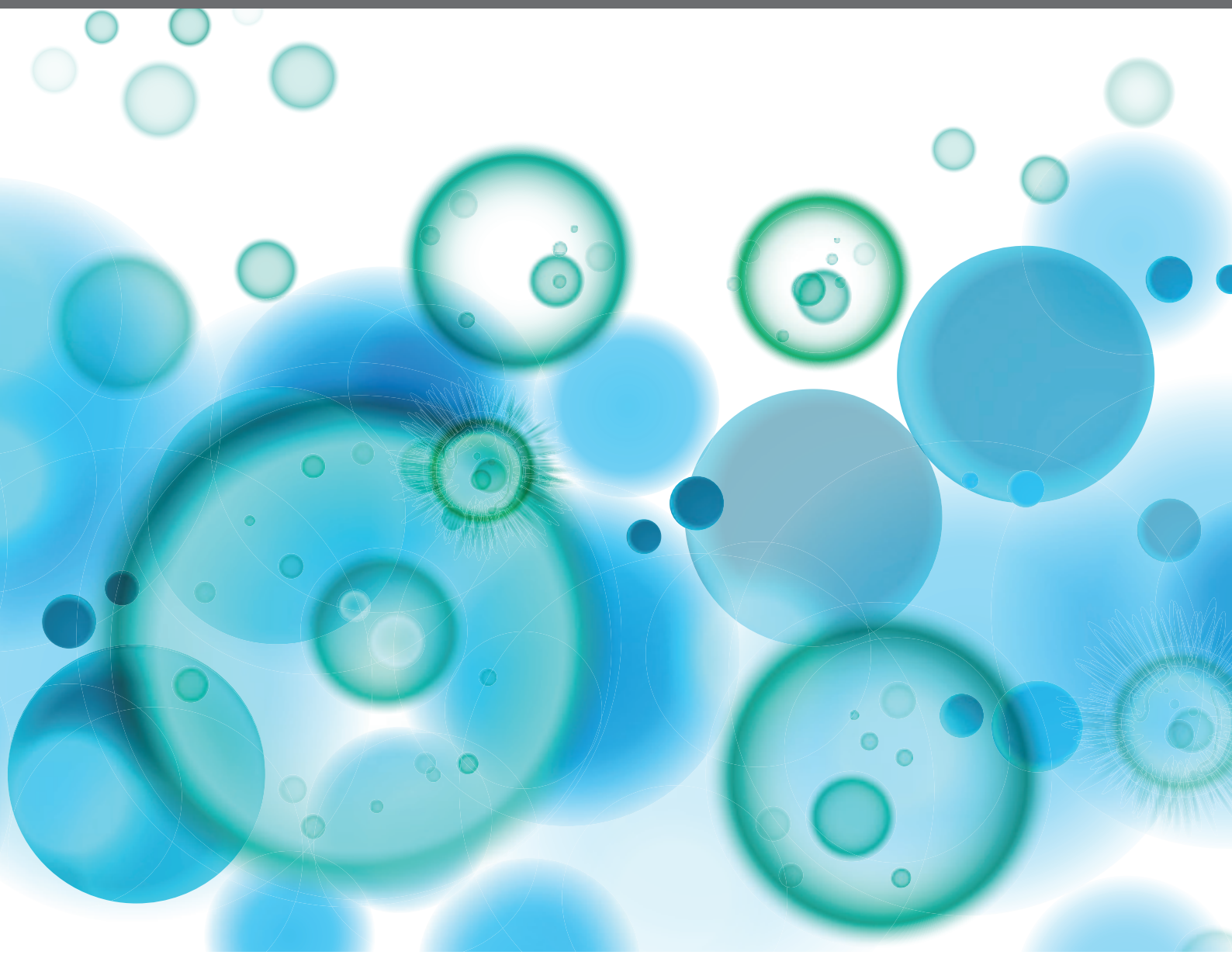

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KIDNEY TRANSPLANTATION AND INNATE IMMUNITY

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Editorial: Kidney Transplantation and Innate Immunity

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Keywords: kidney transplantation, innate immunity, immune cells, complement system, coagulation cascade

Editorial on the Research Topic

Kidney Transplantation and Innate Immunity

INTRODUCTION

Kidney transplantation is the best treatment for End Stage Renal Disease (ESRD). Although the advances in immunosuppressive drugs and protocols have markedly reduced the incidence and relevance of acute rejection, the outcome of kidney grafts is still significantly influenced by the development of chronic cellular or humoral rejection. In this setting adaptive alloimmune response has always been considered as the main, if not the only, player and the role of innate immunity has been disregarded for a long period of time. However, in the last two decades a growing body of evidence suggested that innate immune responses significantly contribute to priming of the rejection machinery and control the activation of alloantigen-specific adaptive immunity.

The principal constituents of innate immunity are represented by cellular components such as phagocytic cells (neutrophils, macrophages), dendritic cells, natural killer (NK) and other innate lymphoid cells, and blood proteins, including members of the complement system and other mediators of inflammation.

Several factors can induce the activation of innate immune responses in clinical kidney transplantation. In Donation after Brain Death (DBD), brain death itself can promote the systemic production and release of pro-inflammatory cytokines such as monocyte chemotactic peptide-1 and interleukin-6, leading to the activation of innate immune pathways such as monocyte recruitment and activation in several organs including the kidneys. Moreover, warm and cold ischemia during kidney retrieval and preservation followed by reperfusion at the time of transplantation is also known to induce, through several molecular mechanisms including oxidative stress and resident cells apoptosis, the activation of innate immune responses. Interestingly, the communication between innate and adaptive immune response in kidney graft rejection is bi-directional since there is now clear evidence that adaptive immune response activation may lead to tissue damage through cellular and molecular components of innate immunity.

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The 17 articles of this Research Topic summarize recent achievements and provide timely reviews in the field of the different components of innate immunity and kidney transplantation.

INNATE IMMUNE CELLS IN KIDNEY TRANSPLANTATION

Different cell types involved in the innate immune response can play an important role in kidney transplantation, and are influenced by the suppression of the recipient's immune. Recognition of allogeneic non-self induces the activation of recipients T lymphocytes, that can inflict direct cytotoxicity on graft cells or can influence other cells of the immune system such as B lymphocytes or macrophages. In this scenario also dendritic cells might play an important role as antigen presenting cells, and their activation can influence innate immune responses in different ways, for example, through activation of natural killer cells. In the present issue, six papers described the characteristic and behavior of different innate immune cells in the setting of kidney transplantation and graft rejection. This section highlights that basic knowledge on innate immune cells in kidney transplantation is continuously emerging with the description of novel functions relying on recently identified cells subsets or on cross-talk with distinct other cell types.

Sablik et al. performed a detailed identification of inflammatory cells in renal allograft biopsies from patients with chronic-active antibody-mediated rejection through multiplex immunofluorescent staining. The glomerular compartment was predominantly characterized by CD8+ cytotoxic T cells (granzyme B+ and CD57+) and M2 macrophages (CD68+ and CD163+). T cells (either CD4+ or CD8+ T cells) and macrophages were also present in the tubular interstitial compartment but relatively few CD8+ T cells expressed granzyme and/or CD57. In the tubular compartment there were also CD3+FoxP3+ cells, and their increased number was significantly associated with a poor renal allograft survival.

Dai et al. provided an up-date on the role of dendritic cells in ischemia injury. Their review includes a description of the dendritic cells "interactome" following renal ischemia reperfusion injury, finally leading to renal tubular epithelial cell apoptosis, glomerular endothelial damage, and fibrin deposition. During post-ischemia reperfusion injury, intra-renal dendritic cells can activate natural killer T cells and amplify the innate immune response. Ischemia reperfusion injury is responsible of increased inflammation in kidney transplantation thus mediating the recruitment of different cell-types, included B cells. In their research, Kreimann et al. observed an increase in the number of cells expressing the CXCL13 receptor CXCR5, the majority of which were B-cells, through a single-cell sequencing analysis. In a mouse model of kidney transplantation, they confirmed that increased levels of systemic serum CXCL13 correlated with length of cold ischemia time.

Ischemia/reperfusion injury induces an inflammatory reaction that is mediated by Pattern Recognition Receptors expressed on both infiltrating immune cells and tubular epithelial cells. In their review Tammaro et al. described how engagement of these innate immune receptors can influence behavior of tubular cells in terms of mitochondrial dysfunction, senescence, cell death and production of pro-fibrotic cytokines. Cross-talk between innate immunity and tubular cell metabolism represents a novel approach in the evaluation of cell fate in ischemia-reperfusion injury.

Among the different cell types involved in the innate immune response, also natural killer cells subsets play an important role in kidney graft damage. The knowledge on natural killer cells has greatly evolved recently and Pontrelli et al. provided an update on their role in kidney transplantation. Their review includes the description of the different natural killer subsets involved in antibody and T-Cell mediated rejection, but also how natural killer cells can contribute to transplant tolerance and the influence of immunosuppression on natural killer cell phenotype. The specific phenotype of infiltrating natural killer cells in human kidney allograft rejection has been confirmed by Kildey et al. through an innovative multi-color flow cytometry-based approach, confirming that natural killer cell subsets are differentially recruited and activated during distinct types of rejection.

CROSS-TALK BETWEEN COMPLEMENT, COAGULATION, AND INNATE IMMUNE CELLS IN KIDNEY TRANSPLANTATION

Innate immune responses in kidney transplantation are also characterized by the activation of complement system, as well as of the coagulation cascade. These key components of innate immunity are tightly connected to each other. The complement system is a crucial mediator of the innate immune response, thus influencing other endogenous systems. The complement cascade can be activated by three major complement activation pathways (classical, alternative, and lectin pathway) that converge into a common sequence leading to the formation of C3- and C5-convertases and generation of the anaphylatoxins C3a and C5a and of the C5b-9 membrane attack complex. The complement system is highly regulated to prevent over-activation, since this may lead to systemic inflammation, dysregulation of coagulation/fibrinolysis, and tissue-damage thus contributing to allograft injury.

In their review, Grafals et al. analyzed how the complement system can influence inflammatory injury of the graft and the response of B and T cells to donor antigens. They also explored the role of complement inhibitory drugs in preventing immune responses against allografts, suggesting their possible use as adjuncts to the currently available anti-rejection drugs in kidney transplantation.

Complement activation in kidney transplantation can be induced by donor-brain death and is associated with a worst renal allograft outcome. In their paper, Jager et al. investigated the role of the alternative pathway in a model of brain-death

induced in Fisher rats. Pre-treatment of rats with anti-factor B exhibited unique complement-regulatory and anti-inflammatory properties and thus extends the emerging field of complement therapeutics. Activation of complement may contribute to the progression of renal failure through tubular C5b-9 formation. Lammerts et al. investigated the alternative pathway complement factor properdin and the terminal sC5b-9 complex in the urine of a previously described cohort of 707 renal transplant recipients. They observed that after kidney transplantation, independent of proteinuria, the urinary presence of properdin and the terminal sC5b-9 complex showed significant impact on the rate of graft failure and graft survival, suggesting them as useful biomarkers of immunological injury and kidney allograft deterioration.

Wang et al. highlighted the role of increased labile heme levels in the kidney, due to prolonged warm ischemia, in the up-regulation of renal inflammation and activation of the complement system. In their paper they used a mouse model of ischemia-reperfusion-injury to demonstrate that prolonged ischemia-reperfusion injury not only increased labile heme concentrations in renal tissue, but also up-regulated C5a receptor, as well as several pro-inflammatory and pro-fibrotic cytokines and induced neutrophil infiltration. Interestingly, heme removal by human serum albumin reduced the expression of pro-inflammatory cytokines, C3a receptor and improved tubular function after ischemia-reperfusion injury.

Beside the pivotal role in innate immunological response, complement activation is also involved in the aging process. The review from Franzin et al. highlights the link existing between complement activation and premature renal senescence in the context of the transition from acute kidney injury to chronic kidney disease, with a special focus on ischemia/reperfusion Injury and antibody-mediated rejection. Strategies to target complement in kidney transplantation, to prevent the development of acute kidney injury and its progression to chronic disfunction, were also discussed both at the experimental and clinical level. Finally, authors provide emerging insights on molecular mechanisms involved in complement-induced renal "inflammaging," including Klotho signaling, Wnt/β catenin pathway, epigenetic changes, and cell cycle arrest.

Systemic activation of the complement cascade as well as of the coagulation system, can be modulated by extracellular vesicles that are known immune-modulators and might play a critical role in kidney transplantation. In their review Quaglia et al. described the involvement of extracellular vesicles in the modulation of innate/adaptive immune systems and their role as shuttle of specific mediators involved in graft tissue injury. Investigation of extracellular vesicles in urinary and serum samples is a very interesting approach for the identification of potential biomarkers of graft damage. Extracellular vesicles might also represent promising therapeutic tools in kidney transplantation, functioning as vehicle of drugs or miRNAs to antagonize specific mediators of inflammation or graft damage.

Stallone et al. highlighted the role of the coagulation cascade and fibrinolytic system in the ischemia/reperfusion injury and in the pathogenesis of tissue damage in acute and chronic rejection. Following ischemia-reperfusion injury, the coagulation cascade is strongly activated, mainly induced by the vascular expression of tissue factor. Fibrin deposition in the kidney graft may also represent a challenging cause of graft dysfunction thus leading to graft rejection. Several interactions between coagulation, fibrinolysis and complement have been proposed in a variety of clinical conditions, including kidney transplantation, opening the way for inhibition of coagulation to modulate innate immunity and to prevent progressive graft damage.

INNATE IMMUNE CELLS AND THERAPY

Innate immune cells such as dendritic cells, monocytes, macrophages, neutrophils and natural killer cells play an important role in most immunological events following kidney transplantation and their behavior can be significantly influenced by immunosuppressive therapies. The definition of the therapeutic range of specific drugs is the final goal for each clinician in order to obtain better outcomes of the graft with reduced adverse events such as graft rejection or occurrence of infections.

Song et al. investigated how the duration of time being within the therapeutic range of tacrolimus-based immunesuppressive regimen might influence the long-term clinical outcomes in living kidney transplantation. Since there is a considerable association between the mantainance within the tacrolimus therapeutic range in the first year and improved long-term outcomes in living kidney transplants, this is a very interesting approach for the future monitoring of tacrolimus exposure.

Differences in therapy regimen can influence cell phenotype and biological effects. Pilon et al. performed a monocentric prospective cohort study of kidney allograft recipients with *de novo* DSA, to evaluate transcriptomic and phenotypic changes in T and B lymphocytes as well as serum cytokines after treatment with high dose intravenous immunoglobulin. High dose intravenous immunoglobulin induced limited modifications in B and T cell phenotype, however results need to be confirmed in a larger population in order to evaluate the clinical use of high dose intravenous immunoglobulin after kidney transplantation.

In their review Zaza et al. discussed, in the setting of kidney transplantation, the effects of currently used immunosuppressive agents on innate immune cells, their direct effects and the effects on induced adaptive immune response. Their overview highlights the possibility that novel drug candidates targeting innate immune cells could be considered in order to prolong allograft function and minimize immunosuppression

One of the most common infections in kidney transplanted patients due to immunosuppression regimen, is represented by Cytomegalovirus. Notably, in the paper by Rahmel et al. authors demonstrated an association between the genetic background of the Aquaporin 5 gene with infection risk, in a cohort of kidney or combined pancreas–kidney transplanted patients from a single center. The presence of a single nucleotide polymorphism in the promoter region of the Aquaporin 5 gene represents an independent risk-factor of post-transplant infections and could open the way to novel strategies for post-transplantation Cytomegalovirus prophylaxis.

CONCLUSION

We hope that this Research Topic will highlight the importance of innate immunity contributing to the mechanisms involved in graft deterioration and rejection and will open the way to novel therapeutic approaches in the management of kidney transplant recipients.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Specialized Roles of Human Natural Killer Cell Subsets in Kidney Transplant Rejection

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Background: Human natural killer (NK) cells are key functional players in kidney transplant rejection. However, the respective contributions of the two functionally distinct human NK cell subsets (CD56^{bright} cytokine-producing vs. CD56^{dim} cytotoxic effector) in episodes of allograft rejection remain uncertain, with current immunohistochemical methods unable to differentiate these discrete populations. We report the outcomes of an innovative multi-color flow cytometric-based approach to unequivocally define and evaluate NK cell subsets in human kidney allograft rejection.

Methods: We extracted renal lymphocytes from human kidney transplant biopsies. NK cell subsets were identified, enumerated, and phenotyped by multi-color flow cytometry. Dissociation supernatants were harvested and levels of soluble proteins were determined using a multiplex bead-based assay. Results were correlated with the histopathological patterns in biopsies—no rejection, borderline cellular rejection, T cell-mediated rejection (TCMR), and antibody-mediated rejection (AMR).

Results: Absolute numbers of only CD56^{bright} NK cells were significantly elevated in TCMR biopsies. In contrast, both CD56^{bright} and CD56^{dim} NK cell numbers were significantly increased in biopsies with histopathological evidence of AMR. Notably, expression of the activation marker CD69 was only significantly elevated on CD56^{dim} NK cells in AMR biopsies compared with no rejection biopsies, indicative of a pathogenic phenotype for this cytotoxic NK cell subset. In line with this, we detected significantly elevated levels of cytotoxic effector molecules (perforin, granzyme A, and granulysin) in the dissociation supernatants of biopsies with a histopathological pattern of AMR.

Conclusions: Our results indicate that human NK cell subsets are differentially recruited and activated during distinct types of rejection, suggestive of specialized functional roles.

Keywords: natural killer cells, innate lymphocytes, kidney allograft rejection, T cell mediated rejection, antibody-mediated rejection

INTRODUCTION

Kidney transplantation is the gold standard treatment for end stage kidney disease, with superior quality of life and patient survival compared to dialysis. Despite advances in kidney transplantation techniques and immunosuppression therapy, immunological rejection continues to account for loss of graft function and eventually graft loss (1). Immune-mediated allograft rejection is classified histopathologically into two types: T cell-mediated rejection (TCMR) and antibody-mediated rejection (AMR) (2, 3). TCMR is characterized by tubulointerstitial inflammation mediated by host alloreactive lymphocytes targeting donor human leukocyte antigen (HLA) molecules in the graft, whilst AMR is a process of microvascular inflammation (glomerulitis, peritubular capillaritis) driven by donor-specific antibodies (DSA) interacting with the allograft endothelium (4–6). Most immuno-biological studies of kidney allograft rejection have focused on conventional T ($CD4^+$ or $CD8^+$) and B cells. Less is known about the roles of innate lymphocytes in the different patterns of immune-mediated allograft rejection.

Natural killer (NK) cells are innate lymphocytes that have an immune surveillance function under homeostatic conditions, but can be rapidly recruited to sites of inflammation under pathological conditions. NK cells are activated by a combination of inhibitory and activating signals orchestrated through cell surface receptors and/or cytokine stimulation. Once activated, NK cells display immediate effector function through the production of pro-inflammatory cytokines and via cytotoxic activity (7).

Human NK cells are classically defined as $CD3^-/CD56^+/CD335$ $(NKp46)^+$ mononuclear cells. They are subcategorised based on expression levels of CD56 (neural cell adhesion molecule) into low-density ($CD56^{dim}$) and high density ($CD56^{bright}$) subsets. The two NK cell subsets differ in phenotypic and functional properties. $CD56^{dim}$ NK cells express high levels of CD16 (low affinity receptor for IgG, FcγRIII) (8) and are considered functionally to be cytotoxic effector cells (9). Upon activation, $CD56^{dim}$ NK cells produce cytotoxic granules containing perforin/granulysin that, when released, create pores in the cell membranes of targeted cells and granzymes that induce apoptosis (9). In contrast, $CD56^{bright}$ NK cells express high levels of CD56, are $CD16^{-/low}$ and mediate immune responses by secreting pro-inflammatory cytokines [e.g., interferon (IFN)-γ] (9).

NK cells are emerging as powerful drivers in immune-mediated kidney allograft rejection. Their functional role has been established in mouse models of kidney allograft injury (10–12). Initial investigations in humans, focused primarily on peripheral blood NK cells from kidney transplant recipients and transcriptomic studies of allograft biopsies, provide compelling evidence supporting a role for NK cells in kidney allograft injury (13–18). Indeed, immunohistochemical (IHC)-based studies report significant associations between human NK cells and both TCMR (19, 20) and AMR (18, 21, 22). However, these IHC-based studies are limited to single antigen (CD56, CD16, or NKp46) labeling to identify human NK cells, a technical approach that cannot unequivocally define this innate lymphocyte population nor differentiate between the distinct NK cell subsets. Thus, to date, the discrete roles of kidney NK cell subsets in different types of human allograft rejection have not been reported.

These technical limitations can be addressed using multi-parameter staining methodologies that accurately identify, enumerate and phenotype human NK cells, in particular, NK cell subsets, in kidney allograft biopsies. In this study, we extend our previously published multi-color flow cytometry-based approach to provide, for the first time, a comprehensive mapping of human NK cell subsets in kidney allograft rejection, ascribing specialized roles during the two patterns of immune-mediated rejection (TCMR vs. AMR).

METHODS

Study Design

Kidney transplant recipients ($n = 56$) were biopsied at the Royal Brisbane and Women's Hospital or Princess Alexandra Hospital between 2015 and 2018. All biopsies were undertaken for clinical indications. Written informed consent for participation in the study was obtained. The Royal Brisbane and Women's Hospital Human Research Ethics Committee (2006/072) and the Princess Alexandra Hospital Ethics Committee (HREC/16/QPAH/214) approved the study.

Kidney Tissue Specimens

Fresh biopsy specimens were taken with either an 18-gauge or 16-gauge biopsy needle (Biopsybell, Mirandola, Italy) and immediately divided for (i) tissue dissociation (1–5 mm of a core biopsy specimen); and (ii) assessment of allograft rejection by specialist renal histopathologists blinded to experimental results. The biopsies were examined for rejection in the pathology departments of participating hospitals.

Samples were graded according to the Banff-classification (23). According to these criteria, biopsies were then grouped into: no evidence of rejection (no rejection), borderline cellular rejection (borderline), TCMR alone, or biopsies with an indication of AMR. Samples that arrived for processing >12 h post collection were excluded. Biopsies that had other diagnoses such as BK nephropathy, recurrent patterns of glomerulonephritis like IgA nephropathy and additional pathology (e.g., amyloidosis) were also excluded from the study.

Tissue Dissociation for Flow Cytometric Analysis

Allograft biopsy specimens excess to clinical diagnostic need were digested within 12 h of collection using our published protocol (24). In brief, kidney cortical tissue was digested with 1 mg/ml collagenase P (Roche, Mannheim, Germany) in the presence of 20 mg/ml DNase I (Roche) for 15 min. Following centrifugation, supernatant was collected for assessment of soluble cytotoxic effector proteins. Tissue was further digested with 10 mg/ml trypsin + 4 mg/ml ethylenediamine tetraacetic acid (EDTA) (Life Technologies, Grand Island, NY) for 10 min.

Flow Cytometry

Single cell suspensions were initially stained with LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Life Technologies) to exclude non-viable cells. Cells were then incubated with Human TruStain FcX Blocking Solution (Biolegend, San Diego, CA) at room temperature for 5 min and then stained on ice for 30 min with combinations of test- (0.25 μg per antibody) (**Table 1**) or isotype-matched control antibodies in cold fluorescence-activated cell sorter buffer (0.5% bovine serum albumin [Sigma-Aldrich, St. Louis, MO] and 0.02% sodium azide [Sigma-Aldrich] in phosphate buffered saline).

Flow-Count Fluorospheres (Beckman Coulter, Brea, CA) were used for direct determination of absolute counts following the manufacturer's recommendations. Briefly, target cell concentrations (cells/μl) were calculated as the total number of target cells counted/total number of fluorospheres counted X Flow-Count Fluorosphere concentration. This value was then multiplied by the total sample volume to obtain absolute counts for each target cell population. Total cell counts were then normalized to cell numbers per cubic centimeter of tissue, in which the volume of renal tissue was calculated as $\pi r^2 \times$ length of biopsy tissue, where the radius (r) of a 16-gauge biopsy specimen is 0.8 mm and 18-gauge biopsy specimen is 0.6 mm. Cell acquisition was performed on an LSR Fortessa (BD Biosciences, San Jose, CA) and data analyzed with FlowJo software (TreeStar, Ashland, OR) to identify immune cell populations as presented in **Figure 1**.

Quantification of Cytokines by Multi-analyte Flow Cytometric Immunoassay

Cytotoxic effector proteins in tissue dissociation supernatants were quantified by the LEGENDplex™ Multi-analyte flow assay kit (human CD8/NK panel) according to the manufacturer's instructions (Biolegend, San Diego, USA). Data acquisition was performed on an LSR Fortessa (BD Biosciences, San Jose, CA). Standard curve and concentrations were calculated with BioLegend's LEGENDplex™ Data Analysis Software (Biolegend, San Diego, USA). Cytokine values were normalized to pg per cm^3 of tissue.

TABLE 1 | Antibodies used for flow cytometric staining.

Antigen	Clone	Flurophore	Source
CD45	HI30	BV510	BioLegend
CD14	M5E2	AF700	BioLegend
CD3	OKT3	BV650	BioLegend
CD19	HIB19	FITC	BD
CD56	HCD56	PerCPCy5.5	BioLegend
CD16	3G8	PE-CF594	BD
HLA-DR	L243	BV785	BioLegend
CD69	FN50	PE	BD

Statistics

All statistical tests were performed using Prism 7.0 analysis software (GraphPad Software, La Jolla, CA). Multiple comparisons were performed using a Kruskal-Wallis test with Dunn's post-test. A Mann-Whitney *u*-test was used for comparisons of non-parametric data from two groups. $P < 0.05$ were considered statistically significant.

RESULTS

Human Population Demographics

As reported in **Table 2**, the mean age of the 56 patients in the study was 52.2 ± 14.5 years (range 20–80 years), with 66.1% (37/56) male. The majority of patients (85.7%; 48/56) undergoing biopsy had not undergone previous transplantation, suggesting a reasonably unsensitised population. Cadaveric transplants accounted for 92.8% (52/56) of all biopsies, with donor after brain death (DBD) being the most common type of cadaveric graft biopsied. The median HLA matching was 4/6. All but one patient underwent basiliximab, mycophenolic acid, tacrolimus, and prednisolone based induction therapy, with the remaining patient receiving thymoglobulin induction. The majority of patients (55.4%; 31/56) were classed as low immune risk at transplantation with undetectable calculated Panel Reactive Antibodies (cPRA 0%), whilst highly sensitized patients with cPRA >95% accounted for only 3.6% (2/56) of total patients. The majority of patients (66.1%; 37/56) underwent biopsy within the first 3 months of transplantation. Mean creatinine at time of biopsy was 219.1 ± 106.7 μmol/L ($n = 41$), with 15 patients who were haemodialysis-dependent at time of biopsy due to delayed graft function or kidney injury excluded from mean creatinine calculations.

The transplant biopsy specimens were categorized based upon histopathological examination by renal histopathologists blinded to experimental results. Samples were graded according to the Banff-classification (23). According to these criteria, the 56 biopsies sorted into groups without histopathological evidence of rejection (no rejection; $n = 17$; 13 males/4 females; mean age of 53.0 ± 13.4 years), borderline cellular rejection (borderline; $n = 22$; 13 males/9 females; mean age of 49.4 ± 14.4 years), T cell-mediated rejection (TCMR; $n = 7$; 4 males/3 females; mean age of 45.1 ± 18.2 years) and antibody-mediated rejection (AMR; $n = 10$; 7 males/3 females; mean age of 58.1 ± 7.4 years). Demographic and clinical characteristics of these cohorts are given in **Table 2**.

Identification of NK Cell Subsets in Human Allograft Kidney Tissue

Human allograft kidney tissue was enzymatically digested to obtain single cells for flow cytometric analysis. Briefly $CD45^+$ leukocytes separated into granulocytes (with higher side scatter) and mononuclear cells (**Figures 1A,B**). The mononuclear cells were further divided into $CD14^+$ monocyte and $CD14^-$ lymphocyte populations (**Figure 1C**). Lymphocytes were then delineated into $CD3^+$ T cells, $CD19^+$ B cells, and $CD3^-$

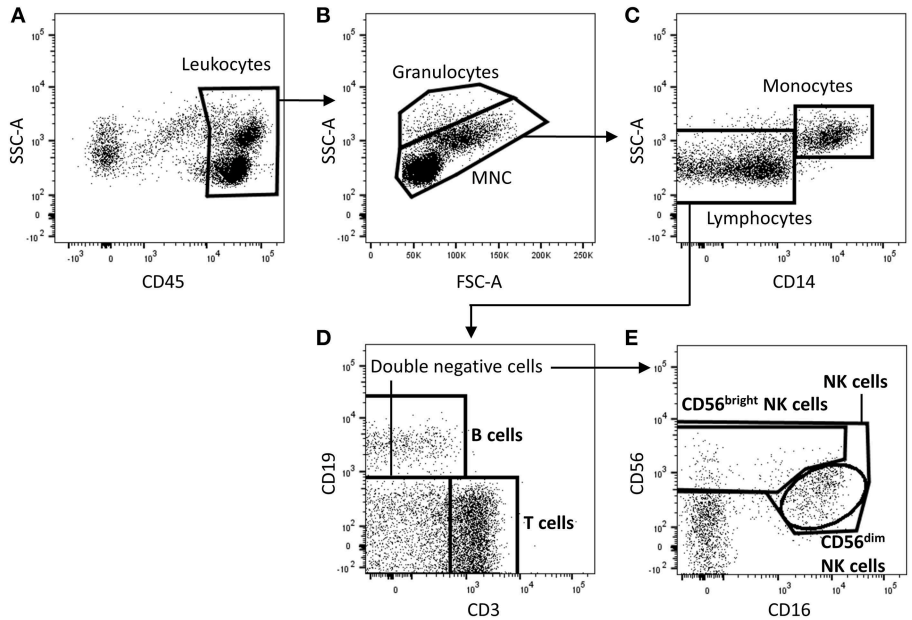

FIGURE 1 | Identification of T cells, B cells, and natural killer (NK) cell subsets in human kidney tissue. Gating strategy used to identify T cells ($CD3^+$), B cells ($CD19^+$), total NK cells ($CD3^-$ $CD19^-$ $CD56^+$ lymphocytes), and NK cell subpopulations ($CD56^{dim}$ and $CD56^{bright}$ NK cells) in human kidney transplant tissue. Single, live, $CD45^+$ mononuclear cells (MNC) and granulocytes are gated on a forward-scatter (FSC)/side-scatter (SSC) plot **(A,B)**. Total lymphocytes are distinguished from granulocytes and monocytes based on low SSC and absent CD14 expression **(C)**. Total lymphocytes are further separated into T cells or B cells by their expression of CD3 and CD19 respectively **(D)**. NK subpopulations, $CD56^{bright}$ and $CD56^{dim}$ NK cells, are identified based on CD56 intensity and CD16 expression **(E)**. Representative flow cytometric data from 1 of 10 individual antibody-mediated rejection (AMR) renal biopsy specimens are shown. An identical gating strategy was used for no rejection, borderline rejection and T cell-mediated rejection (TCMR) biopsies. MNC, mononuclear cells; FSC-A, forward-scatter area; SSC-A, side-scatter area.

$CD19^-$ double-negative cells (**Figure 1D**). Within this double-negative population, total NK cells were identified as $CD56^+$ cells, with $CD56^{bright}$ $CD16^{-/low}$ and $CD56^{dim}$ $CD16^+$ NK cell subsets defined for the first time in human kidney allograft tissue (**Figure 1E**). Importantly, these surface molecules used to identify NK cells subsets in kidney allograft tissue were resistant to proteolytic cleavage as confirmed by enzymatic digestion (with collagenase P and trypsin-EDTA) of peripheral blood mononuclear cells (data not shown).

Significantly Increased Numbers of Total NK Cells in Allograft Biopsy Specimens With TCMR and AMR

In order to profile the major lymphocyte populations in human kidney allograft tissue, we enumerated total T ($CD3^+$) cells, B ($CD19^+$) cells, and NK ($CD3^-$ $CD19^-$ $CD56^+$) cells in patient biopsies. Biopsies were stratified based on the histopathological pattern of rejection, classified as: unsuspicious biopsy (no rejection), borderline cellular rejection, TCMR or AMR.

To establish the independent association between lymphocyte numbers and the severity of cellular rejection as graded using Banff criteria, we firstly performed a subgroup analysis in which patients with a histopathological pattern of AMR were excluded. For this analysis, biopsies were stratified based on the severity of cellular rejection into borderline rejection and TCMR rejection (grades I–II). Quantification using Flow-Count Fluorospheres showed numbers of total T cells, B cells, and NK cells to be significantly increased in biopsy specimens with a histopathological pattern of TCMR compared with biopsy specimens with no evidence of rejection (**Figures 2A–C**; $p < 0.05$).

To independently assess lymphocyte numbers in association with AMR, a second subgroup analysis was performed in which patients with cellular rejection alone were excluded. Total T cell and NK cell numbers were significantly increased in biopsies with a histopathological pattern of AMR compared with no rejection (**Figure 3A**; $p < 0.01$ and **Figure 3C**; $p < 0.001$, respectively), whilst no statistical difference was observed for total B cells (**Figure 3B**). Collectively, these results associate human NK cells with both TCMR and AMR.

Significantly Increased Numbers of $CD56^{bright}$ NK Cells in Allograft Biopsy Specimens With TCMR

We next assessed human kidney NK cells at a subset level. Firstly, we examined the absolute numbers of $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets in allograft biopsies with cellular rejection alone (borderline/TCMR). Notably, only the $CD56^{bright}$ NK cells were significantly elevated in the TCMR group compared with biopsies with no evidence of rejection ($P < 0.01$; **Figures 4A,B**). We also examined the phenotypes of the human kidney NK cell subsets in cellular rejection. Although not reaching statistical significance,

TABLE 2 | Demographic and clinical characteristics of human kidney transplant patients in study cohort.

		All biopsy ($n = 56$)	No rejection ($n = 17$)	Borderline ($n = 22$)	TCMR ($n = 7$)	AMR ($n = 10$)
Mean age (years) [SD]		52.2 [14.5]	53.0 [13.4]	49.4 [14.4]	45.1 [18.2]	58.1 [7.4]
Male : female (*n*)		37:19	13:4	13:9	4:3	7:3
First transplant (*n*)		48	13	20	7	8
Transplant type	DBD (*n*)	34	10	12	5	7
	DCD (*n*)	8	2	4	2	0
	Cadaveric-NOS (*n*)	10	4	4	0	2
	Living donor (*n*)	4	1	2	0	1
Median HLA match		4	4	5	4	4
DSA present (*n*)		5	2	0	0	3
cPRA	0% (*n*)	31	11	14	3	3
	>0–95% (*n*)	4	1	2	0	1
	>95% (*n*)	2	2	0	0	0
	Unavailable	19	3	6	4	6
Biopsy within 3 m of transplantation (*n*)		37	13	14	3	7
Mean creatinine prior to biopsy (μmol/L) [SD]		219.1 [106.7]	284.9 [288.7]	222.0 [135.8]	145.0 [54.4]	267.1 [135.1]
Patients receiving haemodialysis at biopsy (*n*)		15	6	7	1	1

DBD, donor after brain death; DCD, donor after circulatory death; Cadaveric-NOS, Cadaveric-Not otherwise specified; HLA, human leukocyte antigens; DSA, Donor-specific antibodies (pre-existing); cPRA, calculated Panel Reactive Antibodies (at transplantation).

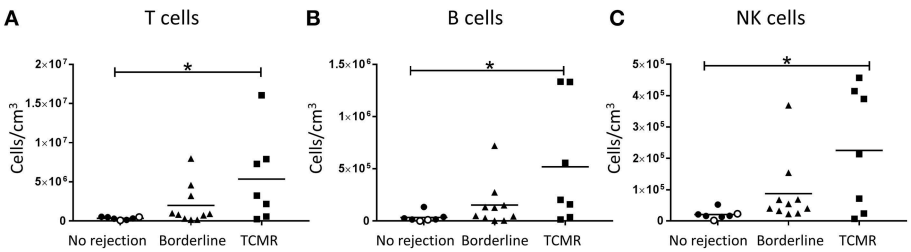

FIGURE 2 | Significantly increased T cells, B cells, and NK cells in TCMR. Absolute numbers of total **(A)** T cells, **(B)** B cells, and **(C)** NK cells in kidney transplant biopsy tissue with histopathological diagnosis of no rejection ($n = 7$), borderline cellular rejection ($n = 10$), and T cell-mediated rejection (TCMR; grades I–II; $n = 7$). Values for individual donors are presented; donors with DSA are identified using open symbols; bars represent means. $^{*}P < 0.05$, Kruskal-Wallis test with Dunn's post-test.

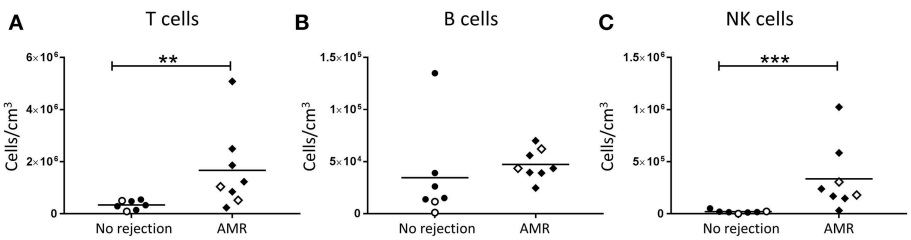

FIGURE 3 | Significantly increased T cells and NK cells in AMR. Absolute numbers of total **(A)** T cells, **(B)** B cells, and **(C)** NK cells in kidney transplant biopsy tissue with histopathological diagnosis of no rejection ($n = 7$) and with indication of AMR (AMR; $n = 8$). Values for individual donors are presented; donors with DSA are identified using open symbols; bars represent means. $^{**}P < 0.01$, $^{***}P < 0.001$, Mann-Whitney test.

expression levels of activation marker CD69 were elevated on CD56^{bright} NK cells in the borderline/TCMR group compared with non-rejecting biopsies (**Figure 4C**), whilst CD69 expression levels on CD56^{dim} NK cells were comparable between the two groups (**Figure 4D**).

CD56^{dim} NK Cells With an Activated Phenotype Associated With AMR

In order to investigate the role of human NK cell subsets in humoral rejection, we examined the absolute numbers of CD56^{bright} and CD56^{dim} NK cells in allograft biopsies with a

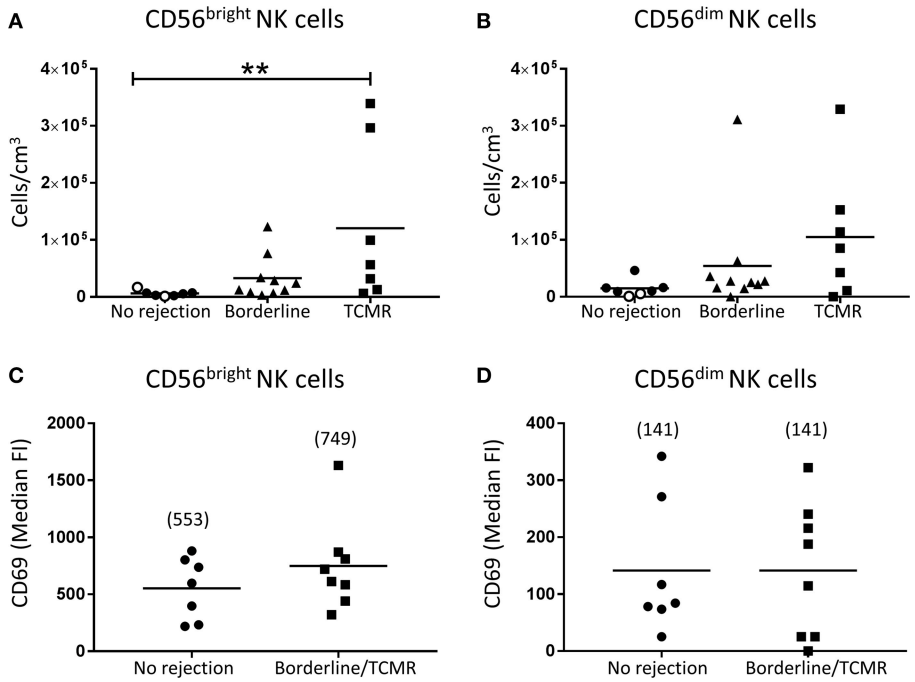

FIGURE 4 | Significantly increased CD56^{bright} NK cells in TCMR. **(A,B)** Absolute numbers of total **(A)** CD56^{bright} NK cells and **(B)** CD56^{dim} NK cells in kidney transplant biopsy tissue with histopathological diagnosis of no rejection ($n = 7$), borderline cellular rejection ($n = 10$) and T cell-mediated rejection (TCMR; grades I–II; $n = 7$). Values for individual donors are presented; donors with DSA are identified using open symbols; bars represent means. **(C,D)** Surface expression of CD69 on **(C)** CD56^{bright} NK cells and **(D)** CD56^{dim} NK cells in kidney transplant biopsy tissue with histopathological diagnosis of no rejection ($n = 7$) and borderline or T cell-mediated rejection ($n = 8$). Median fluorescence intensity (median FI) values for individual donors are presented; bars represent means, with mean values presented in parentheses. $^{**}P < 0.01$, Kruskal-Wallis test with Dunn's post-test.

histopathological pattern of AMR. Interestingly, both CD56^{bright} ($p < 0.001$; **Figure 5A**) and CD56^{dim} NK cell subsets ($p < 0.01$; **Figure 5B**) were significantly increased in the AMR group compared with non-rejecting biopsies.

Expression levels of activation markers on NK cell subsets were again assessed. Whilst CD69 expression levels on CD56^{bright} NK cells were similar between the AMR and no rejection groups (**Figure 6A**), expression of CD69 on CD56^{dim} NK cells was significantly elevated in AMR biopsies ($p < 0.05$; **Figure 6B**), suggestive of an activated phenotype. Human CD56^{dim} NK cells are reported to downregulate CD16 expression and upregulate HLA-DR upon activation (16). Consistent with an activated phenotype, we observed trends toward lower CD16 expression (**Figure 6C**) and higher HLA-DR (**Figure 6D**) on CD56^{dim} NK cells from biopsies with evidence of AMR; however, these did not reach statistical significance. Collectively, these results indicate that CD56^{dim} NK cells shift toward a more activated state in the AMR micro-environment.

Significantly Elevated Levels of Cytotoxic Effector Molecules in AMR Biopsies

The primary function of activated CD56^{dim} NK cells is cytotoxicity through the release of effector molecules perforin, granulysin, and granzymes. Thus, the supernatants from dissociated allograft biopsies were analyzed for levels of these cytotoxic effector molecules. Consistent with a putative role for activated CD56^{dim} NK cells in AMR, we observed significantly elevated levels of perforin ($p < 0.01$; **Figure 7A**), granulysin ($p < 0.05$; **Figure 7B**), and granzyme A ($p < 0.01$; **Figure 7C**) in biopsies with a histopathological pattern of AMR compared with non-rejecting biopsies.

DISCUSSION

Current treatment modalities offer non-specific therapeutic targets in the treatment of TCMR and especially AMR, and while patients can be risk stratified using donor and recipient immunological (cytotoxic and flow cross-match, HLA mismatching, DSAs, and previous recipient sensitization) and non-immunological (ischemia-reperfusion injury, warm and cold ischemic time) parameters, no biomarker exists to predict which patients will develop renal allograft rejection. To more accurately define the local drivers of immune-mediated allograft rejection, we report the use of a multi-color flow cytometric approach to analyse immune cell populations in human renal allograft tissue. Muczynski et al. used this technique to examine the relative proportions (not absolute counts) of immune cell populations in kidney allograft rejection (25). Here, we report, for the first time, the use of this methodology to examine absolute numbers of discrete lymphocyte populations (including NK cell

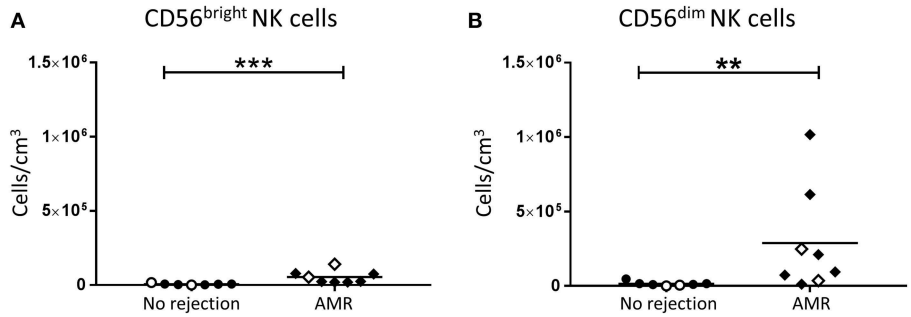

FIGURE 5 | Significantly increased CD56^{bright} NK cells and CD56^{dim} NK cells in AMR. Absolute numbers of total **(A)** CD56^{bright} NK cells and **(B)** CD56^{dim} NK cells in kidney transplant biopsy tissue with histopathological diagnosis of no rejection ($n = 7$) and with indication of AMR (AMR; $n = 8$). Values for individual donors are presented; donors with DSA are identified using open symbols; bars represent means. $^{**}P < 0.01$, $^{***}P < 0.001$, Mann-Whitney test.

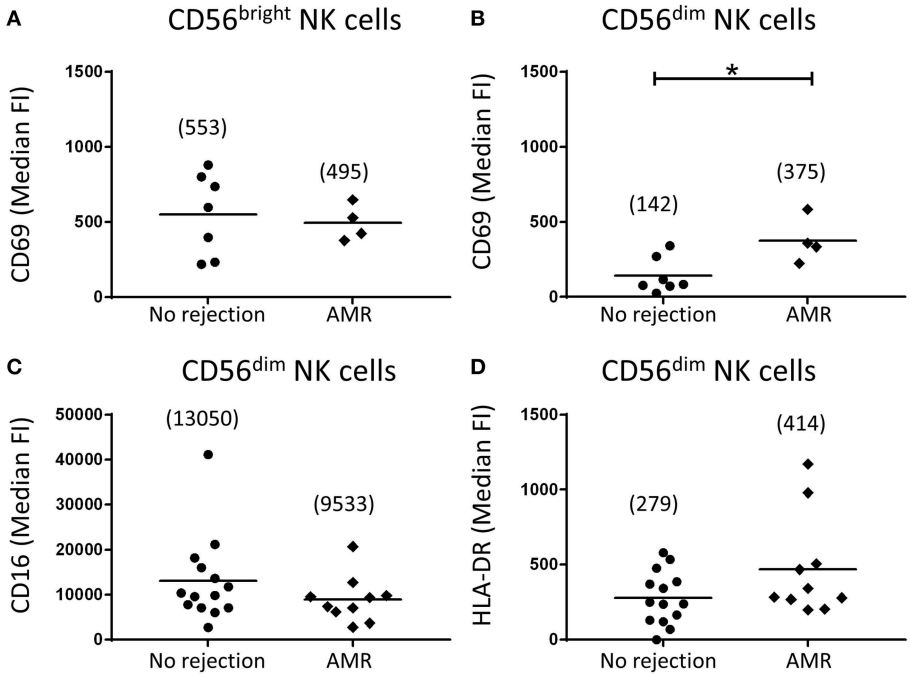

FIGURE 6 | Human CD56^{dim} natural killer (NK) cells in AMR biopsies display an activated phenotype. **(A,B)** Surface expression of CD69 on CD56^{bright} NK cells **(A)** and CD56^{dim} NK cells **(B)** in biopsy specimens without (no rejection; $n = 7$) and with AMR ($n = 4$). **(C,D)** Surface expression of **(C)** CD16 and **(D)** HLA-DR on CD56^{dim} NK cells in biopsy specimens without (no rejection; $n = 14$) and with AMR ($n = 10$). Median fluorescence intensity (median FI) values for individual donors are shown; bars represent means, with mean values presented in parentheses. $^{*}P < 0.05$, Mann-Whitney test.

subsets) in different patterns of immune-mediated rejection (TCMR, AMR).

To focus in on putative drivers of grades of cellular rejection, we performed a subgroup analysis in which AMR biopsies were excluded and remaining biopsies stratified into borderline cellular rejection and TCMR (grades I–II). Using this approach, we observed significantly elevated numbers of total NK cells in TCMR biopsies, consistent with previous transcriptomic and IHC-based studies of cellular rejection. An early transcriptomic investigation of kidney allograft biopsies reported associations between high NK cell transcript expression and histological patterns of TCMR (14). More recently, IHC-based studies showed significant associations between human NK cells and TCMR (19, 20, 22). In agreement with our findings, dos Santos et al. reported significantly increased numbers of CD56^{+} cells associated with histopathological manifestations of interstitial inflammation and tubulitis; both hallmark features of TCMR (20, 22). Another study reported a positive correlation between the number of CD56^{+} cells and the severity of TCMR (19). However, these IHC-based evaluations have identified NK cells based on the expression of a single marker (e.g., CD56) and thus, none of these studies are able to irrefutably exclude the

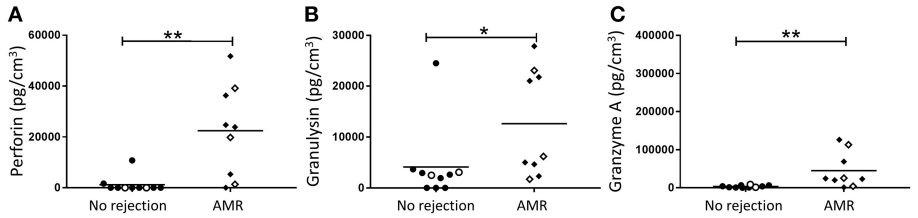

FIGURE 7 | Human kidney tissue from patients with an indication of AMR have an elevated cytotoxic effector molecule profile. Expression of **(A)** Perforin, **(B)** Granulysin, and **(C)** Granzyme A in transplant biopsy specimens without (no rejection; $n = 10$) and with AMR ($n = 9$). Concentrations of effector molecules normalized to cubic centimeters of kidney tissue for individual donors are shown; donors with DSA are identified using open symbols; bars represent means. $^{*}P < 0.05$, $^{**}P < 0.01$, Mann-Whitney test.

interference of $CD3^{+}$ $CD56^{+}$ NK-like T cells or evaluate NK cell subsets.

Through the use of multi-parameter flow cytometry, our group has extended these earlier investigations to unequivocally identify and characterize human $CD56^{bright}$ and $CD56^{dim}$ NK cell subsets in kidney allograft tissue. Indeed, in TCMR biopsies, we observed significantly elevated numbers of only the $CD56^{bright}$ NK cell subset (and not $CD56^{dim}$ NK cells). $CD56^{bright}$ NK cells function to trigger pathological immune responses through the production of pro-inflammatory cytokines (e.g., IFN-γ, TNF-α) and chemokines (e.g., monokine induced by gamma interferon; MIG) (9, 26, 27). It is thus tempting to speculate that $CD56^{bright}$ NK cells play a specialized functional role in TCMR pathology by secreting these pro-inflammatory molecules that, in turn: (1) enhance recruitment of alloreactive T cells (28, 29) and (2) upregulate HLA alloantigens (e.g., MHC class I and II) on target donor kidney cells to make them more susceptible to cytotoxic killing (26).

AMR remains one of the major barriers to graft survival in kidney transplant patients. Diagnoses of AMR center on the detection of DSA, complement deposition and the presence of inflammatory effector cells in the peritubular capillaries and glomeruli (30). Thus, we next identified and examined the role of effector NK cells in AMR pathology. We observed significantly elevated numbers of total NK cells in biopsies with a histopathological pattern of AMR, in line with earlier studies of humoral rejection. Indeed, previous investigations using single marker immunostaining (CD56 or NKp46) reported elevated NK cell numbers associated with peritubular capillaritis (13, 19), glomerulitis (22), microcirculatory inflammation, and peritubular C4d deposition (18), associating NK cells with microcirculatory injury. Furthermore, elevated numbers of $CD56^{+}$ cells have been shown to correlate with AMR and poor graft survival (19). Differential gene expression analyses of allograft biopsies have also demonstrated NK cell-related transcriptomic signatures in biopsies from patients with DSA and microcirculatory damage (14, 18).

We extended the work of these studies by examining human NK cells in AMR biopsies at a subset level. We report here that numbers of both $CD56^{bright}$ and $CD56^{dim}$ NK cells were elevated in biopsies with a histological pattern of AMR. However, only $CD56^{dim}$ NK cells uniquely displayed significantly elevated CD69 levels in biopsy-proven AMR, suggesting this subset specifically is driven to an activated phenotype within the AMR microenvironment. Our results are in line with a recent study by Hoffman et al. showing that NK cells in the peripheral blood of kidney transplant recipients display an activated phenotype (16). In particular, this study reported the presence of activated circulating $CD56^{dim}$ NK cells in kidney transplant recipients, characterized by up-regulated CD69 and HLA-DR, as well as reduced expression of CD16 (16). Similarly, the kidney $CD56^{dim}$ NK cells in our AMR biopsies displayed elevated HLA-DR and reduced CD16 expression levels, although these did not reach statistical significance. Given that only this cytotoxic subset was both elevated in number and displayed an activated phenotype in AMR biopsies, our findings specifically implicate the CD16-expressing $CD56^{dim}$ NK cell subset in the pathogenesis of AMR.

Antibody-mediated activation of NK cells via triggering of CD16 on their cell surface has been proposed to drive antibody-dependent cellular cytotoxicity (ADCC) and thus, allograft rejection (21). Indeed, gene transcript profiling of AMR biopsies has provided initial evidence for NK cell activation and signaling via engagement of Fc receptor CD16 with IgG DSA (17). Critically, in our AMR biopsies, $CD56^{dim}$ NK cells express high levels of cell surface CD16, making them capable of engaging with DSA bound to allograft endothelial cells and, in turn, releasing cytotoxic granules (containing perforin, granulysin, and granzyme A) that can trigger targeted allograft apoptosis. In line with this concept, we found significantly elevated levels of perforin, granulysin, and granzyme A in biopsies with AMR. We speculate that $CD56^{dim}$ NK cells contribute to the burden of cytotoxic molecules observed in biopsies with AMR. In support of our hypothesis, a recent study of the molecular signatures in human AMR biopsies strongly associated endothelial injury and cytotoxic molecules characteristic of $CD56^{dim}$ NK cells [e.g., granulysin (*GNLY*) and *FGFBP2*] with chronic AMR (17). In conjunction with these earlier reports, our collective findings in AMR biopsies strongly support an ADCC-mediated pathogenic function for the human $CD56^{dim}$ NK cell subset in humoral rejection.

Previous studies have reported reduced numbers of $CD56^{dim}$ NK cells in the peripheral blood of kidney transplant recipients with indicative AMR (15, 31). In one study, the presence of DSA was associated with reduced numbers of $CD56^{dim}$ NK cells

in peripheral blood of kidney transplant recipients compared to healthy donors (31). A second, larger cohort confirmed this finding, reporting patients with anti-HLA DSA had lower proportions and absolute numbers of peripheral blood $CD56^{dim}$ NK cells compared to patients without HLA antibodies (15). In the context of our hypothesis, a reduction in circulating $CD56^{dim}$ NK cells might reflect homing of this cytotoxic subset into the rejecting allograft.

It is problematic as to whether human NK cell functions are adequately inhibited by current immunosuppression regimes. An *in vitro* study of peripheral blood NK cells from kidney transplant recipients demonstrated that immunosuppression did not affect the capacity of NK cells to respond to stimulation. In this study, circulating NK cells from recipients receiving immunosuppression secreted equivalent levels of IFN-γ, perforin, and granzyme A in response to stimulation with HLA class I-negative K562 cells compared with NK cells from healthy individuals (16). The inability of current immunosuppression regimes to downregulate activated NK cell functions represents a therapeutic opportunity. Novel treatments with the specificity to target either activated NK cells and/or their effector functions warrant testing.

In addition to our novel human NK cell subset data, our present study also examined the absolute numbers of other lymphocyte populations (e.g., B lymphocytes) in different patterns of immune-mediated rejection. Notably, we demonstrated significant increases in absolute numbers of B lymphocytes in TCMR biopsies (**Figure 2B**), but not in AMR biopsies (**Figure 3B**). Although an unexpected result given the role of B lymphocyte lineage cells (plasma cells) in antibody production, these findings are in fact consistent with previous reports of B lymphocyte infiltration significantly associating with TCMR, but not with AMR (32, 33). These analogous findings from these earlier IHC-based studies further confirm the efficiency and integrity of our multi-color flow cytometric approach.

In summary, we report the first comprehensive characterization of discrete kidney NK cell subsets in human allograft rejection. Our data provide the first evidence that human kidney NK cells may have subset-specific functional roles in the pathobiology of TCMR vs. AMR. Further evaluation of the kidney NK cell compartment in allograft models will test its utility in high precision tissue-based diagnostics. In addition, this will foster therapeutic approaches that specifically target the recruitment (e.g., chemokine receptors) or triggering (e.g., activating receptors) of discrete NK cell subsets dependent on the pathological conditions.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript.

ETHICS STATEMENT

Written informed consent for participation in the study was obtained. The Royal Brisbane and Women's Hospital Human Research Ethics Committee (2006/072) and the Princess Alexandra Hospital Ethics Committee (HREC/16/QPAH/214) approved the study.

AUTHOR CONTRIBUTIONS

Each author has participated sufficiently in the work to take public responsibility for the content. KK, RF, SH, GJ, JU, RW, AK, and HH conceived and designed the study. KK, SH, MH, KG, ES, BL, XW, and AK carried out experiments and analyzed the data. KK, SH, AK, and HH drafted the paper. All authors revised and approved the final version of the manuscript.

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REVIEW
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The Role of Complement in Organ Transplantation

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The current immunosuppressive protocols used in transplant recipients have improved short-term outcomes, but long-term allograft failure remains an important clinical problem. Greater understanding of the immunologic mechanisms that cause allograft failure are needed, as well as new treatment strategies for protecting transplanted organs. The complement cascade is an important part of the innate immune system. Studies have shown that complement activation contributes to allograft injury in several clinical settings, including ischemia/reperfusion injury and antibody mediated rejection. Furthermore, the complement system plays critical roles in modulating the responses of T cells and B cells to antigens. Therapeutic complement inhibitors, therefore, may be effective for protecting transplanted organs from several causes of inflammatory injury. Although several anti-complement drugs have shown promise in selected patients, the role of these drugs in transplantation medicine requires further study.

Keywords: complement, transplantation, alloimmunity, antibody mediated allograft rejection, delayed graft function, therapeutics

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INTRODUCTION

The principal function of the mammalian immune system is to defend the host against infection (1, 2). The immune system consists of two integrated arms—adaptive immunity and innate immunity. The adaptive immune system is primarily comprised of T and B lymphocytes which express highly specific antigen receptors. The diversity of these receptors is generated through somatic gene rearrangement, and T and B cells that express a specific receptor can expand clonally after the cell encounters cognate antigens. Activated T and B cells can also differentiate into memory T cells and B cells, thereby generating long-lived immunological memory of antigens.

Unlike the adaptive system, the innate immune system is comprised of myeloid cells (dendritic cells, monocytes, macrophages, neutrophils), and several other cell types. These cells do not express rearranged receptors, they have limited clonal expansion, and, for the most part, they do not generate memory. Cells of the innate immune system instead express germ-line encoded pattern recognition receptors (PRR) that detect conserved pathogen associated molecular patterns (PAMPs) present in microbes but not shared by healthy mammalian cells (3, 4). The innate immune system also encompasses non-cellular mediators capable of microbial recognition—for example, complement proteins.

Activation of the innate immune system by microbial ligands causes inflammation, the first line of defense against infection, but equally importantly it induces the maturation of antigen-presenting cells (APC) and their migration to secondary lymphoid tissues where they trigger primary T cell and B cell responses. The latter function of the innate immune system is critical for initiating adaptive immunity to infection and vaccines in the naïve host. The innate immune system is therefore responsible for the initial non-self recognition events that

ultimately lead to productive T and B cell immunity. It is also generally accepted that innate immunity represents the first step in allograft rejection mechanisms and guides the development of adaptive immune response in transplantation.

Alloimmunity is considered an adaptive immune response, and it represents acquired immunity against foreign antigens that occurs during the lifetime of an individual. Adaptive immunity is antigen specific and reciprocal cognate interactions by T cells play key roles in the generation of alloimmune responses (1–4). Our current armamentarium of immunosuppressive drugs is designed primarily to keep the adaptive immunity in check. However, the role of innate immunity as a significant driver of alloimmune response is increasingly recognized (5–7). The communication between innate and adaptive immunity mainly involves promoting antigen presentation and co-stimulation of cognate B and T cells (7). It is notable, however, that studies of innate immunity after transplantation have most frequently been performed in the context of ischemia-reperfusion (I/R) injury. The activation of innate immunity in the immediate post-transplant period in the context of I/R injury does not fully explain its role in acute rejection, which typically happens weeks to months after transplantation. There is, therefore, an unmet need for the investigation of innate immunity during an episode of acute rejection, especially in human organ transplants (8, 9).

OVERVIEW OF THE COMPLEMENT CASCADE

The complement cascade is comprised of more than 30 soluble and cell-bound proteins (10). These include PRRs, zymogens that become activating enzymes, biologically active fragments, complement receptors, and complement regulatory proteins. The transplanted organ is exposed to recipient complement proteins as soon as it is reperfused. Conversely, complement proteins and fragments generated within the allograft enter the systemic circulation. Although the complement system is an important effector mechanism for antibody-mediated cytotoxicity, that is only one of its functions. The complement system can be activated in an antibody-independent fashion (discussed below). Complement fragments also modulate T cell differentiation and the B cell response to antigens. Consequently, this system modulates the adaptive immune response, mediates many of the downstream effects of B and T cell immunity, and can function independently of the adaptive immune response. Furthermore, the complement cascade interacts with other biologic systems, including toll-like receptors, the inflammasome, and the clotting cascade (11).

The complement system is activated through three distinct pathways: the classical pathway (CP), lectin pathway (LP), and alternative pathway (AP). These activation pathways can be engaged by different pathologic processes in the allograft, including donor brain death, I/R injury, and antibody mediated rejection. Although these pathologic processes engage the complement system through distinct molecular mechanisms, the same downstream effectors are generated (**Figure 1**). The CP is activated by antibodies bound to their target ligands. This may be particularly important in those transplant recipients with donor specific antibodies (DSA) reactive to polymorphic human leukocyte antigens (HLA) expressed on endothelial cells of the allograft.

The LP is activated when activating proteins bind to sugars expressed on the surface of bacteria. LP activation can be initiated by several different proteins, including mannose binding lectin (MBL), collectins-10 and−11 (and maybe collectin-12), and ficolins 1–3 (12). These pattern recognition molecules are complexed with mannose-associated serine proteases (MASPs). When the pattern recognition molecules bind to target ligands, the MASPs become activated and then cleave C4 or, in some cases, activate the alternative pathway (12). The LP is usually activated by binding of these recognition molecules to sugars expressed on bacteria, but they can also bind to ligands expressed on injured cells. Collectin-11, for example, binds to L-fucose expressed on ischemic tubular epithelial cells (13). Cleavage of C4 by either the CP or LP leads to covalent fixation of C4b to nearby surfaces, and the release of the C4a fragment. Genetic variants in the MBL2 gene affect MBL levels, and lower levels of MBL are associated with increased risk of infection. Because the liver is the primary source of MBL, liver transplant recipients who receive organs from donors with MBL2 polymorphisms or mutations can have low MBL levels post-transplantation. Studies have shown that this acquired MBL deficiency is associated with an increased risk of serious infections in the recipient (14, 15).

In contrast to the CP and LP, the AP is continually and non-specifically activated in plasma through a process called "tick-over" (16). Circulating C3 molecules are hydrolyzed, generating a form of C3 [C3(H_2O)] that can combine with factor B and form a C3 convertase (i.e., an enzyme that cleaves additional C3 molecules). Although C3(H_2O) cannot bind to surfaces, C3b that is generated by the C3(H_2O)Bb convertase can covalently bind to nearby surfaces. This C3b can also form convertase (C3bBb), thereby amplifying alternative pathway activation on the target surface. Because tick-over is a spontaneous process, complement regulatory proteins are critical for controlling AP activation. Patients with mutations in the regulatory proteins are, consequently, susceptible to complement mediated diseases, such as atypical hemolytic uremic syndrome (aHUS) and C3 glomerulopathy. Of note, C3b generated by either the CP or LP can also feed into this process. Amplification through the AP may account for the majority of downstream fragment generation, even when complement activation is initiated through the CP (17).

Complement activation through all three pathways leads to cleavage of the C3 protein, generating the C3a and C3b fragments. C3b has a reactive thioester bond that can bind covalently to hydroxyl and amine groups on nearby surfaces, thereby marking, or "opsonizing," target cells and surfaces. Full complement activation also leads to cleavage of C5, generating soluble C5a and the larger C5b fragment. C5b seeds the formation of the membrane attack complex (MAC, or C5b-9), a multimeric complex that forms a pore in target cells and can cause target cell activation or lysis (18).

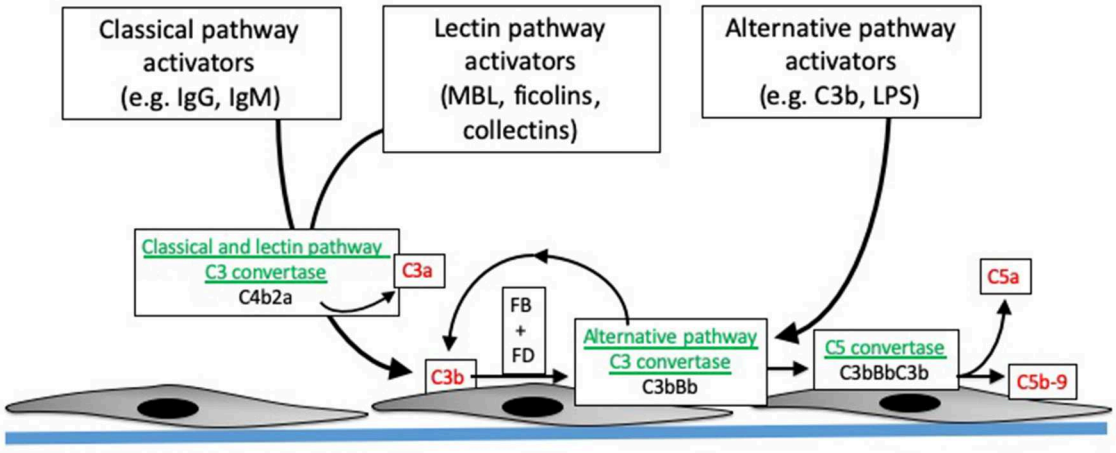

FIGURE 1 | Overview of complement activation. Specific activating molecules engage the classical, lectin, and alternative pathways. Cleavage of C4 and C2 generates C4b2a, the classical and lectin pathway C3 convertases (enzymes that cleave C3). C3 generated by the classical and lectin pathways can combine with factor B (FB), which is then cleaved by factor D (FD), to form C3bBb. C3bBb is the alternative pathway C3 convertase, and can also be generated by spontaneous formation of C3b. The C3 convertases combine with another C3b to form the C5 convertase, which then cleaves C5 into C5b and C5a. C5b combines with C6, C7, C8, and C9 to form C5b-9, or the membrane attack complex. The convertases are depicted in green, and pro-inflammatory molecules generated during complement activation are shown in red.

Complement Receptors

Although the MAC directly affects target cells, most of the biologic effects of complement system are mediated by receptors for the various fragments. The C3a receptor (C3aR) and C5a receptors (C5aR1 and C5aR2) are seven-transmembrane receptors that are expressed on myeloid cells and some parenchymal cells. Expression of these receptors can increase in some conditions (19, 20). C5aR1 expression, for example, increases in rejecting murine renal allografts (21). C3aR and C5aR1 are both G-protein coupled receptors, whereas C5aR2 does not have a G-protein coupling motif. It was initially believed to be a decoy receptor that competed with C5aR1 for C5a, although studies have indicated that C5aR2 does have various functions relevant to transplantation, including generation of induced T regulatory cells (22), mediating I/R injury (23), and inhibition of cellular regeneration after ischemia (24).

Complement receptors 2-4 (CR 2-4) are cell-surface receptors that bind to the C3 degradation fragments (iC3b, C3dg, and C3d). CR2 is a transmembrane protein that binds to C3dg and C3d, as well as several non-complement ligands. It is expressed on B cells and follicular dendritic cells, as well as some T cell subsets (25). CR2 signaling lowers the threshold for B cell activation, thereby increasing the B cell response to C3d-opsonized antigens. CR3 and CR4 are β-integrins that can bind to iC3b as well as other ligands (26). CR3 contains an α chain (CD11b) associated with a β2 subunit (CD18). It is expressed on most myeloid cell populations, and it mediates phagocytosis, cell activation, respiratory burst, and cytokine production. It can also negatively regulate the immune response (27). CR4 is comprised of an α unit (CD11c) associated with a β2 subunit (CD18) and is expressed on myeloid cells and some T and B cells. CR4 binds to iC3b (as well as several other ligands) and can increase phagocytosis and cytokine production (28, 29). In spite of all of these immunomodulatory functions, the role of these complement receptors in allograft tolerance and rejection has not been extensively studied.

Complement Regulatory Proteins

Complement activation is controlled by the specificity of the pattern recognition molecules that initiate activation. Host cells also express several membrane-bound regulatory proteins that negatively regulate activation. These proteins limit complement activation by accelerating the decay of the complement activating complexes ("convertases"), or by inactivating the C3b component of the convertases (30, 31). Several soluble proteins also control complement activation. C1q esterase inhibitor (C1-INH) is the primary inhibitor of the CP and the LP, and C4b-binding protein also controls activation of these pathways. Factor H is an important regulator of the AP. The particular importance of factor H for protecting the body from pathologic AP activation is highlighted by the association of factor H mutations with several diseases (32). Complement-mediated allograft injury indicates that these regulatory proteins can be overwhelmed or subverted in the allograft. Ischemic injury of the kidney, for example, increases local production of activating complement proteins and causes downregulation of regulatory proteins, thereby creating a microenvironment favorable to AP activation (33).

PRO-INFLAMMATORY EFFECTS OF COMPLEMENT IN THE ALLOGRAFT

Once complement is activated within a transplanted organ it can have direct and indirect pathologic effects. As outlined above, multiple different biologically active complement fragments are generated. These proteins and fragments directly affect resident organ cells, they are chemotaxins and activators for neutrophils

and macrophages, and they provide important signals for T and B cell activation. The location of complement activation will vary in different settings. In kidney ischemia, for example, activation primarily occurs in the tubulointerstitium (34), whereas in AMR activation occurs in the peritubular capillaries (35). The location of activation determines which cells will be directly affected by MAC or opsonization with C3b. Soluble fragments such as C3a and C5a can have more distant effects, but the site of activation may affect their access to the circulation and peripheral blood cells. It is useful to understand the contribution of the individual complement fragments to injury, as drugs that target specific fragments are in development.

C3a

Little is known about the specific role of C3a in transplant injury. Nevertheless, studies in mouse models of kidney disease have shown that C3a/C3aR signaling contributes to glomerular and tubular injury (36, 37), and it can promote epithelial to mesenchymal transition (36). C3a also stimulates epithelial cells to produce chemokines which may be an important cause of tissue inflammation (38). There are not currently any specific antagonists of C3a available for clinical use. It is noteworthy, however, that drugs that target complement at the level of C5 will not prevent generation of C3a.

C5a

C5a has several pro-inflammatory effects and is a potent myeloid cell chemoattractant. C5aR deficiency or blockade is protective in models of I/R injury (39), tubulointerstitial injury (40), and anti-neutrophil cytoplasmic antibody (ANCA) vasculitis (41). In a murine kidney transplant model, a small molecule C5aR1 antagonist prolonged the survival of mismatched allografts (21). The agent reduced infiltration by monocytes/macrophages, and also decreased priming of T cells in the recipients. C5aR antagonists have been developed for clinical use.

Membrane Attack Complex

The formation of sublytic MAC on endothelial cells leads to NF- κb activation within the cells (42–44), inducing the cells to produce IL-1α and IL-8 (45). In a heart transplant model, this effect was also associated with activation of allogeneic CD4 T cells (44).

THE ROLE OF COMPLEMENT IN THE ADAPTIVE IMMUNE RESPONSE

Although complement activation can cause direct inflammatory injury of the allograft, it can also enhance the response of B and T cells to donor antigens. Signaling through CR2 increases the B cell response to T-dependent antigens (46). Thus, B cells have a stronger response to antigens that are opsonized with C3d. Complement activation in tissues after I/R amplifies generation of antibodies to foreign antigens, although it is not clear whether this is a specific effect of complement on the B cell response or whether it is indirectly caused by cytokines generated downstream of complement-mediated injury (47). Several studies have linked complement activation in transplanted organs with the development of T cell alloreactivity (21, 48). This may be due to a co-stimulatory effect of complement fragments, but experiments have also shown that C3a and C5a reduce the inhibitory function of T regulatory cells (49). Elegant work has also shown that complement proteins produced by dendritic cells and T cells are activated at the cell-cell interface, and enhance the T cell response to antigen (50). Although complement inhibitors would likely block this mechanism of T cell activation, it is not driven by complement activation within the allograft *per se*.

In some settings, complement activation can also limit the adaptive immune response. Studies in several cancer models have shown that complement activation within tumors can attract myeloid derived suppressor cells (MDSCs) that block T cell anti-tumor immunity (51, 52). Although there are many differences between tumor immunology and transplant immunology, some parallels have been seen. For example, co-stimulatory blockade can induce allograft tolerance in murine models. In this setting, C5aR deficiency reduced infiltration of heart allografts by a myeloid suppressor cell population that is necessary to maintain tolerance, similar of the effect in tumors (53). Another mechanism of complement-mediated immunosuppression has also been identified in the liver. Stellate cells produce iC3b which, in turn, causes dendritic cells to differentiate into MDSCs (54). This finding may explain why greater tolerance is seen in recipients of liver transplants that contain the stellate cells than transplants containing isolated hepatocytes.

Complement in Ischemia/Reperfusion Injury

Numerous studies have shown that complement is activated after I/R, although the mechanisms may vary between different organs. Complement activation in the ischemic heart and intestine may be initiated by immunoglobulin, but it primarily involves the LP (55, 56). In the kidney, complement activation primarily involves the AP and does not require immunoglobulin (34, 57, 58). Studies in which kidneys from C3 deficient mice were transplanted into wild-type recipients revealed that the allograft itself may be an important source of complement proteins involved in tubulointerstitial activation (48, 59).

Complement inhibitory drugs have proven effective in several pre-clinical models of I/R injury. An inhibitory antibody to C5 (which prevents formation of C5a and the MAC) and a small molecule C5a receptor antagonist were each protective in models of cardiac (60, 61) and kidney (39, 62) I/R injury. LP blockade was protective in models of kidney and cardiac I/R injury (56). A monoclonal antibody that inhibits the AP was protective in a model of warm I/R injury of the kidney (58). This same drug also prevented I/R injury in a mouse kidney transplant model, and it also reduced T cell mediated rejection of the organs (63).

In spite of promising pre-clinical data, a trial that enrolled 27 kidney transplant patients at high risk of DGF who were randomized to treatment with an inhibitory monoclonal antibody to C5 (eculizumab) did not show any benefit with treatment (64). Another clinical trial is ongoing, however, in which kidneys treated with an agent that attaches a complement

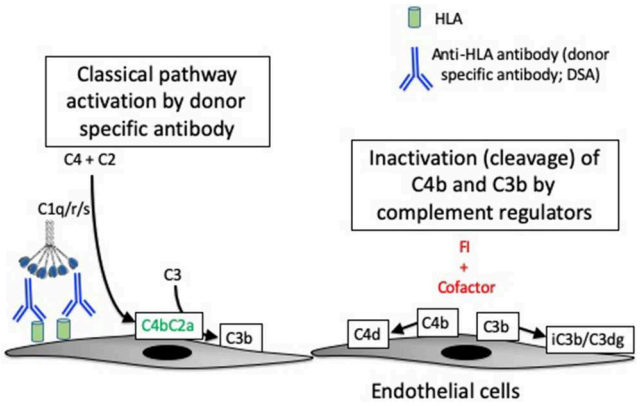

FIGURE 2 | Complement activation in antibody-mediated rejection. Antibody-mediated rejection is caused by binding of antibodies to human leukocyte antigens (HLA) expressed on endothelial cells of the transplanted organ. The antibodies (referred to as donor specific antibodies, or DSA) activate the classical pathway of complement. Classical pathway activation causes the cleavage of C4, and one of the resultant C4 fragments (C4b) is covalently attached to target surfaces. C4b comprises part of the classical pathway C3 convertase, C4b2a. C3b can become covalently attached to target cells, similar to C4b. A protease called factor I (FI) controls complement activation by cleaving the C4b and C3b molecules, thereby stopping convertase activity. Although they are no longer catalytically active, the C4d and C3dg fragments remain bound to the target cells and can be detected by immunostaining of tissue biopsies.

regulator to cell membranes (65). This approach was previously shown to be beneficial in a rat kidney transplant model (66).

ANTIBODY MEDIATED REJECTION

In patients with acute and chronic AMR, DSA binds to donor HLA expressed on endothelial cells and activates the CP. Complement activation on the endothelial cell surface is believed to be an important cause of injury to the capillaries (**Figure 2**) (35, 67). The diagnosis of AMR is based on detecting DSA in the plasma, microvascular inflammation on a biopsy (e.g., glomerulitis or peritubular capillaritis in allografts), and C4d deposition in the peritubular capillaries (68). However, the diagnostic criteria have been modified to account for C4d-negative cases (69, 70). It is not known whether the C4d-negative cases of AMR are caused by non-complement-mediated injurious effects of the DSA, or whether it simply reflects variability in the ability to detect the C4d.

Because complement activation by the DSA is such an important component of AMR, assays have been developed to distinguish the complement activating potential of DSA in the circulation. These assays identify immunoglobulin that binds to specific HLA types, and also tests whether the detected antibodies bind to C1q (71) or carry a C3d molecule (72). Patients with DSA that bind to C1q or to which C3d is bound are at greater risk of developing AMR and they have a worse overall prognosis (73). These findings highlight the importance of the complement system in AMR, and potentially provide a test for identifying patients at risk of AMR. An inhibitory antibody to C5 was protective in a model of heart transplantation in highly sensitized mice, supporting the importance of complement activation in the pathogenesis of microvascular injury (74). Interestingly, treatment with the C5 inhibitor led to long term allograft survival, even though DSA persisted after the treatment was stopped. It is possible that complement inhibition induced "accommodation" in the allograft. Accomodation is a state in which an allograft becomes resistant to AMR. This may occur through upregulation of complement regulatory proteins, altered expression of the target antigens by the allograft, or changes in the isotype of the DSA (75).

Eculizumab has been used in transplant recipients at high risk of developing AMR, as well as patients with active disease refractory to treatment. Positive results have been reported in lung and kidney transplant recipients with AMR (76–78). Larger series in transplant patients have not shown a consistent benefit, however, and the role of eculizumab for preventing or treatment AMR is not yet clear (73, 79, 80). C1-INH is a substrate-like serine protease inhibitor that blocks several proteases, including C1r, C1s, and the MASPs (81). Originally used as a replacement therapy for patients with hereditary angioedema who have deficiency of C1-INH protein, it has also been tested as a treatment of AMR (82). C1-INH appeared to be beneficial in a small trial of six AMR patients who were refractory to conventional therapy (83), and a larger clinical trial is currently underway.

A clinical trial in patients with ANCA-associated vasculitis has shown that a C5aR1 antagonist is beneficial and may reduce the need for corticosteroids in this disease (84). Although the drug has not yet been approved for this indication, the study demonstrated that it can safely be used in patients with kidney disease. Given that there is pre-clinical data showing that C5aR1 blockade may be a beneficial treatment for rejection (21), this approach holds promise as a novel treatment for transplant patients.

Xenotransplantation

The critical shortage of human donor organs limits the number of allotransplants, and there has been a long-standing interest in xenotransplantation as a means of increasing the number of available organs. One of the major obstacles to xenotransplantation is hyperacute rejection of the transplanted organ due to natural antibodies (85). Mammals have a pre-existing repertoire of natural antibodies reactive against several sugar motifs, including anti-Galα1,3Gal (86), which is expressed on pig endothelial cells. Natural antibodies bind these endothelial antigens almost as soon as the xenograft is reperfused and lead to hyperacute rejection. Strategies for preventing this process include deletion of the α1,3-galactosyltransferase gene in the donor animal (87), or transgenic expression of human complement regulatory proteins in the allografts (88). Complement inhibitory drugs may be beneficial in this setting, but they would likely need to be administered long-term as the pathogenic natural antibodies may persist in spite of immunosuppression.

T CELL MEDIATED REJECTION

As outlined above, complement deficiency and/or inhibition can reduce alloreactivity to allografts. Complement-mediated T cell priming may occur at the T-cell/dendritic cell interface, or it may occur downstream of complement activation within the allograft. For example, antibody-induced complement activation on allograft endothelial cells can promote activation of T cells (44).

An interesting discovery was that local production of complement proteins increases after ischemia, and that expression of these proteins by the allograft is associated with T cell mediated rejection (48). Transplantation is a unique setting that allows distinction of local complement production in the allograft from hepatic production in the recipient, as donor C3 may be of a different allotype than recipient C3. C3 allotypes have been defined as fast (F) and slow (S) based on a polymorphism that affects the mobility of the protein on electrophoresis. One study of patients who expressed a different C3 allotype than the allograft they received, reported that the percentage of plasma C3 generated in transplanted kidneys increases during acute rejection episodes (89). Furthermore, C3 generation in organs from brain dead donors may already be increased at the time the organs are harvested, possibly adversely affecting the survival of these organs (90, 91). Interestingly, the C3 allotype expressed by the allograft may affect the long-term prognosis. Patients expressing the C3S allotype had better outcomes if they received allografts that expressed C3F (either C3F/S or C3F/F) (92).

RECURRENCE OF PRIMARY DISEASE IN THE ALLOGRAFT

Most forms of primary glomerulonephritis recur in allografts in spite of immunosuppression. Although the drugs usually employed to protect the allograft may reduce the generation of autoantibodies, they probably do not have much effect on production of complement proteins by the liver or activation of complement by immune complexes. Consequently, if a disease like membranous nephropathy recurs after transplantation, complement activation by deposited immune-complexes will have the same effect that it has in disease of native organ.

C3G and aHUS, two glomerulopathies caused by uncontrolled AP activation, are particularly likely to recur in the transplanted kidney. C3G is among the causes of primary GN with the highest rates of recurrence (93–95). Atypical HUS also frequently recurs in renal transplant patients, particularly in patients with factor H mutations. In a recent case series, 16 of 19 patients had disease recurrence within 7 years of transplantation in spite of treatment with standard immunosuppression (96), and recurrence of aHUS is particularly high in the peri-transplant period (97). This may be due to the inability of these patients, many of whom have molecular defects in AP regulation, to resolve ischemia-induced AP activation in the allograft.

COMPLEMENT BIOMARKERS

During complement activation, complement protein fragments are released into the plasma, and C3 and C4 fragments are covalently fixed to target tissues. Native kidney biopsies are routinely stained for C3 deposits, and in some centers they are also stained for C4 fragments (98). Because C4 is covalently attached to target tissues, C4 deposits provide a durable marker of CP activation. Allograft biopsies are now routinely stained for C4d, and detection of C4d in the peritubular capillaries is interpreted as a marker of classical pathway activation in patients with AMR (99). CP activation on the capillary would also be expected to result in C3 fragment deposition, although C3d deposition seems to be a less sensitive indicator of AMR (100). It is possible that C3d deposition signifies more complete activation of the complement cascade, and one study found that deposition of C3d on the peritubular capillaries was associated with a worse prognosis (101). In contrast to AMR, I/R injury of native kidneys is associated with C3d deposition on the tubules in the absence of C4d, consistent with AP activation at this location (57). Thus, distinct patterns of complement activation may be useful for identifying the underlying cause of organ injury.

Soluble complement fragments can be measured in body fluids by enzyme linked immunosorbent assays (ELISAs). The half-life of these fragments is short, so elevated levels of complement fragments indicates that there is ongoing activation (102). There are assays that can measure many different complement fragments, including C4a, C3a, Ba, Bb, C5a, and soluble sC5b-9. Measurement of these fragments, therefore, can also shed light on the underlying pathologic process. It was recently reported, for example, that Ba fragments are elevated in the urine of patients with ischemic acute kidney injury, indicating that the AP is activated in these patients (103). C4a levels are increased in patients with severe SLE, on the other hand, indicating activation of the CP in this immune-complex disease (104). Other than staining allograft biopsies for C4d, complement biomarkers are not routinely analyzed in transplant recipients. As the use of complement inhibitory drugs expands, however, there will be an increasing need to develop accurate biomarkers.

COMPLEMENT THERAPEUTICS

As described above, studies have tested whether eculizumab is useful for preventing complement activation in the allograft caused by ischemia and AMR. It has also been used in transplant recipients with post-transplant aHUS recurrences (105). C1-INH has also been used to prevent AMR, and it is being tested for treatment of the disease in an ongoing clinical trial. Pre-clinical work has shown that other complement inhibitory agents may be useful in the transplant setting, including an AP inhibitor (63), a LP inhibitor (56), and C5a blockade (21). Many new complement inhibitory drugs are in clinical development, some of which will likely soon become available for clinical use (106, 107). In particular, a C5a inhibitor has shown some efficacy in patients with ANCA associated vasculitis (84).

FUTURE DIRECTIONS

Although immunosuppressive medications have improved short term transplant outcomes, long-term outcomes have not shown an equivalent improvement. Events at the time of transplantation can affect long-term outcomes, including brain death of the donor and I/R injury of the allograft. Acute and chronic AMR are also important causes of allograft failure, and currently there are no specific therapies shown to be effective for AMR. Complement activation may contribute to all of these forms of injury. Furthermore, complement activation provides important signals that enhance the adaptive immune response, thus linking inflammation in allografts with long-term alloimmunity. The standard immunosuppressive medications used to prevent transplant rejection do not directly block the complement cascade. Thus, complement inhibitory drugs may be useful adjuncts to the currently available anti-rejection drugs in several different clinical settings.

Although eculizumab and C1-INH have shown promise in case reports and small series, their role in transplant medicine requires further study. Many additional anti-complement therapeutics are in clinical development, and some of these new drugs block individual activation pathways or specific components of the complement cascade. This could potentially allow clinicians to block the parts of the complement cascade involved in allograft injury while leaving other parts of the cascade active.

However, testing these new agents in the transplant setting poses several challenges. First, all transplant recipients are treated with multiple immunosuppressive drugs. Thus, new drugs will need to be tested as add-on treatments to these other agents. Second, even within a single diagnosis, such as AMR, there is patient heterogeneity. Complement activation may not be an important part of C4d-negative AMR, for example. The development of new complement biomarkers may therefore be critical for selecting patients most likely to benefit from complement inhibitors, and for discerning a response to treatment.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Dendritic Cells as Sensors, Mediators, and Regulators of Ischemic Injury

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Dendritic cells (DCs) are highly specialized, bone marrow (BM)-derived antigen-processing and -presenting cells crucial to the induction, integration and regulation of innate, and adaptive immunity. They are stimulated by damage-associated molecular patterns (DAMPS) via pattern recognition receptors to promote inflammation and initiate immune responses. In addition to residing within the parenchyma of all organs as part of the heterogeneous mononuclear phagocyte system, DCs are an abundant component of the inflammatory cell infiltrate that appears in response to ischemia reperfusion injury (IRI). They can play disparate roles in the pathogenesis of IRI since their selective depletion has been found to be protective, deleterious, or of no benefit in mouse models of IRI. In addition, administration of DC generated and manipulated *ex vivo* can protect organs from IRI by suppressing inflammatory cytokine production, limiting the capacity of DCs to activate NKT cells, or enhancing regulatory T cell function. Few studies however have investigated specific signal transduction mechanisms underlying DC function and how these affect IRI. Here, we address current knowledge of the role of DCs in regulation of IRI, current gaps in understanding and prospects for innovative therapeutic intervention at the biological and pharmacological levels.

Keywords: dendritic cells, ischemic injury, kidney, liver, heart

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AN INTRODUCTION TO DENDRITIC CELLS (DCS)

DCs comprise a heterogeneous population of uniquely well-equipped, bone marrow-derived innate immune cells. They are distributed ubiquitously throughout the body and play an important homeostatic and anti-infectious sentinel role. DCs are highly efficient, antigen (Ag)-acquiring, -processing, and presenting cells, that perform crucial roles in the instigation and regulation of acute and chronic inflammatory responses. While they promote self-tolerance in the healthy steady-state and can be targeted by microbes and tumors to evade immunity, DCs integrate innate and adaptive immunity effectively to combat infection and can also be exploited as anti-cancer vaccines. During autoimmunity and transplant rejection, DCs instigate deleterious immune responses that cause disease; on the other hand, they can be harnessed to silence these conditions using novel targeting and adoptive cell therapy approaches. In the context of ischemic tissue injury that adversely affects short- and long-term transplant outcome, DCs appear to play diverse roles in regulation of the inflammatory response.

DC SUBSETS—PHENOTYPE AND FUNCTION

Distinct subsets of DCs, including myeloid/conventional DCs (cDCs) and type-I IFN-producing plasmacytoid DCs (pDCs) have been described extensively elsewhere (1–3) and are summarized in **Table 1**. Classical DCs, but not monocyte-derived antigen (Ag)-presenting cells (APCs), are critical for central and peripheral regulatory T cell (Treg) induction and the development of tolerance (4) as well as shaping effector T cell responses to Ag. Beyond the classical characterization of DCs, new phenotypic and functional subsets of DCs continue to emerge (5, 6). Moreover, the discovery of new lineage markers and introduction of innovative imaging technologies (including use of reporter mice) have helped to distinguish classical DC subsets from other myeloid cells, particularly macrophages, in tissues such as the kidney (7).

According to their maturation and functional status, DCs can be divided into immature, mature, and regulatory populations. Regulatory DCs (DCregs) have been extensively investigated in transplantation (8–11) and autoimmune disease (12, 13), ranging from pre-clinical models to pilot human clinical trials. Infusion of donor-derived DCregs prior to transplantation has been shown to prolong kidney allograft survival and inhibit donor-reactive $CD8^+$ memory T cell responses in non-human primates (14, 15). First-in-human phase I/II clinical trials of adoptive DCreg therapy in living donor renal and liver transplantation have recently been instigated (8, 16).

TABLE 1 | DC subsets in mouse and human: phenotype, localization, and function.

Human DC subsets	Phenotype	Location	Function
Plasmacytoid DC	CD123 CD303/BDCA-2 CD304/BDCA-4	Blood, tonsil, non-lymphoid tissues	Production of type I and III IFN
Myeloid cDC1	CD141/BDCA-3	Blood, tissues, and lymphoid organs	Present Ag to CD8+T cells and produce type III IFN
Myeloid cDC2	CD1c/BDCA-1 CD2 CD11c CD11b	Blood, tissues and lymphoid organs	Activate Th1/Th2/Th17 and $CD8^+$T cells
Langerhans cells	CD207 CD1a E-Cadherin	Skin (epidermis)	Transfer Ag to afferent lymphatics, stimulate $CD8^+$T cells
Mo-DC	CD11c CD1c/BDCA-1 CD1a	*Ex vivo*-generated	
Murine DC subsets			
Plasmacytoid DC	$CD11c^{int}$ $CD11b^-$ $CD8^-B220^+Gr\text{-}1^+$	Lymphatic and non-lymphoid tissue	IFN-α production
$CD8^+$ cDC	$CD11c^+$ $CD11b^-CD8^+$ $CD24^+$ MR^+	Lymphatic and non-lymphoid tissue	Activate $CD4^+$ and $CD8^+$ T cells, Ag presentation
$CD8^-$ cDC	$CD11c^+$ $CD11b^+CD8^-$ $CD24^-$	All tissues	Activate $CD4^+$ T cells, transport Ag to LN
Langerhans cell	$CD11c^+$ $CD11b^+$ $CD8^-$Langerin$^+$ $CD1a^+$	Skin (epidermis)	Transport Ag from skin to LN

CD, cluster of differentiation; cDC, conventional DC; DC, dendritic cell; Mo-DC, monocyte-derived DC; pDC, plasmacytoid DC; LN, lymph nodes; MR, mannose receptor; IFN, interferon.

ISCHEMIA-REPERFUSION INJURY (IRI)

IRI is a common clinical condition triggered by various physiological derangements (sepsis, cardiogenic shock, vascular surgery, organ retrieval for transplantation). Its pathogenesis has been comprehensively described elsewhere (17–21) but is essentially characterized by endothelial dysfunction, reactive oxygen species (ROS) production, secretion of pro-inflammatory mediators, and recruitment of inflammatory cells which exacerbate/perpetuate tissue injury.

INFLAMMATORY CELLS CHARACTERIZE IRI

Inflammatory cell infiltration after IRI is rapid, peaking 24 h following reperfusion (22). $Gr\text{-}1^+$ neutrophils, which release ROS and proteolytic enzymes, and $NK1.1^+CD161^+$ NKT cells ($CD56^+$ in humans) which elaborate pro-inflammatory cytokines, are the predominant early inflammatory cells that impair organ function within hours of IRI (23–26). However, neutrophil-depleted animals are not protected following ischemic insult to the kidney (27, 28). Neutrophils, NKT cells and DCs intercommunicate to enhance tissue injury through chemokine/cytokine secretion, as well as cell-cell contact (29, 30). These interactions are depicted in **Figure 1** in the context of the early period following renal IRI. While DCs are thought to be crucial to the pathogenesis of IRI, their role in kidney IRI remains unclear, since depletion may be protective (22), deleterious (31), or of no benefit (32). *In situ* targeting of DCs with the vitamin D analog paricalcitol can induce intrahepatic tolerogenic DCs and alleviate $CD4^+$ T cell responses to attenuate hepatocellular damage (33). In contrast, pDC-released type I IFN promotes tissue injury through induction of hepatocyte IFN regulatory factor-1 (IRF-1) to induce apoptosis (34).

Macrophages, NK cells and adaptive immune cells, including T and B cells, infiltrate injured tissues hours after reperfusion (22). Macrophages have been considered to polarize into M1 (classical) and M2 (alternatively-activated) subsets with pro- or anti-inflammatory function, respectively, although recent reassessment suggests a broader functional repertoire for these cells (35). Heme oxygenase 1 (HO-1) negatively regulates M1 polarization and hepatocellular damage in both mouse liver IRI and human liver transplant biopsies (36). Further

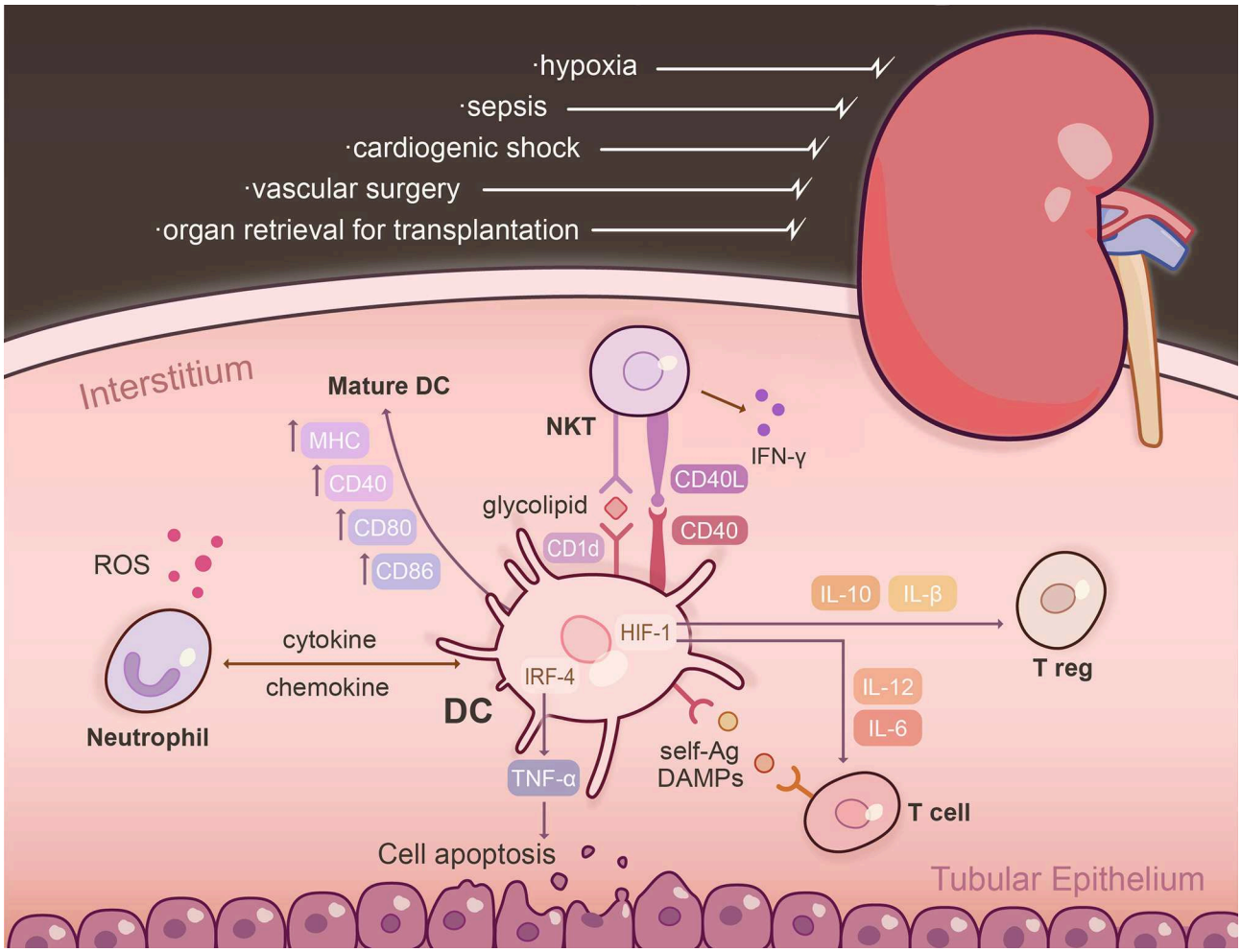

FIGURE 1 | The DC interactome following renal ischemia reperfusion injury (IRI). IRI is a common clinical condition triggered by various physiological derangements including sepsis, cardiogenic shock, vascular surgery, and organ retrieval for transplantation. Following injury, resident, and influxing DCs become activated within the kidney parenchyma and are the dominant TNFα-producing cells. The effect of TNF-α is dependent on the transcription factor IRF-4, promoting renal tubular epithelial cell apoptosis, glomerular endothelial damage, and fibrin deposition. Post-IRI, intra-renal DCs upregulate markers that designate them as mature APCs, including MHC, CD80, CD86, CD40, and CD1d. Activation of NKT cells via CD40 initiates IFN-g to amplify the innate immune response. Renal DCs are capable of presenting self-Ag to a variety of T cells in the context of IRI: $CD11c^+$ DCs cross-present Ag to $CD8^+$ T cells and glycolipids are presented to NKT cells via CD1d. Exposure of DCs to hypoxia/reperfusion augments production of IL-12 and IL-6, and an inflammatory T cell phenotype through HIF-1α transcriptional regulation. Absence of DC-specific HIF-1α limits expression of IL-10 and TGF-β, which are potent inducers of Tregs. DCs intercommunicate with neutrophils and NKT cells to enhance tissue injury through chemokine/cytokine secretion, as well as cell-based contact. Neutrophils release ROS and proteolytic enzymes, and $NK1.1^+CD161^+$ NKT cells elaborate pro-inflammatory cytokines and are the predominant early inflammatory cells that impair organ function within hours of IRI. Ag, antigen; DAMPs, damage-associated molecular patterns; HIF-1, hypoxia-inducible factor-1; IRF-4, interferon regulatory factor-4; ROS, reactive oxygen species.

evidence shows that HO-1 regulates macrophage activation through the Sirtuin/p53 signaling pathway to drive hepatic death during liver IRI (37). Deletion or inhibition of Dectin-1 can suppress M1 macrophage polarization and alleviate myocardial IRI (38). T cells can continue to localize in injured kidneys for 2 weeks and display an effector-memory and activation phenotype characterized by $CD44^{hi}CD62L^-$ and $CD69^+$ expression, respectively (39). Treg function is decreased in aged mice, which contributes to exacerbated liver IRI (40).

HYPOXIC STIMULI

In transplantation, immune-mediated injury is a composite of the innate response to IRI and alloimmune reactivity to foreign Ag. Many clinical studies confirm a link between delayed graft function and a higher rate of acute rejection (41–44). We and others have shown that hypoxia activates DCs (45), as evidenced by their phenotypic maturation, pro-inflammatory cytokine production, and enhanced T cell stimulatory and migratory capacity (46–50).

Altered DCs function under hypoxia has been ascribed to changes in hypoxia-inducible factor (HIF)-1α activity. Longer-term hypoxia (>1 h) followed by reperfusion downregulates the G-protein coupled purinergic receptor P2Y11R through HIF-1α transcriptional regulation, resulting in augmented production of IL-12 and IL-6 and an inflammatory T cell phenotype in response to extracellular ATP (51). Hypoxia, and therefore HIF-1α-dependent upregulation of adenosine receptor A2B (52), or triggering receptor expressed on myeloid cells- (TREM)-1 (53, 54) can induce Th2 polarization and pro-inflammatory cytokine release, respectively. BMDCs lacking functional HIF-1α show deficiencies in type I IFN secretion and fail to activate $CD8^+$ T cells (55). Interestingly, the effect of hypoxia on DCs is attenuated by rapamycin (47) and augmented by concurrent exposure to LPS (56).

Together, these data suggest that DCs are reprogrammed by a hypoxic environment to modulate their inflammation-activating repertoire, however discrepant features in many studies may reflect differences in hypoxia duration and severity. *In vivo*, HIF-1α is crucial for DC-dependent generation of Tregs and Treg homing to inflammatory sites. Absence of DC-specific HIF-1α limits IL-10 and TGF-β expression (both potent inducers of Tregs) and reduces expression of aldehyde dehydrogenase (necessary for catalytic production of retinoic acid) (57). Although HIF-1α is protective against IRI (58), there are no studies assessing its specific effect on DCs in this model.

ISCHEMIC PRECONDITIONING

Ischemic preconditioning (IPC) occurs when brief periods of sublethal ischemia are performed prior to a subsequent prolonged episode and increases organ resistance to IRI (59). However, the underlying mechanism (s) remain elusive. Exposure of renal parenchyma to IPC prior to IRI does not alter the number of infiltrating leukocytes, but reduces $CD11c^+$ DCs (compared to IRI alone), which display lower CCR7 and IL-17 transcript levels, decreased CD80 expression and upregulated IL-10 (60). Elimination of $CD11c^+$ DCs ($CD11b^+$ and $CD8^+$ subsets) using liposomal clodronate is associated with partial loss of preconditioning benefits.

ORGAN CROSS-TALK DURING IRI

IRI rarely occurs in isolation—systemic release of pro-inflammatory cytokines and cell trafficking to primary and secondary lymphoid tissue ensures widespread modulation of innate immunity. Substantial cross-talk between the injured and remote organs manifests clinically as multi-organ failure. The onset of acute lung injury in the context of acute kidney injury (AKI) occurs more frequently than any other organ combination (61). Experimental models have identified a distinct pulmonary genomic signature during AKI, with differentially expressed pro-inflammatory and pro-apoptotic pathways (62). The intestine can also aggravate the systemic inflammatory response syndrome (63). Increased permeability from gut hypoperfusion, modification of microbiota composition, and blood-borne propagation of toxins contribute to the outcome of AKI. Gut microbiota can also modulate the inflammatory response to injury. Short-chain fatty acids such as acetate, propionate, and butyrate are produced by fermentation of complex carbohydrates and have a broad range of anti-inflammatory effects (64). Concurrent incubation of murine BMDCs with short-chain fatty acids and LPS reduces upregulation of maturation markers CD80, CD86, and CD40 (65). Butyrate specifically inhibits production of IL-12 in human monocyte-derived DCs, limiting development of effector Th1 cells (66, 67). Treatment with short-chain fatty acids also protects against AKI, with lower frequencies of infiltrating macrophages and activated DCs (65) in addition to effects on renal tubular epithelial cell apoptosis and ROS production.

ORGAN-SPECIFIC ROLES OF DCS IN IRI

Tissue-Specific Phenomena

The majority of animal data on the function of DCs in IRI is limited to the liver, heart and kidney, and is best characterized in the latter. Robust models of pulmonary, intestinal and pancreatic IRI are lacking due to technical challenges. Human data is also sparse due to inherent difficulties with DC detection and limited tissue availability. *In vivo* targeting of organ-specific DCs to limit IRI and innate immune activation is difficult due to the lack of defining cell-surface markers. **Tables 1**, **2** outline the breadth of available markers, which show significant overlap.

Kidney

$CD11c^+$ DCs are resident within the kidney parenchyma (86), infiltrate following ischemic insult and are the dominant TNFα-producing cells (77) (**Figure 1**). TNF-α is crucial to neutrophil influx post-IRI, renal tubular epithelial cell apoptosis, glomerular endothelial damage and fibrin deposition (87–90). The effect of TNF-α depends on the transcription factor IRF-4 (91). Non-specific elimination of DC using liposomal clodronate or administration of etanercept (a decoy receptor for TNF-α) abrogates AKI in IRF-4 deficient mice (91).

Intra-renal DCs post-IRI constitutively express markers that designate them as professional APCs (MHC, CD80, CD86, CD40, CD54, CD1d), but not tissue macrophage markers [CD169, CD204 (77)]. As early as 4 h post-IRI, higher levels of maturation marker expression are observed, favoring the hypothesis that DC maturation occurs *in situ* rather than by cell replacement (77). Multiple studies have identified a continuum of DC phenotypes that contribute to the innate immune response and IRI. Monocyte subsets migrate to inflamed tissue and differentiate into activated DCs as $CCR2^+CX3CR1^{lo}GR\text{-}1^+Ly6C^{hi}$ cells (92). Resident $CX3CR1^{hi}$ monocytes patrolling the parenchymal space also migrate, differentiate, and participate in inflammation. Both CCR2 (93) and CX3CR1 (94) are essential to this process.

The traditional paradigm has been discrete separation of mononuclear phagocytic cell populations into $CD11b^+$ macrophages and $CD11c^+$ DCs that increase following IRI. More recent thinking has focused on phenotypic heterogeneity and functional plasticity of these cells. They are divided broadly into 5 subsets based on intensity of CD11b and CD11c expression and

TABLE 2 | Identified DC subsets implicated in IRI.

Organ	Mouse	Human	Function
Heart (68)	**Steady-state** **Myeloid DC:** $CD45^+Lin^-CD11c^+MHCII^+$ **cDC1:** $CD103^+CD11b^-$, Clec9a, Flt3/CD135, CD205, CD24, CD283 **cDC2**: $CD103^-$ $CD11b^+$, CD115/M-CSF^{lo}, F4/80^{lo}, CX3CR1^{lo}, Ly6C^{lo} **Double negative (DN) cDC**: $CD103^-CD11b^-$ **Plasmacytoid DC**:$CD45^+$ Lin^- $CD46^-$ MHCII^{lo} $CD11b^-$ CD11c^{lo} $PDCA1^+$ $Ly6C^+$ $SiglecH^+$ $Clec9a^+$	**Steady-state** Hu-mice reconstituted with human stem cells + Flt3L: **Myeloid DC**: $HLA\text{-}DR^+$ $CD11c^+$ $BDCA1^+$ ($CD1c^+$), IRF4 $BDCA3^+$, IRF8 **Plasmacytoid DC:** $CD123^+BDCA2^+$ $LAMP^+$ IRF8	Murine: post-IRI 10-fold increase, DC depletion improves cardiac function post-MI (68), OR worsens LV function (69), increased DC worsen MI outcomes (70, 71), cDC2 increase numbers and CD40 expression in response to MI, prime autoreactive T cells, 4-fold increase, no functional role post-MI
Liver (72)	**mDC:** $CD11c^+CD8\alpha^-CD11b^+$ **CD8α$^+$ DC:** $CD11c^+$ $CD8\alpha^+CD11b^+$ GM-CSF administration: $CD11c^+CD11b^+B220^-CD205^-$ **pDC:** $CD11c^{lo}B220^+Ly6C^+CD11b^-$ **NK-DC:** $CD11c^+NK1.1^+$	Liver perfusate and explanted livers $CD11c^+$ DC subsets (72): $CD141^+$ $Clec9A^+$ (30% of total CD11c population) $ILT3^+$ (38%) $ILT4^+$ (52%) $CD1c^+$ (20% of total CD11c population) **Plasmacytoid DC:** $HLA\text{-}DR^+Lin^-CD11c^-CD123^+$ (15%)	Human: $CD141^+$ cells enriched in healthy livers, secrete CXCL10, IL-1β, IL-17, and IFN-γ; initiate Th1/Th17 responses, express TLR3 Mouse: 65% reduction in cDC post-IRI (73), DC depletion (CD11c-DTR system) worsens IRI (73), Flt3L KO depletes DC and protects against IRI (74), CD11b^{hi} cells increase CD80/86 expression (33), CD40 (DC)-CD154 (T cell) ligation activates innate immunity (75)
Kidney	**Steady-state** **Mononuclear phagocyte subsets** (76): 1. CD11b^{hi}CD11c^{hi}: $MHCII^+$ $CCR2^+$ $CD16^+$ $Zbtb46^+$ 2. CD11b^{hi}CD11c^{lo}: $CCR2^+$ $CSF1R^+$ 3. CD11b^{int}CD11c^{int}: $F4/80^+CD14^+CX3CR1^+CSFR1^+$ $MHCII^+IL\text{-}10^+$ 4. CD11b^{lo}CD11c^{hi}: $CD103^+CCR7^+$ $Zbtb46^+Batf3^+IRF8^+$ 5. $CD11b^-$CD11c^{int} $IRF8^+$ **Post-IRI** $CD45^+CD11c^+$ MHC-II$^+$ $TNF\text{-}\alpha^+CD80^+CD86^+CD40^+$ $CD54^+$ (ICAM), $C1d^+$ $CD8\alpha^-CD4^-CD205^-$ (77)	**Steady-state** (78, 79) **Myeloid DC**: $Lin^-HLA\text{-}DR^+$ **cDC1:** $CD11c^+$ $CD141^+Clec9A^+$ **cDC2:** $CD11c^+CD1c^+$ $CD1a^+$ (subset) **Plasmacytoid DC**: $Lin^-HLA\text{-}DR^+$ $CD11c^-CD123^+$ $CD303^+$	IRI increases: total CD45+ cells, CD45+CD11c–Ly6C+ (monocytes), CD45+CD11c–Ly6G+ (neutrophils), CD45+CD11c+ Ly6C–F4/80– (DC). $CD45^+CD11c^+Ly6C^-F4/80^+$ DC were unchanged (77). $CD11c^+$ DC present Ag to T cells in draining renal lymph node (80).
Pulmonary (81, 82)	**Steady-state** **Myeloid DC:** CD11c^{hi} **Airways:** $CD103^+CD11b^{lo}CD207^+$ (Langerin), $XCR1^+Clec9A^+$ Batf3, ID2, IRF8, Zbtb46 **Beneath basement membrane:** $CD103^-CD11b^+$ RELB, IRF2, IRF4, Zbtb46 **Plasmacytoid DC:** CD11c^{int} MHC II^{int}B220$^+$Ly6C/Gr-1^{hi}Siglec-H$^+$BST-2$^+$ IRF8, E2-2	**Steady state** **Myeloid DC:** $HLA\text{-}DR^+$ $CD1c^+CD11c^+$ $CD14^-$ **Plasmacytoid DC:** $HLA\text{-}DR^+$ $CD123^+$ $CD11c^-CD14^-$ **Maturation:** $CD83^+$	Tightly associated with conducting airways and epithelia (83). No information in IRI models.

(Continued)

TABLE 2 | Continued

Organ	Mouse	Human	Function
Intestine (84, 85)	**Steady state** **cDC1:** $CD103^+$ $XCR1^+$ $CD11b^-CD172^-$ IRF8, Batf3 **cDC2:** $CD103^+CD11b^+CD172^+$ IRF4, Notch2 or $CD103^-CD11b^+CX3CR1^{int}$	**Steady state** **cDC1:** $CD103^+CD141^+$ $XCR1^+DNGR1^+$ **cDC2:** $CD103^+CD172^+$ $CD141^-$ or$CD103^-$ $CD172^+CD141^-$	cDC1 and cDC2 $CD103^+$ cells within epithelium, lamina propria, and draining lymph nodes

Batf, basic leucine zipper transcriptional factor-ATF like; BDCA, blood dendritic cell antigen; Clec, C-type lectin receptor; CSF, colony stimulating factor; FLT3, fms like tyrosine kinase 3; HLA, human leukocyte antigen; MHC, major histocompatibility complex; ICAM, intercellular adhesion molecule; ID, inhibitor of DNA binding; IFN, interferon; IL, interleukin; ILT, immunoglobulin-like transcript; IRF, interferon regulatory factor; IRI, ischemia reperfusion injury; Lin, lineage; MI, myocardial infarction; RELB, v-rel avian reticuloendotheliosis viral oncogene; Siglec, sialic acid binding immunoglobulin like lectin; Zbtb, zinc finger and BTB domain-containing protein.

are further characterized by a comprehensive set of cell surface markers and transcription factors (76). All subsets demonstrate phagocytic capacity but differ in their migratory capacity and cytokine profile as well as their ability to stimulate naïve T cells and alter T cell polarization *ex vivo*. Renal DCs are capable of presenting self-Ag to a variety of T cells in the context of IRI, - $CD11c^+$ DCs cross-present Ag to $CD8^+$ T cells post-IRI (95) and glycolipids are presented to NKT cells via the CD1d cell surface receptor (96). Activation of NKT via CD40 initiates IFN-γ to amplify the innate immune response. Administration of CD1d Ab that blocks NKT-DC interactions or genetic depletion of NKT provides significant protection against renal IRI (96). Renal DCs are also primarily responsible for presenting renal proteins to Ag-specific $CD4^+$ T cells within draining renal lymph nodes. In a model of unilateral IRI where ovalbumin is placed beneath the operated kidney capsule, $CD11c^+$ DCs from the ipsi- or contra-lateral renal lymph nodes induce proliferation of DO11.10 (ovalbumin-restricted) T cells (80). $CD11c^-$ fractions failed to induce T cell stimulation.

Naïve rodent kidneys demonstrate additional DC subsets defined by the expression (or absence) of $CD103^+$. The $CD103^+$ subset is primarily involved in Ag cross-presentation after migrating to lymph nodes (97). Transplantation of syngeneic grafts subjected to negligible or prolonged cold storage leads to depletion of $CD103^+$ DCs, regardless of IRI, but only $CD103^-$ DCs under the former condition (98). Donor cells are replaced by host DCs, accompanied by increases in $CD3^+CD4^+CD62L^-$ T cells, indicative of effector/effector-memory populations.

Heart

Despite advances in percutaneous coronary intervention and use of statin and antiplatelet agents, the incidence of post-infarct heart failure is rising (99). Adverse ventricular remodeling following myocardial infarction increases mortality by precipitating heart failure. Studies of DC subsets in experimental myocardial IRI are relatively uncommon, mostly due to the technical difficulty, high mortality, and significant variations in infarct size associated with left anterior descending artery ligation (100, 101).

DC subsets are found within the $CD45^+$ leukocyte population in healthy myocardium, particularly within the right atrium (68). Conventional $CD11c^+$MHC II^+ DCs (cDC1s) have been defined by CD103 or CD11b expression (or neither) (68). These 2 subsets are also classified by XCR1 or CD172 (SIRP-α) expression, and levels of transcription factors IRF8 and IRF4, respectively (102). Under homeostatic conditions, cDC1s present cardiac self-Ag to α myosin heavy chain-specific $CD4^+$ T cells in mediastinal lymph nodes to induce Treg formation. pDCs ($CD11b^{lo}MHCII^-CD11c^{lo}PDCA\text{-}1^+Ly6C^+$) have also been detected.

DCs infiltrate infarcted myocardium (103), increasing 10-fold, particularly the $CD11b^+$ subset expressing maturation markers such as CD40 (68). Post-myocardial infarction cDCs upregulate CCR7 (the chemokine receptor required for lymph node migration) and CD40 expression; specific activation of the cDC2 subset induces CD86 expression as well as $CD4^+$ T cell proliferation and accompanies IFN-γ and IL-17 production (102). Although mature DC numbers correlate with LV dysfunction (70) and depletion of cDCs reduces infarct size and adverse cardiomyocyte hypertrophy in the border zone (68), a deleterious contribution of DCs is disputed. Prolonged DC ablation leads to impaired LV remodeling, sustained expression of pro-inflammatory cytokines and altered monocyte/macrophage recruitment (69). The administration of liposomal clodronate, which depletes both DCs and macrophages, also impairs wound healing (104). pDC numbers also increase following infarction, but their depletion appears not to affect LV function (68).

DCregs have therapeutic potential in post-infarct healing, modulating Tregs and macrophages. "Tolerogenic DCs" primed with TNF-α and mouse cardiac tissue Ags reduced infarct size, improved LV ejection fraction and increased post-infarct survival (105). This correlated with increased FoxP3+ Tregs in inguinal and mediastinal lymph nodes, as well as in the post-infarct heart. Adoptive transfer of these Tregs into mice post-MI also improved wound healing. Interestingly, troponin- or myosin-primed DCs failed to recapitulate the protective effects seen with tissue-primed DCs.

Liver

Similar roles for DCs have been demonstrated in hepatic IRI. Interestingly, cDCs are depleted from the liver parenchyma, rather than enriched, during maximal injury (12 h post-reperfusion). These DCs display a mature phenotype, with

marked CD86 upregulation, but are also necrotic and apoptotic. Depletion of $CD11c^+$ DCs in CD11c-DTR mice worsens tissue damage and the pro-inflammatory cytokine profile following liver IRI. Adoptive transfer of cDCs into CD11c-depleted mice mitigates this injury, dependent on intrinsic TLR9 activation (from hepatocyte DNA release) and subsequent IL-10 production (73). This is also thought to modulate $CD11b^{int}Ly6C^{hi}$ inflammatory monocyte cytokine production and ROS generation. Augmenting DC numbers with GM-CSF increases susceptibility to liver IRI (106). Post-reperfusion, these DCs exhibited a mature phenotype and enhanced allostimulatory capacity. This effect required release of high motility box group 1 that upregulates DC-expressed TLR4 and interacts with both TLR9 and the receptor for advanced glyosylation end-products (RAGE) to activate DCs (107).

Liver transplant recipients are more likely to develop a tolerant phenotype compared to recipients of other solid organs. This may be due to a comparatively high density of DCs to parenchymal cell populations (108) and the refractory behavior of liver DCs compared with DCs from other tissues in response to TLR ligation (109, 110). Expression of CD39, which drives ATP hydrolysis, is increased on murine liver mDCs but not pDCs, and the levels exceed those in splenic DCs. Not unexpectedly, CD39KO syngeneic liver transplants exhibit worse tissue injury compared to WT grafts, with accompanying upregulation of CD80, CD86, and MHC II, and downregulation of PD-L1 on hepatic mDCs (109). Notably, CD39 expression on freshly isolated human hepatic $CD45^+Lin^-BDCA\text{-}1^+$ DCs is higher than on peripheral blood mDCs.

The discrepant findings suggesting a dichotomous role for DCs in liver IRI imply that tissue-resident and recruited DCs may play distinct roles in the response to injury. DCs are highly motile, and are recruited into the liver in response to macrophage inflammatory peptide-1 (111) (MCP-1 = CCL2), which is produced following IRI. Administration of Flt3L to WT mice increases the DC-resident population 10-fold (112) and enhances liver parenchymal injury; injury is significantly reduced in Flt3L KO mice (74). Adoptive transfer of $CD11c^+$ mDCs or $PDCA\text{-}1^+$ pDCs into Flt3L KO mice at the time of IRI significantly worsens injury. To distinguish the contribution of hepatic-resident vs. infiltrating DCs, a liver transplant model between congenic mice has been used. Donor-derived hepatic DCs upregulated maturation markers to a higher level than on infiltrating (recipient) DCs. Use of Flt3L KO liver donors increased ischemic injury, suggesting a protective effect of liver-resident DCs.

The role of pDCs in liver IRI is poorly defined. IFN-α is produced predominantly by pDCs (113) in response to sensing of self-DNA and TLR9 ligation, which drive the inflammatory response in IRI. IFN-α promotes IRF-1 expression by hepatocytes to induce pro-inflammatory cytokine and death receptor expression (34). Depletion of pDCs using anti-PDCA-1 Ab or use of IFN-α blocking Ab protected the liver against IRI.

The Therapeutic Potential of DC

Despite decades of research and multiple clinical trials, no pharmacological agents are in clinical use for IRI. Cell-based therapy, which capitalizes on the capacity of regulatory cells to modulate adverse immune responses, represents a potential therapeutic option. DCs can exhibit a protective phenotype to modulate pathogenesis of IRI. However, unlike adoptive transfer of Tregs, which can change to a proinflammatory/effector phenotype, depending upon the microenvironment (114, 115), DCregs are maturation-resistant, phenotypically stable and do not need to persist *in vivo* as functional cells to mediate immune response change (116). Pharmacological or genetic manipulation produces a regulatory phenotype (DCregs), secreting low levels of Th1-driving cytokines (particularly bioactive IL-12), and comparatively high levels of anti-inflammatory cytokines (such as IL-10) (117). They are weak T cell stimulators and possess the capacity to induce or expand Tregs. While the beneficial effect of DCregs has been established in multiple pre-clinical models of allograft rejection [reviewed in Thomson et al. (118)], their testing in IRI has been minimal. Elegant experimental work has demonstrated that following adoptive transfer, allogeneic DCregs are rapidly killed by host immune cells (predominantly NK cells), and their effect is mediated by recipient DCs (119, 120). Even in the absence of alloAg, adoptively transferred DCs can direct T cells to produce a regulatory response and mitigate IRI. Thus, while lack of the sphingosine-1-phosphate receptor 3 (S1pr3) protects against renal IRI (121), infusion of $S1pr3^{-/-}$ DCs also protects against IRI which requires functional Tregs, $CD11c^+$ DCs, and $CD169^+$ macrophages (122). In addition, DCs treated with an adenosine 2A receptor agonist protect WT mice from IRI via suppressing NKT cell activation and IFN-γ production (123).

Current Gaps in Knowledge

Variations and limitations in methodology, both for identification and *ex vivo* generation of DCs, have made it difficult to determine whether phenotype and function are consistent between organs affected by IRI. Which self-Ags DCs respond to in IRI is not well-defined and clearly differs according to damaged organ segment, cell type or cellular component (glomerulus vs. tubule, hepatocyte vs. endothelia, myosin heavy chain vs. tubulin). In addition, there is still no consensus to clearly distinguish tissue DCs and macrophages (124, 125). Cell-surface molecules such as CD11c and CX3CR1 are expressed on various myeloid cells and are imperfect DC markers (126). A further caveat is that *ex vivo*-generated DCs do not truly represent a physiological subset of tissue-resident DCs. The majority of studies to date have been conducted in animal models of IRI, and the paucity of human data highlights limitations regarding generalizability of results.

DCregs are currently being tested as cell therapy in clinical liver and kidney transplantation, and the results of these trials are eagerly awaited. If DCregs are to be considered as therapy, it will be necessary to determine cell type (autologous vs. allogeneic vs. donor Ag-loaded), dosage, timing, and frequency of infusion (s) and cost. Other aspects, such as potential sensitization of recipients (with the use of banked allogeneic DCs) and comparison of DCregs with other regulatory cell types, have also not been

addressed. It is also unclear whether the relative efficacy of DC therapy also depends on the organ undergoing IRI. Published research has also focused on the effect of DCs given prior to the onset of IRI, whereas their influence post-injury is not known.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Urinary Properdin and sC5b-9 Are Independently Associated With Increased Risk for Graft Failure in Renal Transplant Recipients

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The pathophysiology of late kidney-allograft failure remains complex and poorly understood. Activation of filtered or locally produced complement may contribute to the progression of renal failure through tubular C5b-9 formation. This study aimed to determine urinary properdin and sC5b-9 excretion and assess their association with long-term outcome in renal transplant recipients (RTR).

Methods: We measured urinary properdin and soluble C5b-9 in a well-defined cross-sectional cohort of RTR. Urinary specimens were taken from a morning urine portion, and properdin and sC5b-9 were measured using an enzyme-linked-immunosorbent assay (ELISA). Cox proportional hazard regression analyses were used to investigate prospective associations with death-censored graft failure.

Results: We included 639 stable RTR at a median [interquartile range] 5.3 (1.8–12.2) years after transplantation. Urinary properdin and sC5b-9 excretion were detectable in 161 (27%) and 102 (17%) RTR, respectively, with a median properdin level of 27.6 (8.6–68.1) ng/mL and a median sC5b-9 level of 5.1 (2.8–12.8) ng/mL. In multivariable-adjusted Cox regression analyses, including adjustment for proteinuria, urinary properdin (HR, 1.12; 95% CI 1.02–1.28; $P = 0.008$) and sC5b-9 excretion (HR, 1.34; 95% CI 1.10–1.63; $P = 0.003$) were associated with an increased risk of graft failure. If both urinary properdin and sC5b-9 were detectable, the risk of graft failure was further increased (HR, 3.12; 95% CI 1.69–5.77; $P < 0.001$).

Conclusions: Our findings point toward a potential role for urinary complement activation in the pathogenesis of chronic allograft failure. Urinary properdin and sC5b-9 might be useful biomarkers for complement activation and chronic kidney allograft deterioration, suggesting a potential role for an alternative pathway blockade in RTR.

Keywords: transplantation, chronic renal failure, properdin, C5b-9, complement activation

INTRODUCTION

Despite improvements in immunosuppressive therapy over the last decades, chronic and irreversible deterioration of a transplanted kidney graft remains a major problem and is responsible for disappointing outcomes in long-term graft survival (1). Even though registry data can be used to define risk factors, chronic allograft failure pathophysiology remains complex and poorly understood, due to difficulty in distinguishing the contribution of several immunological and non-immunological factors (2). Interstitial fibrosis/tubular atrophy (IFTA), presents itself as renal allograft dysfunction (occurring at least 3 months post-transplant) in the absence of active acute rejection, drug toxicity, or other diseases. Due to its multiple possible causes and complex etiology, classification of IFTA is still an ongoing process (3, 4). The clinical diagnosis is usually suggested by gradual deterioration of allograft function, manifested by a slowly rising serum creatinine concentration, worsening hypertension, and increasing proteinuria. Proteinuria is known to be a progression marker and a predictor for renal failure (5, 6). It is thought that proteinuria contributes to the progression of renal failure by various mechanisms. One of these mechanisms is suggested to be leakage of albumin-bound lipids across the damaged glomerular filtration barrier, leading to lipoapoptosis after reabsorption by the downstream proximal tubule (7, 8). Alongside this, activation of filtered or locally produced complement may be harmful to renal tubular cells and contribute to the progression of renal failure by initiating interstitial fibrosis (9, 10). Complement activation leads to the formation of C5b-9 (11), which can be used as a clinical indicator of complement activation in native kidney diseases (12, 13).

Renal proximal tubular cells are known to activate complement via the alternative pathway (AP) (14). Gaarkeuken et al. showed that complement activation on tubular cells is mediated by properdin binding on the tubular brush border (15). Our group identified tubular heparan sulfate as the docking platform for properdin and the consequent AP activation on tubular cells (9). In proteinuric patients, urinary properdin excretion is associated with intrarenal complement activation and poor renal function (16, 17).

Although it has been established that there is a strong relationship between proteinuria, tubulo-interstitial injury and a poor prognosis in kidney disease, to our knowledge no studies have examined the role of urinary complement activation products in kidney transplantation outcomes.

We hypothesized that the AP regulator properdin and the terminal complement complex sC5b-9 play an important role in graft failure and could serve as early biomarkers for late graft failure. Hence, the aim of the present study is to investigate the role of properdin and sC5b9 in renal transplant recipients (RTR) in relation to the development of graft failure over time.

METHODS

Study Population

The study population consisted of a well-characterized and previously described cohort of 707 RTR (18). In short, this cohort comprised RTR (aged $\geq$ 18 years) who visited the outpatient clinic of the University Medical Center Groningen (UMCG), Groningen, The Netherlands, between November 2008 and June 2011, and who had a functional graft for at least 1 year after transplantation. All patients provided written informed consent. Urinary morning samples were collected at inclusion in the study and immediately placed on ice. The samples were centrifuged at 4°C at 4,000 RPM for 15 min to remove components and debris, and the supernatants were stored at −80°C. They were not subjected to freeze/thaw cycles before analysis. There were 639 patients eligible for analysis after we excluded 67 patients with missing urinary samples which precluded the measurement of urinary properdin and sC5b-9 levels. Death-censored graft failure was defined as return to dialysis or re-transplantation. Kidney function was assessed by estimating glomerular filtration rate (eGFR) by applying the Chronic Kidney Disease Epidemiology Collaboration equation (19). Protein excretion of $\geq$0.5 g per day was defined as proteinuria. The study was approved by the UMCG institutional review board (METc 2008/186), adheres to the Declarations of Helsinki and Istanbul and has NCT02811835 as ClinicalTrials.gov identifier.

Quantification of Urinary Properdin

Urinary properdin levels were assessed by a previously described sandwich enzyme-linked-immunosorbent assay (ELISA) (9, 17), with a detection limit of 1.2 ng/mL, a plasma intra-variation of <17% and an inter-variation of <20%. In brief, 96-well ELISA plates (NUNC MaxiSorp^{TM}, Sigma-Aldrich, Saint Louis, MO, USA) were coated overnight at 4°C with monoclonal anti-human properdin (Hycult HM2282, Uden, the Netherlands). Urinary samples were diluted 5 times in DPBS with 0.1% Tween and bovine serum albumin (PTB) and incubated for 1 h at 37°C, followed by secondary antibody; polyclonal rabbit anti-human properdin-biotin (kindly provided by M. R. Daha, Leiden, The Netherlands) and detection with Streptavidin-HRP (Dako P0397, Glostrup, Denmark). Enzyme activity was detected using 2,2′azino-bis (3-ethylbenzo-thiazoline-6-sulphonic acid) (A1888, Sigma-Aldrich, Saint Louis, MO, USA). The optical density was measured at 415 nm using a microplate ELISA reader (Benchmark Plus, Bio-Rad, Veenendaal, The Netherlands). A standard curve was prepared using a serial dilution of zymosan activated serum in PTB with a known concentration of properdin. A reference sample, diluted in PTB with a known concentration of properdin was included as positive control. Potential background signal was assessed and corrected for, with PTB functioning as blank.

Quantification of Urinary Soluble C5b-9

Urinary sC5b-9 levels were assessed by a previously standardized and validated sandwich ELISA (17, 20), with a detection limit of 2.1 ng/mL, a plasma intra-variation of <13% and an inter-variation of <19%. In brief, 96-well ELISA plates (NUNC MaxiSorp^{TM}, Sigma-Aldrich) were coated overnight at 4°C with monoclonal mouse anti-human C5b-9 (Dako M0777). Urinary samples were diluted 1.25 times and incubated for 1 h at 37°C. Secondary antibody polyclonal goat anti-human C5 (Quidel Ca92121, San Diego, CA, USA), followed

by tertiary antibody polyclonal mouse anti-goat IgG HRP (Jackson 205-035-108) were added. Enzyme activity was detected using 3,3′,5,5′-tetramethylbenzidine. The optical density was measured at 450 nm using a microplate ELISA reader. The standard curve, reference sample, and the assessment of a potential background signal was prepared in the same way as the properdin ELISA, with a known concentration of sC5b-9.

Statistical Analyses

Data were analyzed using IBM SPSS software, version 23.0 (SPSS Inc., Chicago, IL, USA) and R version 3.2.3 (Vienna, Austria). Data are expressed as mean ± SD for normally distributed variables and as median [25th−75th interquartile range (IQR)] for variables with a skewed distribution. Categorical data are expressed as number (percentage). Under normal conditions complement factors are not present in the urine. Therefore, we defined urinary properdin and sC5b9 as a negative test when undetectable in the urine and as a positive test when detectable.

We evaluated between-group differences at baseline, comparing RTRs with vs. without detectable properdin and sC5b-9 using Student *t*-test, Mann-Whitney *U*-test, or Chi square test, as appropriate. To visualize the association between urinary properdin and urinary sC5b-9 excretion, we generated a restricted cubic spline plot based on linear regression analyses. Knots were placed on the 10th, 50th, and 90th percentile of ln properdin. To visualize the association between urinary properdin and urinary sC5b-9 excretion with proteinuria, we generated restricted cubic spline plots based on linear regression analyses, with knots placed on the 10th, 50th, and 90th percentile of ln proteinuria. Further, Kaplan Meier curves were used to depict the effect of the presence of urinary properdin and/or sC5b-9 on graft failure and all-cause mortality. Differences in survival rates were tested using the Cox-Mantel log-rank test. To study the prospective association with death-censored graft failure and all-cause mortality, we used Cox proportional hazards regression analysis. Prior to analyses, we first adjusted for statistically significant different parameters at baseline and for other known predictors of graft failure like HLA mismatches. First, death-censored graft failure was adjusted for age, sex, primary renal disease, time since transplantation at inclusion, eGFR, HLA mismatches, and donor type (model 1). Additionally, adjustment was made for high sensitive-CRP (hs-CRP) (model 2); further adjustment for systolic blood pressure, and smoking (model 3); and final adjustment for proteinuria (model 4). Due to skewed distribution, hs-CRP, properdin, and sC5b-9 were natural log-transformed. To determine the optimal cut off value of urinary properdin and sC5b-9 for prediction of graft failure in RTR, the Youden index was used. Finally, we performed mediation analyses to assess whether sC5b-9 was a mediator in the association between properdin and graft failure. For this purpose, we used the method as stated by Preacher and Hayes, which is based on logistic regression (21, 22). These analyses allow for testing significance and magnitude of mediation. For all analyses, a two-sided $P < 0.05$ was considered significant.

RESULTS

Baseline Characteristics

We included 639 RTR (age 53 ± 13 years; 58% males at 5.3 (1.8–12.2) years after transplantation). Mean eGFR was 52.2 ± 20.1 ml/min/1.73 m^2, and urinary properdin excretion was detectable in 161 (27%) RTR with a median [interquartile range] properdin level of 27.6 (8.7–68.1) ng/mL. Urinary sC5b-9 excretion was detectable in 102 (17%) RTR with median sC5b-9 levels of 5.1 (2.8–12.8) ng/mL.

RTR with detectable urinary properdin were more frequently females ($P < 0.001$), had significantly higher: body surface area (m^2) ($P = 0.004$), creatinine ($P = 0.003$), hs-CRP ($P < 0.001$), frequency of proteinuria (≥0.5 g/24 h) ($P < 0.001$), and received a deceased—donor kidney transplant ($P = 0.02$). RTR with detectable urinary sC5b-9 were more frequently males ($P = 0.01$), had higher levels of creatinine ($P < 0.001$), a higher frequency of proteinuria ($P < 0.001$), and a deceased-donor kidney transplant ($P = 0.02$). An inverse association between eGFR and detectable properdin ($P < 0.001$) and sC5b-9 levels ($P < 0.001$) was detected at baseline. No significant differences were found at baseline in HLA mismatches, primary renal disease, history of delayed graft function, and rejection between patients with and without detectable urinary properdin or sC5b-9.

Detectable urinary properdin excretion was present in 11 and 16% of RTR with and without proteinuria, respectively. Detectable urinary sC5b-9 excretion was present in 9 and 8% of RTR with and without proteinuria, respectively (**Figure 1**). Urinary properdin was significantly associated with urinary sC5b-9 excretion in RTR in whom both complement products were detectable (β = 0.25; $P < 0.001$) (**Figure 2**). Urinary

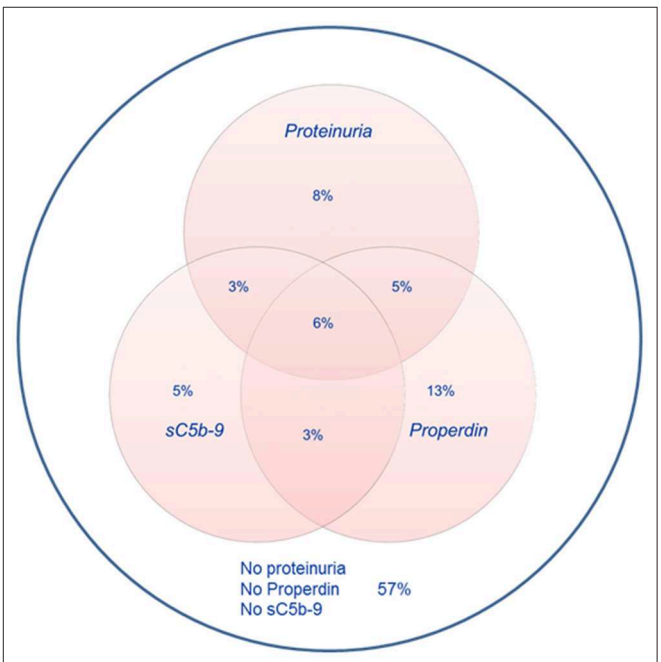

FIGURE 1 | Prevalences of urinary properdin, urinary sC5b-9, and proteinuria.

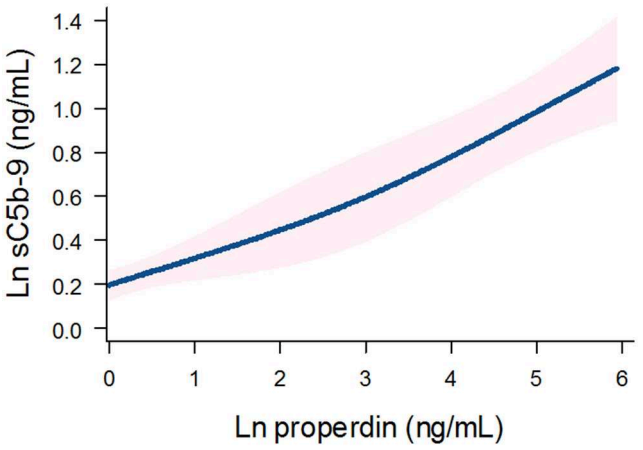

FIGURE 2 | Association between urinary properdin and urinary sC5b-9 excretion in the RTR. A restricted cubic spline is generated based on linear regression analyses. Knots are placed on 10th, 50th, and 90th percentile of ln properdin. Blue line represents the coefficient, and pink band represents the 95% confidence interval.

properdin and urinary sC5b-9 excretion were both significantly associated with proteinuria ($\beta = 0.26$; $P < 0.001$ and $\beta = 0.36$; $P < 0.001$, respectively) (**Supplementary Figures 1, 2**). Further demographics and clinical characteristics dichotomized into detectable or undetectable urinary properdin and sC5b-9 are specified in **Table 1**.

Urinary Properdin and Graft Failure

During a median follow-up of 5.3 (4.5–6.0) years, 75 (12%) RTRs developed death-censored graft failure. As depicted in the Kaplan Meier curves shown in **Figure 3**, RTR with both detectable urinary properdin and sC5b-9 had the highest risk of developing graft failure ($P < 0.001$). RTR with urine in which either properdin or sC5b-9 was detectable, showed an intermediate risk with worse graft survival compared to RTR without detectable urinary properdin or sC5b-9 (**Figure 3**).

In unadjusted Cox regression analysis, detectable urinary properdin was significantly associated with development of death-censored graft failure (HR, 3.08; 95% CI 1.95–4.85; $P < 0.001$), in patients with neither urinary properdin or sC5b-9 as the reference group. In multivariable analyses, detectable urinary properdin remained associated with development of graft failure (HR, 2.30; 95% CI 1.37–3.82; $P < 0.001$, **Table 2**), independent of adjustment for age, sex, primary renal disease, time since transplantation, eGFR, HLA mismatches, donor type, hs-CRP, systolic blood pressure, and smoking. However, the association between detectable properdin and graft failure became borderline significant after further adjustment for proteinuria (HR, 1.47; 95% CI 0.85–2.54; $P = 0.05$).

When we assessed the association between properdin as a continuous variable and graft failure, findings were similar. After adjustment for potential confounders, urinary properdin as a continuous variable was significantly associated with graft failure (HR, 1.25; 95% CI 1.10–1.42; $P < 0.001$) (**Table 3**). After adjustment for proteinuria, the association of properdin as a continuous variable with graft failure remained significant (HR, 1.12; 95% CI 1.02–1.38; $P = 0.008$). The optimal cut-off (Youden index) of urinary properdin for prediction of graft failure was 2.35 ng/mL. At this cut-off value, there was a sensitivity of 59% and a specificity of 79% for prediction of graft failure.

Urinary sC5b-9 and Graft Failure

In unadjusted analysis, detectable urinary sC5b-9 was significantly associated with development of death-censored graft failure (HR, 4.17; 95% CI 2.63–6.63; $P < 0.001$). In multivariable analyses, detectable sC5b-9 remained associated with the development of graft failure (HR, 3.09; 95% CI 1.87–5.11; $P < 0.001$), independent of age, sex, primary renal disease, time since transplantation, eGFR, HLA mismatches, donor type, hs-CRP, systolic blood pressure, and smoking **(Table 2)**. The association between detectable sC5b-9 and graft failure also remained after further adjustment for proteinuria (HR, 2.16; 95% CI 1.30–3.61; $P = 0.003$).

When we assessed the association between sC5b-9 as a continuous variable and graft failure, findings were similar. sC5b-9 as a continuous variable was associated with risk of developing graft failure in the unadjusted analysis and in multivariable analyses, after adjustment for potential confounders, including proteinuria (HR, 1.34; 95% CI 1.10–1.63; $P = 0.004$) (**Table 3**). The optimal cut-off (Youden index) of urinary sC5b-9 for prediction of graft failure was 2.88 ng/mL, there was a sensitivity of 48% and a specificity of 91% for prediction of graft failure.

Mediation Analyses

Since properdin is involved in sC5b-9 complex formation via the alternative complement pathway (9), we aimed to assess whether the association between properdin and graft failure was mediated by sC5b-9. In mediation analyses, sC5b-9 was found to be a significant mediator of the association between properdin and graft failure, 31% of the association between properdin and graft failure was explained by sC5b-9, the *P* value for indirect effect is <0.05 (**Table 4**).

Properdin, sC5b-9, and Mortality

In an unadjusted Cox regression analysis, detectable urinary properdin was significantly associated with an increased risk of mortality (HR, 1.58; 95% CI 1.11–2.25; $P = 0.01$), whereas detectable urinary sC5b-9 was not significantly associated with increased risk of mortality(HR, 1.39; 95% CI 0.92–2.11; $P = 0.12$). After adjustment for potential confounders, the association between properdin and mortality was abrogated, and the association between sC5b-9 and mortality remained non-significant (**Supplementary Table 1**).

In unadjusted Cox regression analyses, both urinary properdin and sC5b-9 as continuous variables were significantly associated with an increased risk of mortality (HR, 1.16; 95% CI 1.05–1.27; $P = 0.003$ and HR, 1.26; 95% CI 1.08–1.48; $P = 0.004$, respectively). However, after adjustment for potential confounders, the associations between properdin and mortality, and between sC5b-9 and mortality were lost (**Supplementary Table 2**).

TABLE 1 | Baseline characteristics according to detectable urinary properdin urinary sC5b-9 levels.

Variables	Urinary properdin		*P*-value	Urinary sC5b-9		*P*-value
	Not detectable (*n* = 478)	Detectable (*n* = 161)		Not detectable (*n* = 537)	Detectable (*n* = 102)	
RECIPIENT						
Age (years)	53 ± 13	53 ± 13	0.96	53 ± 13	53 ± 13	0.88
Male sex (*n*, %)	305 (64)	66 (41)	<0.001	298 (56)	70 (69)	0.01
Body mass index, kg/m^2	26.5 ± 4.2	26.7 ± 5.0	0.65	26.6 ± 4.7	26.3 ± 4.9	0.44
Body surface area (m^2)	1.96 ± 0.21	1.90 ± 0.22	0.004	1.95 ± 0.21	1.94 ± 0.22	0.95
Alcohol use (*n*, %)	387 (82)	124 (77)	0.28	441 (82)	74 (73)	0.69
Current smoking (*n*, %)	55 (12)	20 (12)	0.72	58 (11)	17 (17)	0.06
Primary renal disease			0.34			0.95
Primary glomerular disease (*n*, %)	143 (30)	36 (22)		156 (29)	25 (25)	
Glomerulonephritis (*n*, %)	43 (9)	11 (7)		43 (8)	11 (11)	
Tubulo-interstitial disease (*n*, %)	48 (10)	25 (16)		63 (12)	11 (11)	
Polycystic renal disease (*n*, %)	95 (20)	36 (22)		109 (20)	22 (22)	
Dysplasia and hypoplasia (*n*, %)	19 (4)	6 (4)		22 (4)	4 (4)	
Renovascular disease (*n*, %)	29 (6)	8 (5)		30 (6)	7 (7)	
Diabetic nephropathy (*n*, %)	23 (5)	8 (5)		27 (5)	4 (4)	
Other or unknown cause (*n*, %)	78 (16)	31 (19)		87 (16)	18 (18)	
History of CV-disease (*n*, %)	58 (12)	23 (14)	0.31	71 (13)	10 (10)	0.65
Time since transplantation (years)*	5.3 (1.7–12.0)	6.1 (2.1–12.6)	0.39	5.1 (1.9–11.6)	7.1 (1.7–15.0)	0.07
Delayed graft function (*n*, %)	31 (7)	15 (9)	0.27	36 (7)	10 (10)	0.24
Rejection (*n*, %)	130 (27)	45 (28)	0.82	143 (27)	32 (31)	0.33
Diabetes mellitus (*n*, %)	109 (23)	38 (24)	0.75	124 (23)	23 (23)	0.84
Systolic blood pressure (mmHg)	136 ± 17	135 ± 18	0.84	135 ± 17	139 ± 19	0.05
Diastolic blood pressure (mmHg)	82 ± 11	82 ± 11	0.64	82 ± 11	85 ± 11	0.02
LABORATORY MEASUREMENTS						
sC5b-9 (ng/mL)	0 (0–0)	0 (0–3.8)	<0.001	0 (0–0)	5.1 (2.8–12.8)	
Properdin (ng/mL)	0 (0–0)	27.6 (8.7–68.1)		0 (0–0)	0 (0–32.4)	<0.001
Hemoglobin (mmol/L)	8.3 ± 1.1	7.9 ± 1.0	<0.001	8.2 ± 1.1	8.1 ± 1.2	0.31
Total cholesterol (mmol/L)	5.1 ± 1.1	5.2 ± 1.1	0.60	5.1 ± 1.1	5.2 ± 1.1	0.49
eGFR (ml/min/1.73 m^2)	54 ± 20	47 ± 21	<0.001	54 ± 20	44 ± 21	<0.001
Creatinine (μmol/L)	133 ± 46	154 ± 83	0.003	132 ± 48	172 ± 91	<0.001
Proteinuria (>0.5 g/24 h) (*n*, %)	74 (15)	65 (40)	<0.001	83 (16)	56 (55)	<0.001
hs-CRP (mg/L)	1.5 (0.6–3.7)	2.5 (1.0–7.6)	<0.001	1.6 (0.7–4.3)	2.1 (0.8–6.1)	0.09
TREATMENT						
ACE-inhibitors (*n*, %)	157 (33)	58 (36)	0.47	176 (33)	39 (38)	0.29
Bèta-blocker (*n*, %)	300 (63)	113 (70)	0.08	346 (64)	67 (66)	0.81
Calcium channel blockers (*n*, %)	117 (25)	39 (24)	0.95	128 (24)	28 (28)	0.44
Diuretic use (*n*, %)	189 (40)	72 (45)	0.26	209 (39)	52 (51)	0.02
Calcineurin inhibitor (*n*, %)	281 (59)	92 (57)	0.57	315 (59)	60 (59)	0.44
Sirolimus (*n*, %)	10 (2)	2 (1)	0.33	11 (2)	1 (1)	0.50
Prednisolon, mg/24 h (*n*, %)	468 (99)	161 (100)	0.47	532 (99)	101 (99)	0.53
MMF (*n*, %)	294 (62)	87 (54)	0.10	328 (61)	55 (54)	0.70
Azathioprine (*n*, %)	77 (16)	41 (26)	0.68	93 (17)	27 (27)	0.71
DONOR						
Donor age (years)	46 ± 18	43 ± 15	0.07	43 ± 15	42 ± 16	0.29
Male sex donor (*n*, %)	232 (49%)	90 (56%)	0.11	280 (53%)	45 (46%)	0.19
Deceased type donor (*n*, %)	298 (62%)	117 (73%)	0.02	341 (63%)	77 (75%)	0.02
HLA MISMATCHES (*n*, %)						
Class I						0.46
0 (*n*, %)	102 (22)	27 (17%)		103 (19%)	26 (25%)	

(Continued)

TABLE 1 | Continued

Variables	Urinary properdin		*P*-value	Urinary sC5b-9		*P*-value
	Not detectable ($n = 478$)	Detectable ($n = 161$)		Not detectable ($n = 537$)	Detectable ($n = 102$)	
1 (*n*, %)	113 (24%)	31 (19%)		125 (23%)	21 (20%)	
2 (*n*, %)	169 (36%)	53 (33%)		191 (36%)	33 (32%)	
3 (*n*, %)	43 (9%)	20 (12%)		51 (10%)	12 (12%)	
4 (*n*, %)	21 (4%)	9 (6%)		27 (5%)	3 (3%)	
Class II						0.82
0 (*n*, %)	199 (42%)	60 (37%)		215 (40%)	46 (45%)	
1 (*n*, %)	198 (42%)	66 (41%)		226 (42%)	40 (39%)	
2 (*n*, %)	47 (10%)	14 (9%)		52 (10)	9 (9%)	

Normally distributed data are presented as means ± standard deviation, skewed data as medians (interquartile range), and categorical data as number (percentage). P-values have been calculated by means of independent samples T-test, Mann-Whitney U-test, or Chi-square test. eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; ACE, angiotensin-converting enzyme; MMF, mycofenolaat mofetil; HLA, human leukocyte antigens. * *time since transplantation at inclusion.*

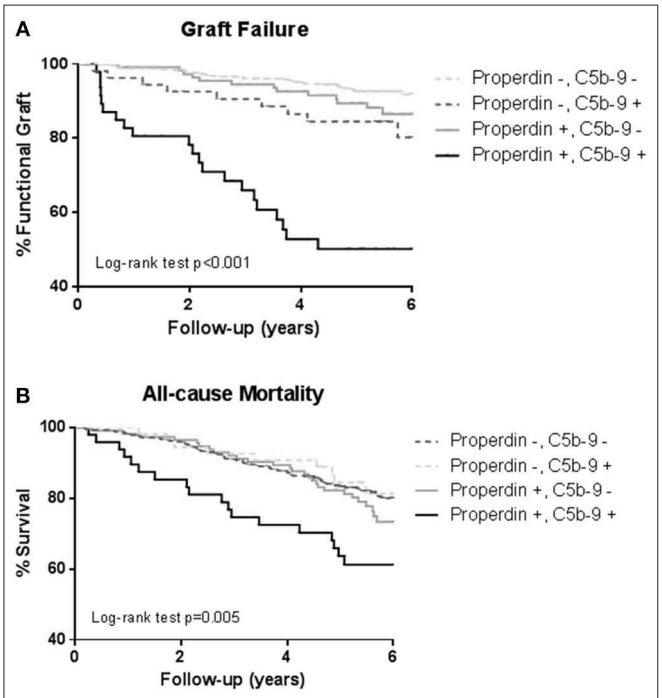

FIGURE 3 | Kaplan-Meier analyses for percentage graft failure **(A)** and survival **(B)** according to no sC5b-9/no properdin, sC5b-9/no properdin, no sC5b-9/properdin, sC5b-9/properdin. Log-rank tests showed that the prevalence of graft failure and survival were significantly higher in the patients with urinary properdin and sC5b-9. Associations between survival and urinary properdin and sC5b-9 did not remain significant after adjustment for potential confounders.

DISCUSSION

In this study, we show that the alternative pathway complement factor properdin and the terminal sC5b-9 complex are detectable in the urine of patients after kidney transplantation and are independently associated with chronic allograft failure. To our knowledge, this is the first report indicating urinary alternative complement pathway involvement in chronic renal allograft failure, independent of potential cofounders including eGFR and proteinuria.

It has long been recognized that patients with high-grade proteinuria are more likely to develop chronic renal failure than patients without proteinuria (23, 24). Urinary proteins elicit pro-inflammatory and pro-fibrotic effects that directly contribute to chronic tubulo-interstitial damage. Additionally, among multiple other pathways complement activation may be an important component leading to fibrogenesis in the kidney. In physiological conditions complement components are not filtered through the glomerular barrier, however complement components are present in the urine of patients with non-selective proteinuria (25, 26). Properdin positively regulates the AP of the complement system and is also a pattern recognition molecule for C3b that subsequently stabilizes the C3bBb complex and thus contributes to C5b-9 formation (27–30). Tubular epithelial cells are especially susceptible to the effects of C5b-9 formation because they lack the membrane-bound complement regulators on the apical cell surface (31). Recently, urinary complement measurements and their clinical value are of increasing interest in transplant medicine. Schröppel et al. showed not long ago the importance of anaphylatoxins C3a and C5a in donor urine and their association with delayed graft function (32), and van Essen et al. recently reviewed the detection of complement biomarkers in urine to monitor local injury in renal diseases, including properdin (33).

In this study, we have shown a potential role of urinary properdin and sC5b-9 in the pathogenesis of chronic allograft failure. Our data show that graft survival is reduced in patients in whom properdin is present in the urine together with sC5b-9. Remarkably, in patients without overt proteinuria, we identified that properdin, sC5b-9 or both properdin and sC5b-9, were also associated with a worse graft survival. More importantly, not only the presence of properdin and sC5b-9 was significantly associated with graft failure, but also properdin and sC5b-9 were robustly associated with graft survival when analyzed as continuous parameters, pointing toward a dose-dependent effect. There are several possible explanations for this association. Properdin is the only known complement protein that is not produced

TABLE 2 | Association of detectable urinary properdin and detectable urinary sC5b-9 with graft failure in renal transplant recipients.

Model	Detectable properdin		Detectable sC5b-9		Both properdin and sC5b-9	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Univariate	3.08 (1.95–4.85)	<0.001	4.17 (2.63–6.63)	<0.001	7.13 (4.30–11.83)	<0.001
Model 1	2.35 (1.44–3.82)	0.001	3.03 (1.86–4.96)	<0.001	8.04 (4.74–13.63)	<0.001
Model 2	2.27 (1.38–3.73)	0.001	2.99 (1.83–4.89)	<0.001	7.63 (4.46–13.10)	<0.001
Model 3	2.30 (1.37–3.82)	<0.001	3.09 (1.87–5.11)	<0.001	6.75 (3.79–12.02)	<0.001
Model 4	1.47 (0.85–2.54)	0.05	2.16 (1.30–3.61)	0.003	3.12 (1.69–5.77)	<0.001

Model 1, *adjustment for age, sex, primary renal disease, time since transplantation at inclusion, eGFR, HLA mismatches, and donor type;* ***model 2****, model 1 + adjustment for hs-CRP;* ***model 3,*** *model 2 + adjustment for systolic blood pressure, and smoking;* ***model 4,*** *model 3 + adjustment for proteinuria.*
Reference group defined as patients with neither urinary properdin or C5b-9, with a hazard ratio of 1.0.

TABLE 3 | Association of continuous natural log transformed urinary properdin and urinary sC5b-9 with graft failure in renal transplant recipients.

Model	Ln properdin		Ln sC5b-9	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Univariate	1.36 (1.21–1.52)	<0.001	1.76 (1.51–2.06)	<0.001
Model 1	1.26 (1.11–1.43)	<0.001	1.61 (1.35–1.91)	<0.001
Model 2	1.25 (1.10–1.42)	0.001	1.61 (1.36–1.92)	<0.001
Model 3	1.25 (1.10–1.42)	0.001	1.63 (1.36–1.96)	<0.001
Model 4	1.12 (1.02–1.28)	0.008	1.34 (1.10–1.63)	0.004

Model 1, *adjustment for age, sex, primary renal disease, time since transplantation at inclusion, eGFR, HLA mismatches, and donor type;* ***model 2****, model 1 + adjustment for hs-CRP;* ***model 3,*** *model 2 + adjustment for systolic blood pressure, and smoking;* ***model 4,*** *model 3 + adjustment for proteinuria.*

TABLE 4 | Mediation analyses of the impact of sC5b-9 on the association between properdin and graft failure.

Potential mediator	Outcome	Effect (path)*	Multivariable model**	
			Coefficient (95% CI)†	Proportion mediated***
C5b-9	**Graft failure**	Indirect effect (*ab* path)	0.08 (0.04;0.13)	31%
		Total effect (*ab* + *c*' path)	0.26 (0.13;0.37)	
		Unstandardized total effect‡	0.22 (0.07;0.38)	

Adjusted for age, sex, primary renal disease, time since transplantation, and hs-CRP.
**The coefficients of the indirect ab path and the total ab + c' path are standardized for the standard deviations of the potential mediators and outcomes.*
***All coefficients are adjusted for age, sex, eGFR, time since transplantation at inclusion, primary renal disease, donor type and proteinuria.*
****The size of the significant mediated effect is calculated as the standardized indirect effect divided by the standardized total effect multiplied by 100.*
†95% CIs for the indirect and total effects were bias-corrected confidence intervals after running 2,000 bootstrap samples.
‡Odds ratios for risk of outcomes can be calculated by taking the exponent of the unstandardized total effect.

in the liver, but synthesized by various other cell types like monocytes, primary T cells, granulocytes, and endothelial cells (34–38). Therefore, it is possible that locally produced properdin and/or filtered properdin with other filtered small complement components, causes intratubular C5b-9 activation leading to progressive renal disease without manifest proteinuria, defined as proteinuria >0.5 g/24 h (39, 40). In kidney transplant patients it is generally believed that small amounts of proteinuria, defined as < 0.5 g/24 h, are harmless (41, 42). Only persistent proteinuria, >0.5 g/24 h for at least 3–6 months is considered significant according to American Society of Transplantation guidelines, and low-grade proteinuria is often referred to as "subclinical" (43). However, low grade proteinuria may be less harmless than originally described. Halimi et al. showed a dose-dependent effect in transplant patients with low grade proteinuria (<0.5 g/24 h) in whom each 0.1 g/24 h difference in proteinuria increased the risk of graft loss by 25% (44). In line with our findings in transplanted patients, Siezenga et al. showed an association between urinary properdin and worse renal function in patients with diabetic nephropathy or glomerular disease. Furthermore, the association of urinary properdin with urinary sC5b-9 was independent of the degree of proteinuria (17).

The fact that adjustment for proteinuria > 0.5 g/24 h did not materially alter the prospective association in the prospective analysis of continuous properdin and sC5b-9 measurements, is supportive of our hypothesis that alternative pathway complement activation might be one of the driving forces of chronic graft failure. More importantly, after correction for other well-known predictors of graft failure, like HLA mismatches and donor type, the association remains. In mediation analysis, we showed that the association between properdin and graft failure was mediated to a considerable extent by urinary sC5b-9.

This may explain why RTR with properdin alone, or sC5b-9 alone in the urine have a better allograft survival compared to both properdin and sC5b9 in the urine. Therefore, it seems that AP complement activation plays an important role in the loss of allograft function of RTR. The possible mechanism of this effect at a tubular level is illustrated by the scheme presented in **Figure 4**. Interestingly, urinary properdin was more frequently detected in females and urinary sC5b-9 was more frequently detected in males. We can only speculate on the causes of these differences. Innate immune function may vary between males and females (45), however a limited number of

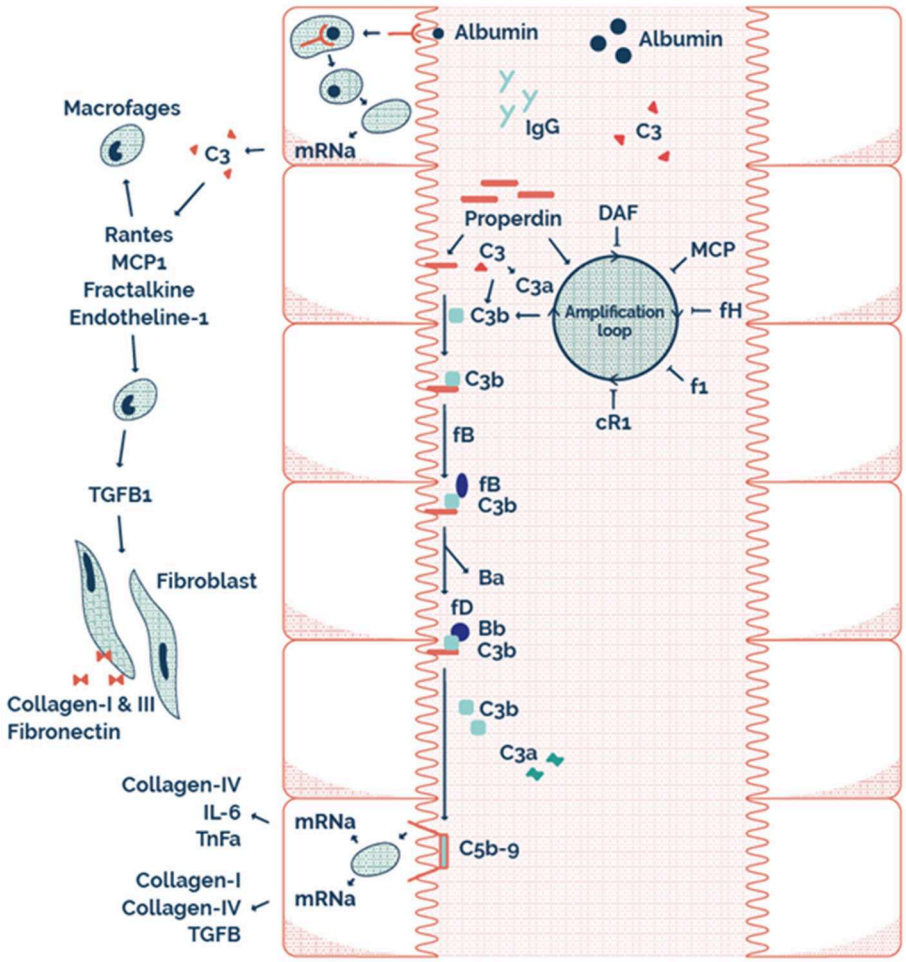

FIGURE 4 | The possible mechanism on a tubular level illustrating tubular alternative pathway complement activation via properdin as pattern recognition molecule.

studies have investigated the influence of sex on the complement system (46–49). Properdin is encoded on the short arm of the X chromosome, and together with hormonal differences between males and females this could be explanations of the sexual differences in properdin (50, 51). However, in a healthy Caucasian population, Gaya da Costa et al. recently found decreased serum properdin and serum C9 in healthy human females compared to males (49). In contrast, animal studies have shown that female mice have a similar serum complement cascade functionality at the level of C3 activation compared to male mice, but a strongly reduced level of serum C9, leading to an inability of female mice to promote inflammation through C5b-9 (46).

Multiple therapeutic modalities to inhibit complement pathway intervention are currently being developed. Our study points toward the potential for complement inhibition at the tubular level in proteinuric patients, which may improve long term outcome in patients with chronic allograft nephropathy.

The main strength of our study is that it comprises a large prospective cohort of stable RTR, in which several renal parameters as well as both urinary properdin and sC5b-9 were measured. In addition, end-point evaluation was complete in all participants despite the long follow-up period. We acknowledge several limitations of the study. First, no gold standard exists for the definition of urinary properdin and sC5b-9. In our study, we defined the detectability of properdin and sC5b-9 as urinary properdin and urinary sC5b-9. Second, complement activation may only be partially reflected by urinary properdin and sC5b-9 excretion, since the excretion may be altered by tubular complement binding and fixation. Third, possible residual confounding in this study cannot be excluded due to the observational status of this single center study. Furthermore, we do not have data on the presence of donor specific antibodies or protocol biopsies in this cohort. Thus, we cannot differentiate between general effects of glomerular filtration of complement products and a specific contribution of alloantibody mediated complement activation. Unfortunately our prospective cohort contained too few events of graft failure to perform analysis for the underlying cause of graft loss and their relation to urinary complement.

We identified that the presence of urinary properdin and sC5b-9 is independently associated with increased risk of late graft failure in RTR, compared to RTR without urinary properdin and sC5b-9. This suggests that urinary properdin and sC5b-9 can serve as useful biomarkers of immunological injury and kidney allograft deterioration. Importantly, urinary properdin and sC5b-9 was associated with graft failure independently of eGFR and significant proteinuria. We suggest that an important part of proteinuria mediated toxicity, is caused by the presence of complement in the primary urine and subsequent activation at the tubular surface. Further studies are needed to unravel the exact interplay between urinary properdin, sC5b-9 and the development of fibrosis, and moreover the potential for therapeutic interventions.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The study was approved by the University Medical Center Groningen institutional review board (METc 2008/186), adheres to the Declarations of Helsinki and Istanbul and has NCT02811835 as ClinicalTrials.gov identifier. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

RL was involved in study design, carrying out assays, interpreting data, statistical analysis, creating tables and figures, and writing of the manuscript. ME was involved in interpreting data, statistical analysis, figure and table design, and manuscript editing. MA was involved in carrying out assays and interpreting data. MD, MS, RP, JB, and J-SS were all involved in interpreting data and manuscript editing. SB and SPB were involved in study design, interpreting data, statistical analysis, and manuscript editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02511/full#supplementary-material

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Blocking Complement Factor B Activation Reduces Renal Injury and Inflammation in a Rat Brain Death Model

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Introduction: The majority of kidneys used for transplantation are retrieved from brain-dead organ donors. In brain death, the irreversible loss of brain functions results in hemodynamic instability, hormonal changes and immunological activation. Recently, brain death has been shown to cause activation of the complement system, which is adversely associated with renal allograft outcome in recipients. Modulation of the complement system in the brain-dead donor might be a promising strategy to improve organ quality before transplantation. This study investigated the effect of an inhibitory antibody against complement factor B on brain death-induced renal inflammation and injury.

Method: Brain death was induced in male Fischer rats by inflating a balloon catheter in the epidural space. Anti-factor B (anti-FB) or saline was administered intravenously 20 min before the induction of brain death ($n = 8$/group). Sham-operated rats served as controls ($n = 4$). After 4 h of brain death, renal function, renal injury, and inflammation were assessed.

Results: Pretreatment with anti-FB resulted in significantly less systemic and local complement activation than in saline-treated rats after brain death. Moreover, anti-FB treatment preserved renal function, reflected by significantly reduced serum creatinine levels compared to saline-treated rats after 4 h of brain death. Furthermore, anti-FB significantly attenuated histological injury, as seen by reduced tubular injury scores, lower renal gene expression levels (>75%) and renal deposition of kidney injury marker-1. In addition, anti-FB treatment significantly prevented renal macrophage influx and reduced systemic IL-6 levels compared to saline-treated rats after brain death. Lastly, renal gene expression of IL-6, MCP-1, and VCAM-1 were significantly reduced in rats treated with anti-FB.

Conclusion: This study shows that donor pretreatment with anti-FB preserved renal function, reduced renal damage and inflammation prior to transplantation. Therefore, inhibition of factor B in organ donors might be a promising strategy to reduce brain death-induced renal injury and inflammation.

Keywords: factor B, complement, renal transplantation, brain death, donation

INTRODUCTION

Although the field of renal transplantation has made huge progress over the last 50 years, one of the main challenges remains the disparity between demand and supply of renal allografts (1). Therefore, increasing efforts are made to expand, but also to optimize the current donor pool. Kidneys are retrieved from living donors, deceased after circulatory death (DCD) donors, and deceased after brain death (DBD) donors. Despite the increasing number of living donors, the majority of kidneys are still retrieved from DBD donors (1). However, brain death induces physiological disturbances characterized by hemodynamic changes, metabolic disturbances, and immunological derangements. Therefore, kidneys retrieved from DBD donors give inferior results, reflected by a higher rate of delayed graft function than their living counterparts (2).

An important denominator in brain death-induced renal inflammation is activation of the complement system (3, 4). The complement system can be activated by three different pathways: the classical pathway (CP), the lectin pathway (LP), and the alternative pathway (AP). Activation of each of these three pathways results in the cleavage of complement component C3 into C3a and C3b. Subsequently, activation of C3 leads to the formation of the C5 convertases, which cleave C5 into C5a and C5b. C5b is the initial protein for the formation of C5b-9, also known as the membrane attack complex (MAC). The MAC induces the formation of pores in the cell membrane, which results in cell lysis. Besides, C5b-9 induces tissue injury via intracellular pro-inflammatory signaling pathways (5). In addition, the anaphylatoxins C3a and C5a are produced, which provoke influx and activation of inflammatory cells (6, 7).

Early studies already have demonstrated the presence of complement C3 in kidneys from DBD rats (8). C3 deposition was seen on endothelial cells and in the glomeruli of DBD-derived kidneys, while no C3 deposition was observed in renal biopsies from living donors. In line with these results, C3d deposition was detected in renal biopsies from human DBD donors before reperfusion (9, 10), which suggest that C3d was deposited as a direct result of brain death itself. Complement C3 activation results in the production of the downstream activation products C5a and C5b-9, which are both systemically and locally upregulated in the DBD donor (11, 12). These studies demonstrate that the complement system is activated in DBD donors. Therefore, inhibition of the complement system might be an attractive strategy to attenuate brain death-induced renal injury (13).

A potential target for intervention in the DBD donor might be complement factor B. Factor B is one of the key components required for activation of the complement AP (9, 14). Bb, an active fragment of factor B, is significantly elevated in plasma from DBD donors compared to living donors (11). These plasma levels of Bb are positively correlated with systemic C5b-9 levels in DBD donors. High systemic C5b-9 levels in DBD donors are associated with a higher incidence of acute rejection in the recipient (11). In addition, local renal expression of factor B was significantly increased in kidneys from DBD donors compared to kidneys from living donors at baseline (15). These studies demonstrate that factor B is both systemically and locally upregulated in kidneys from DBD donors. Therefore, inhibition of factor B could be critical to protect against brain death-induced renal injury. To our knowledge, no studies have investigated whether inhibition of factor B is therapeutically effective in DBD donors.

Our study aimed to investigate whether inhibition of factor B can attenuate brain death-induced renal injury and inflammation. To do so, we pretreated rats with a monoclonal antibody against factor B (anti-FB) and subsequently subjected rats to 4 h of brain death. We found that pretreatment with anti-FB significantly improved renal function, reduced renal damage, and inflammation in brain-dead rats prior to transplantation.

MATERIALS AND METHODS

Experimental Outline

In this study, 22 rats (two rats were excluded because of technical failures and replaced) were randomly divided into the following groups (**Figure 1**):

Brain death with saline (control group) ($n = 8$)
Brain death with anti-factor B (anti-FB) ($n = 8$)
Sham-operation with saline ($n = 4$).

Rats

Adult male Fischer F344/NHsd rats (Envigo, Dublin, VA, USA) between 250 and 300 grams were used. Rats received food and water *ad libitum*. All experiments were performed at the local animal facility of the University Medical Center Groningen according to the Experiments on Animal Act (1996) issued by the Ministry of Public Health, Welfare and Sports of the Netherlands.

Abbreviations: AEC, 3-amino-9-ethylcarbazole; Anti-FB, anti-factor B; AP, alternative pathway; ATN, acute tubular necrosis; CP, classical pathway; DAB, 3,3-diaminobenzidine; DBD, donation after brain death; DCD, donation after circulatory death; HAES, hydroxyethyl starch solution; KIM-1, kidney injury molecule-1; LP, lectin pathway; MAC, membrane attack complex; MAP, mean arterial pressure; PBS, phosphate buffered saline; PEG, polyethylene glycol; TMB, 3,3′,5,5′-tetramethylbenzidine.

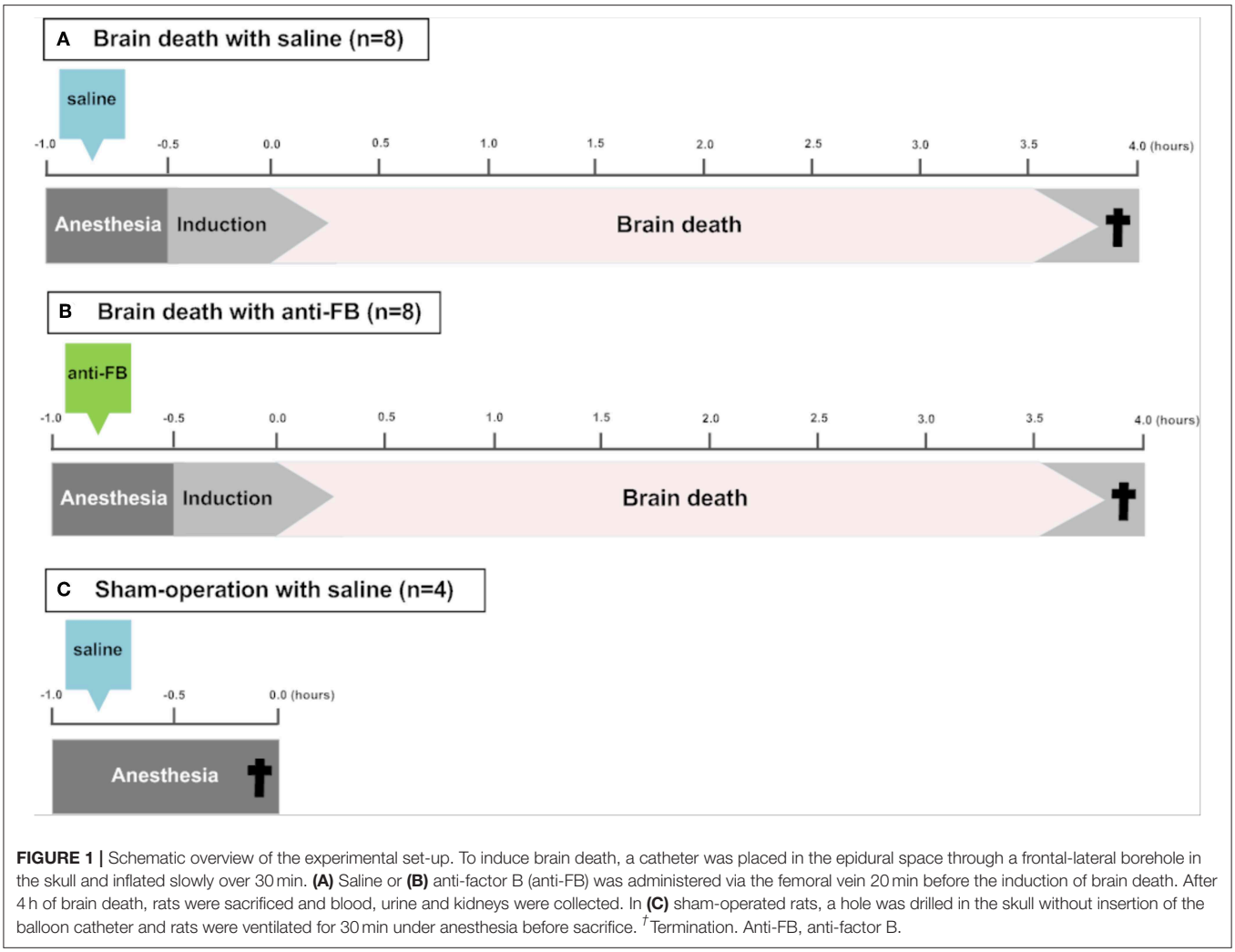

FIGURE 1 | Schematic overview of the experimental set-up. To induce brain death, a catheter was placed in the epidural space through a frontal-lateral borehole in the skull and inflated slowly over 30 min. **(A)** Saline or **(B)** anti-factor B (anti-FB) was administered via the femoral vein 20 min before the induction of brain death. After 4 h of brain death, rats were sacrificed and blood, urine and kidneys were collected. In **(C)** sham-operated rats, a hole was drilled in the skull without insertion of the balloon catheter and rats were ventilated for 30 min under anesthesia before sacrifice. † Termination. Anti-FB, anti-factor B.

TABLE 1 | Overview of the fluids administered, when mean arterial pressure dropped below 80 mmHg, in rats during the 4 h brain death period.

Group	Brain death + saline		Brain death + anti-factor B		*p*-value
	Median	IQR	Median	IQR	
HAES (ml)	1	0–1.5	0.8	0–1.5	0.479
Noradrenaline (ml)	0.295	0–1.9	0.25	0–2.88	0.989
MAP (mmHg)	100	90–110	103	79–115	0.787

IQR, interquartile range; HAES, hydroxyethyl starch solution; MAP, mean arterial pressure.

Brain Death Procedure

To test whether inhibition of the complement AP protects against brain death-induced renal injury, we treated brain-dead rats with a mouse anti-human monoclonal antibody against factor B (FB28.4.2; IgG2b). FB28.4.2 (anti-FB) is produced in the laboratory of prof. Dr. Santiago Rodriquez de Cordoba at the Centro de Investigaciones Biológicas, Madrid, Spain. Anti-FB is an inhibitory antibody, which targets an evolutionary-conserved epitope in the Ba fragment of factor B. Anti-FB efficiently inhibits the formation of the AP C3 proconvertase in humans, rats and other species by blocking the interaction between the Ba domain of factor B and C3b (16). Twenty minutes before the start of brain death induction, 8 mg of purified anti-FB in a total volume of 750 µl, was administered via the femoral vein. Saline, used in the control group and sham-operated group, was also administered via the femoral vein at the same time and in the same volume as anti-FB. Brain death was induced as described previously (17). Briefly, rats were anesthetized, intubated and ventilated. Temperature and mean arterial pressure (MAP) were continuously monitored and regulated. A 4F Fogarty balloon catheter (Edwards Lifesciences LLC, Irvine, USA) was placed in the epidural space through a frontal-lateral borehole in the skull and inflated slowly. Brain death induction was completed after 30 min, and the apnoea test confirmed brain death. Subsequently, anesthesia was stopped and the ventilator was switched to a mix of 50% O_2 and 50% medical air. A MAP above 80 mmHg was considered to be normotensive. When MAP dropped below 80 mmHg, 0.1 kg/L hydroxyethyl starch solution (HAES-steril 10%, Fresenius Kabi, Bad Homburg, Germany) and, if necessary, 0.01 mg/ml norepinephrine were administered. A maximum of

800 μl of fluid was administered during 4 h of brain death. Fluid administration was comparable in all groups (**Table 1**). Blood, urine and kidneys were harvested after 4 h of brain death. Kidneys were embedded in paraffin or snap-frozen in liquid nitrogen and together with the serum and urine stored at −80°C. Sham-operated rats underwent the same surgical procedure, but without the insertion of the balloon catheter, and only received saline. Sham-operated rats were ventilated for 30 min under anesthesia with a mixture of 2.5% isoflurane and 100% O_2. After 30 min, sham-operated rats were sacrificed.

Renal Function

To investigate renal function, serum creatinine levels were measured at the time of sacrifice, using a Roche Modular P system (Roche, Basel, Switzerland).

Renal Morphology

Paraffin sections (4 μm) were stained with Periodic Acid-Schiff stain. Histological injury, reflected by acute tubules necrosis (ATN) was determined semi-quantitatively by two observers using the following scoring system: 0 = no ATN; 1 = 0–10%; 2 = 10–25%; 3 = 25–50% and 4 = ≥50% ATN.

Immunohistochemistry

Immunohistochemical stainings for C3d, C5b-9, and neutrophils (HIS48) were performed on frozen sections (4 μm) fixed in acetone. Immunohistochemistry for kidney injury molecule-1 (KIM-1) and macrophages (ED-1) were performed on formalin-fixed, paraffin-embedded sections. Paraffin sections (4 μm) were deparaffinized and rehydrated. 0.1M Tris/HCl (pH 9) was used as an antigen retrieval buffer. All sections were blocked with hydrogen peroxidase for 30 min and incubated with the primary antibody for 1 h at room temperature (**Table 2**). After washing with phosphate buffered saline (PBS), the slides were incubated with the appropriate horseradish peroxidase-conjugated secondary and tertiary antibodies (Dako, Carpinteria, USA) in 1% BSA solution for 30 min. The reaction was developed by addition of 3-amino-9-ethylcarbazole (AEC; Dako) or 3,3-diaminobenzidine (DAB; Merck, Darmstadt, Germany) and 0.03% H_2O_2. Sections were counterstained with hematoxylin and embedded in Aquatex mounting agent (Merck).

For quantitative evaluation of HIS48 and ED-1 in the renal cortex, 40 snapshots of the renal cortex were taken from the representative sections using ImageJ Software (National Institutes of Health). Cells positive for each marker were then counted using Aperio ImageScope Analysis Software (objective 50x−200x) (Leica Biosystems, Vista, CA, USA). C3d, C5b-9, and KIM-1 sections were scored semi-quantitatively by two observers at a magnification of 20x. C3d and C5b-9 were scored by the following scoring system: 0 = no staining; 1 = 0–25%; 2 = 25–50%; 3 = 50–75% and 4 = 75–100% staining in the renal cortex. KIM-1 was scored by estimating the percentage of KIM-1 expression in the cortical tubules. KIM-1 was scored per field, as described by van Timmeren et al. (18). In brief, 0 = no staining; 1/2 = 0–12.5%; 1 = 12.5–25%; 2 = 25–50%; 3 = 50–75% and 4 = 75–100%.

IL-6 ELISA

Plasma protein levels of IL-6 were determined by a rat enzyme-linked immunosorbent assay (R&D Systems; DY506, Abingdon, Oxon, UK). All samples were analyzed in duplicate and measured at an optical density of 450 nm.

C3d ELISA

Rat C3d ELISA was performed, as described previously, to measure complement activation after 4 h of brain death (19). In brief, rat C3d was captured with a monoclonal mouse anti-C3 antibody (sc-28294, Santi Cruz, CA, USA). A rabbit anti-human C3d was used as detection antibody (Dako) and goat anti-rabbit-HRP (Dako) with 3,3′,5,5′-Tetramethylbenzidine (TMB) K-Blue as substrate. Sample incubation and detection steps were performed for 60 min at 37°C. Before C3d was measured, all samples were polyethylene glycol (PEG) precipitated. PEG precipitation is required since free C3d shares epitopes with intact C3. All plasma samples were 1:1 diluted with 22% PEG in 0.1M borate/EDTA buffer (pH 8.32). Samples were incubated for 3 h on ice and subsequently centrifuged for 10 min at 4000 rpm at 4°C. Then, supernatants were collected and used for C3d quantification. A standard curve was made using zymosan-activated pooled rat plasma. The amount of C3d in the samples was determined from the standard curve and expressed in arbitrary units/ml. Samples were analyzed in duplicate and measured at an OD of 450 nm (VICTOR-3, 1420 multilabel counter, PerkinElmer, Waltham, US).

RNA Isolation

RT-qPCR was performed to investigate the renal gene expression levels of pro-inflammatory cytokines after brain death. Total RNA was extracted from frozen kidneys using the TRIzol method (Invitrogen, Waltham, US) and DNase Amplification Grade (Merck), according to manufacturer's instructions. Genomic DNA contamination was verified by RT-qPCR using β-actin primers, in which the addition of reverse transcriptase was omitted.

cDNA Synthesis and qPCR

cDNA synthesis was performed by the addition of 0.5 μl sterile water, 4 μl 5x first strand buffer (Invitrogen), 2 μl DTT (Invitrogen), and 1 μl M-MLV Reverse Transcriptase (Invitrogen) and primers (**Table 3**). The mixture was then incubated for 50 min at 37°C. After that, the reverse transcriptase was inactivated by incubating the mixture at 70°C for 15 min. The Taqman Applied Biosystems 7900HT RT-qPCR system (Biosystems, Carlsbad, USA) was used to amplify and detect PCR products, using SYBR Green (Applied Biosystems, Foster City, USA). Ct values were corrected for ß-actin and gene expression values were expressed as $2^{-\Delta\Delta CT}$ (Ct: threshold cycle).

Statistical Analysis

Statistical analyses were performed with IBM SPSS Statistics 23 (IBM Corp, Armonk, NY, USA). The Kruskal-Wallis test was performed for multiple group comparisons. The Mann-Whitney U test was used to compare the differences between two groups. Bonferroni correction was used to account for multiple

TABLE 2 | Primary antibodies used for immunohistochemistry.

Antibody	Sections	Host and target species	Supplier	2nd/3rd antibodies
C3d	Frozen	Rabbit polyclonal anti-human C3d	A0063, Dako, Carpinteria, USA	GαRb^{PO}/RbαG^{PO}
C5b-9	Frozen	Mouse monoclonal anti-rat membrane attack complex	Hycult, Uden, the Netherlands	RbαM^{PO}
ED-1	Paraffin	Mouse monoclonal anti-rat macrophages/monocytes	Abcam, Oxford, UK	RbαM^{PO}/GαRb^{PO}
HIS48	Frozen	Mouse monoclonal anti-rat granulocytes	IQProducts, Groningen, the Netherlands	RbαM^{PO}/GαRb^{PO}
KIM-1	Paraffin	Mouse monoclonal anti-human KIM-1 AKG7	Biogen Inc, Cambridge, Massachusetts, USA	RbαM^{PO}/GαRb^{PO}

G, goat; Rb, rabbit; M, mouse; PO, polyclonal; KIM-1, kidney injury molecule-1.

TABLE 3 | Gene-specific qPCR primers.

Primers	Primer sequences	Amplification length
β-actin	5′-GGAAATCGTGCGTGACATTAAA-3′	74
	5′-GCGGCAGTGGCCATCTC-3′	
BAX	5′-GCGTGGTTGCCCTCTTCTAC-3′	74
	5′-TGATCAGCTCGGGCACTTTAGT-3′	
Bcl-2	5′-CTGGGATGCCTTTGTGGAA-3′	70
	5′-TCAGAGACAGCCAGGAGAAATCA-3′	
IL-1β	5′-CAGCAATGGTCGGGACATAGTT-3′	106
	3′-GCATTAGGAATAGTGCAGCCATCT-5′	
IL-6	5′-CCAACTTCCAATGCTCTCCTAATG-3′	89
	5′-TTCAAGTGCTTTCAAGAGTTGGAT-3′	
IL-18	5′-CAACCGCAGTAATACGGAGCATA-3′	62
	5′-CAGGCGGGTTTCTTTTGTCA-3′	
KIM-1	5′-AGAGAGAGCAGGACACAGGCTT-3′	75
	5′-ACCCGTGGTAGTCCCAAACA-3′	
MCP-1	5′-CTTTGAATGTGAACTTGACCCATAA-3′	78
	5′-ACAGAAGTGCTTGAGGTGGTTGT-3′	
P-selectin	5′-TCTCTGGGTCTTCGTGTTTCTTATCT-3′	80
	5′-GTGTCCCCCTAGTACCATCTGAA-3′	
VCAM-1	5′-TGTGGAAGTGTGCCCGAAA-3′	84
	5′-ACGAGCCATTAACAGACTTTAGCA-3′	

β-actin, βeta-actin; BAX, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma protein 2; IL, Interleukin; KIM-1, kidney injury molecule-1; MCP-1, monocyte chemoattractant protein-1; VCAM-1, vascular cell adhesion molecule-1.

comparisons. All statistical tests were 2-tailed and a $p < 0.05$ was considered significant. Non-parametric data are presented as median ± interquartile range and parametric data are displayed as mean ± SD.

RESULTS

Treatment With Anti-factor B Prevents Both Systemic and Local Complement Activation in Rats Subjected to Brain Death

To investigate whether the complement system is activated in our rat brain death model, we determined systemic and local complement activation levels after 4 h of brain death. Systemic C3d levels were significantly increased after the induction of brain death (**Figure 2A**, $p < 0.05$) when compared to sham-operated rats, which indicates that the complement system was indeed activated upon brain death.

Next, we assessed whether treatment with anti-FB was able to prevent systemic complement activation in rats. Pretreatment with anti-FB prevented complement activation significantly, shown by comparable C3d levels as found in sham-operated rats (**Figure 2A**, $p < 0.01$). In addition, we determined whether treatment with anti-FB led to less local complement activation. There was no significant increase in C5b-9 deposition after 4 h of brain death compared to sham-operated rats (**Figure 2B**). However, renal C3d deposition was significantly increased in brain-dead rats compared to sham-operated rats (**Figure 2C**, $p < 0.01$). In addition, brain-dead rats pretreated with anti-FB had significantly less renal C3d deposition than saline-treated rats (**Figures 2C–F**, $p < 0.05$). Overall, anti-FB significantly prevented both systemic and local complement activation on the level of C3 after 4 h of brain death.

Anti-factor B Preserves Renal Function and Attenuates Renal Injury After Brain Death

To determine whether treatment with anti-FB was able to preserve renal function and protect against renal injury, we measured serum creatinine levels, scored for histological injury and investigated kidney injury molecule-1 (KIM-1) levels in the kidney. First, plasma creatinine levels were significantly elevated after brain death compared to sham-operated rats (**Figure 3A**, $p < 0.001$). Pretreatment with anti-FB preserved renal function, reflected by significantly lower serum creatinine levels than saline-treated rats after brain death (**Figure 3A**, $p < 0.01$). However, serum creatinine levels in anti-FB treated rats were still significantly higher than in sham-operated rats. Second, anti-FB treated rats had significant less renal injury than saline-treated brain-dead rats, demonstrated by lower levels of renal tubular necrosis (**Figure 3B**, $p < 0.05$). Third, mRNA expression of KIM-1, a protein which is mainly expressed on damaged renal epithelial cells (18), was significantly upregulated in brain-dead rats compared to sham-operated rats. Pretreatment with anti-FB resulted in significantly lower KIM-1 gene expression levels, which indicates a reduction in renal tubular damage (**Figure 3C**, $p < 0.05$). Lastly, we analyzed renal KIM-1 deposition by performing immunohistochemistry. Brain-dead rats had significant more KIM-1 protein deposition in the proximal renal tubules than sham-operated rats. After brain death, KIM-1 staining was primarily seen in the corticomedullary junction. Anti-FB treated rats had significantly less KIM-1 deposition than saline-treated rats (**Figures 3D–G**, $p < 0.05$). Taken together, these results show that pretreatment with anti-FB

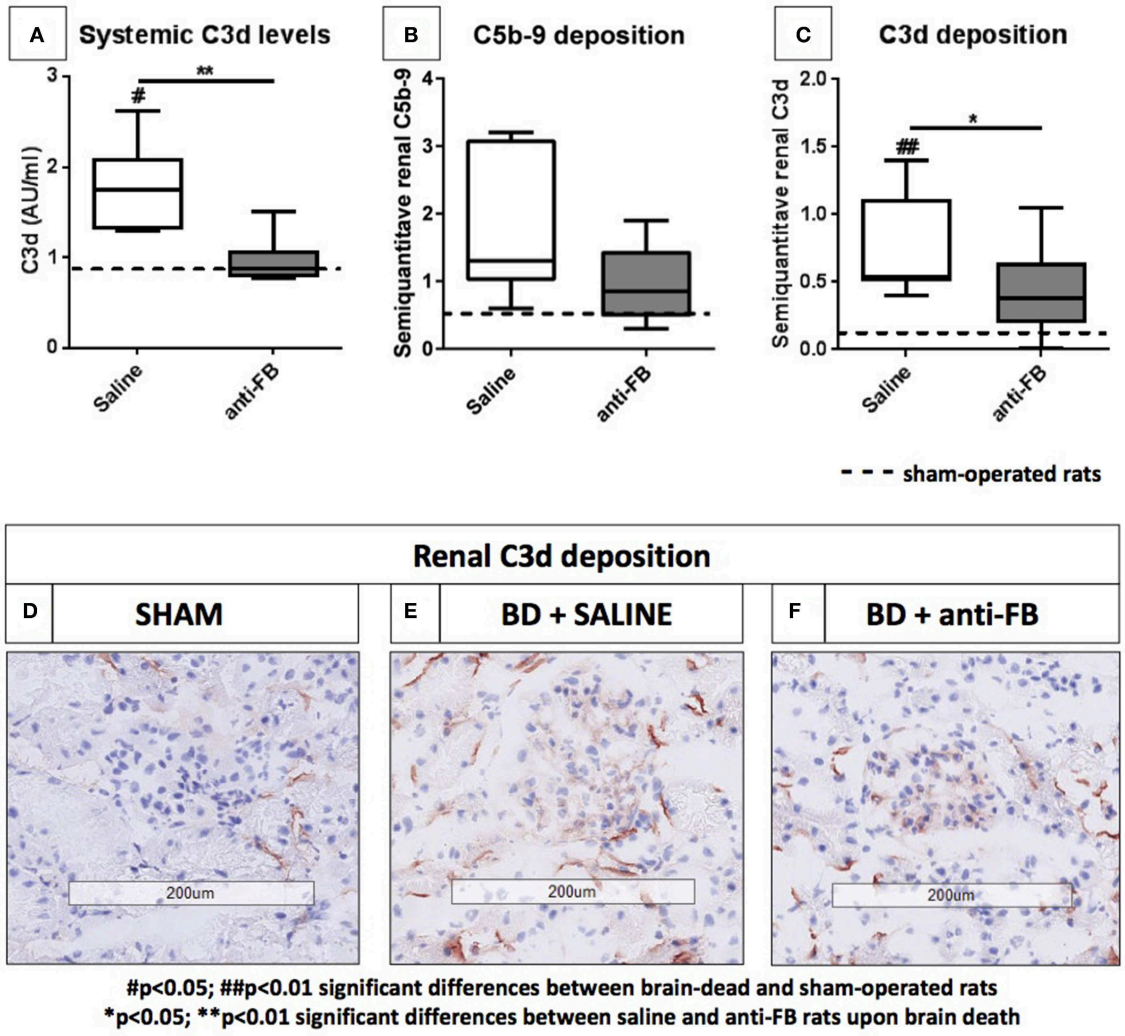

FIGURE 2 | Systemic and local complement levels after 4 h of brain death. **(A)** Systemic C3d levels of brain-dead rats treated with saline or anti-factor B. Plasma C3d levels were determined after 4 h of brain death. C3d was captured by using a monoclonal mouse anti-C3 antibody, detected with a rabbit anti-human C3d antibody and goat anti-rabbit-HRP. **(B)** Renal C5b-9 deposition and **(C)** renal C3d deposition in frozen sections from **(D)** sham-operated rats, **(E)** saline-treated rats, and **(F)** anti-factor B treated rats after 4 h of brain death. Data are shown as median ± IQR. Data were analyzed by Mann Whitney-*U*-test and Bonferroni *post-hoc* test, asterisks above the bars denote significant differences between the brain-dead rats (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). The dashed line represents the mean of the sham-operated rats. #Significant differences between the brain-dead rats vs. sham-operated rats (#$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$). Anti-FB, anti-factor B.

preserved renal function and attenuated brain death-induced renal injury.

Anti-factor B Reduces Systemic IL-6 Levels and Expression Levels of Pro-inflammatory Genes in the Kidney

To assess whether complement inhibition with anti-FB influences brain death-induced renal inflammation, we first determined systemic IL-6 levels after 4 h of brain death. IL-6 plasma levels were significantly higher in saline-treated brain-dead rats than in sham-operated rats (**Figure 4A**, $p < 0.001$). Pretreatment with anti-FB prevented the increase of systemic IL-6 significantly compared to saline-treated rats after brain death (**Figure 4A**, $p < 0.05$). Next, we determined renal mRNA levels of multiple pro-inflammatory genes. After 4 h of brain death, the pro-inflammatory cytokines IL-6, IL-18, and IL-1β, adhesion molecules P-selectin and VCAM-1, chemokine MCP-1, and apoptosis ratio BAX/Bcl-2 were all significantly upregulated compared to sham-operated rats (**Figures 4B–H**). These data demonstrate that our rat brain death model mimics the injury seen in human brain-dead donors (20). Rats treated with anti-FB showed significantly lower renal gene expression levels of pro-inflammatory cytokine IL-6 (**Figures 4B,C**, $p < 0.01$). Besides, renal gene expression levels of VCAM-1 and MCP-1 were significantly lower in anti-FB treated rats than in saline-treated rats upon brain death (**Figures 4F,G**, $p < 0.01$). Gene expression

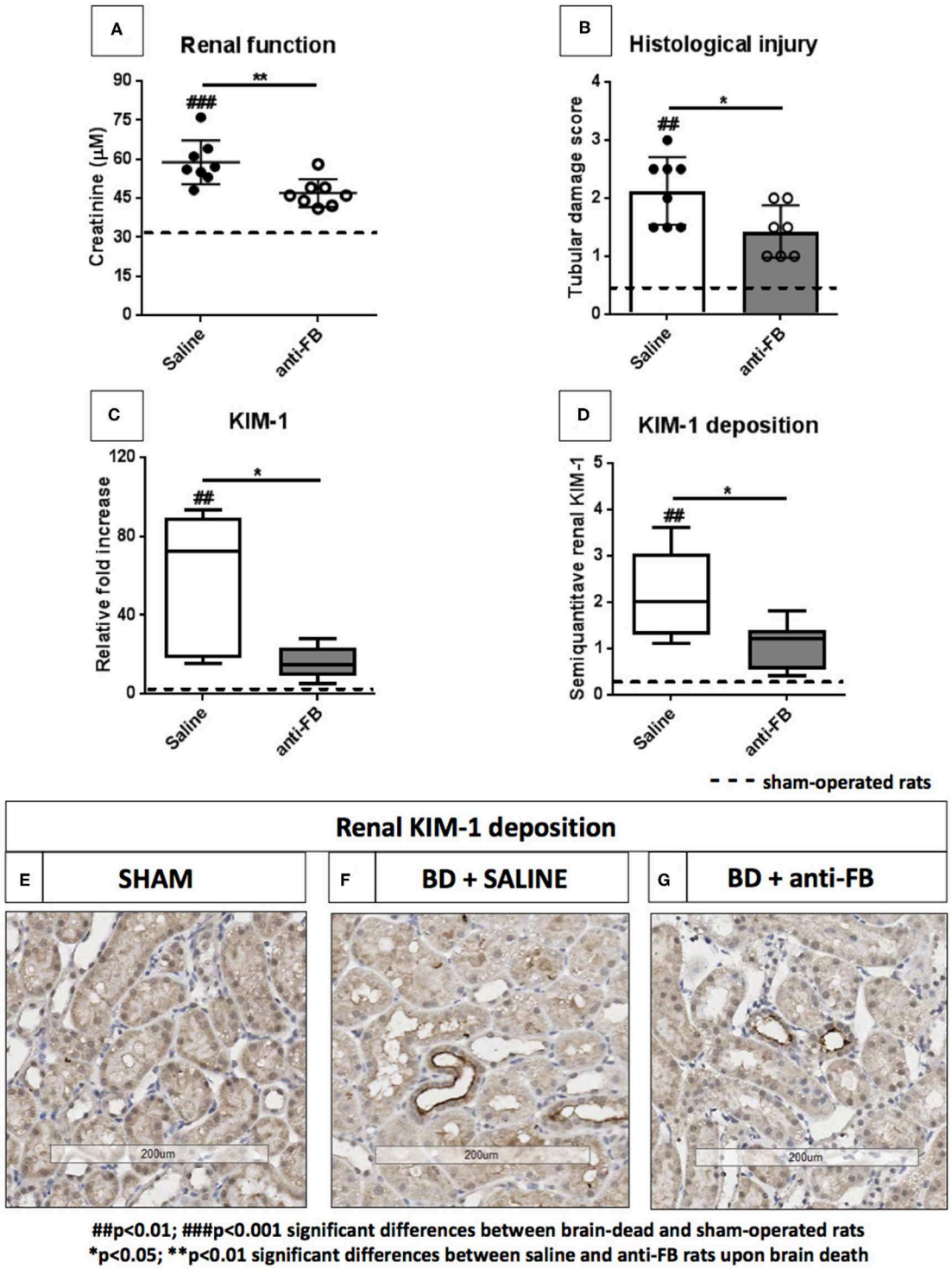

FIGURE 3 | Renal function and renal injury upon brain death with saline or anti-factor B. **(A)** Renal function reflected by plasma creatinine levels of brain-dead rats treated with saline or anti-FB compared to sham-operated rats. Serum creatinine levels were measured at time of sacrifice, using a Roche Modular P system. **(B)** Tubular damage was reflected as a percentage of acute tubular necrosis in the renal cortex using a semi-quantitative method. **(C)** Renal KIM-1 gene expression in brain-dead and sham-operated rats treated with saline or anti-FB was determined by quantitative real-time PCR. The mRNA expression of KIM-1 relative to β-actin was set at 1 in sham-operated rats, the other values, are calculated accordingly. **(D)** Renal KIM-1 deposition after 4h of brain death, KIM-1 was scored by estimating the percentage of KIM-1 expression in the cortical tubules. **(E)** KIM-1 deposition in sham-operated rats, **(F)** in saline-treated rats, and **(G)** anti-FB treated rats. Data are shown as median ± IQR. Data were analyzed by Mann Whitney-*U*-test and Bonferroni *post-hoc* test, asterisks above the bars denote significant differences between the brain-dead rats (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). The dashed line represents the mean of the sham-operated rats. $^{\#}$Significant differences between the brain-dead rats vs. sham-operated rats ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$). Anti-FB, anti-factor B.

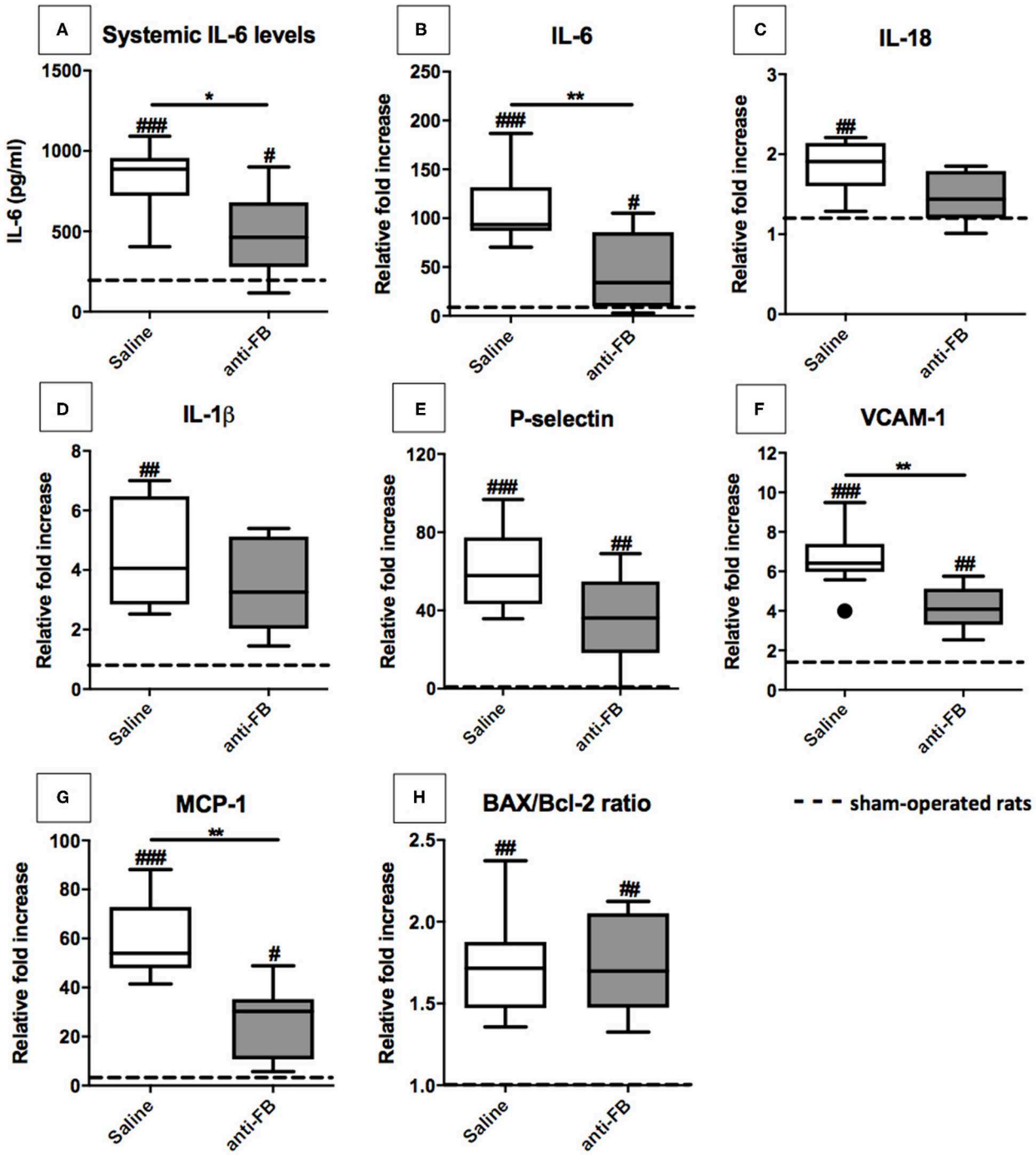

FIGURE 4 | Pro-inflammatory gene expression levels in the kidney after 4 h of brain death. **(A)** Systemic IL-6 levels of brain-dead rats treated with saline or anti-FB compared to sham-operated rats after 4 h of brain death. A rat enzyme-linked immunosorbent assay determined plasma levels of IL-6. All samples were analyzed in duplicate and measured at an OD of 450 nm. Pro-inflammatory gene expressions in the kidneys of brain-dead rats treated with saline or anti-factor B. mRNA expressions of **(B)** IL-6, **(C)** IL-18, **(D)** IL-1β, **(E)** P-selectin, **(F)** VCAM-1, **(G)** MCP-1, and **(H)** BAX/Bcl-2 ratio. Data are shown as expression relative to β-actin as set at 1 in sham-operated rats, the other values, are calculated accordingly. Data are shown as median ± IQR. Data were analyzed by Mann Whitney-*U*-test and Bonferroni *post-hoc* test, asterisks above the bars denote significant differences between the brain-dead rats (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). The dashed line represents the sham-operated rats. #Significant differences between the brain-dead rats vs. sham-operated rats (#$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$). Anti-FB, anti-factor B.

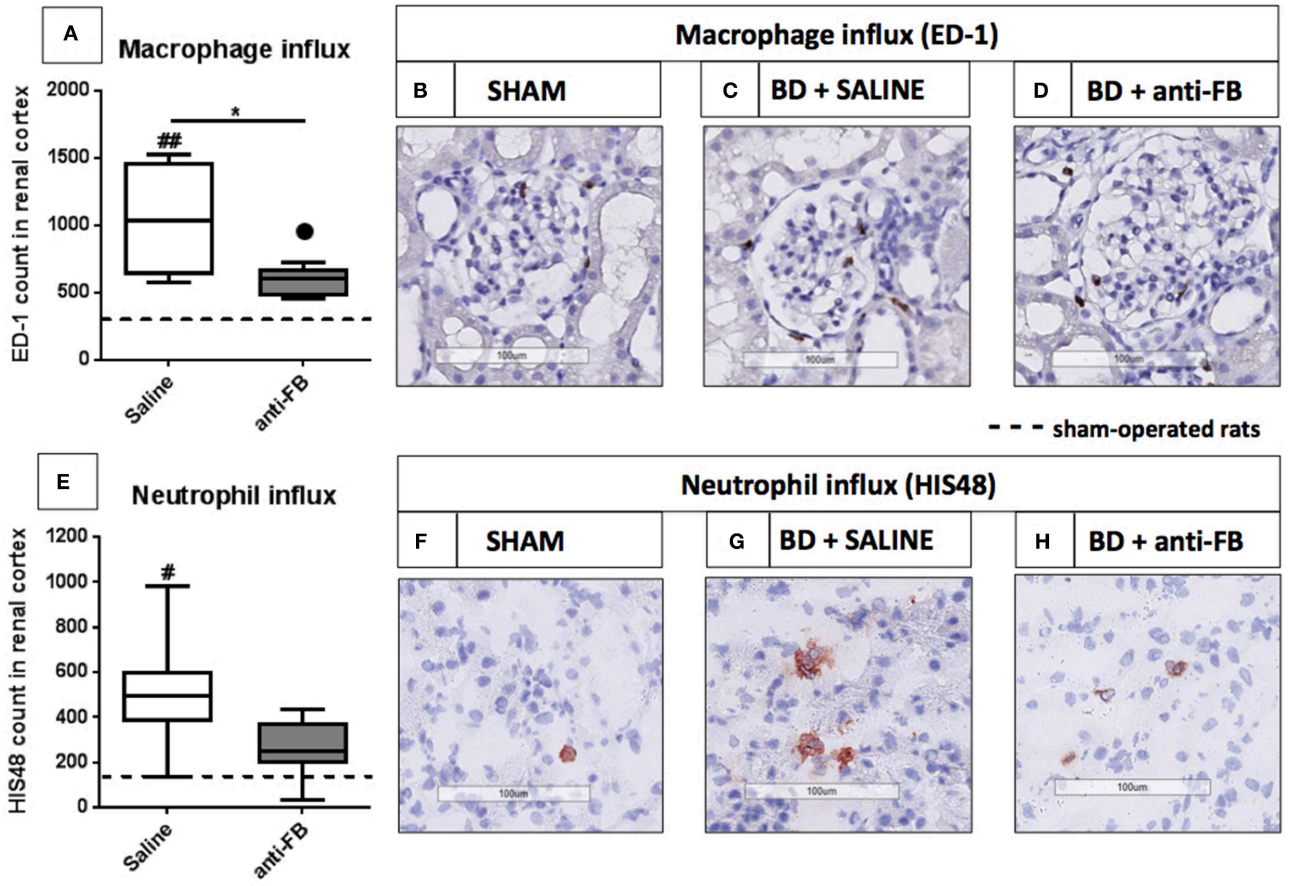

FIGURE 5 | Infiltrated leukocytes in the kidney after brain death in saline or anti-factor B treated rats. Infiltrated leukocytes in kidneys of sham-operated and brain-dead rats treated with saline or anti-factor B. **(A)** Renal neutrophil influx was scored in frozen sections from **(B)** sham-operated rats, **(C)** brain-dead rats treated with saline, or **(D)** brain-dead rats treated with anti-factor B. **(E)** Renal macrophage influx was scored in paraffin-embedded sections from **(F)** sham-operated rats, **(G)** brain-dead rats treated with saline, or **(H)** anti-factor B. Sections were quantified for the number of positive cells in the renal cortex using Aperio Imagescope (objective 50x−200x) and ImageJ Software. All pictures are presented at 200x magnification. Data are shown as median ± IQR. Data were analyzed by Mann Whitney-*U*-test and Bonferroni *post-hoc* test, asterisks above the bars denote significant differences between the brain-dead rats (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$). The dashed line represents the sham-operated rats. #Significant differences between the brain-dead rats vs. sham-operated rats (#$p < 0.05$, ##$p < 0.01$, and ###$p < 0.001$). Anti-FB, anti-factor B.

levels of IL-1β, IL-18, and P-selectin were lower as well, but not significantly (**Figures 4F,G**). Treatment with anti-FB did not reduce the apoptosis ratio BAX/Bcl-2 in the kidney upon brain death (**Figure 4H**). Altogether, the results demonstrate that anti-FB attenuates the pro-inflammatory response upon brain death.

Treatment With Anti-factor B Reduces the Influx of Macrophages After 4 h of Brain Death

To complete the renal inflammatory profile, we determined the influx of neutrophils and macrophages in the kidney by immunohistochemistry. After 4 h of brain death, the number of macrophages (ED-1) and neutrophils HIS(48) in the kidney were significantly increased in brain-dead rats compared to sham-operated rats. Next, we investigated the effect of anti-FB on the influx of leukocytes in the kidney. In brain-dead rats, treatment with anti-FB led to a significantly lower number of macrophages in the kidney than in saline-treated rats (**Figures 5A–D**, $p < 0.01$). In addition, the absolute number of neutrophils in the kidney was lower in anti-FB treated rats than in saline-treated rats (**Figures 5E–H**). In conclusion, pretreatment with anti-FB seems to attenuate the influx of leukocytes, especially macrophages, in the kidney upon brain death.

DISCUSSION

In this study, we investigated whether inhibition of factor B can attenuate brain death-induced renal injury and inflammation

in rats. To achieve this, we pretreated rats with a monoclonal antibody against factor B (anti-FB) and subsequently subjected the rats to 4 h of brain death. We found that anti-FB preserved renal function, reduced renal injury and renal inflammation in brain-dead rats.

First, we studied the effect of anti-FB treatment on systemic and local complement activation. We observed significantly lower plasma levels of C3d in anti-FB treated rats than in saline-treated rats after 4 h of brain death. These results demonstrate that treatment with anti-FB prevents systemic complement activation, which might be important because high systemic complement levels in the donor are associated with acute rejection of human renal allografts (11). In addition, the present study demonstrates that pretreatment with anti-FB resulted in significant less local complement C3d deposition. Of importance, since previous studies have shown that renal complement C3 synthesis is associated with acute renal transplant rejection and acute post-ischemic renal failure (3, 10, 21, 22).

Next, we investigated the effect of treatment with anti-FB on renal function and renal injury after 4 h of brain death. Treatment with anti-FB preserved renal function in brain-dead rats. However, serum creatinine levels in anti-FB treated rats were still higher than creatinine levels measured in sham-operated rats. We ascribe these small differences in serum creatinine levels to the hemodynamic changes seen during brain death, which were not seen in sham-operated rats. Therefore, rats subjected to brain death received more fluid than sham-operated rats, which explains the observed differences in plasma creatinine levels between anti-FB treated rats and sham-operated rats (**Table 1**). In addition, we observed an increase in KIM-1 gene expression levels and KIM-1 deposition in the renal cortex of brain-dead rats compared to sham-operated rats. Similar observations were done by Nijboer et al., who showed that KIM-1 is substantially upregulated in human brain-dead donors (23). The fact that both KIM-1 gene expression levels and KIM-1 deposition were reduced in anti-FB treated rats seems to be important since KIM-1 is known to be an independent predictive marker for renal function in recipients after renal transplantation (24). Lastly, since we observed that treatment with anti-FB led to lower gene expression levels of IL-6, VCAM-1, and MCP-1 after brain death, it is suggested that anti-FB reduces brain death-induced renal inflammation. Interestingly, rats treated with anti-FB had systemic IL-6 levels comparable to sham-operated rats. These low IL-6 levels could be explained by the fact that Ba and Bb fragments have a variety of biological activities independent of the proteolytic activity (25–27).

An important limitation of this study is the fact that anti-FB was not administered after the confirmation of brain death. Anti-FB was administered 20 min before the start of brain death induction, which is impossible to realize in clinical practice (28). Therefore, this study set-up serves as a proof of principle to investigate the effect of anti-FB on brain death-induced renal injury in rats. More research is needed to evaluate the effect of anti-FB during different time points throughout the brain death period. In the current study, we did not include a group with anti-FB administration after the induction of brain death. The optimal time point of intervention would be between 30 and 90 min after brain death induction, which leaves only 2–3 h to evaluate the effect of anti-FB treatment on renal injury in our brain death model for rats. We consider this too short, since the maximum effect of anti-FB treatment is apparent after 4 h (16). Therefore, we consider it as a next step to investigate the effect of anti-FB treatment after the induction of brain death in a larger animal model for brain death. Another limitation of this study is that we only used male rats, to circumvent sex-related differences in complement levels and functionality (14, 29).

A study performed by Thurman et al. already demonstrated the potential of complement factor B inhibition in a mouse model of renal ischemia/reperfusion. An inhibitory monoclonal antibody to mouse factor B was used, which significantly preserved renal function and led to less renal injury (30, 31). Thus, factor B seems to be a promising target to improve renal transplant outcome, in both the donor as well as in the recipient. However, treatment of the donor might be more beneficial than of the recipient, since the complement system is already activated in the donor and as has been shown to affect the function of the renal allograft (11, 32). Taken together, these results create a new window of opportunity for complement-targeted therapies in the renal transplantation setting.

However, when treating the donor, it should be considered that all organs will be treated with the same drug and same dose of treatment. While our study shows that the quality of the kidney improves from treatment with anti-FB in the donor, this is not yet investigated for the other organs. Based on literature, the heart may also benefit from treatment with anti-FB. Chun et al. showed that systemic levels of factor B in both mice and human are positively correlated with myocardial necrosis after cardiac ischemia/reperfusion injury. In addition, absence of factor B resulted in significant myocardial protection after cardiac ischemia/reperfusion (33). Although less is known about factor B in other organs during transplantation, factor B has been described to play a pivotal role in multiple pro-inflammatory disease models, such as retinal injury and arthritis (34, 35). Altogether, these positive findings resulted in the development of therapeutic targets against factor B.

Currently, two drugs that can inhibit factor B are tested in clinical trials. One of them is LPN023, a small molecule that binds the active site of factor B. LPN023 is currently tested in phase II dose-ranging study in IgA nephropathy patients [NCT03373461; (36)]. Recently, Ionis Pharmaceuticals announced to start a phase II study with their antisense drug against factor B in patients with age-related macular disease (37). Various agents that target factor B are currently under development, for example siRNA against factor B [Alnylam; (38)]. These trials, may soon lead to the clinical availability of one or more complement inhibitors that target factor B.

In conclusion, we show that anti-FB pretreatment in brain-dead donor rats preserves renal function and protects against renal injury and renal inflammation. Therefore, anti-FB treatment might be a potential therapy to reduce brain death-induced renal injury prior to transplantation.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Welfare Body of the Institutional Animal Care and Use Committee at the University Medical Center Groningen, University of Groningen, Groningen, The Netherlands.

AUTHOR CONTRIBUTIONS

Research idea and study design by NJ, JZ, MS, MD, SR, FP, and MAS. NJ and JZ planned and conducted the experiments and wrote the manuscript. Data analysis and interpretation of the results by NJ, JZ, HL, MD, FP, and MAS. Statistical analysis by NJ and JZ. All authors were involved in editing the final manuscript. All authors read and approved the final manuscript.

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The Aquaporin 5 −1364A/C Promoter Polymorphism Is Associated With Cytomegalovirus Infection Risk in Kidney Transplant Recipients

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Background: The aquaporin 5 (*AQP5*) −1364A/C promoter single nucleotide polymorphism affects key mechanisms of inflammation and immune cell migration. Thus, it could be involved in the pathogenesis of cytomegalovirus infection. Accordingly, we tested the hypothesis that the *AQP5* promoter −1364A/C polymorphism is associated with the risk of cytomegalovirus infection in kidney transplantation recipients.

Methods: We included 259 adult patients who received a kidney transplant from 2007 and 2014 in this observational study. Patients were genotyped for the *AQP5* promoter −1364A/C single nucleotide polymorphism and followed up for 12 months after transplantation. Kaplan–Meier plots and multivariable proportional hazard analyses were used to evaluate the relationship between genotypes and the incidence of cytomegalovirus infection.

Results: The incidences of cytomegalovirus infection within 12 months after kidney transplantation were 22.9% for the AA genotypes (43/188) and 42.3% for the AC/CC genotypes (30/71; $p = 0.002$). Furthermore, multivariable COX regression revealed the C-allele of the *AQP5* −1364A/C polymorphism to be a strong and independent risk factor for cytomegalovirus infection. In this analysis, AC/CC subjects demonstrated a more than 2-fold increased risk for cytomegalovirus infection within the first year after kidney transplantation (hazard ratio: 2.28; 95% CI: 1.40–3.73; $p = 0.001$) compared to that in individuals with homozygous AA genotypes.

Conclusions: With respect to opportunistic cytomegalovirus infections (attributable to immunosuppression after kidney transplantation), the C-allele of the *AQP5* −1364A/C promoter polymorphism is independently associated with an increased 12-months infection risk. These findings emphasize the importance of genetic variations as additional risk factors of cytomegalovirus infection after solid organ transplantations and might also facilitate the discovery of novel therapeutic targets.

Keywords: *AQP5*, single nucleotide polymorphism (SNP), cytomegalovirus, immunosuppression, infection risk, kidney transplantation

INTRODUCTION

Cytomegalovirus (CMV) is one of the most common opportunistic infections in kidney transplant recipients, which affects transplant rejection and graft function, triggers harmful CMV-associated diseases, and might also influence mortality rates (1, 2). Antiviral chemoprophylaxis seems to be a successful strategy in preventing major complications related to CMV infections, but universal prophylaxis is also detrimental, due to drug toxicity, late CMV disease, and the development of ganciclovir-resistant mutants (3). Therefore, risk-adapted strategies appear to be a cornerstone of modern antiviral chemoprophylaxis and identifying associated risk factors seems to be crucial to improve current post-transplantation care. In this context, the incidence of CMV infections is highly dependent on the serostatus of the recipient (R) and the donor (D) with the highest risk noted in D positive and R negative (D^+/R^-) transplantations (4). However, CMV infection risk cannot be solely attributed to this single risk factor, as some of additional variability might be caused by genetic variations (5).

An interesting candidate for investigations regarding such genetic variations is the single nucleotide polymorphism (SNP; rs3759129) in the aquaporin 5 (*AQP5*) promoter region (−1364A/C). Previously, we described that the substitution of cytosine for adenine at position −1364 is associated with lower AQP5 messenger RNA and protein expression (6). In this context, AQP5 expression mediates water transport across biologic membranes, regulating cellular water fluid homeostasis during inflammation, proliferation, and cell migration, processes that involve the transient formation of membrane protrusions (lamellipodia and membrane ruffles) at the leading edge of the cell (7–9). The crucial effect of this *AQP5* SNP in mediating key mechanisms of inflammation and altering related host–pathogen communication was demonstrated in patients with sepsis and acute respiratory distress syndrome (10–12). In this regard, the *AQP5* −1364A/C promoter SNP was found to affect neutrophil migration into the lungs and the AA genotypes were associated with aggravated pulmonary inflammation in acute respiratory distress syndrome evoked by bacteria (10). Strikingly, increased *AQP5* expression and the AA genotype of the *AQP5* SNP were also shown to be associated with improved bacterial eradication, and therefore an enhanced antimicrobial immune response (10, 13).

Taken together, this *AQP5* polymorphism could contribute to the risk of CMV infection in kidney transplant recipients due to an altered resistance to viral infections, but data addressing this topic are completely lacking. Accordingly, we tested the hypothesis that the *AQP5* promoter −1364A/C polymorphism is associated with the risk of CMV infection in kidney transplantation recipients.

MATERIALS AND METHODS

Patients and Treatments

This study was reviewed and approved by the local ethics board of the Faculty of Medicine, Ruhr-University of Bochum (Bochum, Germany; protocol no. 4870-13). Patients were enrolled in this study upon receiving a kidney or combined pancreas–kidney transplant between 2007 and 2014 at the Department of General Surgery of the University Hospital Knappschaftskrankenhaus Bochum (Bochum, Germany). For study inclusion written informed consent was obtained from all 259 participating patients, according to the Declaration of Helsinki, good clinical practice guidelines and applicable to local regulatory requirements.

Patients were recruited to donate a buccal swab for DNA extraction and the evaluation of *AQP5* SNPs after transplantation. Clinical and demographic data were gathered upon study inclusion and patients were observed for 1 year after organ transplantation. All patients received immunosuppressive induction and maintenance therapy according to locally specific standard operating procedures, which included steroids, calcineurin inhibitors, and mycophenolic acid (**Table 1**), as well as risk-adapted perioperative and post-operative antiviral chemoprophylaxis with ganciclovir or valganciclovir. In this context, 59 high-risk patients (D^+/R^-) received chemoprophylaxis for 6 months (except five patients in this group with unknown or shorter duration), 144 medium-risk patients (D^+/R^+ and D^-/R^+) received prophylaxis for 3 months (except 10 patients in this group with unknown or shorter duration), and 41 low-risk patients (D^-/R^-) received perioperative prophylaxis, for whom chemoprophylaxis was expanded to 3 months in 20 cases, for example, due to CMV-positive blood transfusions.

Routine surveillance for viral reactivation or infection comprised weekly determinations of CMV viremia based on whole blood samples via PCR, until hospital discharge from index-admission and continuing monthly thereafter and when clinically indicated. Additionally, all patients were screened for CMV infection at the 1-year follow up examination after transplantation. Delayed graft function was defined as the necessity for dialysis in the first week after surgery.

DNA Genotyping

DNA was extracted from buccal swabs using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany). To genotype the −1364A/C *AQP5* promoter SNP, a nested polymerase chain reaction was performed with the forward AQP5-SE 5′-CCCAGACCAGGGGTAGAAGA-3′, and the reverse AQP5-AS 5′-TCTTCCTGCTAGAAGCCCCT-3′ primers followed by tetra-primer ARMS-PCR with Forward inner primer (A allele): 5′-GAGAGAGACAGAGAGACTAAGACAGCGAA-3′, Reverse inner primer (C allele): 5′-CATTTTCTGTTTTTCCT TCCTGCTTG-3′, Forward outer primer 5′-GACCACATGTAA GAGAGAGAGACATGGA-3′ and Reverse outer primer 5′-CTGTCAGTCAGTCTTTGCAAAACCCTAT-3′ resulting in a 223 base pair fragment for A allele and a 189 base pair fragment for C allele.

Study Groups and End Points

Study patients were assigned to two groups (AA genotype vs. AC/CC genotype) depending on the −1364A/C SNP in the *AQP5* promoter. The AC and CC genotypes were combined because of the low frequency (3.1%; 8/289) of the CC genotype.

TABLE 1 | Characteristics of kidney transplantation patients ($n = 259$) at baseline stratified by *AQP5* −1364 A/C genotype.

Variable	AA $n = 188$ (73%)	AC/CC $n = 71$ (27%)	*P*-value
Age (y), mean (range/±SD)	53.3 (23–89/±12.6)	53.0 (28–77/±11.3)	0.890
Male sex, *n* (%)	120 (63.8%)	45 (63.4%)	0.908
Body mass index (kg/m^2), mean (± SD)	25.7 (± 4.4)	26.2 (± 4.6)	0.404
Ethnicity, *n* (%)			1.000
Caucasian	184 (97.9%)	70 (98.6%)	
Other	4 (2.1%)	1 (1.4%)	
Etiology of end-stage renal disease, n (%)			0.675
Glomerular disease	49 (26.1%)	14 (19.7%)	
Diabetes	45 (23.9%)	21 (29.6%)	
Hypertension	21 (11.2%)	6 (8.5%)	
Polycystic kidney disease	25 (13.3%)	12 (16.9%)	
Other/unknown	48 (25.5%)	18 (25.3%)	
Pre-transplantation renal replacement therapy, n (%)	166 (88.3%)	63 (88.7%)	0.922
Transplantation, n (%)			0.908
Kidney	131 (69.7%)	50 (70.4%)	
Combined pancreas + kidney	57 (30.3%)	21 (29.6%)	
Cold ischemia time (min), mean (±SD)	688 (± 315)	674 (± 262)	0.736
First kidney transplantation, *n* (%)	173 (92.0%)	62 (87.3%)	0.245
Previous kidney transplantation, *n* (%)	15 (8.0%)	9 (12.7%)	
HLA-mismatches, median (IQR)	3 (2:5)	4 (2:5)	0.731
0–1, *n* (%)	21 (11.2%)	13 (18.3%)	0.283
2–4, *n* (%)	109 (57.9%)	33 (46.5%)	
≥5, *n* (%)	46 (24.5%)	21 (29.6%)	
Missing, *n* (%)	12 (6.4%)	4 (5.6%)	
Donor			0.558
Age (y), mean (range/± SD)	52.4 (4–85/± 16.3)	49.1 (8–87/± 18.6)	
Male sex, *n* (%)	92 (48.9%)	41 (57.7%)	0.130
Living donor, *n* (%)	21 (11.2%)	10 (14.1%)	0.519
Cadaveric donor, *n* (%)	167 (88.8%)	61 (85.1%)	
Delayed graft function, *n* (%)	52 (27.7%)	23 (32.4%)	0.454
eGFR 1-year after transplantation (ml/min/1.73 m^2), median (IQR)	46.4 (32.9:59.1)	47.1 (29.8:57.7)	0.613
Biopsy-proven acute rejection, *n* (%)	57 (30.3%)	22 (30.9%)	0.917
Induction with ATG, *n* (%)	155 (82.4%)	56 (78.9%)	0.509
Initial immunosuppressive regimen, *n* (%)			0.684
MPA, prednisone, and tacrolimus	171 (91.0%)	62 (87.3%)	
MPA, prednisone, and cyclosporine	13 (6.9%)	7 (9.9%)	
Other	4 (2.1%)	2 (2.8%	
Usage of mTOR inhibitors, *n* (%)	30 (16.0%)	7 (9.9%)	0.239
CMV infection, *n* (%)	43 (22.9%)	30 (42.3%)	0.002
Time of transplantation to CMV infection (days), median (IQR)	169 (106:265)	115 (70:188)	0.012
CMV disease, *n* (%)	10 (5.3%)	11 (15.5%)	0.007
CMV pneumonia	0	2 (18.2%)	
CMV syndrome	6 (60.0%)	4 (56.3%)	
CMV gastrointestinal disease + hepatitis	4 (40.0%)	2 18.2%)	
Other	0	3 (27.3%)	
Indication of anti-CMV therapy, *n* (%)			0.776
Prophylactic–perioperative	21 (11.2%)	8 (11.3%)	
Prophylactic−3 months	123 (65.4%)	42 (59.1%)	
Prophylactic−6 months	40 (21.3%)	19 (26.8%)	
None/unknown	4 (2.1%)	2 (2.8%)	
Anti-CMV therapy, *n* (%)			0.867
Ganciclovir	18 (9.6%)	8 (11.3%)	
Valganciclovir	166 (88.3%)	61 (85.9%)	
None/unknown	4 (2.1%)	2 (2.8%)	
CMV serology at transplantation, n (%)			0.973
D^+/R^-	45 (23.9%)	19 (26.8%)	
D^+/R^+	68 (36.2%)	25 (35.2%)	
D^-/R^+	45 (23.9%)	16 (22.5%)	
D^-/R^-	30 (16.0%)	11 (15.5%)	

IQR, Interquartile Range with 25th and 75th percentile; HLA, human leukocyte antigen; eGFR, Glomerular filtration rate was estimated by using Modification of Diet in Renal Disease (MDRD) study equation; ATG, antithymocyte globulin; MPA, mycophenolic acid; mTOR, mechanistic target of rapamycin; CMV, cytomegalovirus; D^+, CMV sero-positive donor; D^-, CMV sero-negative donor; R^+, CMV sero-positive recipient; R^-, CMV sero-positive recipient. Missing data were excluded from the analysis: six cases were missing for body mass index and one case was missing for cold ischemia time.

The primary end point was CMV-free survival in the first year after kidney transplantation. The key secondary end point was the effect of chemoprophylaxis duration on the time of CMV infection onset.

Clinical Definitions and Diagnostics

CMV infection was defined as the detection of viral nucleic acid in accordance to the definition of Ljungman and colleagues (14). CMV DNA was evaluated using a commercially available PCR assay (Roche Ampliprep Assay; Roche Molecular Diagnostics, Pleasanton, CA, USA) as per the manufacturer's instructions and calibrated to the World Health Organization International Standard for Human CMV.

CMV disease and related entities (e.g., CMV pneumonia and CMV syndrome) were defined as the presence of CMV in the blood based on a local assay plus the presence of compatible symptoms as described by Ljungman and colleagues (14).

Statistical Analysis

The characteristics of patients at baseline (timepoint of transplantation) were reported as percentages for categorical variables and as means with standard deviations (±SD) or medians with interquartile ranges (25th; 75th percentile) for continuous variables, as appropriate. Categorical variables were

compared with chi-square or Fisher's exact tests, and continuous variables were compared with a parametric Student's *t*-test or non-parametric Wilcoxon-Mann-Whitney-Test. The *AQP5* −1364A/C SNP distributions were tested for deviations from the Hardy–Weinberg equilibrium (exact two-sided *P*-value; significance value, 0.05). Explorative comparisons based on *AQP5* −1364A/C genotypes (AC/CC vs. AA) were performed for several clinical patient characteristics (**Table 1**).

CMV infection probabilities were graphically assessed by the Kaplan–Meier method. The log-rank test was used to evaluate the univariate relationship between the *AQP5* −1364A/C genotype and incidence of CMV infection. Next, we performed Cox regression analyses assessing the joint effect of the *AQP5* −1364A/C genotype and potential predictors on CMV-free survival. At first, Cox regression was performed with several models based on a single predictor (**Table 3**, left column). Thereafter, multiple variable Cox regression was performed with an initial model investigating multiple predictors simultaneously (**Table 3**, right column). To avoid overfitting, a restricted model with only four predictors was assessed subsequently using only those predictors with a *P*-value 0.05 or lower based on either the single or multiple predictor comparisons (**Table 4**). Confidence intervals (CI) were calculated with a coverage of 95%. All reported *P*-values were nominal and two-sided with an a priori α error of <0.05. All analyses were performed using SPSS (version 24, IBM, USA); for graphical presentations, GraphPad Prism 7 (Graph-Pad, USA) was used.

RESULTS

The baseline characteristics of the 259 kidney transplant recipients stratified for the *AQP5* −1364A/C promoter SNP are presented in **Table 1**. The mean age of the recipients at the time of transplantation was 53.2 ± 12.2 years and most were male (63.7%; 165/259). The observed 1-year CMV infection rate of the entire cohort was 28.1% (73/259) and the median duration of CMV infection onset after transplantation was 150 days [90; 217]. Regarding the distribution of genetic variations according to the Hardy–Weinberg equilibrium of the *AQP5* SNPs, we observed a frequency of 188 for the AA-genotype (expected: $n = 186$), 63 for the AC-genotype (expected: $n = 67$), and eight for the CC-genotype (expected: $n = 6$) in our cohort. Accordingly, no deviation from the Hardy–Weinberg equilibrium was observed ($p = 0.8475$).

In addition, 69.9% (181/259) received a kidney and 30.1% (78/259) received a combined pancreas and kidney transplantation, without statistically significant distribution among AA and AC/CC genotypes ($p = 0.908$; **Table 1**). Furthermore, we found no evidence of statistically significant associations between the *AQP5* −1364A/C genotypes and age ($p = 0.890$), sex ($p = 0.908$), etiology of end-stage renal disease ($p = 0.675$), rate of pre-transplantation renal replacement therapy ($p = 0.922$), delayed graft function ($p = 0.454$), and CMV serology at transplantation ($p = 0.973$). Cases of ganciclovir-resistant CMV strains were not detected among the study patients.

One-year CMV infection risk was significantly associated with the *AQP5* −1364A/C genotypes ($p = 0.001$; **Figure 1**). CMV infection rates were 23% (43/188) for the AA genotype and 42% (30/71; $p = 0.001$) for the AC/CC genotypes. In addition, CMV disease was more common in individuals with the AC/CC genotypes (15.5%; 11/71), when compared to the association with the AA genotype (5.3%; 10/188; $p = 0.007$). Further, stratifying patients according to the presence of CMV infections and CMV-associated diseases, there were no statistically significant

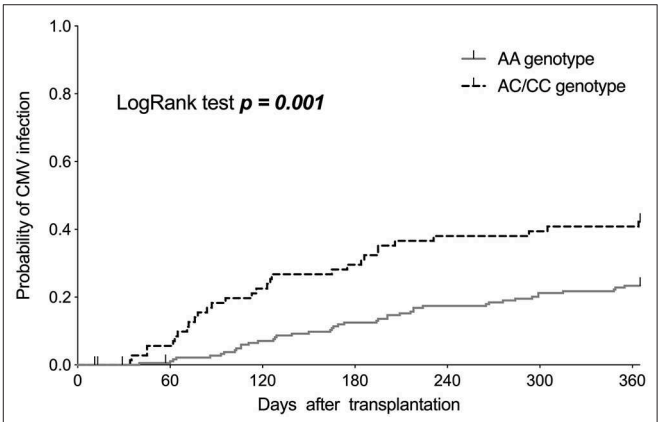

FIGURE 1 | Kaplan–Meier curves showing the incidence of cytomegalovirus (CMV) infections in the first year after kidney transplantation, stratified based on the AA and AC/CC genotypes of the *AQP5* −1364A/C single nucleotide polymorphism.

TABLE 2 | Characteristics of kidney transplantation recipients ($n = 259$) stratified by frequencies and time of CMV infection onset.

Variable	Total frequencies: n_{total} (%)	Frequency of CMV infection: n_{CMV} (%)	Time to CMV infection, median (IQR)
AQP5 −1364A/C Genotype			
AA	188 (72.6%)	43 (22.8%)	169 (106:265)
AC	63 (24.3%)	25 (39.7%)	116 (64:191)
CC	8 (3.1%)	5 (62.5%)	84 (77:238)
p-value		$p = 0.003$	$p = 0.041$
Duration of prophylactic Anti-CMV Therapy			
Perioperative, *n* (%)	29 (11.2%)	5 (17.2%)	64 (35:113)
3 months, *n* (%)	165 (63.7%)	41 (24.8%)	126 (94:174)
6 months, *n* (%)	59 (22.8%)	25 (42.4%)	209 (156:289)
None/unknown, *n* (%)	6 (2.3%)	2 (33.3%)	172 (45:299)
p-value		$p = 0.032$	$p = 0.001$
CMV Serology at Transplantation, *n* (%)			
D^+/R^-	64 (24.7%)	29 (45.3%)	201 (96:280)
$D^{+/-}/R^+$	154 (59.5%)	39 (25.3%)	126 (93:173)
D^-/R^-	41 (15.8%)	5 (12.2%)	103 (61:266)
p-value		$p = 0.001$	$p = 0.073$

CMV, cytomegalovirus; D^+, CMV sero-positive donor; D^-, CMV sero-negative donor; R^+, CMV sero-positive recipient; R^-, CMV sero-positive recipient.

differences between the AC (39.7%; 25/63 and 14.2%; 9/63, respectively) and CC (62.5%; 5/8; $p = 0.269$ and 25.0%; 2/8; $p = 0.601$, respectively) genotypes (**Table 2**).

Multivariate Cox regression analysis revealed the *AQP5* −1364A/C genotype was both an independent and strong (due to the estimated effect size) risk factor for CMV infection (**Tables 3**, **4**). In this context, C-allele carriers had a more than 2-fold greater risk of CMV infection in the first year after kidney transplantation (hazard ratio 2.28; 95% CI: 1.40–3.73; $p = 0.001$) compared to that with the AA genotype. Furthermore, the D^+/R^- CMV serostatus (hazard ratio 8.61; 95% CI: 2.0–5.7; $p = 0.003$) was confirmed as an important risk factor for CMV infection based on our cox-regression model.

Strikingly, the duration of prophylactic anti-CMV therapy did not seem to affect the sustainably of the 1-year CMV infection

TABLE 3 | Univariable and multivariable Cox regression analysis of kidney transplantation recipients regarding the effect on cytomegalovirus infection risk.

(Co) variable	Univariable			Multivariable		
	p-value	HR	95% CI	*p*-value	HR	95% CI
Aquaporin 5 −1364A/C genotype						
AA	–	1		–	1	
AC/CC	0.001	2.196	1.377–3.502	0.001	2.331	1.394–3.899
Recipient age [per year]	0.792	0.997	0.979–1.016	0.400	0.989	0.964–1.015
Recipient sex						
Female	–	1		–	1	
Male	0.989	1.003	0.624–1.614	0.796	1.071	0.637–1.801
Donor age [per year]	0.637	0.997	0.983–1.010	0.533	0.994	0.976–1.013
Donor sex						
Female	–	1		–	1	
Male	0.526	1.160	0.733–1.836	0.768	0.926	0.577–1.540
Cold ischemia time [per h]	0.379	1.020	0.976–1.066	0.353	1.027	0.970–1.088
Transplanted organ						
Kidney	–	1		–	1	
Kidney + pancreas	0.726	1.092	0.667–1.790	0.318	0.703	0.353–1.403
Living donor	–	1		–	1	
Cadaveric donor	0.413	1.385	0.635–3.019	0.339	1.502	0.652–3.458
Delayed graft function [no]	–	1		–	1	
Delayed graft function [yes]	0.513	1.179	0.720–1.932	0.769	1.087	0.622–1.901
BPAR [no]	–	1		–	1	
BPAR [yes]	0.633	1.125	0.694–1.824	0.519	1.188	0.703–2.007
HLA mismatch [per 1]	0.020	1.195	1.028–1.390	0.019	1.234	1.036–1.471
Immunosuppressive regimen						
MPA, prednisone and cyclosporine	–	1		–	1	
MPA, prednisone, and tacrolimus	0.410	0.720	0.330–1.572	0.208	0.590	0.259–1.343
Other	0.909	1.096	0.228–5.278	0.446	2.375	0.257–21.990
CMV risk status						
D^-/R^-	–	1		–	1	
$D^{+/-}/R^+$	0.089	2.241	0.883–5.686	0.109	2.672	0.803–8.892
D^+/R^-	0.003	4.248	1.644–10.981	0.004	10.744	2.153–53.628
Agent for anti-CMV prophylaxis						
Ganciclovir	–	1		–	1	
Valganciclovir	0.226	1.866	0.680–5.118	0.605	0.520	0.043–6.221
Prophylactic anti-CMV therapy						
Perioperative	–	1		–	1	
3 months	0.483	1.394	0.551–3.528	0.871	1.193	0.141–10.085
6 months	0.041	2.482	1.017–6.329	0.514	0.493	0.059–4.124

HR, odds ratio point estimates, 95% CI, and p-values (two-sided) are reported; BPAR, biopsy-proven acute rejection; HLA, human leukocyte antigen; MPA, mycophenolic acid; CMV, cytomegalovirus; D^+, CMV sero-positive donor; D^-, CMV sero-negative donor; R^+, CMV sero-positive recipient; R^-, CMV sero-positive recipient; six cases with unknown or no prophylactic anti-CMV therapy were excluded from analysis; 16 cases with missing HLA mismatch scores were excluded from analysis; omnibus test of model coefficients: Chi-square 35.9, p =0.007; Homer-Lemeshow statistics for multivariable approach were as follows: $\kappa^2 = 7.9$; $p = 0.445$.

risk (**Table 4**, **Figure 2**). In this context our Kaplan–Meier estimations showed the highest CMV infection rate after 1 year in patients treated with anti-CMV chemoprophylaxis for 6 months (42%, 25/59) compared to that with treatment for 3 months (25%; 41/165) and only perioperative treatment (17.2%; 5/29, $p = 0.043$).

DISCUSSION

This study shows that the C-allele of the *AQP5* −1364/A/C single nucleotide promoter polymorphism is associated with a marked increase in CMV infection and CMV disease risk in the first year after kidney transplantation. Furthermore, this SNP represents an independent and clinically meaningful risk factor of post-transplant CMV infection, with an estimated hazard ratio of nearly 2.3 for the AC/CC-genotypes. Hence, the *AQP5* −1364A/C promoter SNP might play a pivotal role in the management of post-transplantation CMV prophylaxis.

TABLE 4 | Restricted multivariable Cox regression analysis of kidney transplantation recipients with respect to the effect on cytomegalovirus infection risk.

(Co) variable	Multivariable-restricted		
	p-value	**HR**	**95% CI**
Aquaporin 5 −1364A/C genotype			
AA	–	1	
AC/CC	0.001	2.282	1.396–3.732
HLA mismatch [per 1]	0.047	1.161	1.002–1.347
CMV risk status			
D^-/R^-	–	1	
$D^{+/-}/R^+$	0.127	2.335	0.787–6.932
D^+/R^-	0.003	8.613	2.079–35.685
Prophylactic anti-CMV therapy			
Perioperative	–	1	
3 months	0.676	0.793	0.268–2.347
6 months	0.159	0.359	0.086–1.491

HR, odds ratio point estimates, 95% CI, and p-values (two-sided) are reported; CMV, cytomegalovirus, D^+, CMV sero-positive donor; D^-, CMV sero-negative donor; R^+, CMV sero-positive recipient; R^-, CMV sero-positive recipient; six cases with unknown or no prophylactic anti-CMV therapy were excluded from analysis, 16 cases with missing HLA mismatch score were excluded from analysis; omnibus test of model coefficients: Chi-square 29.4, $p < 0.001$; Homer-Lemeshow statistics for multivariable approach were as follows: $\kappa^2 = 7.4$; $p = 0.494$.

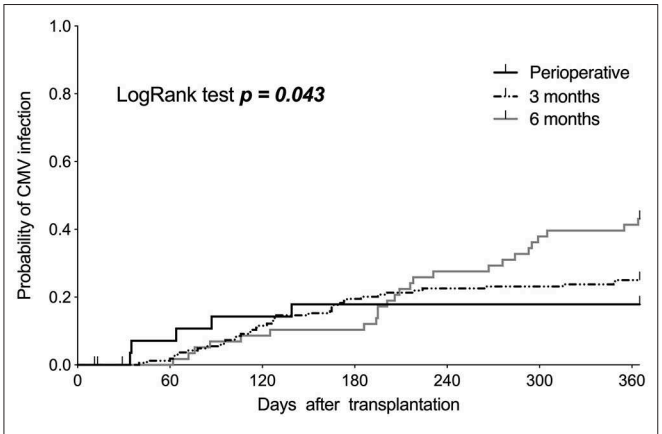

FIGURE 2 | Kaplan–Meier curves showing the incidence of cytomegalovirus (CMV) infections in the first year after kidney transplantation, stratified based on the duration of applied anti-CMV prophylaxis.

Since CMV infections continue to have a tremendous effect on outcome in kidney transplant recipients, anti-CMV chemoprophylaxis is a cornerstone of modern post-transplantation management (2). Antiviral prophylaxis involves the administration of antiviral drugs, preferably valganciclovir (15), to all patients at-risk of CMV infection, and is given for up to 6 months, in accordance with the IMPACT trial (16). In this context, the decision on antiviral prophylaxis duration is usually based only on the serostatus of the donor and recipient. However, one major drawback of current antiviral chemoprophylaxis is late-onset CMV infection and disease that is most commonly observed among high-risk CMV D^+/R^- patients after the completion of antiviral prophylaxis (17). This is in line with our results demonstrating a median CMV infection onset time of 129 and 209 days after receiving prophylactic anti-CMV therapy for 3 and 6 months, respectively. Therefore, most cases of CMV infections in patients who received antiviral prophylaxis occur after the cessation of antiviral drug administration, and still predominantly occur in the high-risk D^+/R^- group (17, 18). In this regard, we also found the highest infection rate of 42% 1 year after transplantation, despite the fact that anti-CMV chemoprophylaxis was applied for 6 months in 92% of the D^+/R^- cases. Thus, there seems to be room to further improve the current anti-CMV approaches for post-transplantation management (19, 20). In this context, a recent study elucidates that assessing the cell-mediated anti-CMV immunity could help to identify patients at-risk of developing late-onset CMV infections supporting a guided decision-making to safely stop or better continue antiviral treatment (21). Hence, advances in the field of post-transplantation anti-CMV management will partly be facilitated by the development of first, a better diagnostic assay including genetic variations to the stratify risk of CMV infection, and second, new antiviral agents with unique mechanisms of action and ideally less toxicity.

A promising candidate for further investigation is the common *AQP5* −1364A/C promoter SNP, potentially addressing the aforementioned issues. Obviously, the exact mechanisms associated with genotype-related increased mortality, associated with the AA and AC/CC genotypes, cannot be pinpointed by our study due to absence of profound mechanistical and immunological examinations. However, based on our clinical data and considering previous evidence (8, 22, 23), we speculate that the *AQP5* −1364A/C SNP or rather altered *AQP5* expression might shape the efficiency of immune responses, thereby influencing the efficacy of microbial clearance, and with respect to our study, CMV elimination.

The immune response to CMV infection is highly complex and includes innate and adaptive immune responses (24). Accordingly, CMV infection is first detected by the innate immune system, which seems to be crucial during the early phase of an CMV infection (25). Surprisingly, an important

role has been suggested for neutrophils as potent antiviral effector cells that restrict viral replication and associated pathogenesis (26). In this context, it is of note that *AQP5* expression significantly affects the migration and associated activity of neutrophil granulocytes (8, 10). *AQP5*-knockout mice exhibit the attenuated migration of neutrophil granulocytes, which was also associated with higher survival compared to those in wild-type animals after intraperitoneal LPS injection (8). Furthermore, the target-oriented migration of human neutrophils *in vitro* was found to be slower and occurred to a lesser extent with reduced AQP-5 expression. In patients suffering from acute respiratory distress syndrome, attributed to bacterial pneumonia, the AA genotype of the *AQP5* promoter SNP was associated with aggravated pulmonary inflammation accompanied by a significant increase in neutrophil counts in the bronchoalveolar lavage fluid (10). Thus, the AA-genotype of the *AQP5* genotype seems likely to be associated with better neutrophil granulocyte reactivity, which could at least in part explain the lower risk of CMV infection described by this study.

In addition, the sustained control of CMV infection is largely driven by adaptive immunity, involving broadly targeted CMV-specific T-cells to achieve viral control (27). Furthermore, patients with the delayed emergence of CMV-specific $CD4^+$ T-helper cells are more likely to develop a CMV infection (28). In addition, evidence from kidney transplantation has confirmed that the frequency of CMV-specific T-helper cells is inversely correlated with the incidences of CMV replication, high CMV load, and onset of CMV-related disease (29–31).

Strikingly, *AQP5* expression also seems to profoundly affect the T-cell response. A recent study demonstrated that T-cell specific cytokines are significantly down-regulated in *AQP5*-knockout mice (32), thus suggesting the crucial contribution of *AQP5* to the effectiveness of T-cell driven immune responses. More recently, the relationship between the *AQP5* deletion and elevated IFN-α and IL-2 production was shown, indicating an effect on the shift from type 2 T-helper cells toward a type 1 phenotype (33). Considering these results, it can be suggested that the *AQP5* −1364A/C promoter SNP critically shapes the innate and adaptive immune response in response to CMV infections. These hypotheses are in line with our results demonstrating that AC/CC genotypes of the *AQP5* −1364A/C SNP are strong and independent risk factors of CMV infection, as compared to the risk with AA genotypes.

Our results could be considered contradictory as the AA genotype of the *AQP5* −1364A/C SNP was found to be associated with worse outcome in our previous studies on sepsis (11) and ARDS (10). In contrast, the present study reports that the AA-genotype can diminish the risk of CMV infection and thus can potentially confer protective effects for kidney transplant recipients. However, sepsis and ARDS are phenomena in which an exaggerated immune response prevails, and therefore, the collateral damage observed with AA genotypes would be in the foreground and caused by a more potent immune system. In kidney transplantation recipients, exactly the opposite must be presumed, because of the profound immunosuppression. In this context, the enhanced immunoreactivity observed with the AA genotypes might mediate immunological benefits in immunosuppressed patients.

Nevertheless, these relationships, and especially the mechanistic associations, must be elucidated in the future, since this approach might also offer a new therapeutic target. In this context, it has been demonstrated that dexamethasone and ambroxol can upregulate AQP-5 expression *in-vitro* (34). Modulating *AQP5* expression depending on the genotype could be an interesting focal point for additional or rather optimized CMV prophylactic strategies. However, whether this approach offers therapeutic or prophylactic benefits, needs to be elucidated in future investigations.

Limitations

The limitations of this study must also be mentioned. First, unrecognized selection bias, inherent to many genetic association studies, cannot be entirely excluded. Second, our study was almost exclusively conducted on patients of European-Caucasian descent, and therefore, findings cannot be generalized to subjects of other ancestries. Third, although all patients were treated with a rather standardized multimodal regimen, undetected confounding factors might have distorted the results because of the multidimensionality of solid organ transplantation, immunosuppression, and immune responses against CMV infection. However, the single center nature of this study might be an advantage as it limits the varied protocols that can be used when treating kidney transplant recipients. Finally, the observational design, the absence of a reasonable control group, and lack of histologic and mechanistic examinations precludes verification of the causality and underlying mechanisms. Additional studies, especially to uncover mechanistic insights, are needed to further asses the effect of *AQP5* expression on inflammation and immune cell migration, as it relates to CMV infection risk.

Conclusions

During opportunistic CMV infections attributed to immunosuppression after kidney transplantation, the C-allele of the *AQP5* −1364A/C promoter polymorphism is independently associated with an increased 12-months infection risk. These findings emphasize the importance of genetic variations as additional risk factors of CMV infection after solid organ transplantation, which might also facilitate the discovery of novel therapeutic targets. Consequently, increasing *AQP5* expression in AC and CC genotypes could be an interesting therapeutic approach for organ transplant recipients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

TR, HN, PS, RV, MA, and LB: conceived and designed the research. TR, HN, KR, BK, and LB: performed the experiments.

KR and BK: contributed the reagents. TR, PS, RV, MA, and LB: collected and provided the clinical data. TR, HN, BK, PS, MA, and LB: interpreted the data. TR, HN, MA, and LB: performed the statistical analysis. TR, HN, PS, and LB: wrote the initial draft. All authors critically revised and approved the manuscript and are accountable for the accuracy and integrity of the work.

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MINI REVIEW
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Effects of Antirejection Drugs on Innate Immune Cells After Kidney Transplantation

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Over the last decades, our understanding of adaptive immune responses to solid organ transplantation increased considerably and allowed development of immunosuppressive drugs targeting key alloreactive T cells mechanism. As a result, rates of acute rejection dropped and short-term graft survival improved significantly. However, long-term outcomes are still disappointing. Recently, increasing evidence supports that innate immune responses plays roles in allograft rejection and represents a valuable target to further improve long-term allograft survival. Innate immune cells are activated by molecules with stereotypical motifs produced during injury (i.e., damage-associated molecular patterns, DAMPS) or infection (i.e., pathogen-associated molecular patterns, PAMPs). Activated innate immune cells can exert direct pro- and anti-inflammatory effects, while also priming adaptive immune responses. These cells are activated after transplantation by multiple stimuli, including ischemia-reperfusion injury, rejection, and infections. Data from animal models of graft rejection, show that inhibition of innate immunity promotes development of tolerance. Therefore, understanding mechanisms of innate immunity is important to improve graft outcomes. This review discusses effects of currently used immunosuppressive agents on innate immune responses in kidney transplantation.

Keywords: innate immunity, kidney transplantation, calcineurin inhibitors, mTOR-inhibitors, mycophenolate mofetil, glucocorticoids

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INTRODUCTION

For many years, strategies to prevent allograft rejection have focused purely on preventing adaptive immunity. Recent evidence has increasingly indicated that pure focus on T and B cells is not sufficient to improve long-term renal transplant outcomes (1–3). Innate immune cells (e.g., dendritic cells, monocytes, macrophages, neutrophils, NK cells), via numerous mechanisms, play an important role in all major immunological events following kidney transplantation (4). During the peri-transplant period, innate immunity is activated by donor brain death, ischemia-reperfusion injury, immunosuppression non-adherence, and infections—all of which increase risk for acute rejection (4–6). Late post-transplant innate immune cells produce an inflammatory microenvironment either in response to ongoing adaptive immune responses (e.g., chronic antibody mediated rejection) or independently, that enhances chronic allograft damage (7).

Innate immune cells are activated by common mechanisms. Molecules with stereotypical motifs produced during injury (i.e., damage-associated molecular patterns, DAMPS) or infection

(i.e., pathogen-associated molecular patterns, PAMPs) initiate a variety of inflammatory events, including diapedesis, inflammatory cytokine production, and cell death (8). These pattern recognition receptor (PRR)-mediated inflammatory responses are necessary for microbial clearance. However, occurring post-transplant and resulting from release of endogenous PRR ligands, so-called "sterile inflammation," they can lead to severe and often irreversible graft tissue damage and fibrosis (8, 9). Importantly, these phenomena provide a link to adaptive immune responses through induced costimulatory molecule expression and cytokine-mediated "help."

Herein, we will review relevant literature regarding the impact of the main immunosuppressive agents employed in the maintenance phase of kidney transplantation (calcineurin inhibitors, mycophenolate mofetil/mycophenolic acid, corticosteroids, and mTOR inhibitors) on innate immune responses.

CALCINEURIN INHIBITORS

Calcineurin inhibitors (CNI), such as tacrolimus (TAC, FK-506) and cyclosporine A (CsA), still represent the mainstay of immunosuppression in kidney transplantation. Their dominant mechanism of action is the inhibition of Nuclear Factor of Activated T-cells (NFAT) phosphorylation, with consequent reduction of IL-2-mediated T lymphocyte activation and proliferation (10, 11). CNI may also inhibit cytokines secretion and effective antigen presentation in innate immune cells, reducing their T cell priming capacity (12, 13). All these activities are primarily involved in the pathogenesis of acute rejection and other transplant-associated comorbidities.

Evidence shows that CsA blocks NFAT binding to the inducible NO synthase (iNOS) promoter, causing a reduction of iNOS expression and nitrite production in macrophages (14, 15). CsA can also down-regulate the enzyme cyclooxygenase-2 (COX-2) in the kidney, which converts arachidonic acid into prostaglandin E2 (PGE2), an inflammatory mediator that modulates vascular permeability to expedite immune cell recruitment (16, 17).

CsA reduces the secretion of pro-inflammatory cytokines tumor necrosis factor (TNF)-α and IL-12 induced by LPS in human DCs (12, 18–20) and murine Langerhans cells (21, 22). On the other hand, CsA is able to increase the production of anti-inflammatory IL-10 in bone marrow derived DCs (BMDCs) and human blood-derived DCs induced by LPS (12, 18, 21). The inhibition of IL-12 and the induction of IL-10 mediate the ability of CsA to promote an anti-inflammatory phenotype on these DCs with consequent differential regulation of effector T cell subsets.

This effect could be enhanced in patients treated with anti-thymocyte globulins. As reported by Naujokat et al. (23), DCs are potential targets of anti-thymocyte globulins (ATGs). These agents can bind cell surface receptors on DCs and regulate some of their major immunological functions.

CNI have also an inhibitory effect on Toll-like receptors (TLRs) dependent activation of monocytes/macrophages. In monocytes/macrophages from liver transplant recipients, therapeutic concentrations of CsA impaired IL-6 production in response to TLR2 and TLR7/8 activation, and TNF-α synthesis due to TLR7/8 stimulation, more than TAC (24). In renal transplant recipients, a switch from CsA to TAC caused a large monocytes/macrophages response, measured as TNF-α, IL-1β, IL-6, and IL-10 production, further supporting the higher inhibitory effects of CsA on monocytes compared to TAC (25). The impairment of TLR function affects the risk of graft rejection, infection, and disease recurrence after transplantation, and the difference impact of CsA and TAC on monocytes should be considered in the choice of immunosuppressant therapy in order to improve the outcomes (24).

Moreover, although there are not conclusive findings regarding the effects of maintenance immunosuppressive drugs on innate immunity and their impact on ischemia reperfusion, the study of Yang et al. (26) suggested that CsA was ineffective to control innate immunity following ischemia reperfusion injury (IRI). In fact, this medication increased the infiltration of Endothelin-1 (ED-1+) (a specific rat monocyte/macrophage marker) cells in tubulointerstitium and periglomerular areas in rat kidneys undergoing IRI. Centrally, TAC had an opposite effect. A similar trend was seen for several inflammation cytokines (26).

CNI may also influence immune cells by affecting their mitochondrial function (27). In macrophages, mitochondrial cardiolipin, ROS, and DNA trigger IL-1β secretion by activating the NLRP3 inflammasome (28) and mitochondrial antiviral signaling protein (MAVS) oligomerization, inducing type I IFN production (29) and NFκB activation (30). CsA inhibits inflammasome activation preventing mitochondrial membrane permeability transition (MPT), thereby reducing inflammatory cytokine secretion (28).

In neutrophils CNI are able to inhibit ROS generation and the formation of Neutrophil Extracellular Traps (NET) (31), causing important functional or pathological effects. In $Rag2^{-/-}$ mice, lacking B and T cells, CNI treatment induced a rapid development of *Candida albicans* infections, indicating that CsA impairs specific anti-fungal functions in innate immune cells (32). More specifically, mice lacking calcineurin activity in neutrophils were defective in the ability to kill *Candida albicans* indicating that CsA may directly influence neutrophil killing processes (32).

Currently, overall mortality due to fungal infections in transplant patients varies between 25 and 80%, with Candida and Cryptococcus species being the most commonly identified yeasts (33).

The higher doses of immunosuppressive medications in the first 6 months after transplantation are major causes of fungal infections. *Ex vivo* studies revealed that CsA damages human neutrophil clearance of *Aspergillus fumigatus* (another important cause of post-transplant opportunistic infections) (34), and that this effect is more evident in patients reaching high CNI trough levels. Inhibition of neutrophils activity by CNI may be, at least in part, responsible for increased risk of post-transplant fungal infections.

CNI do also affect NK cells in kidney transplant recipients (35). Zhang et al. have demonstrated that the expression levels

of TNF-related apoptosis-inducing ligand (TRAIL) and FasL, potent apoptosis inducers, increase in NK cells at day 5 after transplantation, while their levels return to baseline on day 13 post-kidney transplantation (36). The authors also demonstrated that in supernatants generated from mixed lymphocytes culture (MLC) and on the surface of activated lymphocytes (particularly on NK cells) there was a significant increment of the expression of TRAIL and FasL. This condition was considerably reduced by adding CsA (500 ng/mL) at the beginning of MLC, an effect that could, at least in part, be implicated in the antirejection properties of CsA (36).

CsA inhibits the NK cells proliferation in a dose-dependent manner (37). Morteau et al. showed that *ex vivo* treatment of NK cells from healthy controls with CNI inhibits their degranulation and IFN-γ production. Similar functional impairment was observed in NK cells from CNI-treated patients. This could have dramatic effects on the NK cells capacity of killing transformed or virus-infected cells and producing pro-inflammatory cytokines and could, at least in part, explain the increased risk of opportunistic infections and tumors of CNI-treated patients (38).

MYCOPHENOLATE MOFETIL/MYCOPHENOLIC ACID

Currently, mycophenolate mofetil (MMF) and its active metabolite mycophenolic acid (MPA), are the most widely used drugs in transplantation (39, 40). MMF/MPA are considered specific anti-lymphocytes agents, since they reduce the *de novo* guanosine nucleotide synthesis by selectively inhibiting the inosine monophosphate dehydrogenase (IMPDH), mainly expressed by T- and B- cells (41, 42).

When exposed to MMF/MPA, monocytes show lower levels of pro-inflammatory cytokine IL-1β and altered polarization, with enhanced expression of surface markers (like CD163 and CD200R), generally associated with an anti-inflammatory function (M2 phenotype) (43). Additionally, MMF/MPA-exposed monocytes down-regulate several adhesion molecules, like ICAM-1, and display a weaker binding to cultured human umbilical vein endothelial cells (HUVEC) (44). Treating HUVECs alone with MMF/MPA does not reduce the adhesion of activated monocytes, reinforcing the idea of a direct effect of these compounds on monocytes (45).

In a mouse model of renal IRI, MMF down-regulated TLR4 expression on monocytes surface, along with plasma level of several cytokines (IL-6, MCP-1, and TNF-α). This resulted in milder kidney damage, as defined by creatinine levels and histological findings at 48 h after IRI (46).

MMF also reduces the LPS-induced expression of MHC-II on monocyte surface, suggesting a reduced activity as antigen presenting cells (44). In the presence of increasing MMF concentrations, human monocyte-derived dendritic cells (hMDDC) showed progressively less reactive phenotype. MMF treatment lowers the expression of costimulatory molecules (CD40, CD80, CD86), adhesion proteins (ICAM-1) and maturation markers (CD83, CD206), and decreases the synthesis of proinflammatory cytokines (TNF-α, IL-10, IL-12, IL-18) and alloreactive T-cells stimulation (47). When exposed to MMF, monocytes do also display higher rates of apoptosis (48).

MPA and MMF have similar effects on hMDDCs activation and maturation, but MMF reduces, instead of increasing, IL-10 synthesis. This may support the concept that MPA has stronger protolerogenic effects on monocytes compared to MMF (49). It is likely that these effects are independent of IMPDH inhibition.

MMF/MPA have also modulating effects on NK cell activity. Similarly to mTOR inhibitors, they significantly reduce the proliferation of these cells and inhibit the expression of CD56, associated with a highly reactive phenotype (50, 51). Accordingly, NK cells treated with these agents lose their cytotoxicity against K562 bone marrow target cells and reduced IFN-γ production upon target encounter (50, 51).

Taking together, these data suggest that MMF/MPA impair differentiation, maturation and function of various innate immunity cells, which may represent an additional mechanism of their immunosuppressive effects. Whether similar mechanisms are shared with azathioprine, an antiproliferative agents with similar antirejection effects (52), is unclear.

GLUCOCORTICOIDS

Glucocorticoids (GCs) are anti-inflammatory drugs employed in both induction and maintenance phase of immunosuppression after kidney transplantation. They inhibit the inflammatory response and leukocyte migration into inflamed tissues. They also accelerate resolution of inflammation by inhibiting vascular permeability and leukocyte distribution/trafficking, and by modulating death/survival and cellular differentiation programs (53, 54).

Until recently, it has been thought that the anti-inflammatory effects of GCs were linked to their ability to inhibit regulator of genes encoding pro-inflammatory cytokines (e.g., NFκB and AP-1) through a mechanism called "transrepression" (55). However, additional mechanisms include: (1) transcription of genes able to negatively interfere with the synthesis of inflammatory mediators; (2) repression of genes mediating immune cells activation; (3) synergism between glucocorticoid receptor and transcription factors leading to the induction of anti-inflammatory genes (56, 57).

Glucocorticoids may also have direct effects on innate immune cells. *In vitro*, methylprednisolone-treated monocytes show increased expression of anti-inflammatory cytokines, like IL-10, with concomitant down-regulation of TNF-α, IL-1β, and IL-12 (58–60). Furthermore, GC-treated monocytes show lower expression levels of CD80 in response to inflammatory stimuli, which impairs their antigen-presenting activity (61). *In vivo*, data from methylprednisolone-treated kidney transplant patients, show increased numbers of $CD14^{++}CD16^{-}$ (classical) and $CD14^{++}CD16^{+}$ (intermediate) monocytes, while the $CD14^{+}CD16^{++}$ (non-classical) population is declined compared to patients receiving CNI, MMF/MPA or mTOR inhibitor (62). This is consistent with recent observations showing a downregulation of TLR4 level on the surface of GC-treated monocytes. TLR4 is a pivotal element of the monocyte activation

during sepsis, as well as in the acquired immune response to transplanted organs (63). GCs reduce the *in vitro* expression of TLR4 and the response to endotoxin in monocytes through the mediation of micro-RNA (MiR) 511-5p, a keystone in the anti-inflammatory effect of GCs (64).

GC affects also DC differentiation and maturation. In fact DC differentiated from human monocytes in presence of dexamethasone expressed lower levels of CD83 and CD86, lower APC function and a lower capacity to secrete TNF-α and IL-1β induced by CD40L and LPS than untreated cells (65, 66).

It is well-known that the administration of GCs induces neutrophilic leukocytosis, in particular by promoting neutrophil maturation and mobilization (67), an effect that is blocked by simultaneous inhibition of the L-selectin adhesion protein (68, 69).

The entire neutrophil activation process is also inhibited by GCs that reduce the expression of enzymes related to respiratory burst, such as NADPH oxidase, iNOS and COX-2 (70–73), as well as processes of chemotaxis, phagocytosis, and cytokines secretion (74, 75). In neutrophils, GCs simultaneously inhibit transcription factors related to pro and anti-inflammatory genes. The net effect is an increase in the expression of some receptors for interleukins and pro-inflammatory leukotrienes, such as IL1R1 and BLT1, (76–78), as well as a reduced sensitivity to apoptosis which increases neutrophils average life span (79).

NK are also sensitive to the effects of endogenous glucocorticoids under stress conditions, when steroids reduce NK cytolytic activity (80–82). Recent evidence shows that GCs can also induce the synthesis of pro-inflammatory cytokines through an epigenetic mechanism in NK cells. In particular, the expression of IL-6 and INF-γ is increased, along with a greater histone acetylation in the enhancer regions of these genes, which are thus more easily accessible to activating transcription factors (83, 84).

THE MAMMALIAN TARGET OF RAPAMYCIN INHIBITORS: SIROLIMUS AND EVEROLIMUS

The mammalian target of rapamycin (mTOR) is part of 2 different complexes (mTORC1 and mTORC2) with diverse signaling networks. mTORC1 promotes anabolic cellular metabolism stimulating synthesis of proteins, lipids, and nucleotides and, at the same time, inhibits catabolic processes, such as lysosome biogenesis and autophagy. mTORC2 controls cell survival, cytoskeleton organization, lipogenesis, and gluconeogenesis (85). In organ transplantation mTOR inhibitors, Sirolimus, and Everolimus, exert their immunosuppressive functions by preferentially inhibiting mTORC1 (86) thereby ostensibly halting protein translation necessary for effector T cell proliferation. Additional experimental and clinical experience with mTOR inhibitors support that they exert effects on graft survival, both beneficial and detrimental, in part by acting on innate immune cells (87, 88). Via changes in antigen presentation and costimulatory molecules, cytokine production, and metabolic pathways, mTOR inhibitors produce extensive, and sometimes conflicting, effects on innate immune cells.

The mTOR network allows innate immune cell maturation and costimulatory molecule expression during inflammation (89). As might be predicted, treatment with mTOR inhibitor impairs DCs maturation after LPS stimulation by reducing translation, including that of MCH-II and costimulatory molecules (90). Rapamycin hampers functional and phenotypic maturation of DCs prompted by IL-4, LPS, or CD40 ligation (91–93) and impairs their ability to stimulate effector T cell proliferation. Similarly, DC development induced by fms-like tyrosine 3 kinase ligand (Flt3L), a powerful DC growth factor, is inhibited by rapamycin (93, 94). Accordingly, the DCs antigens uptake activity is impaired which further contributes to damaged allogeneic T lymphocytes stimulation (90, 95).

Conversely, mTOR inhibitors indirectly inhibit regulation of autophagy and promote this degradation with immunoregulatory capabilities. Importantly autophagy is a well-known contributor to both MHCII presentation and MHCI cross-presentation of exogenous peptides (96, 97). Increased antigen presentation increases the risk of activating adaptive immune responses and is an unintended and unwanted consequence of mTOR inhibitor use. In a murine liver transplant model, use of autophagy inhibitors improved graft and animal survival, although whether this was mediated by MHC presentation effects is unknown. Regardless, induction of innate immune cell autophagy is potential counterproductive side effect of mTOR inhibitor (98).

In immature DCs, mTOR inhibitors induce apoptosis by blocking the granulocyte-macrophage colony-stimulating factor (GM-CSF) signaling. Disruption in GM-CSF/PI3K/mTOR pathway produces a pro-apoptotic state, unbalancing anti- and pro-apoptotic mediators by reduction of the mitochondrial membrane potential (99). In mature DCs, PI3K/mTOR inhibition with increasing drug concentrations down-regulates progressively several pro-inflammatory cytokines of the monocytic/macrophagic repertoire, in parallel with the reduction of phosphorylated Akt and p706K levels (90, 100, 101). In addition, mTOR inhibitor causes apoptosis in both human monocyte-derived and CD34+-derived DCs, without any effect in macrophages or myeloid cell lines (102).

mTOR inhibitors inhibit NK cell inflammatory capabilities by inhibiting their cytokine expressing and cytotoxic function. In particular, rapamycin impaired growth of the $CD56^{bright}CD16^{+/-}$ NK cell subset (associated with enhanced cytokine production) without affecting the amount of $CD56^{dim}CD16^{+}$ cells subset (with more cytotoxic capacity). With regards to the cytotoxic subset, mTOR inhibitors prevented NK cell expression of NKG2A and NCR (51). Absence of receptor ligation (if present) by target cells induces NK cell cytotoxic activity against the target cell. Prevention of their expression by mTOR inhibitors, therefore, impairs NK cell cytotoxic functioning. Overall, these results demonstrate that, mTOR inhibitors have distinct deleterious effects in immune cells which may have important implications in transplantation (51).

TABLE 1 | Main effects of the immunosuppressive drugs on innate cells.

Drug	Dendritic cells	Phagocytes	Natural Killer (NK)
CNIs	Reduce LPS-induced secretion of pro-inflammatory cytokines TNF-α, IL-12 (12, 18–20).	Impair IL-6 and TNF-α production in response to TLR2 and TLR7/8 activation in **monocytes/macrophages** (24).	Reduce the expression levels of TNF-related apoptosis-inducing ligand (TRAIL) and FasL (36).
	Increase LPS-induced production of IL-10 in bone marrow derived DCs (BMDCs) and human blood-derived DCs (12, 18, 21).	Inhibit inflammasome activation preventing membrane permeability transition (MPT) in **monocytes/macrophages** (28).	Inhibit proliferation of NK cells in a dose-dependent manner (37).
	These effects may promote an anti-inflammatory phenotype on DCs that may lead to differential regulation of effector T cells subsets.	Inhibit **neutrophil's** reactive oxygen species generation and the formation of Neutrophil Extracellular Traps (NET) (31). This effect on neutrophil activity may be responsible for increased risk of post-transplant fungal infections.	Inhibit degranulation and IFN-γ production (38).
MMF/MPA	Lower the expression of costimulatory molecules (CD40, CD80, CD86), adhesion proteins (ICAM-1) and maturation markers (CD83, CD206) (47).	Inhibit IL-1β production and enhance the expression of surface markers of M2 phenotype (CD163 and CD200R) in **monocytes** (43).	Reduce proliferation of NK cells and inhibit the expression of CD56 (50, 51).
	Decrease the synthesis of proinflammatory cytokines (TNF-α, IL-10, IL-12, IL-18) (47).	Down-regulate adhesion molecules, like ICAM-1 in **monocytes** and inhibit their adhesion to endothelial cells (44).	Reduce cytotoxicity against K562 bone marrow target cells and IFN-γ production upon target encounter (50, 51).
	MMF reduces IL-10 synthesis (49).	Down-regulate TLR-4 expression on **monocytes** surface in a mouse model of Ischemia reperfusion injury resulting in milder kidney damage (46).	
		Reduce the LPS-induced expression of MHC-II on **monocyte** surface (44).	
		Induce apoptosis in **monocytes** (48).	
GCs	Reduce the production of TNF-α, IL-1β induced by CD40L and LPS (65, 66).	Increase expression of anti-inflammatory cytokines (IL-10) with concomitant down-regulation of TNF-α, IL-1β, IL-12 in **monocytes** (58–60).	Reduce NK cytolytic activity (80–82).
	Inhibit the LPS-induced up-regulation of costimulatory molecules (e.g., CD40, CD80, CD83, CD86, and MHC-II) (65, 66).	In **monocytes** GCs reduce the expression of CD80 in response to inflammatory stimuli which impairs their antigen-presenting activity (61).	Through an epigenetic mechanism GCs induce the synthesis of pro-inflammatory cytokines (83, 84).
	DC differentiated in the presence of GC are not able to induce the proliferation of allogeneic CD4 T cells (65, 66).	In kidney transplant patients, increase the number of CD14++CD16- and CD14++CD16+ **monocytes** while the CD14+CD16++ population is declined compared to patients receiving CNI, MMF/MPA or mTOR inhibitor (62).	
		Down-regulate TLR4 expression on the surface of **monocytes** and their response to endotoxin (64).	
		Inhibit activation process of **neutrophils** by reducing the expression of NADPH oxidase, iNOS, COX-2 (70–73).	
		Reduce chemotaxis, phagocytosis and cytokines secretion in **neutrophils** (74, 75).	
		Increase the expression of some receptors for interleukins and pro-inflammatory leukotrienes such as IL1R1 and BLT1 in **neutrophils** (76–78).	
		Reduce sensitivity to apoptosis which increases **neutrophils** average life span (79).	
mTOR inhibitors	Impair DC maturation after LPS stimulation by reducing translation, including that of MHC-II and costimulatory molecules (90).	In LPS-stimulated human **monocytes** reduce chemokines synthesis such as MCP-1, RANTES, IL-8, and MIP-1 (103).	Inhibit NK proliferation and cytotoxicity capacity (51).
	Prevent phenotypic and functional maturation induced by IL-4, LPS, or CD40 ligation (91–93).		

(Continued)

TABLE 1 | Continued

Drug	Dendritic cells	Phagocytes	Natural Killer (NK)
	Inhibit DC development induced by Flt3L (93).		
	Impair antigen uptake contributing to damage allogeneic T lymphocytes stimulation (95).	Induce the up-regulation of pathways involved in production of nitric oxide, reactive oxygen species and IL-12 in **macrophages** (105).	Inhibit the shift toward an overall NKG2A+KIR-NCR+ phenotype and maintain an overall NKG2A-KIR+NCR+/– (51).
	Disinhibit autophagy that contributes to both MHCII presentation and MHCI cross-presentation of exogenous peptides (96, 97).		
	Induce apoptosis in immature DC by blocking GM-CSF signaling (99).		
	Increase surface expression of chemokine receptor CCR7 promoting DC migration into lymphoid tissue (108).		

An essential innate immune cell role involves production of cytokines. The mTOR inhibitors have pleiotropic effects that depend on the cells and circumstances studied. In LPS-stimulated human monocytes, mTOR inhibitors reduce several pro-inflammatory chemokines synthesis such as MCP-1, RANTES, IL-8, and MIP-1 (103). Fine-needle aspiration biopsy (FNAB) samples (containing mononuclear cells together with kidney parenchymal cells) obtained from kidney transplant recipients receiving sirolimus showed lower synthesis of many proinflammatory cytokines, including IL-6 and MCP-1, and higher production of TGF-β than samples from patients whose regimen contained MMF (104). Conversely, switch from a CNI based to a mTOR inhibitor-based regimen may worsen post-transplant inflammation. Gene expression profile on kidney samples showed the upregulation of pathways involved in production of NO, ROS, and IL-12 in macrophages and the activation of the adaptive immune response. Histological analysis confirmed a higher macrophages infiltration (105). Similarly, after shift from CsA to Sirolimus, the transcriptomic analysis on peripheral blood leucocytes showed a significant enrichment in pro-inflammatory pathways related to NFκB and specific transcripts for monocyte and NK cells (106). It is noteworthy that the concomitant administration of mTOR inhibitors and GCs seem to cause a state of innate immune cell hyper-responsiveness, as if GCs action is override by the inhibition of mTOR (107).

mTOR inhibitor modulation of innate immune cells may contribute to a pro-tolerogenic state in the early phases of transplantation. Sordi et al. (108) showed that sirolimus, at clinically relevant concentrations and in contrast to calcineurin inhibitors, enhances the expression of CCR7 on the surface of human and mouse derived DCs with consequent expedite migration of DCs into lymphoid tissue. This condition may promote the tolerogenic effect of mTOR inhibitors, because these immune cells may reach appropriate T cell areas in the lymphoid tissue (109). Recent evidence challenged long-held notions that immunological memory is a feature exclusively for adaptive immunity. Evidence in monocytes showed that beta-glucan (a fungal antigen) experienced monocytes developed epigenetic changes spurred by accumulation of a cholesterol intermediate, mevalonate (110). Epigenetic changes were dependent on activation of mTOR to induce necessary downstream metabolic and histone changes. Importantly, using a strategy that included innate immune targeting rapamycin loaded nanoparticles, Braza et al. prevented macrophage trained immunity and extended graft survival indefinitely. These findings lead to an intriguing possibility that short term myeloid-specific nanoimmunotherapy that targets mTOR inhibitor in post-transplant may extend graft survival by preventing trained immunity generation (111).

There are controversial data about the impact of the m-TOR inhibitors on the regulation of I/R injury-related innate immune system in kidney transplantation. Several authors have suggested that these drugs may impair recovery of kidney function (112–114) because of an anti-proliferative effects on tubular cells and an hyper-expression of several pro-inflammatory cytokines (e.g., IL1-β, IL-12, TNF-α) and an inhibition of the production of anti-inflammatory cytokines as IL-10. On the contrary, Macedo et al. have reported that m-TOR inhibitors may protect from innate immunity activation (115). In particular the inhibition of mTOR may induce resistance to phenotypic maturation of DCs induced by inflammation and may facilitate the production of regulatory tolerogenic DCs.

CONCLUSIONS

In the past, great strides in allograft survival prolongation were attributed to successful suppression of adaptive immune responses (**Table 1**). A great body of literature, both clinical and basic science, attests to profound and diverse effects of modern immunosuppressive agents on innate immune cells. To make further progress improving transplant outcomes requires a more complete understanding of these effects and attempts to blunt current insufficiencies or vulnerabilities. As an example, clinical trials using monoclonal antibodies against innate immune receptors TLR2 (NCT01794663) and/or TLR4 (NCT01808469) to prevent delayed graft function and innate immune cell based therapies (including administration of regulatory macrophages and tolerogenic DCs) (116) may lead to new therapeutics that become standard of care to decrease the need for, or

even completely replace, current immunosuppression regimens. These efforts to enlarge the post-transplant armamentarium by targeting innate immune cells will ideally lead to prolonged allograft function and minimized immunosuppression that extend allograft longevity without overly immunosuppressing and endangering the patient.

AUTHOR CONTRIBUTIONS

GZ, JL, and PC searched the literature and wrote the manuscript. LS contributed to the literature search and literature analysis. GG and PC revised the manuscript. All authors read and approved the final manuscript.

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Labile Heme Aggravates Renal Inflammation and Complement Activation After Ischemia Reperfusion Injury

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Background: Ischemia reperfusion injury (IRI) plays a major role in solid organ transplantation. The length of warm ischemia time is critical for the extent of tissue damage in renal IRI. In this experimental study we hypothesized that local release of labile heme in renal tissue is triggered by the duration of warm ischemia (15 vs. 45 min IRI) and mediates complement activation, cytokine release, and inflammation.

Methods: To induce IRI, renal pedicle clamping was performed in male C57BL/6 mice for short (15 min) or prolonged (45 min) time periods. Two and 24 h after experimental ischemia tissue injury labile heme levels in the kidney were determined with an apo-horseradish peroxidase assay. Moreover, renal injury, cytokines, and C5a and C3a receptor (C5aR, C3aR) expression were determined by histology, immunohistochemistry and qPCR, respectively. In addition, *in vitro* studies stimulating bone marrow-derived macrophages with LPS and the combination of LPS and heme were performed and cytokine expression was measured.

Results: Inflammation and local tissue injury correlated with the duration of warm ischemia time. Labile heme concentrations in renal tissue were significantly higher after prolonged (45 min) as compared to short (15 min) IRI. Notably, expression of the inducible heme-degrading enzyme heme oxygenase-1 (HO-1) was up-regulated in kidneys after prolonged, but not after short IRI. C5aR, the pro-inflammatory cytokines IL-6 and TNF-α as well as pERK were up-regulated after prolonged, but not after short ischemia times. Consecutively, neutrophil infiltration and up-regulation of pro-fibrotic cytokines such as CTGF and PAI were more pronounced in prolonged IRI in comparison to short IRI. *In vitro* stimulation of macrophages with LPS revealed that IL-6 expression was enhanced in the presence of heme. Finally, administration of the heme scavenger human serum albumin (HSA) reduced the expression of pro-inflammatory cytokines, C3a receptor and improved tubular function indicated by enhanced alpha 1 microglobulin (A1M) absorption after IRI.

Conclusions: Our data show that prolonged duration of warm ischemia time increased labile heme levels in the kidney, which correlates with IRI-dependent inflammation and up-regulation of anaphylatoxin receptor expression.

Keywords: ischemia reperfusion injury, AKI, HO-1, C5aR, C3aR, complement

INTRODUCTION

Ischemia reperfusion injury (IRI) is a major complication in solid organ transplantation (1). Cold ischemia time (CIT) for kidney allografts after post mortal donation can reach >20 h which mediates delayed graft function (DGF). In contrast, living donation has only 2–3 h CIT and lower rates of about 5% DGF (2). Notably, prolonged warm ischemia times have been linked to DGF observed in obese recipients who have undergone complicated surgeries (3). Additionally, IRI-mediated acute kidney injury (AKI) is a frequently encountered complication in other forms of solid organ transplantation such as lung transplantation (4).

The kidney is very sensitive to hemolysis-mediated damage (5). Hemolysis- associated hemoglobin cast nephropathy has been reported in renal biopsies of patients with various conditions such as autoimmune hemolytic anemia, paroxysmal nocturnal hemoglobinuria, transfusion of incompatible blood, disseminated intravascular coagulation (DIC) and in hemoglobinopathies (6). Hemolytic uremic syndrome (HUS) also leads to chronic kidney disease (CKD) and even end stage renal disease (ESRD) (7). Enhanced nephrotoxicity has also been documented in an experimental model of trauma hemorrhage (8). Upon hemolysis extracellular hemoglobin gets oxidized to methemoglobin and releases its prosthetic heme group. This fraction of "free or loosely" bound heme also termed "labile" is biologically active and high levels of labile heme are considered to be cytotoxic and to aggravate inflammation and tissue injury (9, 10). In support of this notion are the findings that the deficiency of the heme-degrading enzyme heme oxygenase-1 aggravates renal injury in different models of nephrotoxicity (11, 12).

In the current study we hypothesized that labile heme contributes to IRI-induced kidney injury. To test this hypothesis we determined labile heme levels in the kidney in a mouse model of renal IRI. Further, we also investigated the correlation between IRI-induced labile heme levels, inflammation and AKI. A panel of early and late inflammatory and histopathological markers after short (15 min) and prolonged (45 min) warm ischemia times were determined. In addition, the effect of administration of human serum albumin (HSA) as a heme scavenger was investigated in the 45 min IRI model.

MATERIALS AND METHODS

Animals

All experiments were performed with adult male C57Bl/6 mice (11–13 weeks of age, bodyweight 23–28 g). Mice were housed and bred in the Institute of Laboratory Animal Sciences, Hannover Medical School. Mice had free access to drinking water and food. The day/night cycle was 14/10 h. Mice were monitored daily for the physical condition after surgery. The experiments were approved by the local animal protection committee of the Lower Saxony State department for animal welfare and food protection (33.19-42502-04-14/1657) which are in line with the National Institutes of Health guidelines.

Renal Ischemia Reperfusion Injury

Mice were anesthetized with isoflurane (3% induction, 1–2% maintenance), for analgesic treatment butorphanol 1 mg/kg bodyweight was given subcutaneously. IRI was induced by unilateral renal pedicle clamping with a micro aneurysm clip (Aesculap, Germany) for either 15 or 45 min. Afterwards, reperfusion was visually controlled. The contralateral not clipped kidney and sham surgery with opening of the abdominal cavity served as controls. For inhibitor experiments human albumin (Kendrion Biopharma, purity 98%), was diluted by sterile Phosphate Buffered Saline (PBS) to a final dose of 4 mg /mouse given i.v. 10 min before IRI 45 min surgery. The vehicle group received PBS injection.

Organ Preservation

Mice were sacrificed at two and 24 h after IRI and organ retrieval was done in deep general anesthesia (5% isoflurane). After midline laparotomy whole body perfusion with ice-cold 0.9% PBS via the left cannulated ventricle resulted in a circulatory arrest. Organs were dissected and fixed in RNA later, 4% paraformaldehyde or shock frozen in liquid nitrogen.

Renal Morphology and Immunohistochemistry

After paraffin embedding 2 μm sections were cut and stained. For overall morphology Perjodic Acid Schiffs (PAS) stain was done. Semi-quantitative scoring for signs of AKI was done: 0 = none or focal AKI, <5% of tubuli affected. 1 = mild AKI, 5–25% of tubuli affected. 2 = moderate AKI, 26–50% of tubuli affected. 3 = severe AKI, 51–75% of the tubuli affected. 4 = very severe AKI > 75% of tubuli affected. Immunohistochemistry was done with the following antibodies: Gr-1$^+$ for neutrophils (Ly-6G/Ly-6C$^+$, Serotec, UK), Alpha 1 Microglobulin (A1M, gift from Dr. Grams Lund University, Sweden) which is a marker of tubular transport and heme oxygenase-1 (HO-1, Enzo life sciences, Switzerland). Neutrophil infiltration was scored semi-quantitatively. 0: <5 cells/view field (VF), 1: 5–10 cells/VF, 2: 11–20 cells/VF, 3: 21–50 cells/VF and 4: >50 cells/VF. HO-1 and A1M were semi-quantitatively expressed in percentage of the affected tubuli in 10 different areas. Images were captured with

the same magnification. Five to six mice per group were used for all experiments. Analysis was conducted using a Leica imaging microscope at 200-fold magnification. Investigators were blinded to the animal group assignment.

Pro-inflammatory Cytokine Expression

For cytokine analysis total mRNA was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany). Then, cDNA was synthetized with Prime Script Reverse Transcriptase reagent (Takara, Japan) from DNase-treated total RNA. qPCR was conducted on a LightCycler 96 (Roche, Penzberg, Germany) using sybrgreen primers. The following primers were used: Interleukin-6 (IL-6, Qiagen, #QT00098875), TNFα (Qiagen, #QT00104006), Monocyte Chemoattractant Protein-1 (MCP-1, Qiagen, #QT00167832), Plasminogen-Aktivator-Inhibitor Type 1 (PAI 1, Bio Tez Berlin-Buch GmbH, fwd: 5′-ATGTTTAGTGCAACCCTGGC-3′, rev: 5′-CTGCTCTTGGTCGGAAAGAC-3′), Connective tissue growth factor (CTGF, Qiagen, #QT00096131) and HO-1 (Qiagen, #QT00159915). Complement 5a receptor 1 (C5aR1, accessionnumber: NM_007577, fwd: 5′CAGGTGACCGGGG TGATGATAGC3′rev: 5′GTAGGCCAGGGACACGCACAGG3′ and Complement 5a receptor 2 (C5aR2, accession number: NM_001173550, fwd: 5′GCTGCATACAGCACAAGCA3′, rev: 5′ACCACCACCGAGTATTATGACT3′). Complement 3a receptor (C3aR, accession number: NM_009779, fwd: 5′-GTG CTT GAC TGA GCC ATG GAG T-3′, rev: 5′-CAG CAC CAG CCC ATT GCC TA-3′), Hypoxanthine phosphoribosyl transferase (HPRT, Qiagen, #QT00166768) was used as house keeper for normalization. Five to six mice per group were analyzed.

Protein Isolation and Western Blotting

Frozen tissue samples were lysed in RIPA lysis buffer on ice, and protein was isolated. Protein quantification was done by BCA assay (Thermo Scientific, USA). Fifty micrograms of protein was loaded on a 10% polyacrylamide gel for electrophoresis. Proteins were blotted to polyvinylidene fluoride membranes (Millipore, Darmstadt, Germany). After blocking in 3% bovine serum albumin (BSA), incubation with primary antibodies was done overnight at 4°C. The following antibodies were used: pERK (44/42 kDa, 9102S), ERK1/2 (44/42 kDa, 9106S, both from Cell Signaling Technologies, USA). 14-3-3 served as loading control (28 kDa, K-19, Santa Cruz Biotechnology, USA). After washing in PBS horseradish peroxidase (HRP) conjugated secondary antibodies (Dianova, Hamburg, Germany) were incubated for 1 h at room temperature. Proteins were visualized by incubation with SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, USA). To enhance signal intensity SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, USA) was used according to the manufacturer's instructions. Bands were immediately captured by VersaDoc MP 400 System (Bio Rad, USA). Five mice per group were analyzed.

Determination of Labile Heme With an Apo-Horseradish Peroxidase (Apo-HRP) Assay

Labile heme assay was performed in 96 well plates as previously described (13, 14) with minor adaptations for measurement in tissue samples. Immediately after sacrifice the kidney tissue samples were weighed and minced in 1 ml Hank's Balanced Salt solution (HBSS). The supernatant was centrifuged at 1,500 rpm for 5 min to remove residual tissue aggregates and transferred to a fresh tube and stored at −80°C or directly applied for the assay. Briefly, 5–20 μl of the supernatant was added in a final volume of 100 μl HBSS reaction mixture containing 0.75 μM apo-HRP (BBI Solutions, Gwent, UK) and incubated for 10 min at 4°C. In parallel hemin standards (0.25–2.5 nM) in a final volume of 100 μl reaction mixture (HBSS + apo-HRP) prepared from a stock solution of 25 nM hemin (Frontier Scientific, Logan, UT, USA) were also incubated. Then, 5 μl of samples and standards were transferred to a new 96 well plate and the assay was initiated by adding 200 μl of TMB substrate. The absorbance at 652 nm was kinetically read for 2–3 min and the time point at which the highest hemin standard gave an absorbance from 1.6 to 2 was chosen for determining the concentration of labile heme in samples. The calculated concentrations of heme were normalized with the tissue weight and expressed as pmol/mg tissue wet weight. Five mice per group were analyzed.

In vitro Studies With Mouse Bone Marrow Derived Macrophages (BMDM)

Mouse bone marrow cells were differentiated into macrophages using r-MCSF as described previously (15). After 7 days of differentiation macrophages were seeded at a density of 5×10^5 cells in each well of a 6-well plate and allowed to rest overnight. Stimulation with LPS (1 μg/ml) was performed in the presence or absence of hemin at the indicated doses in medium containing 1% serum for 16 h. The cells were lysed and processed for RNA isolation.

Statistical Analysis

For statistical analysis GraphPad prism software (GraphPad Software Inc. 5.0, San Diego, CA) was used. Differences between groups were determined by one way ANOVA or student's *t*-test if two groups were compared. Data are shown as mean ± standard error (SEM). Significant differences were defined as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

RESULTS

Increased Levels of Labile Heme After Prolonged Renal Ischemia Times—Correlation With HO-1 Expression

IRI is worsened by the length of the warm ischemia time (16, 17). To investigate whether labile heme levels are detectable after renal IRI we used an apo-HRP based assay on renal tissue samples subjected to short (15 min) and long (45 min) time periods of warm IRI. Labile heme levels were significantly increased in IRI kidneys with prolonged (45 min) compared to

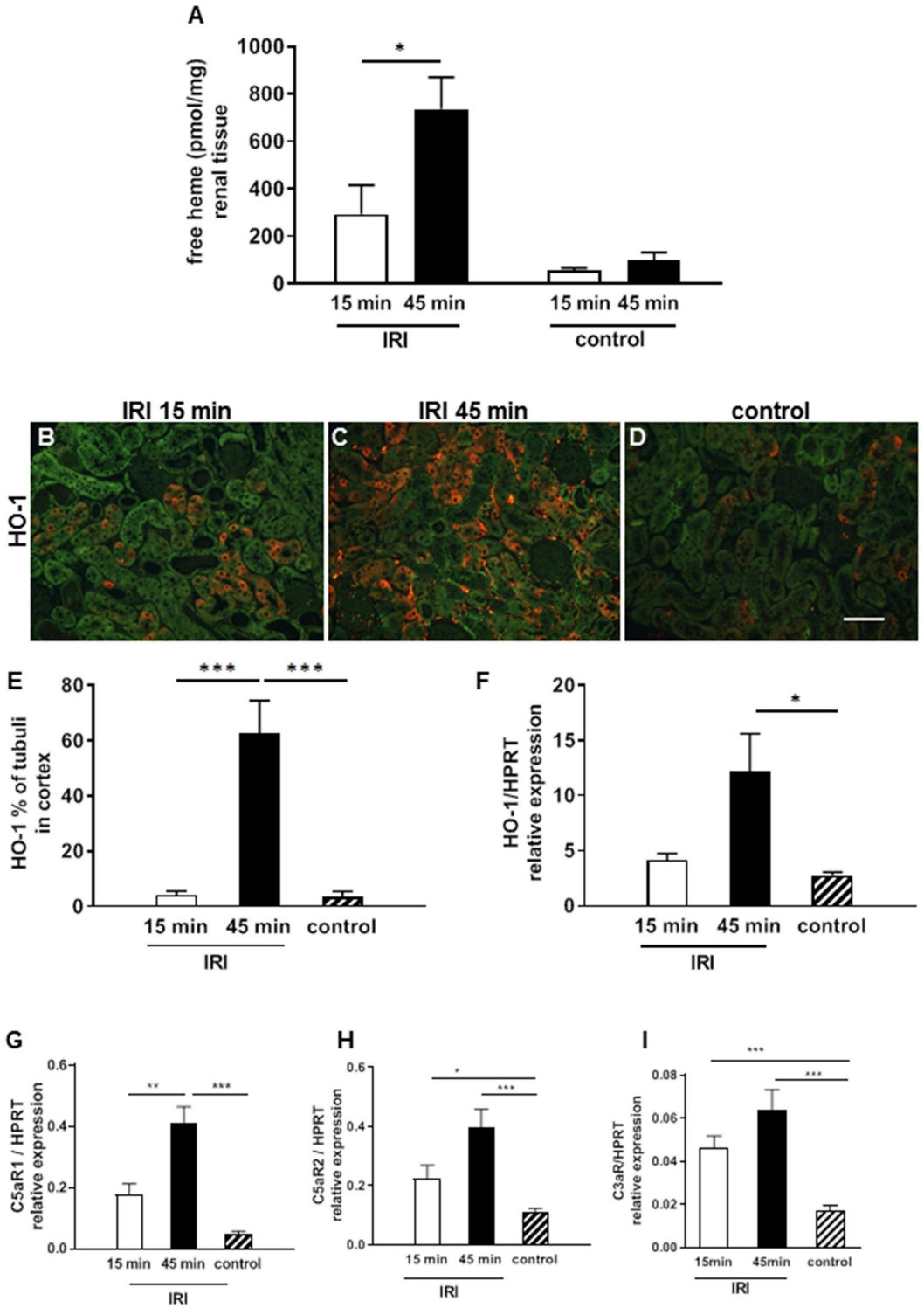

FIGURE 1 | Labile heme release and complement activation after IRI. In renal tissue labile heme was elevated after 2 h in the 15 min but even more in the 45 min IRI model **(A)**. HO-1 mRNA expression increased significantly after 45 min IRI **(F)** and also the expression of HO-1 protein on proximal tubular epithelial cells was significantly enhanced in the 45 min IRI group (**B–E**, bar: 100 μm). The anaphylatoxin receptor C5aR1mRNA expression was significantly higher after prolonged ischemia time at 24 h after IRI **(G)**. C5aR2 and C3aR showed enhanced mRNA expression after 15 and 45 min IRI compared to controls but the IRI groups did not differ **(H,I)** $n = 5$ mice/group, one way ANOVA. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

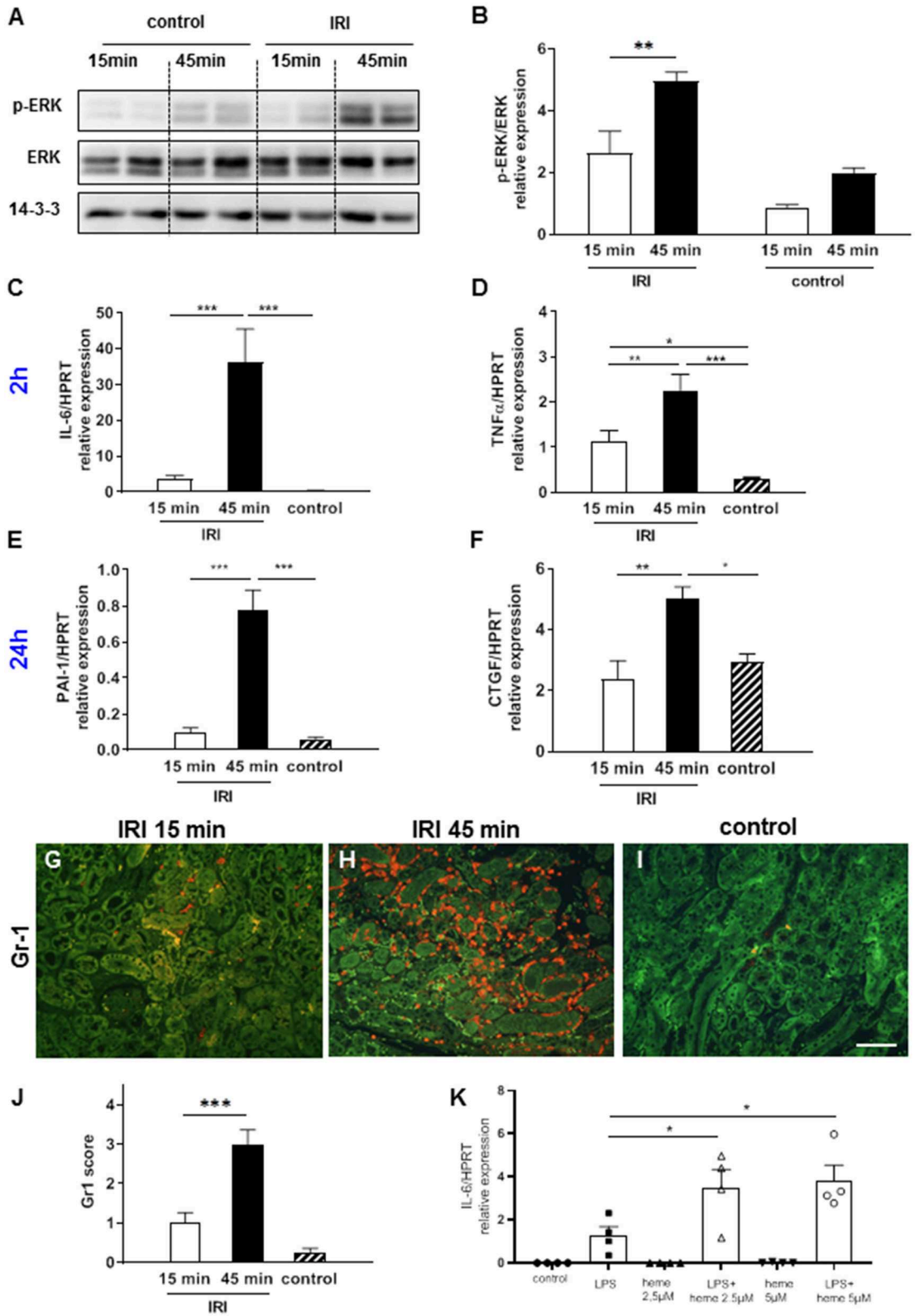

FIGURE 2 | ERK activation, cytokine expression and neutrophil infiltration after IRI. Already at 2 h after IRI ERK activation was significantly enhanced in the kidneys after 45 min compared to 15 min IRI (**A,B**, $^*p < 0.05$). Pro-inflammatory cytokines IL-6, TNF-α at 2 h **(C,D)** after IRI and pro-fibrotic cytokines PAI-1 and CTGF at 24 h

(Continued)

FIGURE 2 | (E,F) after IRI were significantly increased in 45 min compared to 15 min IRI. Neutrophil infiltration was mainly observed in the outer medulla and was more prominent after prolonged ischemia time at 24 h after IRI (**G–J**, bar: 100 μm, mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 6$ mice/group, one way ANOVA). Co-stimulation experiments with bone marrow-derived mouse macrophages revealed that heme amplified the LPS induced up-regulation of IL-6 mRNA (**K**, $^{*}p < 0.05$ LPS vs. LPS+heme, one way ANOVA).

15 min IRI (**Figure 1A**, $^{*}p < 0.05$). In contrast, labile heme levels in kidney samples after sham surgery or in contralateral non-clipped kidneys which served as controls were markedly lower. To investigate whether the determined levels of labile heme in kidneys after IRI are biologically relevant, we also determined renal expression of HO-1, which is the inducible isoform of the heme-degrading enzyme HO and is highly up-regulated by heme (18, 19). Interestingly, HO-1 protein was induced in proximal renal tubuli after prolonged, but not after short ischemia times (**Figures 1B–E**, $^{***}p < 0.001$). Similarly, HO-1 mRNA expression was significantly up-regulated in 45 min IRI (**Figure 1F**, $^{*}p < 0.05$).

In addition, the mRNA expression of anaphylotoxin receptors C5aR1, C5aR2, and C3aR were analyzed as markers of complement activation. The expression of all three receptors was significantly induced after IRI (**Figures 1G–I**, $^{***}p < 0.001$). Of note, only for C5aR1 expression the difference between 15 min and 45 min IRI reached statistical significance (**Figure 1G**, $^{**}p < 0.05$).Taken together, the data show that increased levels of labile heme after prolonged warm ischemia time correlates with up-regulation of the heme-inducible gene HO-1 and the anaphylatoxin receptors in IRI kidneys.

Regulation of Inflammation Markers After Short and Prolonged Ischemia Times in Renal IRI

Heme has previously been shown to cause activation of the MAP kinase ERK1/2 (20) and TLR4-mediated up-regulation of pro-inflammatory cytokines TNF-α and IL-6 (21, 22). Hence, we hypothesized that an increase in labile heme after prolonged IRI will also lead to ERK1/2 activation and up-regulation of TNF-α and IL-6. Accordingly, prolonged IRI but not short IRI caused significant up-regulation of pERK at the 2 h time point (**Figures 2A,B**, $^{**}p < 0.01$). Similarly, at this time-point the expression of the pro-inflammatory cytokines IL-6 and TNF-α were also markedly up-regulated after prolonged IRI in comparison to short IRI (**Figures 2C,D**, $^{**}p < 0.01$, $^{***}p < 0.001$). At 24 h expression of pro-fibrotic cytokines were also altered: PAI-1 and CTGF showed significant differences between short and prolonged IRI (**Figures 2E,F**). We also determined renal invasion of neutrophils as a marker of inflammation. Infiltration of neutrophils was most prominent in the outer medulla and Gr1 scores were 3-fold higher after 15 min IRI and 10-fold higher in 45 min IRI kidneys compared to controls at 24 h after IRI (**Figures 2G–J**, $^{***}p < 0.001$). IRI causes activation of the TLR4 signaling pathway (23) and heme might play a role in amplifying TLR4-mediated inflammatory response. To test if heme can amplify TLR-4 mediated responses we stimulated bone marrow-derived macrophages (BMDM) *in vitro* with the TLR4-agonist lipopolysaccharide (LPS) in the presence or absence of heme. The LPS-induced up-regulation of IL-6 markedly increased in a dose-dependent manner in the presence of heme (**Figure 2K**, heme at 2.5 and at 5 μM, $^{*}p < 0.05$). To summarize, these results indicate that increased labile heme correlates with the increase in inflammatory parameters after prolonged IRI.

Acute Kidney Injury After Short and Prolonged IRI

At 2 h after short IRI some signs of mild AKI with partial loss of the brush border membrane and tubular dilatation were detected (**Figures 3A,D**). As expected, prolonged IRI resulted in more severe AKI with flattening of the tubular epithelial cells, tubular cell detachment and cast formation. Major tissue damage was observed in the outer medulla where hypoxia is most severe due to the physiological lower oxygen saturation compared to the cortex (24). At 24 h after prolonged IRI the outer medulla had very severe AKI with tubular casts and interstitial inflammation, which was hardly detectable after short term IRI (**Figures 3G–K**).

Impairment of Tubular Function After Prolonged IRI

A1M is a circulating protein that is synthesized in the liver, filtered in the glomeruli and reabsorbed by healthy proximal tubular epithelial cells (pTEC). A1M was located in small vesicles in the cytoplasma of 60% of the healthy pTECs in the control groups. Already at 2 h after IRI tubular reabsorption was disturbed and A1M expression was reduced after short and virtually abolished in prolonged IRI (**Figures 4A–C,G**). The reduction of tubular reabsorption was even more pronounced and almost absent after 24 h in the prolonged IRI model. The profound tubular damage caused protein cast formation in the outer medulla which also contained A1M after prolonged IRI (**Figure 4E**).

Treatment With the Heme Scavenger Human Serum Albumin Attenuated IRI

Human serum albumin (HSA), which is routinely applied in the clinic for various medical indications, is known to bind heme with high affinity and can therefore be considered as a potent heme scavenger. To investigate whether HSA may interfere with the putative heme-mediated effects of renal IRI, HSA was administered intravenously 10 min prior to prolonged (45 min) IRI. Treatment with HSA blocked the expression of pro-inflammatory cytokines IL-6 and TNF-α (**Figures 5A,B**, $^{*}p < 0.05$). Similarly, the expression of the anaphylatoxin receptor C3aR was significantly reduced by albumin treatment (**Figures 5C,D**, $^{*}p < 0.05$). In addition, A1M staining was increased in the HSA treated IRI group (**Figures 5E–G**) indicating improved maintenance

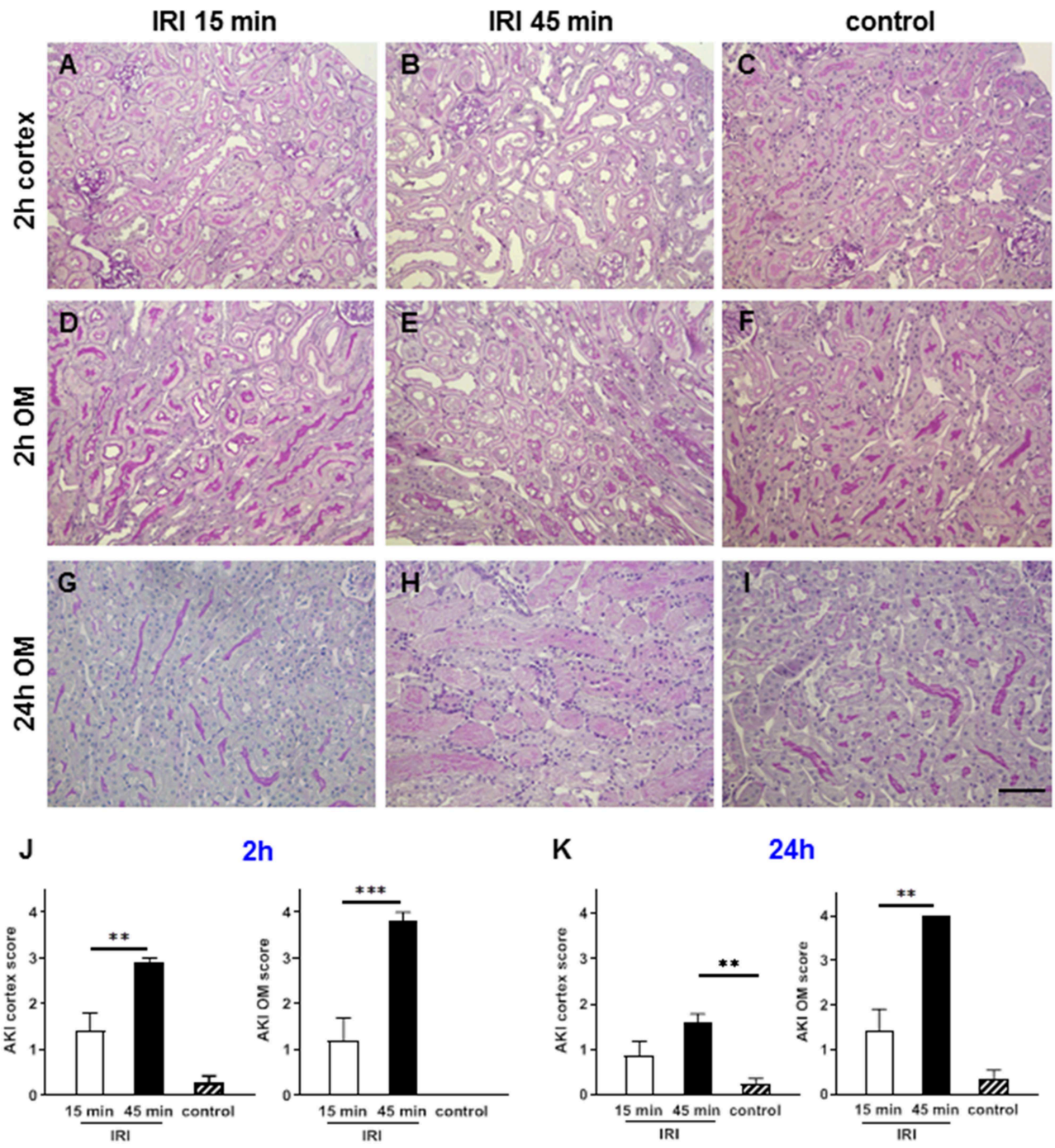

FIGURE 3 | Renal morphology at two and 24 h after IRI. PAS staining revealed that mild AKI was present already after 2 h in short term IRI (15 min, **A,D,G,J**). Longer ischemia time of 45 min caused more severe AKI mainly in the outer medulla **(B,E,H,K)**. Sham kidneys served as controls and showed normal renal morphology **(C,F,I)**. Signs of AKI were less at 24 h after IRI in this short IRI group and more pronounced in the prolonged IRI group (**G–I**, mean ± SEM, $^{**}p < 0.01$, $^{***}p < 0.001$, bar: 100 μm, $n = 6$ mice/group, one way ANOVA).

of tubular function by absorption of A1M. To summarize, these results indicate that treatment with albumin may at least partially prevent IRI-mediated inflammatory activation and damage.

DISCUSSION

Heme release has been proposed to play a role in IRI related kidney injury based on the findings that heme levels and

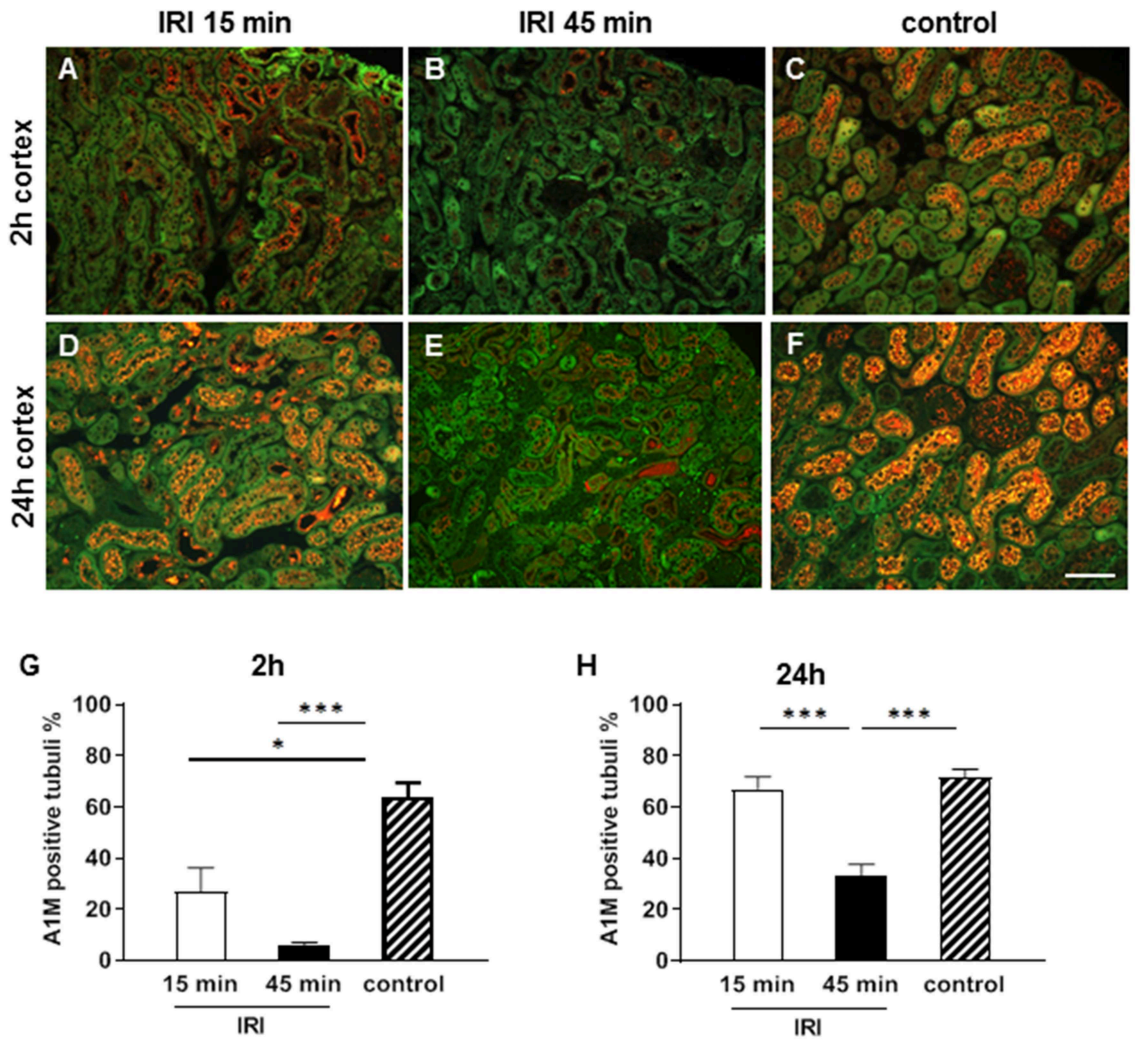

FIGURE 4 | Tubular function at two **(A–C,G)** and 24 h **(D–F,H)** after IRI. A1M is reabsorbed from the intact tubuli and is stained in small vesicles in the cytoplasm of 70% of the proximal tubuli in the cortex in the healthy state **(C,F)**. Already after 2 h after short IRI A1M expression was moderately reduced **(A)** and after 45 min IRI normal vesicular A1M staining was almost absent in the pTECs **(B)**. At 24 h vesicular A1M staining was normal again in the short term IRI group **(D)** and significantly reduced in the prolonged IRI group (**E**, bar: 100 μm, mean ± SEM, $^{*}p < 0.05$, $^{***}p < 0.001$, $n = 6$ mice/group, one way ANOVA).

HO-1 expression in the microsome (heme-rich) fraction of kidneys are increased after IRI (25). However, due to lack of a suitable method it was not possible to distinguish between bound-heme (i.e., heme in hemoproteins) and un-bound or loosely bound heme (i.e., labile heme). In particular, the latter fraction of heme has been proposed to play a detrimental role in pathophysiological conditions (26). In the current study, we extend these earlier findings and report that IRI causes an increase of the labile heme fraction in renal tissue. Importantly, the levels of labile heme correlated with the duration of warm ischemia time and with the severity of inflammation following IRI. Furthermore, treatment with the heme-scavenger albumin reduced the expression of IRI-related inflammatory markers.

Source of Labile Heme in the Kidney Following IRI

Kidney after heart has the highest mitochondrial abundance (27) and it is feasible that IRI alters mitochondrial heme synthesis to increase labile heme levels. In support of this notion are the findings that failing human hearts have increased levels of heme accompanied by increased expression of the heme synthesizing enzyme 5′-aminolevulinate synthase 2 (ALAS2) (28). Moreover, increased heme synthesis led to exacerbated injury after coronary ligation in ALAS2 transgenic mice (29). It remains to be evaluated if ischemic injury also alters heme synthesis in the kidney. Independently, an increase in labile heme levels can also be a result of heme release from unstable hemoproteins such as cytochromes due to mitochondrial damage. Corroborating this

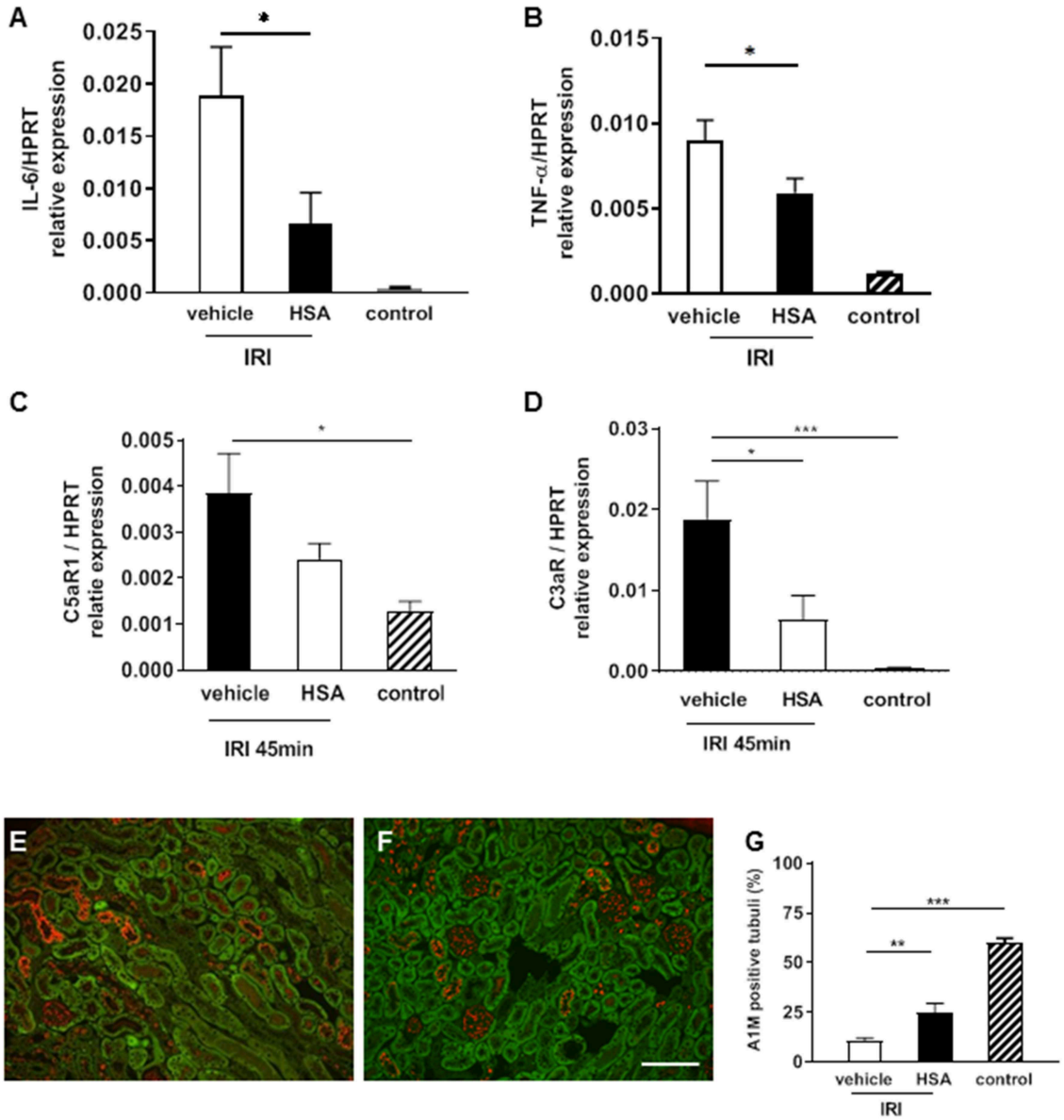

FIGURE 5 | Albumin treatment to attenuate IRI. Human serum albumin (HSA) was given intravenously 10 min prior to IRI (45 min). The pro-inflammatory cytokine IL-6 and TNF-α mRNA expression was significantly decreased in the albumin treatment group compared to the vehicle at 2 h after IRI (**A,B** $^{*}p < 0.05$). C5aR1 expression was significantly higher in vehicle treated IRI kidneys compared to control kidneys (**C**, $^{*}p < 0.05$). C3aR mRNA expression was significantly reduced by albumin treatment compared to vehicle (**D**, $^{*}p < 0.05$). The tubular function marker A1M showed higher expression after albumin treatment in proximal tubular epithelial cells after IRI (**E–G**, bar: 100 μm mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $n = 6$ mice/group, one way ANOVA). $n = 5$ mice/group, one way ANOVA.

notion are the findings that inhibitors of cytochrome P450 prevent cell injury by attenuating (heme-derived) iron release and hydroxyl radical formation after reoxygenation of kidney tubular epithelial cells *in vitro* (30). Alternatively, local hemolysis that occurs during IRI or myoglobin released during tissue injury could also contribute to the pool of extracellular labile heme. We have previously shown that prolonged IRI caused substantial impairment of renal perfusion (31) which may result in trapping and degradation of erythrocytes.

Underlying Mechanisms of Heme-Mediated Inflammation After IRI

The findings of this study indicate that the increase in labile heme levels correlates with the degree of inflammation after prolonged ischemia time. Heme can promote and aggravate inflammation by diverse mechanisms. In endothelial cells, heme induces the production of various adhesion molecules, including E-selectin, P-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1) (32, 33),

thus promoting leukocyte infiltration. In accordance with this notion, neutrophil infiltration was substantially increased after prolonged ischemia time. Additionally, heme has also been implicated in the secretion of the neutrophil chemoattractant CXCL2 (34). Neutrophil infiltration was mainly observed in the outer medulla, the area with the lowest oxygen tension and consecutively with the highest extent of hypoxia (24).

Our findings also indicate that renal expression of the anaphylatoxin receptors (C5aR1, C5aR2, and C3aR) is induced by prolonged ischemia. Both C3a and C5a are known to promote IRI (35) and C3aR and C5aR deficient mice are protected from IRI-induced damage (35, 36). As a mediator of complement activation, heme has gained increasing appreciation in recent years. Intravascular hemolysis has been shown to activate the complement system via the alternative pathway, which in turn has been implicated in the pathogenesis of atypical hemolytic uremic syndrome (aHUS) (37, 38). More recently, heme-mediated complement activation via the alternative pathway has been shown to be involved in the pro-inflammatory activation of leukocytes (39). Noteworthy, it has been reported that kidney biopsies from sickle cell disease nephropathy patients showed deposits of C3 and C5, which was also observed in a mouse model of sickle cell disease (38). It may well be that the increased expression of C5aR1, C5aR2, and C3aR observed in this study are secondary effects of heme-mediated complement activation. Further studies are required to elucidate the interplay of labile heme and anaphylatoxin receptors by using genetically modified mouse models in renal IRI and kidney transplantation (36, 40). Heme has also been proposed to be a danger associated molecular pattern (DAMP) (41) that can amplify TLR4 signaling. Accordingly, the *in vitro* findings of the current study show that the LPS-induced expression of the pro-inflammatory cytokine IL-6 is aggravated in the presence of heme in bone-marrow derived macrophages which is in accordance with a previous report in peritoneal macrophages after LPS stimulation (21).

Scavenging Labile Heme as a Therapeutic Strategy Against IRI-Mediated Kidney Injury

In this study treatment with the heme scavenger human serum albumin (HSA) prior to IRI reduced pro-inflammatory cytokine release and C3aR expression in the renal tissue after prolonged IRI. HSA is broadly used in the clinic for the treatment of patients with ascites and chronic liver disease (42, 43) and in the context of plasmapheresis in certain renal diseases (44). However, it is noteworthy that the serum protein hemopexin has a markedly higher binding affinity for heme than albumin. While hemopexin has been shown to counteract heme-mediated complement activation and pro-inflammatory responses in mouse models it has not been approved for therapeutic applications in clinical practice (45, 46). It might well be that preventive strategies using hemopexin could be more beneficial than albumin and future comparative studies are required to understand if heme scavengers can prevent renal IRI-mediated inflammation or delayed graft function after kidney transplantation. Due to the local malperfusion after IRI elaborated strategies to deliver heme scavengers to the kidney might be necessary. In the context of transplantation normothermic machine perfusion strategies (47) might prevent the release of labile heme in allografts. Additionally, the method can also be used for drug delivery to allografts prior to implantation (48). Introducing heme scavengers at the time of *ex vivo* perfusion either directly or in a targeted fashion using drug-delivery systems such as liposomes (49) might also be a viable therapeutic strategy to counter labile heme.

Limitations

Our study focuses on the early time points after IRI and has only a short follow-up of 2 h after albumin treatment. Further experiments are needed to investigate heme-binding strategies in the context of IRI and to investigate long term effects on renal function such as progressive inflammation and renal fibrosis.

In conclusion, our results indicate that prolonged ischemia time after IRI enhances labile heme levels in renal tissue, which contribute to complement activation, inflammation and AKI. Future studies are warranted to understand if scavenging labile heme can attenuate AKI and delayed graft function after kidney transplantation. Developing clinical applicable therapeutic strategies to reduce the burden of labile heme in the ischemic organ might result in improved long term outcome after solid organ transplantation.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

ETHICS STATEMENT

The animal study was reviewed and approved by Lower Saxony State department for animal welfare and food protection.

AUTHOR CONTRIBUTIONS

FG: designed, supervised all aspects of the study and drafted the manuscript. LW, NS, VV, RC, SR, KM, AT, PP, RG, M-SJ, and FG: experimental conduct. FG, LW, VV, and SI: discussion and wrote the manuscript. RC and SR: animal surgeries. IT, JB, NM, KD, MH-L, CK, RL, and HH: discussion of results. All authors participated in the interpretation of data, editing, and approval of the manuscript.

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Increasing Time in Therapeutic Range of Tacrolimus in the First Year Predicts Better Outcomes in Living-Donor Kidney Transplantation

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Background: The aim of the present study was to investigate the impact of time in therapeutic range TTR on long-term outcomes of living kidney transplants.

Methods: We included 1,241 living kidney transplants and randomized them into development and validation cohorts with a ratio of 2:1. The tacrolimus TTR percentage was calculated by linear interpolation with a target range (5–10 ng/ml months 0–3, 4–8 ng/ml months 4–12). The optimal TTR cutoff was estimated by the receiver operating characteristic curve analysis on the basis of acute rejection (AR) within 12 months in the development cohort. Outcomes were analyzed between patients with high TTR and low TTR in the development and validation cohorts, respectively. The TTR was also compared with other tacrolimus measures.

Results: The optimal TTR cutoff value was 78%. In the development cohort, patients with TTR > 78% had significantly higher rejection- and infection-free survival. TTR < 78% was an independent risk factor for AR (OR: 2.97, 95%CI: 1.82–4.84) and infection (OR: 1.55, 95%CI: 1.08–2.22). Patient and graft survival were significantly higher in those with TTR>78%, and TTR<78% was associated with graft loss (OR: 3.2, 95%CI: 1.38–7.42) and patient death (OR: 6.54, 95%CI: 1.34–31.77). These findings were confirmed in the validation cohort. Furthermore, we divided all included patients into a high and low TTR group. TTR was more strongly associated with patient and graft survival than mean level, standard deviation, and intrapatient variability (IPV).

Conclusions: Increasing the TTR of tacrolimus in the first year was associated with improved long-term outcomes in living kidney transplants, and TTR may be a novel valuable strategy to monitor tacrolimus exposure.

Keywords: tacrolimus, time in therapeutic range, kidney transplantation, development and validation, acute rejection, infection, graft loss, patient death

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INTRODUCTION

Tacrolimus-based regimens are the most commonly used immunosuppressive therapies, preventing T-cell and antibody-mediated rejection after kidney transplantation (1); however, a narrow therapeutic index limited their clinical application. Overexposure can result in toxicity and severe infection, and underexposure can lead to graft rejection (2, 3). Therapeutic drug

monitoring to maintain the intensity and stability and a constant tacrolimus trough level allows for the achievement of optimal immunosuppression. Mean level, intrapatient variability (IPV), and variability of the standard deviation of the tacrolimus trough level are associated with acute rejection (AR) and graft loss (4–6). However, these indexes did not consider whether the tacrolimus trough level achieved a target therapeutic window and exposure time. As failure to maintain the tacrolimus trough level in target ranges is a risk factor for inferior short- and long-term outcomes (7, 8), it seems more practical and clinically relevant to develop a new indicator to combine the tacrolimus trough level with variation and the corresponding maintaining time.

Percent time in therapeutic range (TTR), defined as the percentage of time within the therapeutic range over time, takes stability, intensity, and constancy into consideration simultaneously. TTR is a validated method for assessing effective warfarin therapy and has been used as a tool to risk stratifying (9, 10). Limited studies have investigated the use of TTR in transplantation, but a low tacrolimus TTR was associated with significantly increased acute cellular risk in lung transplants (11) and *de novo* donor-specific antibodies (dnDSAs) in kidney transplants (12). These facts indicated that the tacrolimus TTR might have potential as a prognostic indicator in organ transplantation, but evidence of its impact on the long-term outcomes of living kidney transplants is lacking. The present study investigated whether patients with a high tacrolimus TTR had better clinical results than those with a low tacrolimus TTR.

METHODS

Patient Population

The clinical data of patients who received a living-related kidney transplant at West China Hospital between August 2007 and April 2017 were retrospectively analyzed. The Ethics Committee of West China Hospital approved the study. Patients who were <18 years of age, with an initial calcineurin inhibitor (CNI) other than tacrolimus, tacrolimus switch or withdrawal in first 12 months, receiving an ABO-incompatible kidney transplant, with organ transplant history, a follow-up of <1 year, or with three or more consecutive missing measures of tacrolimus trough level, according to our monitoring protocol, were excluded.

Data Collection

We retrieved information from medical records, including patient age, sex, body mass index at the time of transplantation, duration of pretransplantation dialysis, organ transplant history, panel reactive antibody (PRA), human leukocyte antigen (HLA) mismatch, induction therapy, delayed graft function (DGF), and cold ischemic time. DGF was defined as the need for dialysis in the first week after transplantation. AR, infection, graft loss, and patient death were the clinical outcomes of interest. AR was diagnosed clinically based on a 50% or more significant increase in serum creatinine levels within 3 days that was not explained by some other cause and that was confirmed by biopsy when necessary. AR was treated primarily with bolus doses of methylprednisolone and with antithymocyte globulin if refractory. Infection was defined as any infectious symptoms needing medication intervention, including wound, pulmonary, urinary tract, and skin infections. Re-establishment of long-term dialysis therapy or estimated glomerular filtration rate (eGFR) of <15 ml/min was considered as graft loss. Allograft survival was censored at the earliest of the following events: loss to follow-up or patient death. The definition of graft failure did not include patient death with a functioning graft. Renal function was assessed by eGFR, calculated using the Modification of Diet in Renal Disease (MDRD) equation for Chinese and adjusted for body surface area (13).

Immunosuppression Regimen

The immunosuppression therapy used at our hospital has been previously described (14). Briefly, rabbit antihuman thymocyte immunoglobulin (ATG) (1 mg/kg administered for 3–7 days) or monoclonal anti-CD25 monoclonal antibody (IL-2R antibody) (20 mg on days 0 and 4 post-transplant) were used as induction therapy. Maintenance immunosuppressive therapy consisted of tacrolimus, mycophenolate mofetil/enteric-coated mycophenolate sodium (MMF/EC-MPS), and corticosteroids. Tacrolimus was initiated at 1.5 mg bid on day two post-transplantation and maintained at 5–12 ng/mL. The tacrolimus trough level was measured by the enzyme multiplied immunoassay technique (EMIT, Dade-Behring, NY, USA) in blood samples collected weekly during months 0–3, every 2 weeks during months 4–6, and monthly thereafter in the first year. Tacrolimus trough levels were obtained before breakfast and dose administration in the morning. Any tacrolimus levels that were <2 or > 15 ng/mL were individually reviewed and excluded if they were not valid. MMF was started on the night before the operation at 1,000 mg and maintained at 1,000 mg bid. The area under the curve (AUC) for mycophenolate mofetil was 30–70 mg/h·L^{-1}. EC-MPS 720 mg was administrated the night before the operation and at 720 mg bid after that. The EC-MPS AUC was not measured. Methylprednisolone 500 mg was administered intravenously during the surgery, and 300 mg was given daily for the next 3 days. It was then replaced by 60 mg of prednisone, which was tapered by 10 to 5–10 mg/day for maintenance.

Time in Therapeutic Range

We randomized all patients into development and validation cohorts with a ratio of 2:1. The process was finished by SPSS 24.0 software. First, SPSS gave each individual a random number. Then, these number were ranked in order from large to small. Last, the first 2/3 was used as the developed cohort and the latest 1/3 was used as the validation cohort. The tacrolimus TTR percentage was calculated by linear interpolation as described by Rosendaal in the development cohort (15). The linear relationship between each tacrolimus trough level and the TTR was calculated by summing the time during which the value fell within the target tacrolimus range of 5–10 ng/ml during months 0–3 and 4–8 ng/ml during months 4–12. The tacrolimus TTR was compared with the tacrolimus standard deviation (SD), mean, and the IPV. The IPV was calculated by dividing SD by the mean level (16).

TABLE 1 | Clinical characteristics of the development cohort and validation cohort.

Characteristics[a]	Development (*N* = 827)	Validation (*N* = 414)	*P*-value
Donor age (years)	47.3 (±9.8)	47.4 (±9.7)	0.88
Donor sex (Male)	33.00%	31.50%	0.72
Donor eGFR	109.4 ± 13.7	115.6 ± 17.5	0.63
Recipient age (years)	33.1 (±8.3)	32.7 (±8.7)	0.43
Recipient sex (Male)	71.70%	71.90%	0.95
Recipient BMI, Kg/m^2	21.5 (±3.5)	21.8 (±18)	0.72
Cold ischemic time (h)	2.5 (±0.9)	2.5 (±0.9)	0.88
DGF	1.10%	1.50%	0.48
Induction therapy			0.46
No	33.90%	31.60%	
ATG	11.95%	10.60%	
IL-2 R antibody	54.20%	57.90%	
HLA mismatches			0.11
≤3 mismatch	11.50%	15.00%	
>3 mismatch	88.50%	85.00%	
Pre-transplant PRA >20%	2.90%	2.90%	0.95
Duration of dialysis, months	13.4 (±14.8)	13.4 (±15.8)	0.94
Transplant year			0.56
2007–2012	266	140	
2013–2017	561	274	

ATG, antithymocyte globulin; BMI, body mass index; eGFR, estimated glomerular filtration rate; DGF, delayed graft function; HLA, human leukocyte antigen; PRA, panel reactive antibody.

[a]*Continuous data are presented as mean ± standard deviation (SD), and categorical data as percentage of the total, unless otherwise noted.*

Statistical Analysis

Descriptive statistics were used to describe the baseline characteristics of the patients in the development and validate cohorts. Categorical variables were compared using the χ^2-test or Fisher's exact test; continuous variables were compared using a Student's *t*-test. A receiver operating characteristic (ROC) curve was built from the calculated TTR value in development cohort to determine the optimal TTR cutoff value that can discriminate patients with or without AR in the first year best. The area under the ROC curve with sensitivity and specificity was computed, and the TTR with the greatest AUC is the optimal cut-off value (17).

Time to AR, infection, graft loss, and recipient death was analyzed by the Kaplan–Meier method, and between-group differences were assessed for significance by the log-rank test. Cox Proportional regression was used to identify predictors of AR, infection, graft loss, and patient death. Variables with $p < 0.1$ in the univariate analysis were included in the multivariate analysis. The statistical analysis was performed using SPSS 24.0 (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered significant.

RESULTS

From August 2007 to April 2017, 2,048 patients received a living related kidney transplant in West China Hospital, Sichuan University. A total of 807 patients were excluded: follow-up time was <1 year ($N = 227$); CNI was not tacrolimus ($N = 275$); lost in the follow-up ($N = 82$); organ transplantation history ($N = 15$); ABO-incompatible kidney transplantation ($N = 38$); and consecutive missing of tacrolimus trough level 3 times or more ($N = 170$). A total of 1,241 patients were included with 827 in the development cohort and 414 in the validation cohort. The process of enrollment of patients is shown in the flow chart (**Supplementary Figure 1**). The clinical characteristics of the development cohort and validation cohort are shown in **Table 1**. The median follow-up for the entire cohort was 42 months [interquartile range (IQR): 26–59 months]; 42 months (IQR: 26–59 months) for the development cohort, and 42 months (IQR: 25–59 months) for the validation cohort.

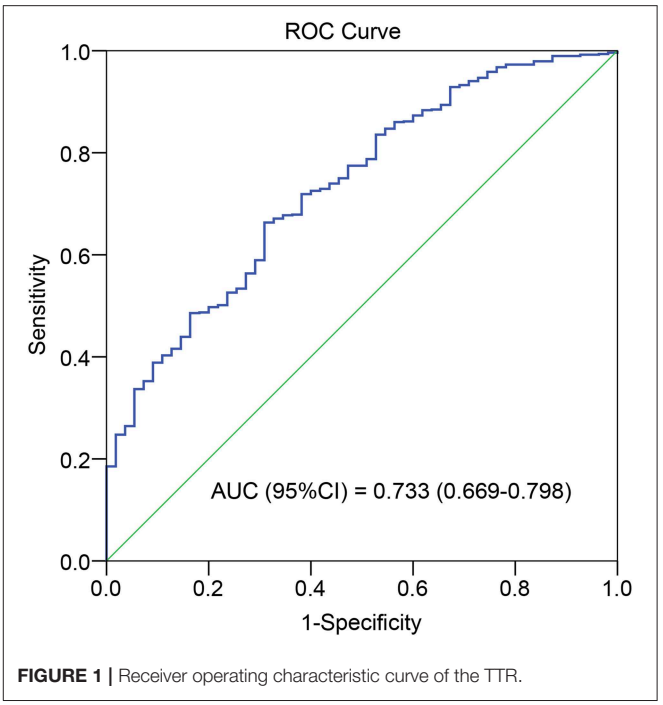

FIGURE 1 | Receiver operating characteristic curve of the TTR.

The optimal cutoff value for TAC TTR was 78% (AUC = 0.733; sensitivity = 66.3%; and specificity = 69.1%) (**Figure 1**). We thus divided patients in the development and validation cohort into a high TTR group and low TTR group. Baseline characteristics between the high and low TTR groups in both cohorts are summarized in **Table 2**. All variables were comparable between high and low TTR groups in the development cohort; however, in the validation cohort, patients in the low TTR group received kidneys from older donors ($P = 0.02$) and had more HLA mismatches ($P = 0.02$). There was no difference in the tacrolimus trough levels between high and low TTR groups within the first year in the development and validation cohort. No difference was detected in renal function between the two groups during the follow-ups in the development cohort, while the high TTR group in validation cohort had a litter higher eGFR in first 3 years (mean difference 4∼5 ml/min/1.73 m^2) (**Figure 2**).

Graft Survival

In the development cohort, more patients experienced graft loss (15/283, 5.3%) in the low TTR group than those in the high TTR (9/544, 1.7%) ($P < 0.001$). The Kaplan–Meier curve indicated

TABLE 2 | Characteristics between the high and low TTR groups in development and validation cohorts.

Characteristics	Development		*P*-value	Validation		*P*-value
	TTR<78% (*N* = 283)	TTR>78% (*N* = 544)		TTR<78% (*N* = 145)	TTR>78% (*N* = 269)	
Donor age (years)	46.8 (±10.2)	47.7 (±9.5)	0.31	46 (±9)	48.5 (±9.8)	0.09
Donor sex (Male)	30.70%	32.70%	0.61	28.20%	36.90%	0.22
Donor eGFR	111.3 ± 11.6	108.4 ± 14.9	0.46	118.6 ± 20.8	114.0 ± 11.8	0.57
Recipient age (years)	32.9 (±8.7)	32.8 (±8.5)	0.87	34.4 (±8.5)	32.4 (±8.1)	0.02
Recipient sex (Male)	72.00%	71.80%	0.95	71.05%	72.10%	0.81
Recipient BMI, kg/m^2	21.4 (±3)	21.9 (±18)	0.53	21.5 (±3)	21.4 (±3.7)	0.74
Cold ischemic time (h)	2.5 (±0.9)	2.6 (±0.9)	0.13	2.5 (±0.9)	2.6 (±0.9)	0.52
DGF	1.40%	1.00%	0.7	1.30%	1.60%	0.37
Induction therapy			0.21			0.21
No	35.50%	29.70%		37.90%	31.30%	
ATG	9.20%	11.30%		13.80%	10.80%	
IL-2 R antibody	55.30%	59.00%		48.30%	57.80%	
HLA mismatches			0.1			0.02
≤3 mismatch	86.20%	78.70%		82.60%	90.90%	
>3 mismatch	13.80%	21.30%		17.40%	9.10%	
Pre-transplant PRA >20%	3.00%	2.80%	0.95	2.80%	2.60%	0.93
Duration of dialysis, months	13.2 (±14.9)	13.5 (±15.8)	0.76	14.4 (±15.7)	12.8 (±14.3)	0.29
Transplant year			0.53			0.52
2007–2012	87	179		52	88	
2013–2017	196	365		93	181	

ATG, antithymocyte globulin; BMI, body mass index; eGFR, estimated glomerular filtration rate; DGF, delayed graft function; HLA, human leukocyte antigen; PRA, panel reactive antibody.

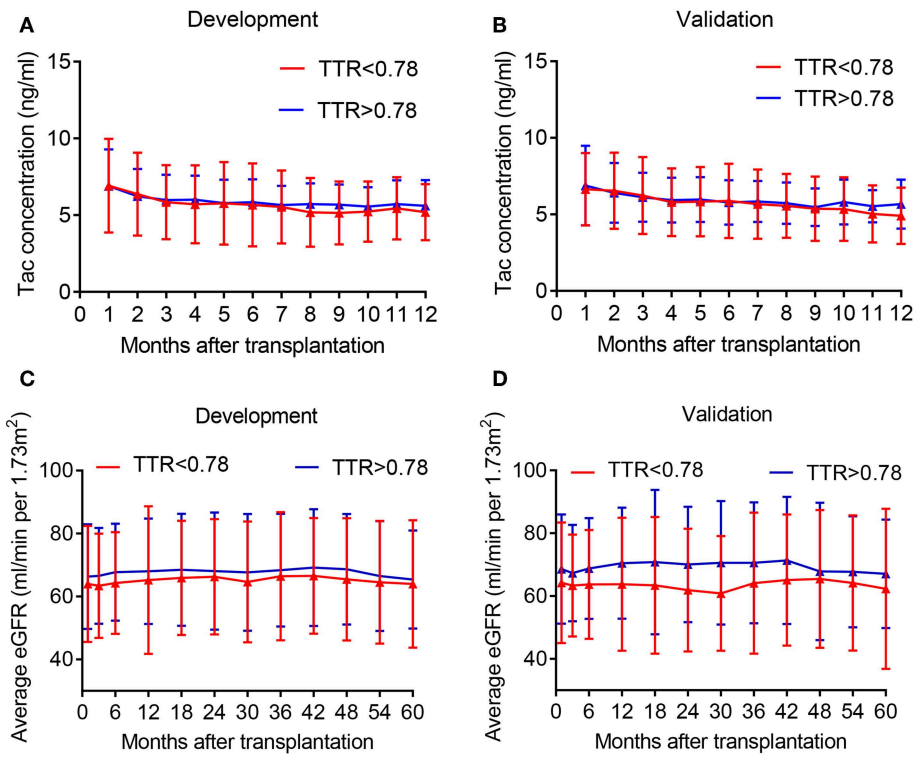

FIGURE 2 | Average tacrolimus concentration and eGFR distribution in the development and validation cohort at different time point. **(A)** Tac concentration in the development cohort; **(B)** Tac concentration in the validation cohort; **(C)** eGFR in the development cohort; **(D)** eGFR in the validation cohort. Tac, tacrolimus.

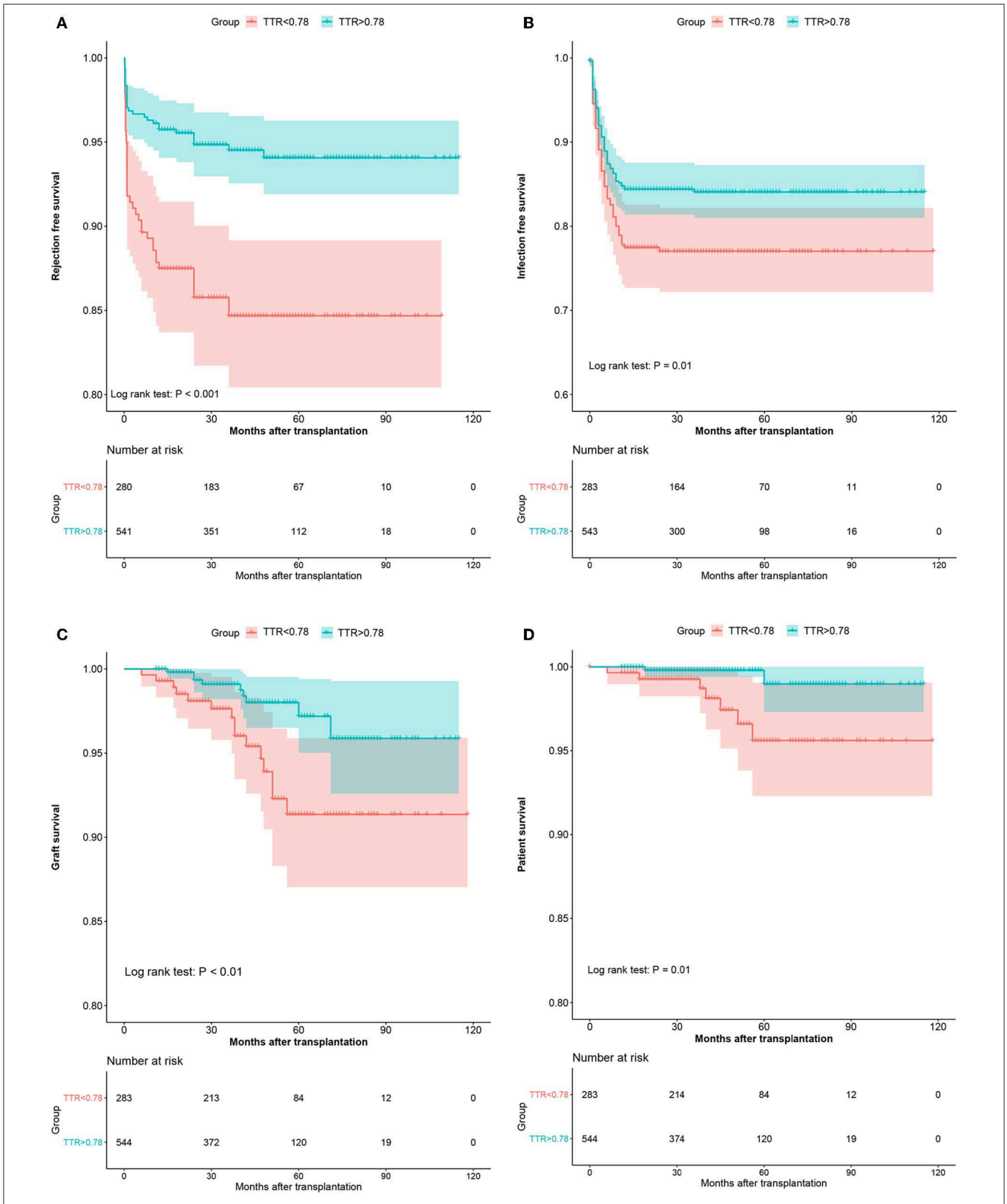

FIGURE 3 | The Kaplan–Meier curves of the rejection-free survival **(A)**, infection-free survival **(B)**, graft survival **(C)**, and patient survival **(D)** in the development cohort.

that the graft survival in the high TTR group was significantly higher than that in the low TTR group (**Figure 3C**). The 3- and 5-year graft survival was 97.5 and 96.6% as well as 93.5 and 92.3% for the high and low TTR group, respectively. Lower TTR was an independent graft-loss contributor in the multivariate analysis (OR: 3.2, 95%CI: 1.38–7.42). A younger age also seemed to be protective for graft survival (OR: 0.95, 95%CI: 0.90–1.00) (**Table 3**).

Similarly, in the validation cohort, more patients have experienced graft loss (6.2 vs. 2.6%, $P = 0.069$) in the low TTR group. Kaplan-Meier estimation indicated graft survival in high TTR group was significantly higher than that in low TTR group (**Figure 4**). Lower TTR was associated with a higher risk of graft loss (OR: 5.09, 95%CI: 1.28–23.65). Also, younger recipients (OR: 0.87, 95%CI: 0.78–0.96) and lower PRA (OR: 0.07; 95%CI: 0.01–0.56) were found to have independent protective effects on graft survival (**Table 4**).

Recipient Survival

In the development cohort, during the follow-up, 7 (2.5%) and 2 deaths (0.4%) were recorded in the low TTR group and high TTR group ($P = 0.016$), respectively. The Kaplan–Meier estimation found that the high TTR group had significantly better patient survival than that of low TTR group (**Figure 3D**). The 3- and 5-year graft survival was 99.2 and 99.2% as well as 97.2 and 94.8% for the high and low TTR group, respectively. Lower TTR was an independent risk factor for graft loss in the multivariate analysis (OR: 6.54, 95%CI: 1.34–31.77) (**Table 3**).

In the validation cohort, a similar trend was observed with more patients experiencing patient death (4.1 vs. 0.7%, $P = 0.043$) in the low TTR group. Lower TTR was associated with a higher risk of patient death (OR: 6.8, 95%CI: 1.34–34.61) (**Table 4**). The Kaplan–Meier estimation indicated patient survival in the high TTR group was significantly higher (**Figure 4**).

Acute Rejection

In the development cohort, 44 patients (15.5%) developed AR in the low TTR group compared to 32 (5.9%) in the high TTR group ($P < 0.001$). Patients without developing DGF (OR: 0.21, 95%CI: 0.06–0.77) were also observed to develop less AR. A multivariate analysis indicated that TTR $\leq$ 78% was an independent risk factor for AR (OR: 2.97, 95%CI: 1.82–4.84) (**Table 3**). Rejection-free survival was significantly higher in the high TTR group than that in the low TTR group (**Figure 3**).

In the validation group, similar results were observed. There were significantly more patients experiencing AR (12.4 vs. 2.6%, $P < 0.001$) in the low TTR group. Multivariate regression showed that TTR $\leq$ 78% was associated with a higher incidence of AR (OR: 2.97, 95%CI: 1.82–4.84) (**Table 4**). The Kaplan–Meier estimation indicated that rejection-free survival was significantly higher in the high TTR group than that in the low TTR group (**Figure 4**).

Infection

For the development cohort, a total of 148 patients developed infection at least once with 63 (22.3%) in the low TTR group and 85 (15.6%) in the high TTR group ($P = 0.018$). Low TTR was associated with a higher risk of infection in the multivariate analysis (OR: 1.55, 95%CI: 1.08–2.22) (**Table 3**). The Kaplan–Meier estimation revealed that the infection-free survival was significantly higher in the high TTR group than that in the low TTR group (**Figure 3**).

Furthermore, in the validation group, we confirmed the results that more patients experienced infection episodes (24.1 vs. 13.8%, $P = 0.008$) in the low TTR group. TTR $\leq$ 78% was associated with a higher incidence of infection (OR: 2.00, 95%CI: 1.19–3.34) in the multivariate regression (**Table 4**). Infection-free survival in the Kaplan–Meier curve was significantly higher in the high TTR group than that in the low TTR group (**Figure 4**).

Comparison to Other Tacrolimus Measures

Multivariate regression was also used to examine alternative measures for the characterization of tacrolimus exposure. We separated 1,241 patients into high and low TTR groups by TTR cut-off value. In 1,241 patients overall, increasing the TTR by 10% was associated with reduced patient death (OR = 0.73, 95%CI: 0.63–0.86) and graft loss (OR = 0.53, 95%CI: 0.36–0.80) (**Table 5**). Increasing IPV was an independent risk factor for graft loss, and all alternative tacrolimus measures were predictors for patient death. However, none of the tacrolimus measures were significantly associated with patient and graft survival in the high TTR group. In the low TTR group, increasing TTR by 10% remained independently protective for both patient (OR = 0.60, 95%CI: 0.40–0.93) and graft survival (OR = 0.51, 95%CI: 0.28–0.92). Only increasing IPV was associated with a higher incidence of graft loss (OR = 1.04, 95%CI: 1.01–1.07).

DISCUSSION

To the best of our knowledge, this is the first study to assess the effect of tacrolimus TTR on long-term outcomes in living kidney transplantation. In the current study, we found that a TTR above 78% was not only associated with improved graft survival but also with reduced risk of AR and infection. Tacrolimus TTR was more strongly associated with patient and graft survival than mean level, standard deviation (SD), and IPV.

The first reported use of TTR was for the therapeutic use of warfarin, a drug with considerable inter- and intra-patient variability and a narrow therapeutic index, much like tacrolimus. The warfarin TTR cut-off value was arbitrarily determined as 75% (10) or was recommended by European guidelines as being 70% or greater (18). Two studies investigating the use of tacrolimus TTR also arbitrarily set the TTR threshold as 30% in lung transplant (11) or 60% in kidney transplant (12). Although they found that increasing tacrolimus TTR was associated with improved clinical outcomes, the optimal TTR was not determined. The primary aim of maintaining the tacrolimus within the therapeutic range is to control rejection, and so we utilized the ROC curve to estimate the optimal TTR value based on the AR episodes in the first year. The cut-off value was 78%, and the AUC was 0.733. Though the AUC value was fair, the TTR of 78% can differentiate patients with increased risk of graft failure and patient death in the development group very well. Additionally, we further validated the TTR cut-off value and its

TABLE 3 | Univariate and Multivariate analysis of clinical characteristics for acute rejection, infection, and patient and graft survival in development cohort.

Characteristic	Acute rejection				Infection				Graft survival				Patient survival			
	Unadjusted	***P***	**Adjusted**	***P***	**Unadjusted**	***P***	**Adjusted**	***P***	**Unadjusted**	***P***	**Adjusted**	***P***	**Unadjusted**	***P***	**Adjusted**	***P***
TTR	3.36(2.21–5.11)	<0.001	2.97 (1.82–4.84)	<0.001	1.55 (1.08–2.22)	0.019	1.55 (1.08–2.22)	0.019	3.33 (1.44–7.7)	0.005	3.2 (1.38–7.42)	0.007	6.87 (1.42–33.31)	0.017	6.54 (1.34–31.77)	0.02
Donor age	1.01 (0.98–1.04)	0.58			1.02 (0.99–1.05)	0.277			1.02 (0.96–1.09)	0.538			1.02 (0.94–1.11)	0.638		
Donor sex	0.67 (0.34–1.32)	0.24			0.59 (0.31–1.13)	0.112			1.27 (0.36–4.43)	0.707			0.44 (0.05–3.78)	0.452		
Recipient age	0.98 (0.96–1.01)	0.17			0.99 (0.97–1.01)	0.196			0.95 (0.9–1)	0.052	0.95 (0.9–1)	0.07	1.04 (0.97–1.12)	0.226		
Recipient sex	1.21 (0.76–1.93)	0.43			1.15 (0.77–1.72)	0.506			1.18 (0.46–3)	0.733			3.15 (0.39–25.35)	0.28		
Recipient BMI	0.99 (0.93–1.05)	0.79			0.99 (0.97–1.02)	0.72			0.95 (0.8–1.13)	0.586			1 (0.98–1.03)	0.812		
Time of dialysis	1 (0.98–1.01)	0.56			1 (0.99–1.01)	0.69			1 (0.98–1.03)	0.687			1.01 (0.99–1.04)	0.291		
DGF	0.21 (0.07–0.7)	0.01	0.21 (0.06–0.77)	0.018	0.43 (0.13–1.44)	0.172				0				0		
HLA mismatch	1.49 (0.7–3.2)	0.3			0.99 (0.59–1.65)	0.974			1.93 (0.45–8.3)	0.379			1.38 (0.17–11.17)	0.761		
PRA	1.17 (0.27–5.06)	0.83			1.15 (0.39–3.4)	0.802			0.71 (0.09–5.47)	0.741				0		
Cold ischemic time	0.73 (0.55–0.95)	0.02	1 (0.98–1.01)	0.91	0.92 (0.75–1.13)	0.427			1 (0.63–1.58)	0.996			0.48 (0.21–1.1)	0.082	0.5 (0.22–1.15)	0.102
Induction therapy		0.15				0.254				0.297				0.838		
No	Reference				Reference				Reference				Reference			
ATG	1.65 (0.99–2.73)				0.81 (0.54–1.22)				0.42 (0.14–1.26)				0.6 (0.12–3.02)			
IL-2R	1.24 (0.55–2.76)				1.33 (0.77–2.32)				0.96 (0.28–3.36)				0.91 (0.11–7.66)			
Transplant year		0.36				0.62				0.48				0.56		
2007–2012	Reference				Reference				Reference				Reference			
2013–2017	0.85 (0.64–1.13)				0.94 (0.76–1.38)				0.89 (0.43–1.84)				0.91 (0.58–1.43)			

ATG, antithymocyte globulin; BMI, body mass index; eGFR, estimated glomerular filtration rate; DGF, delayed graft function; HLA, human leukocyte antigen; PRA, panel reactive antibody; TTR, time in therapeutic range.

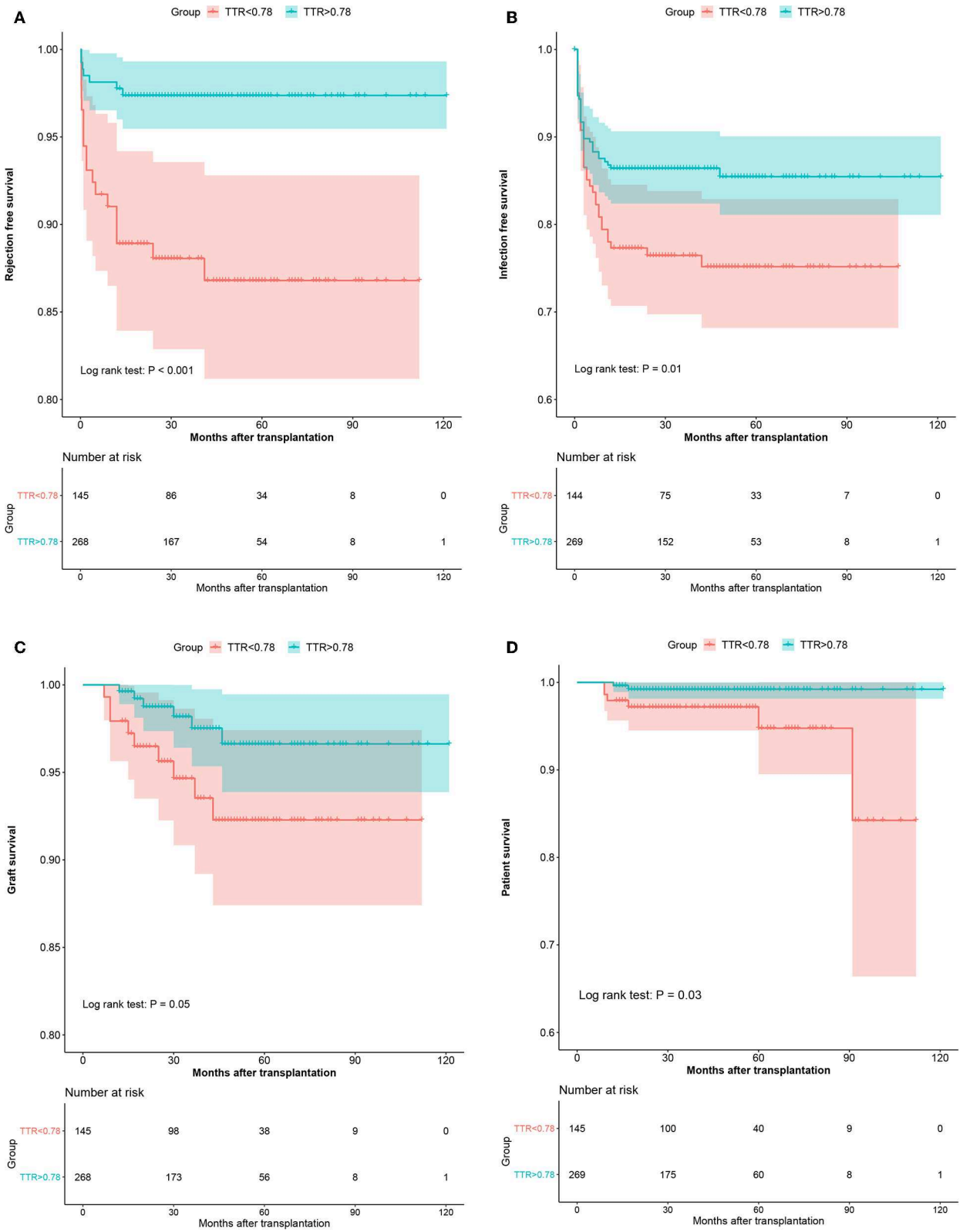

FIGURE 4 | The Kaplan–Meier curves of the rejection-free survival **(A)**, infection-free survival **(B)**, graft survival **(C)**, and patient survival **(D)** in the validation cohort.

TABLE 4 | Univariate and Multivariate analysis of clinical characteristics for acute rejection, infection, and patient and graft survival in validation cohort.

Characteristic	Acute rejection				Infection				Graft survival				Patient survival			
	Unadjusted	***P***	**Adjusted**	***P***	**Unadjusted**	***P***	**Adjusted**	***P***	**Unadjusted**	***P***	**Adjusted**	***P***	**Unadjusted**	***P***	**Adjusted**	***P***
TTR	5.3 (2.16–13.03)	<0.001	5.36 (2.17–13.21)	<0.001	2 (1.19–3.34)	0.009	2 (1.19–3.34)	0.009	2.66 (0.95–7.46)	0.064	5.09 (1.28–23.65)	0.022	5.76 (1.15–28.93)	0.033	6.8 (1.34–34.61)	0.021
Donor age	1.01 (0.95–1.08)	0.731			0.99 (0.95–1.04)	0.751			0.95 (0.89–1.01)	0.085	0.93 (0.86–1)	0.066	0.92 (0.84–1.01)	0.086	0.91 (0.82–1.01)	0.089
Donor sex	0.64 (0.17–2.47)	0.521			0.81 (0.33–1.97)	0.643			0.69 (0.21–2.26)	0.538			/	/		
Recipient age	1 (0.95–1.05)	0.965			1 (0.97–1.03)	0.9			0.88 (0.8–0.96)	0.003	0.87 (0.78–0.96)	0.006	0.93 (0.83–1.03)	0.145		
Recipient sex	0.68 (0.29–1.59)	0.378			1.34 (0.74–2.42)	0.336			2.84 (0.64–12.71)	0.171			/	/		
Recipient BMI	0.91 (0.79–1.06)	0.222			1.01 (0.93–1.1)	0.769			1.02 (0.87–1.19)	0.815			1.01 (0.81–1.25)	0.957		
Time of dialysis	0.97 (0.93–1.01)	0.157			0.99 (0.98–1.01)	0.524			0.97 (0.91–1.03)	0.26			0.98(0.92–1.05)	0.566		
DGF	/	/			/	/			/	/			/	/		
HLA mismatch	0.67 (0.22–2.04)	0.477			0.93 (0.43–2.02)	0.861			0.96 (0.21–4.35)	0.956			0.96 (0.12)	0.969		
PRA	0.27 (0.06–1.33)	0.109	0.25 (0.05–1.36)	0.109	2.14 (0.27–16.97)	0.472			0.16 (0.03–0.82)	0.028	0.07 (0.01–0.56)	0.013	0.18 (0.02–1.58)	0.12		
Cold ischemic time	1.14 (0.72–1.79)	0.575			1.06 (0.8–1.4)	0.704			0.92 (0.53–1.6)	0.759			0.97 (0.45–2.1)	0.936		
Induction therapy		0.334				0.578				0.347				0.237		
No																
ATG	1.85 (0.76–4.47)				0.9 (0.51–1.59)				2.23 (0.76–6.57)				4.16 (0.8–21.74)			
IL-2R	1.91 (0.57–6.37)				1.38 (0.65–2.94)				1.55 (0.3–7.94)				2.32 (0.21–26.14)			
Transplant year		0.21				0.73				0.32				0.63		
2007–2012	Reference				Reference				Reference				Reference			
2013–2017	0.76 (0.51–1.13)				0.96 (0.82–1.13)				0.85 (0.63–1.15)				0.92 (0.67–1.26)			

ATG, antithymocyte globulin; BMI, body mass index; eGFR, estimated glomerular filtration rate; DGF, delayed graft function; HLA, human leukocyte antigen; PRA, panel reactive antibody; TTR, time in therapeutic range.

TABLE 5 | Adjusted Cox regression models for patient and graft survival using different measures of tacrolimus exposure.

	Overall patient		High TTR group		Low TTR group	
	Adjusted	***P***	**Adjusted**	***P***	**Adjusted**	***P***
GRAFT SURVIVAL						
increasing TTR by 10%	0.73 (0.63–0.86)	<0.001	0.91 (0.44–1.91)	0.809	0.60 (0.40–0.93)	0.02
Increasing mean level	0.79 (0.55–1.13)	0.194	0.59 (0.26–1.36)	0.215	0.82 (0.45–1.50)	0.518
Increasing standard deviation	1.33 (0.66–2.54)	0.461	1.46 (0.06–37.04)	0.820	0.54 (0.14–2.09)	0.37
Increasing IPV%	1.05 (1.03–1.07)	<0.001	0.77 (0.48–1.24)	0.278	1.04 (1.01–1.07)	0.003
PATIENT SURVIVAL						
increasing TTR by 10%	0.53 (0.36–0.80)	0.002	1.16 (0.28–4.88)	0.836	0.51 (0.28–0.92)	0.026
Increasing mean level	2.49 (1.08–5.75)	0.032	1.79 (0.36–8.96)	0.476	2.44 (1.14–5.23)	0.021
Increasing standard deviation	2.49 (1.06–5.87)	0.037	0.94 (0.13–6.90)	0.952	2.70 (0.81–9.02)	0.107
Increasing IPV%	1.06 (1.00–1.13)	0.05	0.32 (0.003–28.87)	0.671	1.05 (0.96–1.16)	0.299

IPV, intra-patient variability; TTR, time in therapeutic range.

role as an informative predictor in the validation cohort. Of note was the fact that, to accurately estimate TTR, we excluded cases with three or more consecutive missing values of tacrolimus. As the TTR was calculated by the Rosendaal method, which assumed a linear relationship exists between each measured value, that linear interpolation method was used to estimate the missing values (15). When there are three or more consecutive missing values, it is not reliable to apply interpolation, and this is due to the limitation of this technique.

We found that a higher tacrolimus TTR was strongly associated with better graft survival and rejection-free survival. These observations were consistent with the findings from previous kidney transplant and lung transplant analyses, which used the TTR to characterized tacrolimus exposure (11, 12). Notably, we did not find any difference in mean tacrolimus trough level between the high and low TTR groups. However, the high TTR group had much lower SD (1.9 vs. 1.4) and IPV (24.1 vs. 34.2%) of tacrolimus levels than that in low TTR group. Together with the facts that increasing SD and IPV of tacrolimus levels are independent risk factors for rejection and graft loss (6, 19), a higher TTR can thus predict better outcomes.

Interestingly, we found that TTR > 78% was associated with reduced infection, which was corroborated by the lung transplant study that found that increasing the TTR by 10% was associated with a decreased likelihood of infection (OR 0.81, 95%CI: 0.67–0.97) (11). We previously found that the increasing tacrolimus trough level at the first month was associated with infection, and those that had a tacrolimus trough level >7.15 ng/ml experienced a much higher incidence of infection (20). In the high TTR group, of 9,756 tacrolimus measures in 12 months, 8.0% had level >8 ng/ml, significantly lower than that in the low TTR group (14.4%, $p < 0.001$). Similar findings were confirmed in the first 3 months (3.2 vs. 6.4%, $p < 0.001$), indicating that the low TTR group had more patients with over-immunosuppression. Thus, the low TTR group may have a higher incidence of infection. However, when infection is diagnosed, a dose reduction is usually required. That erratic change of the tacrolimus level will result in a lower TTR.

The effects of several tacrolimus exposure characteristics have been investigated in kidney transplantation. A single measurement of an increased tacrolimus trough level soon after a kidney transplant has been associated with a decreased risk of AR and biopsy-proven AR (21, 22). However, a single time-point measurement may not be meaningful because tacrolimus trough levels are produced by dynamic rather than stable metabolic processes. Recently, a pooled analysis across four randomized trials found that the average tacrolimus level in first 12 months <4.0 ng/ml was associated with an increased incidence of BPAR (HR = 6.33, $p < 0.00001$) (7). Sapir-Pichhadze et al. conducted a retrospective cohort investigation of kidney transplants, examining the effect of the SD of tacrolimus levels on the composite endpoint (late allograft rejection, glomerulopathy, and total graft loss). They found that, for every 1-unit increase in SD, there was a 27% increase in the adjusted hazard of the composite endpoint (HR 1.27, 95%CI: 1.03–1.56) (6). Additional study using the mean level and SD to characterize tacrolimus exposure yielded similar findings (23, 24). Importantly, we found that the tacrolimus TTR was more strongly associated with patient and graft survival than mean level and SD. A subgroup analysis of the high TTR group found the predictive effect of all alternative measures disappeared. SD may poorly characterize levels that are consistently above or below the target range, and mean value cannot differentiate erratic changes between high and low levels, but TTR accounts for the concentration, variability, and the time between levels simultaneously. Together with our finding that a higher TTR can eliminate a large part of SD (1.4 vs. 1.9, $p < 0.001$), we found that that the TTR may better characterize exposure compared to the mean value and SD.

Increasing the IPV of tacrolimus is also a risk factor for inferior graft survival. In a retrospective analysis of 310 adult kidney transplants, an IPV >30% independently related to death censored graft loss (HR = 2.613, 95%CI: 1.361–5.016) and dnDSAs (HR = 2.925, 95%CI: 1.473–5.807) (25). However, we found the high TTR group had a much lower IPV than that in low TTR group (24 vs. 34%, $p < 0.001$), and tacrolimus TTR is more strongly associated with patient and graft survival than IPV, indicating that the TTR may be a better index of tacrolimus monitoring. What's more, the real-time clinical utility of IPV is limited because it cannot be generally computed at the bedside nor making clinical decisions based on the result.

The ease of estimating TAC TTR supports its use as a risk-stratification and decision-making tool. Additionally, high IPV is mainly due to non-adherence (26), and the utilization of TTR may also stratify those with a high non-attendance rate in the outpatient clinic. As suggested by studies involving Warfarin, a less frequent dose change and better adherence contributed to a higher TTR level (27). Interventions to minimize the non-adherence, including the timing and dosing, may also improve tacrolimus TTR in kidney transplants and consequently better long-term allograft outcomes. In addition, a high-fat meal and administration of CYP3A4-interfering medications had a significant impact on the rate of TAC absorption and metabolism (28, 29), and so avoiding these factors may improve the TTR as well.

There are several limitations when interpreting our results. First, due to its retrospective nature, we could only establish an association between the TTR and the clinical outcomes. These findings should be confirmed with a prospective assessment. Second, tacrolimus was measured at discrete time points, and missing values were estimated by linear interpolation method. Though we excluded those with three or more consecutive missing values, estimated tacrolimus values may not have accurately reflected the real exposures. Third, the cut-off value of the TTR and its predictive role were based on our target range, whether the TTR remains associated with patient and graft survival in other tacrolimus target ranges was unknown. Finally, we only analyzed living kidney transplants; these observations therefore need to be externally validated in other transplant categories, such as deceased donor and ABO-incompatible kidney transplants.

CONCLUSION

Tacrolimus TTR monitoring was predictive in achieving well-managed tacrolimus-based immunosuppression in living kidney transplants. Future prospective investigations should be conducted to confirm these findings.

DATA AVAILABILITY STATEMENT

The datasets analyzed in this article are not publicly available. Requests to access the datasets should be directed to kidney5@163.com.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of West China Hospital, Sichuan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

TS, SY, and TL participated in research design. TS and SY participated in the writing of the paper. YJ, ZH, JL, ZW, XL, and JZ participated in the data collection and data wash. YF, SY, TS, XW, and LL participated in data analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02912/full#supplementary-material

Supplementary Figure 1 | A flow chart to describe the enrollment of patients.

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Phenotypic and Transcriptomic Lymphocytes Changes in Allograft Recipients After Intravenous Immunoglobulin Therapy in Kidney Transplant Recipients

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High dose intravenous immunoglobulin (IVIG) are widely used after kidney transplantation and its biological effect on T and B cell phenotype in the context of maintenance immunosuppression was not documented yet. We designed a monocentric prospective cohort study of kidney allograft recipients with anti-HLA donor specific antibodies (DSA) without acute rejection on screening biopsies treated with prophylactic high-dose IVIG (2 g/kg) monthly for 2 months. Any previous treatment with Rituximab was an exclusion criterion. We performed an extensive analysis of phenotypic and transcriptomic T and B lymphocytes changes and serum cytokines after treatment (day 60). Twelve kidney transplant recipients who completed at least two courses of high-dose IVIG (2 g/kg) were included in a median time of 45 (12–132) months after transplant. Anti-HLA DSA characteristics were similar before and after treatment. At D60, PBMC population distribution was similar to the day before the first infusion. $CD8^+$ $CD45RA^+$ T cells and naïve B-cells ($Bm2^+$) decreased ($P = 0.03$ and $P = 0.012$, respectively) whereas Bm1 (mature B-cells) increased ($P = 0.004$). RORγt serum mRNA transcription factor and CD3 serum mRNA increased 60 days after IVIG ($P = 0.02$ for both). Among the 25 cytokines tested, only IL-18 serum concentration significantly decreased at D60 ($P = 0.03$). In conclusion, high dose IVIG induced limited B cell and T cell phenotype modifications that could lead to anti-HLA DSA decrease. However, no clinical effect has been isolated and the real benefit of prophylactic use of IVIG after kidney transplantation merits to be questioned.

Keywords: kidney transplantation, high-dose intravenous immunoglobulin, donor specific antibodies, lymphocytes phenotype, immunomodulation

INTRODUCTION

Intravenous immunoglobulin (IVIG) is the most highly used therapies for immunodeficiencies, autoimmune, and inflammatory diseases (1). Despite the wide utilization of IVIG, the mechanisms supporting their immunomodulation properties remain not fully elucidated and somewhat controversial. Low-doses IVIG (0.3 g/kg) in common variable immunodeficiency induces

proliferation and immunoglobulin synthesis from B cells (2). In the context of autoimmune and inflammatory diseases, dose of IVIG were elevated (2 g/kg) and their mechanisms of action were described to depend on Fc and/or F(ab')2 fragments (3). IVIG inhibit the activation and function of various innate immune cells such as dendritic cells, monocytes, macrophages, neutrophils cells, and NK cells (3) and neutralize activated complement components (3). In addition, IVIG modulate B-cell functions with a significant reduction in serum levels of BAFF (4) and plasma cells. In turns, IVIG enhances and restores the functions of Treg cells, induces apoptosis of activated effector T lymphocytes and down regulates the production of inflammatory cytokines (3). In addition, IVIG inhibits activation of endothelial cells, expression of adhesion molecules and secretion of soluble mediators (5).

In the field of kidney allograft transplantation, a number of preventive treatments in sensitized patients before transplant have been reported including high-dose IVIG and/or anti-CD20 antibody and/or plasmapheresis and/or bortezomib and/or monoclonal antibody to C5 (6–12). In the only randomized double-blinded trial analyzing high-dose IVIG in sensitized kidney allograft recipients, IVIG group displayed lower panel reactive antibody (PRA), higher rate of deceased-donor transplants, shorter time to transplantation, and similar 2-year graft survival albeit at the expense of higher rate of rejection (8). In another randomized clinical trial, adding rituximab to high-dose IVIG induced a significant decrease in acute antibody mediated rejection (ABMR) and improved kidney allograft function after transplant compared to IVIG alone (12). A retrospective study analyzed the benefit of adding plasmapheresis and rituximab to high-dose IVIG and depicted less microvascular inflammation and histological changes of chronic ABMR in the more intensive treated group compared to a historical cohort of control patients (11). Finally, the accurate role of IVIG in such clinical context remains difficult to establish and analysis of their action on humoral response is difficult to define since IVIG are mostly used in combined therapy strategies. Moreover, in the context of kidney transplant recipients receiving maintenance immunosuppressive drugs, the biological effect of high-dose IVIG treatment on T and B cell phenotype was not evaluated yet. Here, we proposed to analyze *in vivo* phenotypic and transcriptomic lymphocytes changes in kidney allograft recipients treated monthly with prophylactic high-dose IVIG (2 g/kg) because of *de novo* anti-HLA DSA (*dn* DSA) or preexisted DSA.

PATIENTS AND METHODS

Study Design and Patients

We designed a monocentric prospective cohort study of kidney allograft recipients with significant anti-HLA DSA (before transplant (presensitized) or *dn* DSA) without acute rejection on protocol kidney allograft biopsy. A part of the cohort was treated with prophylactic high-dose IVIG (2 g/kg) monthly during 2 months between January 2013 and January 2014 and none were treated before with Rituximab. Prophylactic treatment was decided because of significant anti-HLA DSA (before transplant (presensitized) or *dn* DSA). Protocol kidney allograft biopsies were performed in our center to follow kidney allograft recipients with DSA before transplantation and *dn* DSA as acute ABMR is significantly higher in those patients (13, 14). Demographic and clinical information were collected before and after kidney transplantation. Tolerance of IVIG treatment were collected. Glomerular filtration rate (eGFR) was estimated with MDRD formula (15). Acute rejections were biopsy-proven in all cases and classified according to updated Banff classification (16). Allograft loss was defined with eGFR < 15 ml/min/1.73 m^2 or the need for dialysis. This study was reviewed and approved by the Paris-4 institutional review board (CPP-APHP_2021).

HLA Typing and Anti-HLA Donor Specific Antibodies Identification

HLA type was determined using high resolution typing for all donors and recipients. Participants were typed for class I loci (A, B, and CW) and class II loci (DR, DQ, and DP). Serum samples were systematically collected before IVIG treatment and 1 month after the last course of high dose IVIG to evaluate HLA sensitization. All serum samples were assessed with Luminex assays to determine the specificity of HLA class I and II IgG donor specific antibodies (DSA) (One Lambda Inc, CA). A baseline mean fluorescence intensity (MFI) value > 500 was considered positive. DSA characteristics analyzed included the absolute number, the highest MFI (MFImax) and the sum of MFI (MFIsum).

Human Cell Isolation and Flow Cytometry

Peripheral blood was obtained from patients before each high-dose IVIG infusion (day 0 and day 30) and 1 month after completion of the two courses (day 60). Peripheral blood mononuclear cells (PBMCs) were isolated with lymphocyte separation medium (Laboratoires Eurobio, Les Ulis, France) and resuspended in phosphate-buffered saline (PBS; Life Technologies; Thermo Fisher Scientific, Waltham, MA) with 3% fetal bovine serum (FBS; Gibco, Life Technologies; Thermo Fisher Scientific). PBMCs were stained with various mAb combinations for 20 min at 4°C in staining buffer (PBS with 3% FBS). The directly conjugated mAbs anti-CD19-V500 (clone HIB19), CD56-APC (clone B159), CD14-PE-Cy7 (clone M5E2), CD3-V450 (clone UCHT1), CD4-PE (clone RPA-T4), CD8-APC (clone RPA-T8), CD45RA-FITC (clone L48), CD45RO-PerCP (clone UCHL1), CD38-PE-Cy7 (clone HB7) were supplied by BD Biosciences (France), IgD-FITC (clone IADB6), CD27-PE (clone IA4CD27) by Beckman Coulter (France), and Foxp3-eF450 (clone PCH101) by eBioscience (Thermo Fisher Scientific). Data were processed using FlowJo soft-ware (FlowJo LLC, Ashland, OR).

The gating strategy of the different cells subsets is presented in **Supplemental Figure 1**.

RNA Isolation, Preamplification, and Reverse Transcription–Quantitative Polymerase Chain Reaction

Expression levels of 13 genes were analyzed using quantitative polymerase chain reaction (qPCR). Messenger RNA (mRNA) was extracted from PBMCs lysate (day 0, day 30, and day 60) using the RNeasy MiniKit (Qiagen, Hilden, Germany), according to the manufacturer's instructions and quantified on a nanodrop spectrophotometer. Total RNA was then reverse transcribed to complementary DNA (cDNA) with reverse transcriptase (Thermo Scientific, Courtaboeuf, France). Real-time quantitative PCR was performed with 13 commercially available primers and probe sets (Applied Biosystems, Foster City, CA) (HPRT: Hs99999909_m1, CD19: Hs00174333_m1, CD32a: Hs00234969_m1, CD32b: Hs00269610_m1, BAFF-R: Hs00606874_g1, BAFF: Hs00198106_m1, RORγT: Hs01076122_m1, Tbet: Hs00203436_m1, GATA-3: Hs00231122_m1, CD3: Hs00174158_m1, TGFβ1: Hs00998133_m1, Fas: Hs00236330_m1, FasL: Hs00181225_m1, CD4: Hs01058407_m1). This mechanistically informative panel of 13 mRNAs was designed based on our single center experience and as informed from the literature (17–19). The $2^{-\Delta\Delta ct}$ method was used to calculate the abundance of mRNAs in the samples and relative to reference controls. All samples were tested in duplicate in 96-well plates with the 7900HT fast real-time PCR system (Applied Biosystems). HPRT1 was used as an endogenous control to normalize RNA amounts.

Cytokine Detection in Serum

Serum were harvested after centrifugation of whole blood (collected without any additives) from each patient at day 0, 30 and 60, and stored at −80°C. Cytokines were quantified using the Cytokine 25-Plex human ProcartaPlex Panel 1B with Luminex-based technology as specified by manufacturer (Thermo Scientific, Courtaboeuf, France). The following cytokines were analyzed: GM-CSF; IFN-α; IFN-γ; IL-1 α; IL-1 β; IL-1RA; IL-2; IL-4; IL-5; IL-6; IL-7; IL-9; IL-10; IL-12 p70; IL-13; IL-15; IL-17A; IL-18; IL-21; IL-22; IL-23; IL-27; IL-31; TNF-α; TNF-β/LTA.

Statistical Analysis

Each patient was his own control. Continuous variables were expressed in mean Standard Deviation (SD) or median Interquartile Range (IQR) as appropriate. Categorical variables were expressed in N (%). Statistical analyses were adapted to data distribution (Mann–Whitney test, and unpaired or paired *t*-test). A *P*-value below than 0.05 was considered to be significant. Statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA).

RESULTS

Patients Characteristics

A total of 21 patients were included in the study. Among those, 12 were treated with prophylactic high-dose IVIG and 9 were not. All patients from treatment group completed at least two courses of high-dose IVIG (2 g/kg). Reason of prophylactic IVIG treatment was anti-HLA DSA detection without acute ABMR lesions on protocol biopsy. Clinical characteristics and transplant courses are depicted in **Table 1**. DSA characteristics are presented in **Table 2**. Both groups were comparable (**Supplemental Table 1**).

TABLE 1 | Patients and kidney transplant characteristics.

Variables	IVIG patients $N = 12$	IVIG free patients $N = 9$	*P* value
Demographic			
Women, *N* (%)	6 (50)	2 (22)	0.36
Age at the time of transplant, years, mean (SD)	48 ± 13	55 ± 15	0.29
Initial nephropathy			
Genetic disease, *N* (%)	1 (8)	2 (22)	0.09
Glomerular disease, *N* (%)	8 (67)	3 (33)	
Diabetes—Hypertension, *N* (%)	0 (0)	3 (33)	
Others, *N* (%)	3 (25)	1 (12)	
Donor			
Deceased, *N* (%)	12 (100)	8 (89)	0.43
Age, years, mean (SD)	50 ± 18	52 ± 14	0.74
Mismatch number, median (IQR)			
Class I	2.5 (2–4)	2 (2,3)	0.17
Class II	2 (0–3)	2 (1–4)	0.79
Cold ischemia time, hour, mean (SD)	18 ± 4	19 ± 6	0.89
Immunosuppressive treatment			
Induction, *N* (%)	8 (67)	9 (100)	0.10
R-Il2 antibody	4 (50)	4 (44)	0.67
Thymoglobulin	4 (50)	5 (66)	
Calcineurin inhibitors, *N* (%)	12 (100)	9 (100)	
Mycophenolate mofetil, *N* (%)	12 (100)	9 (100)	1.00
Steroids, *N* (%)	12 (100)	9 (100)	
End of follow-up			
eGFR, ml/min/1.73 m2, median (IQR)	49 (37–67)	61 (34–70)	0.60
Proteinuria, g/day, median (IQR)	0.1 (0–0.1)	0.2 (0.1–0.3)	0.97
Allograft loss, *N* (%)	1 (8)	0 (0)	1.00
Patient survival, *N* (%)	12 (100)	8 (89)	1.00

Regarding IVIG treated patients, median delay of treatment was 45 (12–132) months after kidney transplant. Among the $N = 8$ patients receiving induction therapy, $N = 4$ were treated with thymoglobulin and $N = 4$ with RIL-2 antibody. One patient was treated 1 month before IVIG. Three patients presented with one episode of acute T cell mediated rejection, 24, 51, and 67 months before high dose IVIG treatment. All of them received steroids. The one occurring 24 months before was also treated with thymoglobulin. Delay from acute rejection to IVIG treatment and immunophenotyping analysis seemed to be reasonable as it was always more than 1 year. None of them received Rituximab before high-dose IVIG infusions. Delay between thymoglobulin and IVIG treatment was more than 12 months in all but one patient. Screening biopsies at the time of anti-HLA DSA detection were available in all patients (**Supplemental Table 2**). Histological analysis was strictly normal in nine patients. Two

TABLE 2 | Anti-HLA donor specific antibodies (DSA) characteristics evolution in both groups.

Variables	IVIG patients		*p*-value	IVIG-free patients		*p*-value
	At the time of immunomodulatory treatment	At day 90 after treatment		At the time of immunomodulatory treatment	At day 90 after treatment	
Patients, *N*	12	12		9	9	
Delay from transplant, months, median (IQR)	45 (12–132)			53 (25–72)		0.98
CLASS I						
Number, median (IQR)	0.5 (0–1)	0.5 (0–1)	1.00	1 (0.5–2)	0.5 (0–1.5)	1.00
MFI max, median (IQR)	2,647 (1,590–7,177)	1,896 (1,363–4,886)	0.09	4,334 (2,254–12,119)	7,267 (1,605–8,693)	0.68
MFI sum, median (IQR)	2,646 (1,028–10,886)	2,228 (1,237–9,031)	0.19	6,295 (3,814–1,3351)	7,267 (3,202–10,866)	1.00
CLASS II						
Number, median (IQR)	1 (1,2)	0.25 (1–2.75)	0.75	0.5 (1,2)	0.5 (1–1.5)	1.00
MFI max, median (IQR)	1,735 (973–9,544)	1,888 (773–13,909)	1.00	5,880 (848–10,506)	6,499 (1,369–9,489)	0.22
MFI sum, median (IQR)	1,911 (1,516–12,610)	3,310 (1,641–14,694)	0.36	6,967 (848–11,010)	7,511 (1,369–10,706)	0.37

biopsies presented with isolated peritubular capillaritis grade 1 with no C4d positive staining nor glomerulitis. Chronic lesions included transplant glomerulopathy ($N = 1$), grade III IFTA ($N = 3$), and grade II IFTA ($N = 2$). DSA characteristics at the time of first IVIG infusion and 60 days after were described in **Table 2**. No difference can be isolated between the DSA before and after treatment (as in IVIG-free group—**Table 2**). One patient developed an acute ABMR 12 months after IVIG treatment completion. Clinical tolerance of high-dose IVIG was good without any adverse events besides benign headache in $N = 4$ patients. None of them presented with acute kidney injury.

PBMC Phenotype Evolution After High Dose IVIG

We first analyzed the 21 patients included treated or not. Results are shown in **Table 3**. No difference could be isolated neither in PBMC population at day 30 and day 60 compared to day 0 (T-cell, B-cell and NK-cell) nor in subtypes.

Then, as shown in **Figure 1** and **Table 4**, we performed a matched analysis in the 12 treated patients and PBMC population distribution was similar whatever time before or after treatment and whatever the population considered: monocytes (CD14), B-cell lymphocytes (CD19), NK cells (CD56), NKT cells ($CD3^+CD56^+$) and/or T-cell lymphocytes (CD4 and CD8 among CD3). We next analyzed T-cell lymphocytes: $CD45RA^+$ T cells (probably naive), $CD45RO^+$ T cells (probably memory) and regulatory T cells ($CD4^+CD25^{++}Foxp3^+$). Only CD8+ $CD45RA^+$ cells decreased significantly at D60 (**Figure 1C**). We also analyzed T reg subtypes according to Miyara description (20). No changes could be isolated at day 30 and 60. We studied B-cell lymphocytes using Bm1-5 classification (**Figure 1B** and **Table 3**) (21). Two subgroups of B-cells were modified along the treatment. While Bm1 (mature B-cells) increased significantly ($P = 0.004$), Bm2 (naïve B-cells) decreased significantly ($P = 0.012$). No significant changes were observed on the other B-cells subsets. Next, we performed a discriminative analysis based on the induction therapy in the treated patients. According to small number of patients in each group (RIL2 antibody-group 2, thymoglobulin—group 1 and no induction—group 0), we analyzed variation considering each patient as his own control. No significant results could be highlighted excepted increase of Bm1 proportion in no induction treatment group at day 60 after treatment (**Supplemental Table 2**).

PBMC mRNA Quantification Changes After High Dose IVIG

Transcript levels of molecules involved in inhibitory B-cell profiles (CD32 isoforms and the B-cell scaffold protein ankyrin repeats 1 BANK1), in B-cell survival (the B-cell activating factor BAFF and its receptor: BAFF-R), in T-cell differentiation (Th1, Th2, and Th17) and in apoptosis in the PBMC were analyzed in the treated patients. We justified the choice of targeted genes in **Supplemental Data**. Data were analyzed before treatment, at day 30 and at day 60 after two high dose IVIG infusions (**Table 3**). Levels of RORγt mRNA transcription factor and CD3 mRNA increased significantly 60 days after IVIG treatment beginning ($P = 0.02$ and $P = 0.02$, respectively). Correlation coefficient between CD3 transcriptomic and proteomic/cellular analysis was not significant (**Supplemental Figure 2**).

Discriminative analysis according to induction therapy did not reveal significant difference at day 30 and 60 compared to baseline (day 0) (**Supplemental Table 3**).

Circulating Serum Cytokines Along High Dose IVIG Treatment

Among the 25 cytokines analyzed in patients serum treated with IVIG, 11 where undetectable (IL-1α, IL-2, IL-5, IL-9, IL-10, IL-12p70, IL-13, IL-23, IL-31, IFNα, TNFβ), 9 where sporadically

TABLE 3 | PBMC populations distribution in patients treated or not with IVIG.

Populations	D0 (%.[Q1–Q3]) $N = 21$	D30 (%. [Q1–Q3]) $N = 12$	D30-0 p-value	D60 (%.[Q1–Q3]) $N = 10$	D60-0 p-value
Monocytes ($CD14^+$)	19.7 [14.4–33.9]	22.0 [16.6–32.3]	0.73	18.9 [7.6–35.3]	0.42
B cells ($CD19^+$)	5.9 [2.5–10.6]	6.6 [3.4–11.1]	0.90	7.8 [4.0–9.3]	0.98
Bm1	18.2 [10.9–34.1]	17.6 [9.3–22.2]	0.67	20.7 [13.6–28.8]	0.37
Bm2	34.4 [26.0–63.0]	43.1 [29.2–58.1]	0.69	37.9 [24.6–56.9]	0.85
Bm2'	0.9 [0.6–2.2]	1.8 [0.8–3.0]	0.77	0.8 [0.7–1.8]	0.48
Bm3+4	0.7 [0.4–0.9]	0.6 [0.4–0.9]	0.96	0.7 [0.6–0.8]	0.89
Bm5	18.7 [8.5–27.7]	18.1 [11.0–26.3]	0.84	18.3 [8.9–27.8]	0.79
eBm5	8.1 [6.4–14.1]	10.0 [8.4–18.2]	0.15	10.5 [8.4–11.6]	0.53
T cells ($CD3^+$)	50.7[32.6–61.2]	48.5 [39.1–58.8]	0.90	47.4 [44.5–69.2]	0.85
$CD4^+$	55.3 [45.8–65.3]	54.3 [48.9–66.7]	0.83	53.6 [40.0–59.9]	0.53
CD45RA	25.1 [16.1–43.1]	22.6 [20.2–41.2]	0.86	21.0 [13.4–37.7]	0.54
CD45RO	53.8 [38.9–61.5]	51.8 [34.8–84.3]	0.93	58.2 [43.8–68.2]	0.49
Treg	6.5 [3.8–11.1]	5.6 [4.1–13.5]	0.94	6.8 [5.1–10.1]	0.57
$CD8^+$	33.6 [28.1–42.3]	31.2 [27.7–42.8]	0.85	39.3 [32.0–46.2]	0.35
CD45RA	59.7 [43–72.9]	56.8 [42.8–68.5]	0.95	54.8 [37–71.1]	0.76
CD45RO	10.8 [6.6–20.9]	14 [7.6–21]	0.88	12 [8.2–28]	0.66
NK cells ($CD56^+$)	12.7 [7.6–18.5]	12 [7.8–21]	1.00	11.7 [9.1–21.7]	0.59
NKT cells ($CD3^+CD56^+$)	5.7 [1.5–16.5]	6.2 [2.1–9.8]	0.89	8.8 [2–15.4]	0.44

detected by one or two patients (IL-1β, IL-4, IL-6, IL-15, IL-17A, IL-21, IL-22, IL-27) and 5 were detected in more than 5 patients and are shown in **Figure 2** and **Table 3** (IL-7, IL-1Rα, IL-18, IFNγ, TNFα). Only IL-18 serum concentration significantly decreased at D60 ($P = 0.03$).

Considering discriminative analysis according to induction therapy, we could not analyze cytokines in each group due to small number of positive patients.

DISCUSSION

Prophylactic high dose IVIG has been used at the time of transplantation in high immunological risk recipients but analysis of their true action on humoral response is difficult to define since IVIG is mostly used as combined therapy (8, 22). In the context of *de novo* DSA in kidney allograft recipients, prophylactic use of high dose IVIG did not prevent acute ABMR and had minimal effects on DSA outcome (17). We provided here an extensive *in vivo* phenotypic and transcriptomic analysis in 12 patients treated with prophylactic high dose IVIG after kidney transplantation in patients with anti-HLA DSA without acute rejection and therefore without any other treatment associated. So more, the absence of acute rejection means the absence of immunological mechanisms activation or subclinical which will not interfere with IVIG actions.

Considering B lymphocytes, we showed first that proportion of naïve B-cells (Bm2) decreased significantly after high dose IVIG. This population was associated before and after transplant with anti-HLA DSA development within the first year after transplant (23, 24). In addition, belimumab, an anti-B lymphocyte stimulator (BLyS) antibody, associated at the time of transplant with standard transplant immunosuppression, decreased significantly naïve B-cells 24 weeks after transplant and *de-novo* IgG antibody formation (25). High dose IVIG could via the decrease of naïve B-cells (Bm2) limits *de-novo* IgG DSA formation. However, in our study, no early effect on DSA MFI has been reported. A later analysis merits to be performed in the future to look for a sustained effect on naïve B-cells and DSA.

In our study, high dose IVIG increased significantly, the proportion of memory B cells. Lower proportion of memory B cell before transplant, has been associated with high immunization levels (24) while higher proportion of memory B cell after transplant, has been described in operationally tolerant patients (no DSA and no immunosuppressive treatment) (26). In addition, in patients treated with belimumab proportion of memory B cell increased throughout the treatment with lower *de-novo* DSA (25). Whether increasing memory B-cell could decrease anti-HLA DSA production in our patients with immunomodulatory effects should be analyzed further.

High dose IVIG on T cell highlighted the decrease of the proportion of $CD8^+$ $CD45RA^+$ T cell after two infusions suggesting decrease of naïve T cell. Decreased frequency of $CD8^+$ $CD45RA^+$ and $CD4^+$ $CD45RA^+$ T cells have never been associated with acute rejection but with infection in older kidney recipients (> 60 years) (27). Increasing naïve T cells after transplantation could decrease memory T cells, interactions between T and B cells and may prevent not only alloantibody formation but also generation of long-lived memory T cells improving allograft survival. It is now well established that $CD4^+$ and $CD8^+$ memory T cells could contribute to allograft rejection and pose a major barrier to tolerance induction (28, 29).

RORγt mRNA transcripts, one nuclear factor involved in Th17 cells generation (30), increased significantly after high dose IVIG but without any changes in Th17 pathway cytokines. However, we did not analyzed RORγt using flow cytometry. Th17

TABLE 4 | PBMC populations distribution, serum cytokines and PBMC mRNA transcripts evolutions after IVIG treatment.

Populations	**D0 (%.[Q1–Q3]) *N* = 12**	**D30 (%. [Q1–Q3]) *N* = 12**	**D30-0 *p*-value**	**D60 (%.[Q1–Q3]) *N* = 10**	**D60-0 *p*-value**
Monocytes ($CD14^+$)	19.9 [14.4–32.9]	22.0 [16.6–32.3]	0.79	18.9 [7.6–35.3]	0.16
B cells ($CD19^+$)	9.4 [3.0–14.2]	6.6 [3.4–11.1]	0.15	7.8 [4.0–9.3]	0.11
Bm1	14.8 [9.9–19.0]	17.6 [9.3–22.2]	0.73	20.7 [13.6–28.8]	0.004
Bm2	43.8 [32.6–65.4]	43.1 [29.2–58.1]	0.15	37.9 [24.6–56.9]	0.01
Bm2'	1.1 [0.7–3.2]	1.8 [0.8–3.0]	1.00	0.8 [0.7–1.8]	0.64
Bm3+4	0.6 [0.3–0.8]	0.6 [0.4–0.9]	0.20	0.7 [0.6–0.8]	0.03
Bm5	16.7 [8.5–22.7]	18.1 [11.0–26.3]	0.18	18.3 [8.9–27.8]	0.09
eBm5	9.3 [7.1–14.1]	10.0 [8.4–18.2]	0.02	10.5 [8.4–11.6]	0.65
IgD+CD27- (naives)	60.7 [42.1–74.3]	56.4 [41.3–73.4]	0.46	55.8 [42.1–69.3]	0.34
Unswitched	6.8 [3.8–9.3]	9.6 [3.7–11.8]	0.46	7.6 [3.5–18.2]	0.31
IgD^+CD27^+ Switched IgD-$CD27^+$	13.3 [8.5–31.7]	15.5 [9.7–28.6]	0.41	16.5 [10.6–32.2]	0.25
T cells ($CD3^+$)	41.7[31.4–61.2]	48.5 [39.1–58.8]	0.73	47.4 [44.5–69.2]	0.06
$CD4^+$	55.5 [46.2–63.6]	54.3 [48.9–66.7]	1.00	53.6 [40.0–59.9]	0.24
CD45RA	25.2 [21.6–43.7]	22.6 [20.2–41.2]	0.56	21.0 [13.4–37.7]	0.23
CD45RO	50.9 [38.9–57.4]	51.8 [34.8–84.3]	0.04	58.2 [43.8–68.2]	0.08
Treg	7 [3.6–15.8]	5.6 [4.1–13.5]	1.00	6.8 [5.1–10.1]	0.91
$CD45RA^+$ $Foxp3^+$ (I)	0.7 [0.4–1.7]	0.8 [0.6–1.5]	0.42	0.6 [0.6–1.0]	0.82
CD45RA- $Foxp3^{hi}$ (II)	1.2 [0.2–2.0]	1.7 [0.8–2.3]	0.42	1.3 [0.7–1.7]	0.05
CD45RA- $Foxp3^{lo}$ (III)	4.8 [2.2–13.1]	4.2 [3.5–7.8]	1.00	4.6 [4.1–5.9]	0.25
$CD8^+$	32.6 [29.1–38.3]	31.2 [27.7–42.8]	0.92	39.3 [32.0–46.2]	0.24
CD45RA	59.9 [47.5–75.1]	56.8 [42.8–68.5]	0.63	54.8 [37–71.1]	0.03
CD45RO	13.9 [6–20.9]	14 [7.6–21]	0.70	12 [8.2–28]	0.43
NK cells ($CD56^+$)	12.9 [7.6–22.4]	12 [7.8–21]	0.79	11.7 [9.1–21.7]	0.43
NKT cells ($CD3^+CD56^+$)	4.2 [1.9–15.7]	6.2 [2.1–9.8]	0.73	8.8 [2–15.4]	0.77
Serum cytokines	**D0 (pg/ml. [Q1–Q3]) *N* = 11**	**D30 (pg/mL. [Q1–Q3]) *N* = 11**	**D30-0 *p*-value**	**D60 (pg/mL. [Q1–Q3]) *N* = 8**	**D60-0 *p*-value**
IFNγ	6.85 [0.86–9.21]	6.17 [0–12.1]	1.00	9.88 [5.92–12.5]	0.38
IL-1RA	172 [16.9–524]	159.1 [65.5–240]	1.00	11.33 [0–343.8]	0.16
IL-7	7.55 [0.69–12.4]	7.49 [3.7–13.3]	0.94	7.54 [1.1–12.25]	0.64
IL-18	1.46 [0–9.97]	0 [0–3.68]	0.06	0 [0–5.6]	0.03
TNFα	0 [0–0.69]	0 [0–0.93]	0.50	0.47 [0–1.52]	0.07
Genes	**D0 (Fold increase. [Q1–Q3]) *N* = 11**	**D30 (Fold increase. [Q1–Q3]) *N* = 11**	**D30-0 *p*-value**	**D60 (Fold increase. [Q1–Q3]) *N* = 8**	**D60-0 *p*-value**
RORγT	1.00	1.5 [0.3–3.7]	0.29	2.0 [1.4–4.5]	0.02
Tbet	1.00	1.0 [0.5–1.6]	0.83	1.0 [0.6–3.6]	0.36
Gata-3	1.00	0.5 [0.3–1.0]	0.12	1.1 [0.3–2.0]	0.70
CD3	1.00	1.2 [0.8–2.6]	0.21	2.2 [0.8–3.2]	0.02
CD32a	1.00	1.0 [0.4–2.2]	0.90	0.9 [0.4–1.9]	1.00
CD32b	1.00	0.9 [0.3–2.5]	0.52	1.2 [0.6–2.3]	0.28
CD19	1.00	0.8 [0.6–1.5]	0.90	1.7 [0.7–2.9]	0.10
BAFF	1.00	1.3 [0.9–2.5]	0.06	1.6 [0.6–3.1]	0.10
BAFF-R	1.00	1.2 [0.6–3.0]	0.36	1.6 [0.8–2.0]	0.28
TGFβ	1.00	2.0 [0.6–3.0]	0.06	0.6 [0.4–1.6]	0.95
Fas	1.00	0.7 [0.3–3.3]	1.00	0.7 [0.2–1.4]	0.43
FasL	1.00	0.9 [0.3–1.2]	0.58	2.0 [0.6–4.6]	0.12
CD4	1.00	1.3 [0.4–2.2]	0.29	1.3 [0.9–1.8]	0.09

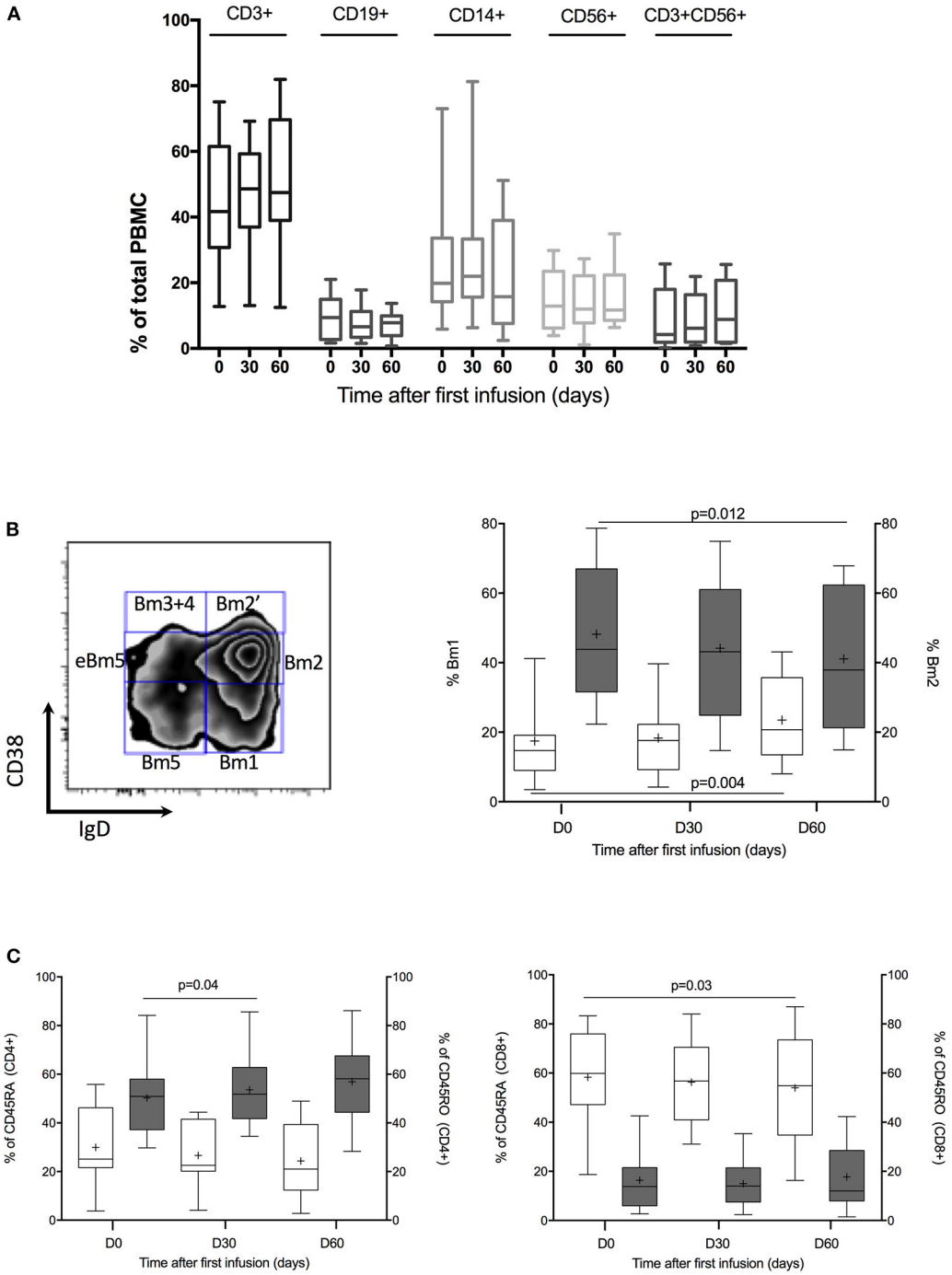

FIGURE 1 | PBMC cells phenotypic analysis. **(A)** Kinetic of $CD3^+$, $CD19^+$, $CD14^+$, $CD56^+$, and $CD3^+CD56^+$ proportion throughout IVIG treatment. Box plot represent % of positive cells with 5–95% Whiskers. **(B)** B-cell population analysis. On the left, representative dot plots of B cells using the Bm1-5 classification based on CD38 and IgD expression. Gating strategies is shown. On the right % of Bm1 B cells (blanked) vs. % of Bm2 B cells (dark gray) at day 0, 30, and 60. Box plot represent % of positive cells with 5–95% Whiskers. Results were compared with Wilcoxon signed rank test. **(C)** CD45RA and CD45RO analysis in CD4 and CD8 cells. On the left, % of CD45RA (blanked) vs. % of CD45RO (dark gray) in $CD4^+$ cells. On the right, % of CD45RA (blanked) vs. % of CD45RO (dark gray) in $CD8^+$ cells. Box plot represent % of positive cells with 5–95% Whiskers. Results were compared with Wilcoxon signed rank test.

cells and their cytokines, IL-17, IL-22, IFNγ, IL-6 and TNF-α, play a significant role in the development of acute and chronic allograft injury after organ transplantation (31). CD3 mRNA transcripts increased significantly while CD3 cells proportion remained stable. Only five cytokines could be analyzed because of low expression of cytokines in our patients. IL-18, which is a potent pro-inflammatory cytokine involved in the host defense by upregulating both innate and acquired immune

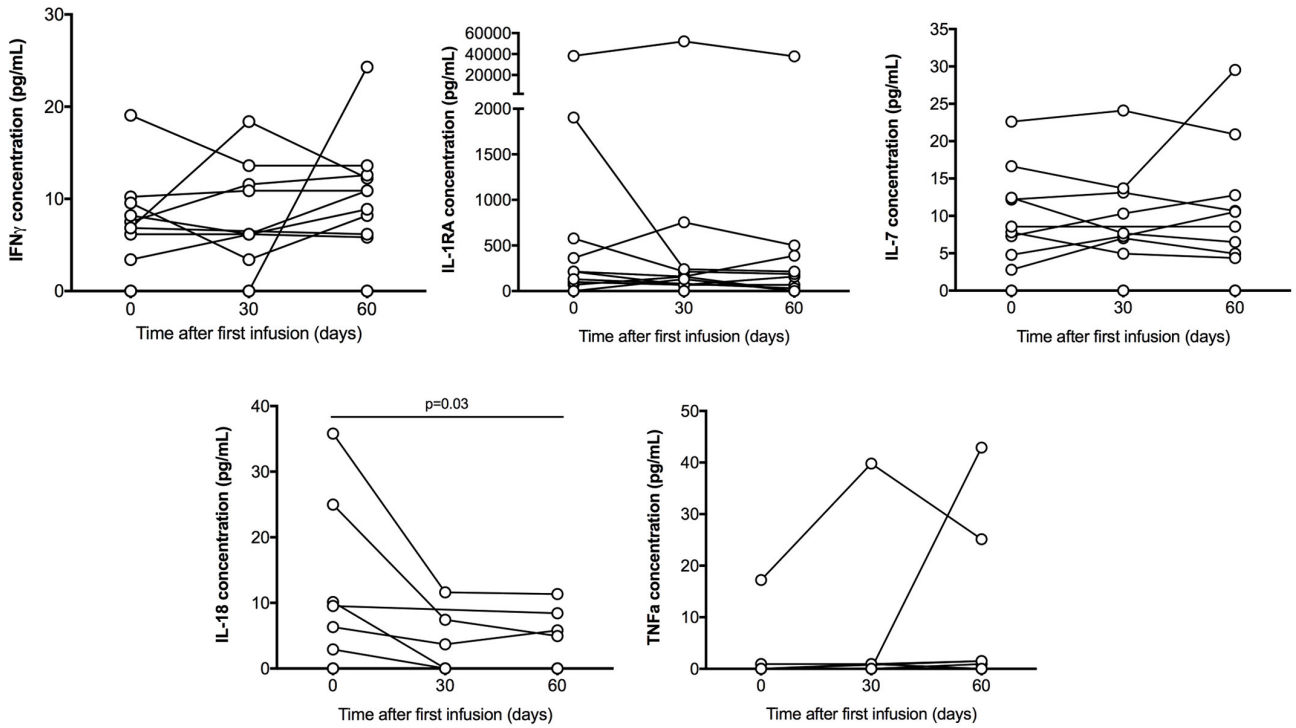

FIGURE 2 | Serum cytokines multiplex analysis. IFNγ, IL-1R1A, IL-18, IL-7, and TNFα concentration (pg/mL) detected in patient throughout the treatment, on day 0, 30, and 60. Only IL-18 serum concentration significantly decreased at D60 ($P = 0.03$). Results were compared with Wilcoxon signed rank test.

responses especially Th1 responses (32), decreased significantly after IVIG high dose infusion. The main role of IL-18 is to crucially stimulate lymphocytes to produce the IFN-γ and regulate macrophages activity (32). However, in our study, serum levels decrease of IL-18 was not followed by a significant effect on serum levels of IFN-γ. Conclusion about transcripts levels modifications and cytokines expression after high dose IVIG could not be formulated.

Recently, Yabu et al. showed that immune profiles could predict response to desensitization therapy in highly HLA-sensitized kidney transplant candidates (33). More precisely, authors depicted a combination of transitional B cell and regulatory T cell frequencies before initiation of desensitization therapy that could distinguish responders from non-responders. The response to therapy was assessed by a predefined decrease of 5% or greater in cumulative calculated panel reactive antibodies. So more, one transcript, TRAF3IP3 could also distinguish responders from non-responders. However, we could not isolate responders from non-responders in our cohort because of the small number of patients, the absence of significant changes in DSA and only one acute ABMR episode after treatment.

The major limit of our study was the small number of patients included. However, after kidney allograft transplantation, high dose IVIG are never infused alone. Indications after kidney transplantation include (i) the prevention of acute ABMR in presensitized patients associated with thymoglobulin induction therapy and plasmapheresis and/or rituximab (ii) treatment of acute ABMR associated with plasmapheresis (11). In the first indication, no study evaluating prophylactic treatment in kidney deceased donor recipients is available. So more, prophylactic high doses IVIG are not used in clinical practice in patients with *dn* DSA and we stopped our pilot study because of failure to prevent acute ABMR (17). Considering clinical data without a net benefit and the cost of high dose IVIG, ∼$6,500 for a single 140 g IVIG treatment (34), we could not treat more patients and our data *in vivo* could not be expanded. Meanwhile, we would like to highlight that this is the sole study analyzing high dose IVIG *in vivo* effect after kidney transplantation.

Taken together, our data (clinical and experimental) suggested that the benefits of high dose IVIG after kidney transplantation are limited. Furthermore, high dose IVIG have been associated with more tubular macrovacuoles and chronic tubulointerstitial changes after kidney transplantation (35, 36). Our results remain to be confirmed in a larger population which could lead to deep modifications of the current use of high dose IVIG in clinical practice after kidney transplantation.

In conclusion, we present here the first study analyzing *in vivo* prophylactic high dose IVIG effects on T and B cell phenotype in kidney allograft recipients with *de novo* DSA. Our results trend to suggest that high dose IVIG induce limited modifications in B and T cell phenotype including decrease of naïve B cell and $CD8^+$ T cell and increase of memory B cell. These modifications could lead to immunomodulation and limit anti-HLA DSA production. However, our previously clinical data suggested that kidney allograft recipients with DSA treated preventively with high dose IVIG showed similar AMR rate than non-treated group (17) and no DSA modifications could be isolated in this study.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by APHP-Saint Louis. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

CP, JB, CG, PG, and MM: conceptualization, methodology, and data curation. CP: software. CP, JB, CG, PL, JC, AT, PG, and MM: validation and formal analysis. MM: resources and supervision. CP and MM: writing—original draft preparation. CP, JB, AT, JC, PG, and MM: writing—review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00034/full#supplementary-material

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ORIGINAL RESEARCH
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Immune Cell Infiltrate in Chronic-Active Antibody-Mediated Rejection

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Background: Little is known about immune cell infiltrate type in the kidney allograft of patients with chronic-active antibody-mediated rejection (c-aABMR).

Methods: In this study, multiplex immunofluorescent staining was performed on 20 cases of biopsy-proven c-aABMR. T-cell subsets (CD3, CD8, Foxp3, and granzyme B), macrophages (CD68 and CD163), B cells (CD20), and natural killer cells (CD57) were identified and counted in the glomeruli (cells/glomerulus) and the tubulointerstitial (TI) compartment [cells/high-power field (HPF)].

Results: In the glomerulus, T cells and macrophages were the dominant cell types with a mean of 5.5 $CD3^+$ cells/glomerulus and 4 $CD68^+$ cells/glomerulus. The majority of T cells was $CD8^+$ (62%), and most macrophages were $CD68^+CD163^+$ (68%). The TI compartment showed a mean of 116 $CD3^+$ cells/HPF, of which 54% were $CD8^+$. Macrophage count was 21.5 cells/HPF with 39% $CD68^+CD163^+$. $CD20^+$ cells were sporadically present in glomeruli, whereas B-cell aggregates in the TI compartment were frequently observed. Natural killer cells were rarely identified. Remarkably, increased numbers of $CD3^+FoxP3^+$ cells in the TI compartment were associated with decreased graft survival ($p = 0.004$).

Conclusions: Renal allograft biopsies showing c-aABMR show a predominance of infiltrating $CD8^+$ T cells, and increased numbers of interstitial $FoxP3^+$ T cells are associated with inferior allograft survival.

Keywords: kidney transplantation, pathology, chronic rejection in renal transplant, antibody-mediated allograft rejection, immune cell

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INTRODUCTION

The renal biopsies of patients with chronic-active antibody-mediated rejection (c-aABMR) have defining histomorphological lesions (1). These characteristic lesions include double contours of the glomerular basement membrane (transplant glomeropathy) and/or severe peritubular capillary basement membrane multilayering. In addition, evidence of current or recent antibody interaction with the vascular endothelium is provided by either linear C4d staining in peritubular capillaries or moderate microvascular inflammation [(g + ptc) $\geq$ 2] (2). The presence of donor-specific antibodies underlies c-aABMR, but the influx of inflammatory cells and rate of loss of renal function may vary substantially.

Previously, different studies have reported on the inflammatory cell types involved in renal allograft rejection. Hidalgo et al. provided evidence for a possible effector role for natural killer (NK) cells in endothelial injury during ABMR (3). In addition, ABMR is associated with both glomerular and interstitial monocyte infiltration (4–7). Mengel et al. showed the importance of inflammatory infiltrates in protocol biopsies, as they were independently associated with allograft survival, regardless of their location (8). Furthermore, activated cytotoxic T cells are known to play an important role in the inflammatory activity and destruction of allograft tissue (9). The increased presence of cytotoxic granules such as granzyme B have been associated with acute rejection (10–12). Similarly, $FoxP3^+$ T cells [T regulatory cells (Tregs)] have been identified as potential crucial players in inflammatory disease and allograft rejection (13–15).

However, the majority of studies has focused on acute rejection (both TCMR and ABMR), and there is currently a lack of data on the presence and clinical relevance of inflammatory cells in renal allografts of patients with c-aABMR.

In the current study, we used multiplex immunofluorescent (IF) staining to assess the inflammatory cell composition in the renal allograft biopsy of patients with c-aABMR. We report on the presence of various T-cell subtypes, B cells, NK cells, and myeloid cells in both glomeruli and the tubulointerstitial (TI) compartment. In addition, the association between location and quantity of the different inflammatory cells with allograft survival was analyzed.

MATERIALS AND METHODS

Study Population and Material

Twenty patients with biopsy-proven c-aABMR between 2010 and 2014 were included in this retrospective study. Sufficient formalin-fixed paraffin-embedded (FFPE) material after diagnostic process was needed to be included for further analysis. Renal transplant biopsies were all for cause and deemed necessary by the treating nephrologist due to a unexplained decline in renal allograft function with or without new onset proteinuria. All stained slides were re-evaluated and scored by two blinded pathologists according to the Banff 15 criteria (16). Donor-specific antibodies (DSA) were retrospectively assessed for all patients. The study was approved by the Medical Ethical Committee of the Erasmus MC (MEC-2019-0308).

Staining

For routine diagnostic analysis, 3 μm sections were cut from the FFPE tissue and stained by H&E, periodic acid–Schiff–diastase, and Jones according to standardized diagnostic protocol at the Department of Pathology at the Erasmus Medical Center. Immunofluorescence staining was performed on snap-frozen tissue sections [immunoglobulin G (IgG), IgA, IgM, C3, C1q, kappa, and lambda] to exclude immune-complex-mediated disease.

Additional quadruple multiplex IF staining was performed on 4 μm FFPE sections. Multiplex IF staining was performed using the antibody combination of CD3, CD8, and granzyme B for $CD4^+$ T cells, $CD8^+$ cytotoxic T cells both with or without granzyme B presence (**Figure 1**). Second, CD20 was used as a B-cell marker, and for the identification of macrophages, the pan macrophage marker CD68 was used in combination with CD163 to distinguish the M2 macrophage subtype (profibrotic) (**Figure 2**). Lastly, a combination of CD3, FoxP3, and CD57 was used to identify $CD3^+FoxP3^+$ regulatory T cells and NK cells (**Figure 3**). 4′,6-Diamidino-2-phenylindole (DAPI) was used in all stainings to visualize the nuclei. Multiplex IF was performed as previously described by Punt et al. (17). In brief, after deparaffinization, ethylenediaminetetraacetic acid (pH 9) antigen retrieval was performed in a microwave. Next, three different combinations of primary antibodies were applied to the slides. The first mixture consisted of the antibodies anti-CD3 (rabbit polyclonal, ab828; Abcam), anti-CD8 (mouse monoclonal IgG2b,

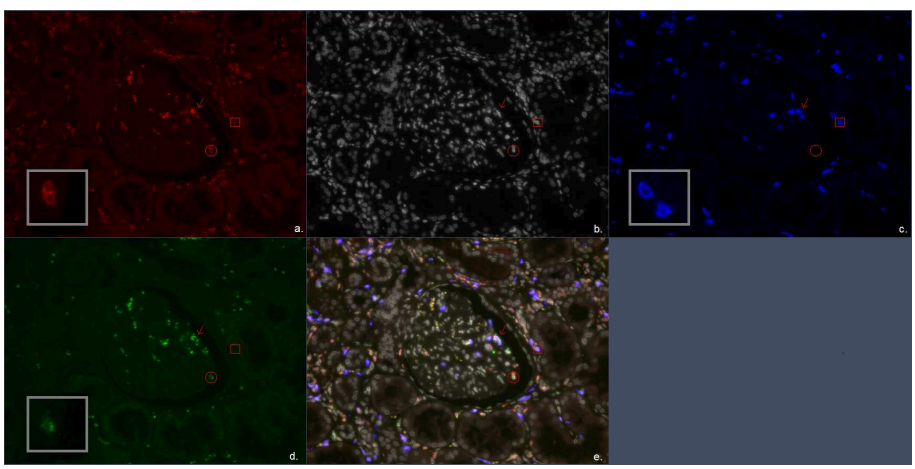

FIGURE 1 | Multiplex immunofluorescent (IF) staining image of the antibody combination CD3, CD8, and granzyme B. **(a)** Red, CD3; **(b)** white, DAPI; **(c)** blue, CD8; **(d)** green, granzyme B; **(e)** combination of CD3, CD8, granzyme B, and DAPI. The arrow marks a $CD3^+CD8^+$granzyme B^+ cell representing a $CD8^+$ cytotoxic T cell. The circle encloses a $CD3^+$granzyme B^+ cell representing a $CD4^+$ cytotoxic T cell. The square encloses a $CD3^+CD8^+$ cell representing a $CD8^+$ T cell.

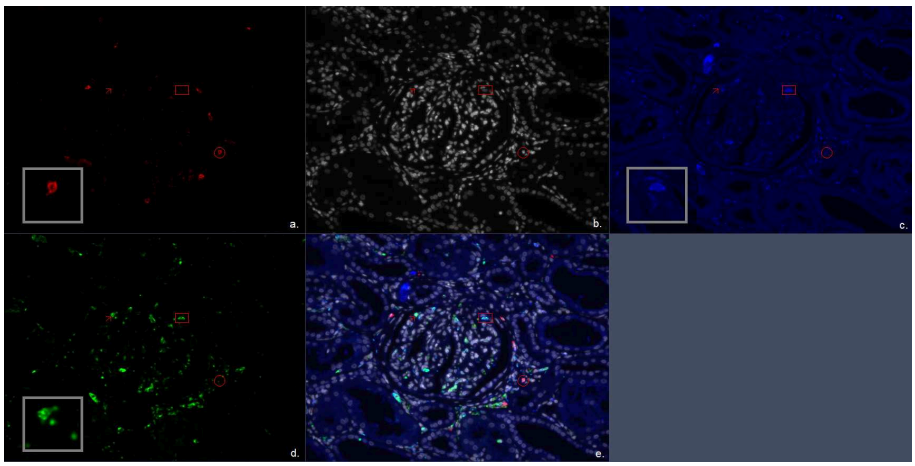

FIGURE 2 | Multiplex immunofluorescent (IF) staining image of the antibody combination CD20, CD68, and CD163. **(a)** Red, CD20; **(b)** white, DAPI; **(c)** blue, CD163; **(d)** green, CD68; **(e)** combination of CD20, CD68, CD163, and 4′,6-diamidino-2-phenylindole (DAPI). The arrow marks a $CD68^+$ cell representing a single positive CD68 macrophage. The circle encloses a $CD20^+$ cell representing a B cell. The square encloses a $CD68^+CD163^+$ cell representing a double positive M2 macrophage subtype.

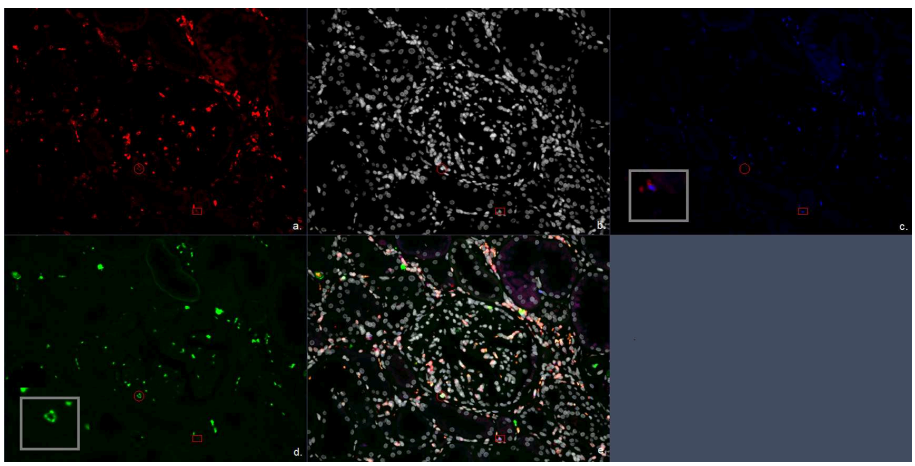

FIGURE 3 | Multiplex immunofluorescent (IF) staining image of the antibody combination CD3, CD57, and FoxP3. **(a)** Red, CD3; **(b)** white, DAPI; **(c)** blue, FoxP3; **(d)** green, CD57; **(e)** combination of CD3, CD57, FoxP3, and 4′,6-diamidino-2-phenylindole (DAPI). The circle encloses a $CD3^+CD57^+$ cell representing a $CD3^+CD57^+$ cytotoxic T cells. The square encloses a $CD3^+FoxP3^+$ cell representing a $FoxP3^+$ T regulatory cell.

4B11; Novocastra), and anti-Granzyme B (mouse monoclonal IgG2a, GRB-7, DAKO). The second mixture consisted of the antibodies anti-CD3 (rabbit polyclonal, ab828; Abcam), anti-FoxP3 (mouse monoclonal IgG1, 236A/E7, Abcam), and anti-CD57 (mouse monoclonal IgM, HNK-1; developed in Leiden University Medical Center). The third mixture comprised the antibodies anti-CD20 (mouse-IgG2a, L2G, Ventana), anti-CD163 (rabbit, ab100909, Abcam), and anti-CD68 (mouse-IgG1, 123C3, Ventana). The first combination was visualized using a combination of fluorescent antibody conjugates (Molecular Probes, ThermoFisher): goat antirabbit IgG–Alexa Fluor 546, goat antimouse IgG2b–Alexa Fluor 647, and goat antimouse IgG2a–Alexa Fluor 488. The second combination was visualized using goat antirabbit IgG–Alexa Fluor 546, goat antimouse IgG1–Alexa Fluor 647, and goat antimouse IgM–Alexa Fluor 488. The third combination was visualized using goat antimouse IgG2a–Alexa Fluor 546, antirabbit IgG–Alexa Fluor 647, and goat antimouse IgG1–Alexa Fluor 488. The panels were optimized using normal human tonsil and normal renal cortical tissue.

Detection and Analysis

Images of the slides were taken for further analysis on a confocal laser scanning microscope in multitrack setting (Zeiss LSM700, Zeiss, Jena, Germany). An LCI Plan-Neofluar 25×/0.8 Imm Korr DIC objective (Zeiss) was used.

If possible, tissue sections were scanned in their entirety. For the glomerular cell counts, all available glomeruli per silver-stained tissue were included. The glomerular compartment cell

counts were represented as the average number of whole cells per glomeruli. Tubulo-interstitial compartment cell counts were represented as the average number of whole cells per high-power field (HPF, 40×). A blinded and random selection of 3 HPF was analyzed for the TI compartment cell counts. All cells were counted using the Zen 2.3 SP1 software.

Statistical Analysis

Normally distributed data are expressed as mean ± SD, non-normally distributed data as median (interquartile range). All statistical analysis were performed using Graphpad Prism 6 and SPSS software version 24. Statistical significance was calculated using unpaired *t*-test for continuous variables, Mann–Whitney *U*-test for ordinal variables, and chi-squared or Fisher exact test for categorical variables. A $P < 0.05$ was considered statistically significant.

Graft survival curves, starting at time of c-aABMR diagnosis, were censored for death with functioning graft and analyzed by Kaplan–Meier with log-rank test. For the analysis of association of inflammatory cells with allograft survival, both the glomerular and TI compartment cell count were divided dichotomously based on the mean cell count.

TABLE 1 | Main clinical features at time of chronic-active antibody-mediated rejection (c-aABMR) diagnosis.

		***N* = 20**
Recipients age, years (IQR)		54 (44–63)
Male, *n* (%)		14 (70)
Donor age, years (IQR)		52 (40–59)
Prior transplantation, *n* (%)		7 (35)
Living donation, *n* (%)		13 (65)
HLA mismatch, median (IQR)		3 (2–4)
Time post-transplantation, years (IQR)		3.6 (1.8–7.5)
eGFR, ml/min/1.73 m^2 (IQR)		29 (24–38)
Proteinuria, g/L (IQR)		0.75
DSA positive, *n* (%)		9 (45)*
	HLA class I	2
	HLA class II	8
C4d positive, *n* (%)		10 (50)
Renal disease, *n* (%)	Diabetic nephropathy	5 (25)
	Hypertensive nephropathy	2 (10)
	Reflux nephropathy	2 (10)
	Chronic pyelonephritis	2 (10)
	Cystic kidney disease	2 (10)
	Other	7 (35)
Immunosuppressive therapy, *n* (%)	Tacrolimus	16 (80)
	MMF	18 (90)
	Corticosteroids	9 (45)
	Other	1 (5)

**One patient had both HLA class I and HLA class II DSA present.*

RESULTS

Baseline Characteristics

Clinical and histological characteristics of the included patients are shown in **Table 1** and **Figure 4**. The mean age of the patients was 54 years at the time of transplant biopsy. Mean time point of biopsy post-transplantation was 3.6 years. Patients were predominantly treated with an immunosuppressive regimen using a combination of calcineurin inhibitors (mainly tacrolimus, 80%) and mycophenolate mofetil (90%). Mean follow-up was 3.4 years (range, 0.7–8.3 years) or until graft failure (either retransplantation or return to dialysis). Two patients died with a functioning graft during follow-up.

Forty-five percent of patients were DSA positive at the time of biopsy, 50% showed linear positive C4d staining of the peritubular capillaries, and all biopsies showed microvascular inflammation. Furthermore, all patients had severe double contours of the glomerular basement membrane (cg) present in their biopsy with moderate to severe chronic damage [interstitial fibrosis and tubular atrophy (IFTA)].

$CD3^+$, $CD8^+$, Granzyme B^+ T Cells

First, we analyzed the number of glomerular T cell ($CD3^+$ T cells), $CD8^+$ T cells ($CD3^+CD8^+$ T cells), $CD4^+$ T cells ($CD3^+CD8^-$ T cells), $CD8^+$granzyme B^+ T cells, and $CD4^+$granzyme B^+ T cells by quadruple immunostaining. **Figure 5** shows the distribution as well as the mean number of inflammatory cells per glomerulus per biopsy. A mean total of 5.5 $CD3^+$ cells were present per glomerulus with a range of 0.8–9.6 $CD3^+$ cells. $CD8^+$ T cells were predominant, making up 61.7% of $CD3^+$ T cells with a mean of 3.4 cells per glomerulus. A minority of $CD8^+$ T cells (46%) and $CD4^+$ T cell (23%) showed cytotoxic potential as measured by coexpression of granzyme B. Inflammatory cell counts were then performed for the TI compartment (**Figure 6**). Although substantial variations were observed in the number of infiltrating cells among the biopsies, large areas of $CD3^+$ T cells were abundantly present within the TI compartment. The TI showed a mean $CD3^+$ cell count of 116.2 per HPF. The majority of $CD3^+$ T cells present in the TI

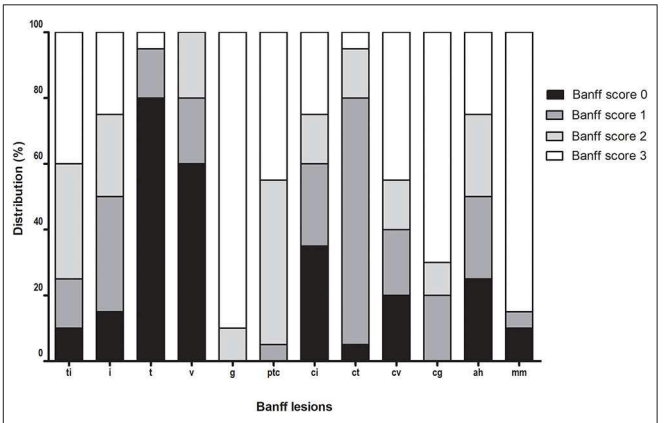

FIGURE 4 | Distribution of Banff lesions at time of chronic-active antibody-mediated rejection (c-aABMR) diagnosis.

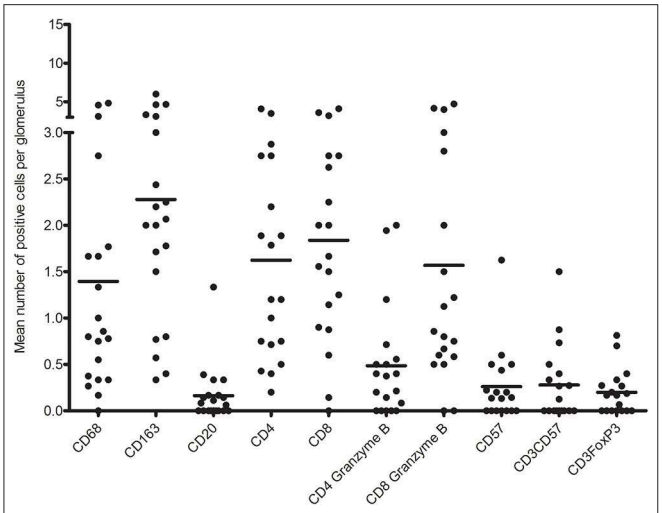

FIGURE 5 | Distribution of mean number of inflammatory immune cells per glomerulus per biopsy.

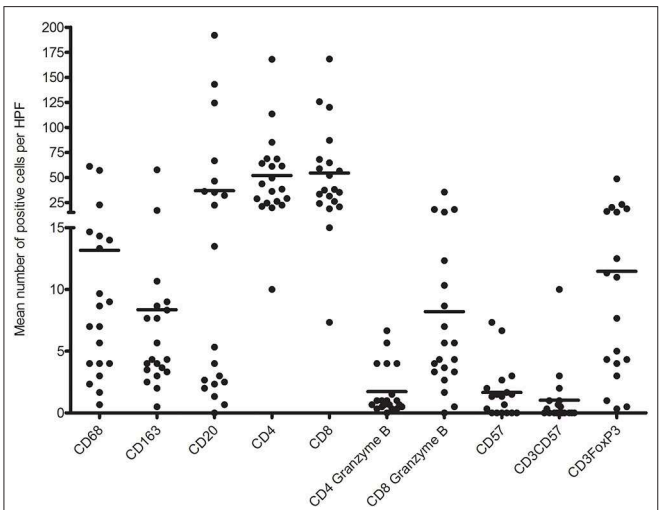

FIGURE 6 | Distribution of mean number of inflammatory immune cells per high-power field (HPF) per biopsy.

represented $CD8^+$ T cells (53.9%) with an average cell count of 62.6 per HPF followed by $CD4^+$ T cells (46.2%) with 53.6 positive cells per HPF. In contrast to the glomeruli, there were hardly any $CD8^+$ granzyme B^+ T cells (7% of total $CD3^+$ T cells, 8.2 cells per HPF) and $CD4^+$ granzyme B^+ T cells (1.5% of total $CD3^+$ T cells, 1.7 cells per HPF) present.

No significant association was found for the presence of $CD3^+$ T cells and the T-cell subsets in the glomeruli and TI compartment with regard to allograft survival or DSA presence.

$CD3^+$, $FoxP3^+$ T Cells, and $CD57^+$ NK Cells

The second quadruple multiplex IF staining identified $CD57^+$ NK cells ($CD3^-$), $CD3^+FoxP3^+$ regulatory T cells, and $CD3^+CD57^+$ T cells.

The glomeruli showed low counts of $CD57^+$ and $CD3^+CD57^+$ cells in this multiplex IF staining (**Figure 5**). Almost 40% of biopsies showed no presence of $CD57^+$ or $CD3^+FoxP3^+$ cells in the glomeruli. The remaining biopsies showed a mean of 0.67 $CD57^+$, 0.56 $CD3^+CD57^+$, and 0.32 $FoxP3^+$ cells per glomerulus. $CD3^+CD57^+$ cytotoxic T cells accounted for 6.7% of $CD3^+$ cells in the glomerulus.

The TI compartment, however, showed a much higher presence of $CD3^+FoxP3^+$ T cells with an average of 11.5 cells per HPF. These cells accounted for 8.5% of the $CD3^+$ cells. A clear distinction into two groups was noticeable within the biopsies for $CD3^+FoxP3^+$ T cells (high vs. low mean cell count). Fifty percent of biopsies had a high mean cell count of 19.6 positive cells per HPF vs. a low mean cell count of 3.4 positive cells per HPF. The increased presence of $CD3^+FoxP3^+$ T cells was significantly associated with a decreased allograft survival (**Figure 7A**). Patients with high $CD3^+FoxP3^+$ T cell rates had a graft survival of 2.1 vs. 5.3 years in the patients with low FoxP3 presence ($p = 0.004$).

Similar to what was observed for the glomeruli, the $CD57^+$ cell count in the TI was low with a mean of 1.7 cells per HPF and $CD3^+CD57^+$ T cells accounted for only 0.8% of $CD3^+$ cells in this compartment.

$CD68^+$, $CD163^+$ Macrophages, and $CD20^+$ B Cells

The third multiplex IF staining panel included markers for macrophages ($CD68^+$), M2 macrophages ($CD68^+CD163^+$), and B cells ($CD20^+$).

$CD20^+$ cells were sporadically present in glomeruli with a mean number of 0.16 positive cells per glomerulus. Interestingly, 45% of biopsies hardly contained any B cells in the glomeruli. The macrophages ($CD68^+$ cells) represented mean number of almost four cells per glomerulus. The majority (68%) was $CD68^+CD163^+$ with a mean positive cell count of 2.3 per glomerulus. A scattered distribution of macrophages was visible with ranges of 0–6 positive cells per glomerulus. No significant association with graft function or DSA presence was found for macrophage or B cell presence in the glomeruli (data not shown).

In contrast to the glomeruli, the TI compartment showed a higher percentage of $CD68^+$ cells (61%) with a mean positive cell count of 13.2 per HPF. $CD68^+CD163^+$ macrophages accounted for 39% of macrophages with a mean of 8.4 positive cells per HPF. The presence of total $CD68^+$ and $CD68^+CD163^+$ macrophages in the TI compartment showed a near significant inverse association with graft survival ($p = 0.08$) (**Figure 7B**).

Furthermore, a mean number of 36.8 positive CD20 cells was counted in the tubulointerstitium. However, as with the $CD3^+FoxP3^+$ T cells, a clear distribution into two groups was visible. Forty-five percent of the biopsies were found to present $CD20^+$ cells in nodular formation with a mean of 74.5 CD20-positive cells per HPF. The remaining biopsies reached a mean of 3.4 $CD20^+$ cells per HPF. The distribution in B cell was not significantly associated with graft survival ($p = 0.13$). However, patients with increased numbers of B cells in the TI compartment

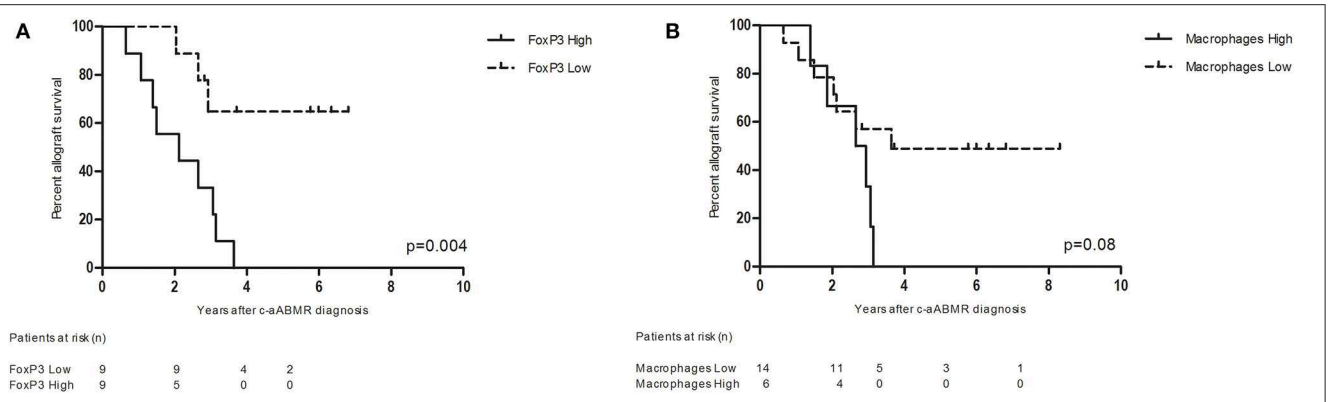

FIGURE 7 | (A) Renal allograft survival after chronic-active antibody-mediated rejection (c-aABMR) diagnosis in relation to $CD3^+$ $FoxP3^+$ cell presence in the tubulointerstitial compartment; **(B)** renal allograft survival after c-aABMR diagnosis in relation to overall macrophage ($CD68^+$ and $CD163^+$) presence in the tubulointerstitial compartment.

had the tendency to have DSA present in the serum at time of biopsy. However, this was not statistically significant ($p = 0.078$).

DISCUSSION

No detailed description on inflammatory cells in renal allograft biopsies showing c-aABMR is currently available. Through multiplex IF staining, we evaluated inflammatory cell presence in glomeruli and TI compartment and related their presence to renal allograft survival. Our study is the first detailed report on the localization and immunophenotypic composition of inflammatory cells in c-aABMR of the renal allograft. Most notably T cells and macrophages were observed within the glomeruli and/or in the TI compartment. Of interest is the increased presence of $FoxP3^+$ T cells in the TI compartment, which is significantly associated with inferior allograft survival.

Renal biopsies showing c-aABMR demonstrated vast areas of inflammatory cells denoting the presence of an ongoing immune-mediated process. Substantial numbers of T cells and macrophages were present in the glomeruli and TI compartment.

The glomerular compartment predominantly comprised of $CD8^+$ cytotoxic T cells (granzyme B^+ and $CD57^+$) and M2 macrophages ($CD68^+$ and $CD163^+$), but there was a relatively low amount of single positive $CD57^+$ NK cells in the glomeruli. Previous reports have provided evidence for the presence of NK cell transcriptomes marking NK cells as an important player in the pathogenesis of c-aABMR (3, 18, 19). Our study using multiplex IF staining could not confirm the presence of these cells as only a small number of NK cells were present in allografts within the setting of c-aABMR. However, Parkes et al. previously demonstrated that over 50% of increased transcripts in CD16a-activated NK cells are also increased in activated $CD8^+$ T cells. The NK and T cells shared transcripts for effector cytokines, chemokines, and other molecules related to effector cell function (18). Of interest was the presence of $CD3^+CD57^+$ T cells in the glomeruli. Several biopsies showed infiltrates of $CD3^+CD57^+$ T cells, and their presence accounted for 6.7% of $CD3^+$ cells in the glomerulus. Although these cells have not been further phenotyped, they might indicate the presence of terminally differentiated effector $CD8^+CD57^+$ cytotoxic T cell, possessing NK cell-like properties. These cells are known to have strong cytotoxic potential with high expression of both granzyme B and perforin. The expression of these cytolytic molecules is considerably higher in $CD8^+CD57^+$ T cells than in their $CD57^-$ counterpart (20). This type of T cell is presumed to increase in frequency with chronic immune activation as well as during normal aging (21–23). In addition, Björkström et al. proposed that in the setting of chronic immune activation terminal, $CD8^+$ T cells can acquire the ability to express FcγRIIIA (CD16) (24). CD16 expressed on $CD8^+$ T cells functioned independently from the T-cell receptor and was able to generate effector cytokine production as well as degranulation. CD16 function has been well-studied on NK cells and is best characterized for its role in antibody-dependent cellular cytotoxicity (25). The presence of these cells in combination with the high percentage of granzyme B^+ T cells found in the glomeruli suggest possible severe cytotoxic damaging effects in the glomerular compartment (26, 27).

The glomerular compartment also contained high numbers of macrophages with widespread distribution in number of $CD68^+$ cells per glomerulus. This finding is in accordance with previous literature in which macrophages were significantly associated with clinical rejection and severity, regardless of type of rejection (5–7). Although not statistically significant, the increased presence of macrophages in the TI compartment showed a possible association with graft survival. In addition, it was van den Bosch et al. who provided evidence for increased glomerular infiltration with $CD68^+CD163^+$ macrophages in c-aABMR compared to ABMR and TCMR (7). In accordance with this finding, $CD68^+CD163^+$ macrophages were the predominant subset in the glomeruli (68%), contrary to the TI compartment (39%).

T cells and macrophages were also the main inflammatory cells in the TI compartment but with a different

immunophenotypic distribution. The predominant T cells were either $CD4^+$ or $CD8^+$ T cells and of interest was the observation that, in contrast to the glomeruli, relatively few $CD8^+$ T cells expressed granzyme and/or CD57. The areas of the TI compartment with diffuse cellular infiltrates could suggest a possible contributing role for T cells in c-aABMR. Previously, Mengel et al. identified the importance of inflammatory cell infiltrates in protocol biopsies, specifically in areas of IFTA (8). These areas are now classified as i-IFTA/t-IFTA and have also been incorporated into the latest Banff meeting report (2). Although we did not find an association for T-cell infiltrates with (inferior) graft survival, further study is needed to assess if i-IFTA and t-IFTA play an important role in the outcome of graft with c-aABMR.

Of interest in the TI compartment are the $CD3^+FoxP3^+$ cells. We demonstrated that patients with increased $FoxP3^+$ Tregs in the TI compartment had inferior renal allograft survival compared to those with few Tregs in the TI compartment. Initially, FoxP3 was identified as the transcription molecule required for Treg development (28). Its presence in certain autoimmune diseases suggests an important role in immune tolerance (29, 30). Similarly, several studies regarding transplantation have shown upregulation of FoxP3 during episodes of acute rejection (15, 31, 32). However, the significance of $FoxP3^+$ T cell presence remains uncertain as these studies have shown conflicting data on the relation with graft survival (15, 33–35). For instance, Yapici et al. and Veronese et al. found a correlation between decreased graft survival and an increased density of Foxp3-expressing cells in acute rejection (35, 36).

Our data showing increased presence of $FoxP3^+$ Tregs in c-aABMR might be interpreted as an end result of widespread T-cell activation, rather than the cause of poor allograft survival (34). This is in accordance with Bunnag et al., who presented FoxP3 presence as a time-dependent feature reflecting chronic inflammation and showed association with IFTA, rather than FoxP3 as an independent predictor of outcome (34).

In addition, the association with inferior graft survival might be related to the potential of these $FoxP3^+$ T cell to easily differentiate into interleukin (IL)-17 producing T helper 17 (Th17) cells, as previously demonstrated in other autoimmune-mediated inflammatory diseases (37). Under normal circumstances, the transcription of FoxP3 in T cells is mainly induced by transformation growth factor-β. However, under proinflammatory conditions involving IL-6, believed to be present in renal allografts during rejection, T cells from a regulatory pathway can be adapted to an inflammatory pathway resulting in Th17 (38, 39). Th17 possess highly proinflammatory capacities rather than immune-regulatory functions.

This is the first study of this nature, describing inflammatory cell presence in glomeruli and the TI compartment of patients with c-aABMR. However, it does have a small number of samples. The presented data are of great interest and provide further insight into the possible pathogenesis of c-aABMR.

In conclusion, the predominant renal-infiltrating immune cells in renal biopsies of c-aABMR are $CD8^+$ T cells and M2 type macrophages in both the glomeruli and the TI compartment. Interestingly, in c-aABMR, increased numbers of FoxP3-expressing T cells are significantly associated with poor renal allograft survival.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusion of this article will be made available upon request. Requests to access the datasets should be directed to Kasia A. Sablik, k.sablik@erasmusmc.nl.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by MEC-2019-0308. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

KS: conception and design of the work, acquisition, analysis, and interpretation of data, and writing of the manuscript. EJ: conception of the work, acquisition and analysis of data, and critical revising of the manuscript. NP: acquisition and analysis of data and critical revising of the manuscript. MC: conception and design of the work, acquisition of data, and writing, and critical revising of the manuscript. MB: conception and design of the work, analysis and interpretation of data, and writing and critical revising of the manuscript.

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REVIEW
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Extracellular Vesicles as Mediators of Cellular Crosstalk Between Immune System and Kidney Graft

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Extracellular vesicles (EVs) are known immune-modulators exerting a critical role in kidney transplantation (KT). EV bioactive cargo includes graft antigens, costimulatory/inhibitory molecules, cytokines, growth factors, and functional microRNAs (miRNAs) that may modulate expression of recipient cell genes. As paracrine factors, neutrophil- and macrophage-derived EVs exert immunosuppressive and immune-stimulating effects on dendritic cells, respectively. Dendritic cell-derived EVs mediate alloantigen spreading and modulate antigen presentation to T lymphocytes. At systemic level, EVs exert pleiotropic effects on complement and coagulation. Depending on their biogenesis, they can amplify complement activation or shed complement inhibitors and prevent cell lysis. Likewise, endothelial- and platelet-derived EVs can exert procoagulant/prothrombotic effects and also promote endothelial survival and angiogenesis after ischemic injury. Kidney endothelial- and tubular-derived EVs play a key role in ischemia–reperfusion injury (IRI) and during the healing process; additionally, they can trigger rejection by inducing both alloimmune and autoimmune responses. Endothelial EVs have procoagulant/pro-inflammatory effects and can release sequestered self-antigens, generating a tissue-specific autoimmunity. Renal tubule-derived EVs shuttle pro-fibrotic mediators (TGF-β and miR-21) to interstitial fibroblasts and modulate neutrophil and T-lymphocyte influx. These processes can lead to peritubular capillary rarefaction and interstitial fibrosis–tubular atrophy. Different EVs, including those from mesenchymal stromal cells (MSCs), have been employed as a therapeutic tool in experimental models of rejection and IRI. These particles protect tubular and endothelial cells (by inhibition of apoptosis and inflammation–fibrogenesis or by inducing autophagy) and stimulate tissue regeneration (by triggering angiogenesis, cell proliferation, and migration). Finally, urinary and serum EVs represent potential biomarkers for delayed graft function (DGF) and acute rejection. In conclusion, EVs sustain an intricate crosstalk between graft tissue and innate/adaptive immune systems. EVs play a major role in allorecognition, IRI, autoimmunity, and alloimmunity and are promising as biomarkers and therapeutic tools in KT.

Keywords: extracellular vesicles, acute rejection, ischemia-reperfusion syndrome, autoimmunity, renal transplant, biomarker, miRNA

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INTRODUCTION

Extracellular vesicles (EVs or microparticles) is a general term that refers to membrane structures released by all cell types through different biogenesis pathways; EVs are secreted after fusion of endosomes with the plasma membrane (exosomes), shed from plasma membrane (microvesicles), or released during apoptosis (apoptotic bodies). These three entities differ in size (exosomes, 30–150 nm; shedding microvesicles, 150 nm−1 μm; apoptotic bodies, 1–5 μm) and partly in content (1–4). In this review, we will employ the umbrella term "EVs" to include all the above-mentioned types of secreted membrane vesicles.

After cellular shedding, EVs are rapidly taken up by neighboring or distant target cells (paracrine and endocrine effects) through a variety of mechanisms, such as endocytosis, phagocytosis/pinocytosis, membrane fusion, and receptor-mediated endocytosis (2).

EVs are involved in a wide range of physiological and pathological processes (4–7), including acute kidney injury (AKI), chronic kidney disease (CKD), thrombotic microangiopathies, and vasculitis (2, 3). EVs play a key role in all these settings by shuttling their bioactive cargo between cells. Most of their effects are mediated by microRNAs (miRNAs), which modulate gene expression in target cells and induce epigenetic reprogramming (3). Additionally, EVs carry a wide variety of immune modulatory molecules (e.g., cytokines, costimulatory/inhibitory molecules, and growth factors). Packing of nucleic acids and other contents into EVs is coordinated by multiple signals from EVs themselves or from cellular/extracellular environment (8–10). For example, TNFα modulates miRNA content of endothelial particles (11). Of interest, most EVs do not express human leukocyte antigens (HLAs) and escape the immune system; moreover, they cross numerous biological barriers (8), including glomerular endothelium basement membrane (12). Homing and uptake of EVs are mediated by signals and receptors on target cells (13) and influenced by local factors such as pH and electric charge (14). After intake, their complex biocargo exerts multiple effects: mRNAs are translated; miRNAs activate or silence protein expression (1, 2, 8); surface receptors are transferred from one cell to another (15, 16) and bacterial, viral, or graft alloantigens can be exchanged among immune cells (17, 18). A detailed analysis of EV general properties has been covered by recent reviews (1, 6, 8) (**Figure 1**).

EVs released from innate immune cells, such as macrophages, dendritic cells (DCs), or natural killer (NK) cells, are involved in the regulation of innate immune response mainly as pro-inflammatory and paracrine mediators (4, 19). However, their immunomodulatory role is probably far more complex and includes anti-inflammatory and immunosuppressive effects.

The role of innate immunity as a trigger for acute rejection has been the focus of intense research over the last years (20, 21), and the possibility of manipulating EVs as a therapeutic tool or employing them as biomarkers is opening new paths in solid organ transplantation (22).

The aim of this review is to outline the role of EVs in innate immunity by analyzing different aspects of kidney transplantation (KT) biology. After analyzing EVs as mediators among different innate immune cell types, we will describe the role in complement and coagulation, two pivotal systems in innate immunity, and in other key settings such as allorecognition, ischemia–reperfusion injury (IRI), and the autoimmune component of antibody-mediated rejection. Finally, we will review recent evidence about the role of EVs as potential therapeutic tools and biomarkers in KT.

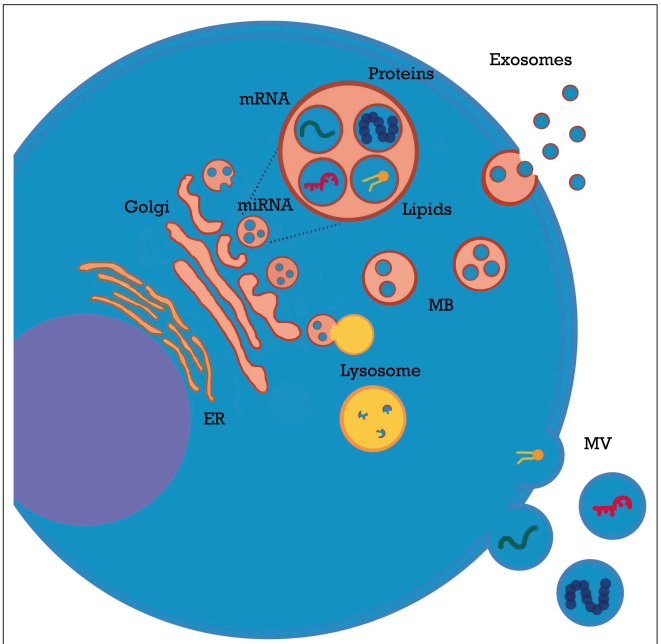

FIGURE 1 | Extracellular vesicle (EV) biogenesis.

A general overview of immune-modulating effects of innate cell-derived EVs on different immune system cells or molecular targets is outlined in **Table 1**.

NEUTROPHIL-DERIVED EXTRACELLULAR VESICLES

Far from being mere final effectors of the inflammatory response, neutrophils [or polymorphonuclear cells (PMN)] exert several modulating effects on both innate and adaptive immune cells and can migrate to secondary lymphoid organs. These actions are partly mediated by EVs (23).

In general, PMN-derived EVs have anti-inflammatory and immunosuppressive effects, mainly on DCs and macrophages. EVs released from apoptotic PMNs also extend their actions on T-lymphocyte subsets, blunting their activation (24).

Neutrophil-derived EVs can inhibit lipopolysaccharide (LPS)-activated DCs and macrophages by reducing their phagocytic capacity, their maturation, and the release of pro-inflammatory cytokines (IL-8, IL-10, IL-12, and TNFα) while increasing TGF-β1 excretion. This cytokine plays a key role in suppressing immune response: it promotes anti-inflammatory DC, suppresses $CD4^+$ and $CD8^+$ T cells and induces T reg expansion (25, 26).

TABLE 1 | Immune-modulating effects of innate cell-derived EVs on other immune system cells or molecular targets.

Cell of origin	Cellular/molecular target	EV-surface proteins and content	Main biological effects	References
PMN	DC/macrophage	Phosphatidylserine Annexin 1 MPO CD11b/CD18 Lactoferrin Elastase	Reduced DC phagocytosis, maturation and capacity to stimulate T-cell proliferation; reduced production of pro-inflammatory cytokines and increased release of TGF β1 by DC and macrophage (tolerogenic profile)	(23, 24) (25, 26)
	T lymphocytes	CD11b/CD18 Annexin V Elastase	Reduced release of TNFα by naive and activated effector T cells; reduced IL2 secretion and CD25 expression by resting T helper cells	(27) (28) (29) (30) (31) (32)
	Cytokines/membrane receptors	Catepsin G Proteinase 3	Cleavage of cytokines and their membrane receptors	(33) (34)
	L-Arginine	Arginase-1	Reduced T-cell proliferation and function	(30)
	Neutrophil	LT B4 and enzymes for its synthesis; C5R1	PMN chemotaxis	(35) (36)
Macrophage	Macrophage and DC	p-MHC; Microbial and viral antigens; Hsp-70; IL1β; TNFα; CCL2-5; C3 fragments; Proteins of the leukotriene pathway IL 36y miR-223	Transfer of p-MHC, antigens and activating signals to DCs; DC maturation, activation and migration; release of Th1 - (M1 macrophages- derived EVs) or Th2-promoting cytokines (M2 macrophages-derived EVs)	(37) (38) (39) (40) (41) (42)
	PMN	Enzymes of the leukotriene biosynthesis	PMN chemotaxis	(43)
	T lymphocytes	IL1β TNFalfa and CCL2-5 proteins of the leukotriene pathway IL 36y	Increased T cell expansion and differentiation; induction of IFNy and IL 17 producing CD4+ T cells (T helper 17); inhibition of Treg	(40) (44) (45) (46) (35) (47)
	B lymphocytes	C3 fragments IL 36y	Increased B cell expansion and differentiation	(41, 48) (47)
DC	DC	TLR4 p-MHC; Costimulatory or inhibitory molecules; miRNA (miR-148a, miR 451)	Amplification of antigen spreading among APCs and antigen presentation to T lymphocytes	(49, 50) (17, 51) (52)
	PMN	Enzymes of the leukotriene biosynthesis	Neutrophil chemotaxis	(43)
	T lymphocytes	p-MHC; MHC II; Microbial or tumoral antigens; adhesion molecules (ICAM-1); costimulatory molecules (B7 family members)	Activation (mature DCs) or inhibition (immature DCs) of CD4/CD8 pos T lymphocytes	(4, 49) (53) (54) (55) (56)
	B-lymphocytes	Complement fragments, microbial or tumoral antigens		(20, 52)
MC	DC	p-MHC; FcεRI Hsp 60, Hsp 70; PLA2, PLC, PLD; PGD2,PGE2	Transfer of p-MHC II and IgE-antigens complexes; antigens activation and DC maturation; generation of neolipid antigens	(51) (57) (58) (59) (60) (61)
	T lymphocyte	Proteases	Cytokine inactivation, T helper 2 induction	(51)
	B lymphocyte	CD 40	EVs binding; IL-10 competent B cells	(62)
Eosinophil	DC	MBP EPO	DC maturation; DC-driven Th2 response	(63) (64)
NK	T lymphocyte	Perforin FasL	Cell lysis	(65)

EVs released by apoptotic human PMN suppress T-cell proliferation, IL-2 production, and IL-2 receptor upregulation on activated T cells (27). The binding of these EVs to activated T cells seems to occur through Mac-1 (CD11b/CD18), an integrin also involved in immunological synapse formation (28).

The bioactive cargo of PMN-derived EVs includes numerous immune modulatory molecules: annexin V (induction of Tregs) (29), arginase-1 (depletion of arginine with inhibition of T-cell proliferation), lactoferrin (inhibition of DC migration to lymph nodes) (30), myeloperoxidase (inhibition of DCs) (31), elastase (conversion of human immature DCs into TGF-β1-secreting cells) (32), and other proteases such as cathepsin G and proteinase 3, which can inactivate pro-inflammatory cytokines (IL-2, IL-6, and TNFα) (33) and cleave their receptors from the plasma membrane (34). Additionally, PMN EVs regulate inflammatory cell trafficking; leukotriene B4 (LTB4) activates PMN chemotaxis and is particularly enriched in their EVs (35). Conversely, during sepsis, PNM shed C5a receptor 1 into their EVs and reduce their response to complement activation (36).

MACROPHAGE-DERIVED EXTRACELLULAR VESICLES

In general, macrophage-derived EVs exert pro-inflammatory effects, mainly directed toward DCs, macrophages, PMNs, and T lymphocytes.

Infected macrophages release EVs loaded with pathogens' proteins that can activate other antigen-presenting cells (APCs). Depending on the microenvironment, targeted macrophages activate either M1 or M2 polarization (37), whereas DCs process and present the antigens to T cells, thus promoting allorecognition and adaptive immunity. In addition to microbial or viral antigens, macrophage-derived EVs also carry peptide–major histocompatibility complex (MHC) complexes and costimulatory molecules, further enhancing alloantigen spreading among innate immune cells (38).

The cargo of macrophage-derived EVs includes several molecules with immunomodulatory functions, such as Hsp 70 (pro-inflammatory or tolerogenic effect depending on coexistent signals) (39), IL-1 β (DC migration and expansion of T/B lymphocytes) (40, 41), TNFα, and several chemokines (CCL2, CCL3, CCL4, and CCL5) (44–46). Complement C3 fragments are expressed on EV surface and interact with T cells during antigen presentation (48). Proteins involved in leukotriene synthesis were isolated in human macrophages, converting LTA4 into LTB4 and LTC4 and potentially activating DCs and CD4/CD8 T cells (43). IL-36γ was found in EVs released by infected pulmonary macrophages, with possible impact on DC maturation and T-cell activation [T helper (Th)1 or Th17 development and inhibition of Tregs] (47). Finally miR-223, a regulator of myeloid differentiation, was found in macrophage-derived EVs (42).

Zhang et al. stimulated macrophages *in vitro* with different protocols and performed an extensive proteomic profiling of their EVs. When the inflammasome complex was activated, EVs had a higher immunogenicity and induced NF-κB signaling in neighboring immune cells, thus amplifying inflammation (44). The inflammasome is a multimeric caspase-activating complex that can modulate a wide range of pathways in response to pathogens and activate both innate and adaptive immunity.

This is relevant to KT because IRI determines tissue damage, release of EVs, and inflammasome activation (44). These aspects will discussed in *Extracellular Vesicles in Ischemia–Reperfusion Injury and in the Autoimmune Component of Rejection*.

Finally, glucocorticoid therapy and long-term LPS exposure (mimicking chronic infection) can trigger macrophage release of toll-like receptor-2-containing EVs; these particles act as decoy receptors to antagonize toll-like receptor-2 signaling and blunt inflammation (66).

DENDRITIC CELL-DERIVED EXTRACELLULAR VESICLES: EARLY INFLAMMATORY RESPONSE AND T-LYMPHOCYTE ACTIVATION

Dendritic Cell Extracellular Vesicle and Innate Immunity

DCs highly express pattern recognition receptors and represent a pivotal link between innate and adaptive immunity (49). Toll-like receptors belong to pattern recognition receptors family and play a key role in the early inflammatory response; indeed, toll-like receptors avidly bind damage-associated molecular patterns, a wide group of molecules released by damaged tissues (e.g., during IRI) (67, 68). Toll-like receptor 4 is transferred *via* EVs among bone marrow DCs (BM-DCs) and activate NF-κB signaling pathway (50). Moreover, EV-mediated transfer of miRNAs among DCs contributes to enhance their mutual activation during inflammation (17, 69).

As described above (PMN paragraph), DC-derived EVs also carry enzymes of the leukotriene biosynthesis, which stimulate PMN chemotaxis (43).

Antigen Presentation to T Lymphocytes

DC-derived EVs also play a pivotal role in allorecognition (4, 49). DCs capture EVs released from graft tissue. Graft particles carry surface class I and II MHC molecules, non-HLA donor antigens, costimulatory and adhesion molecules, and pro-inflammatory cytokines such as IL-1β (52). The DC–EVs axis plays a pivotal role in all the three antigen presentation pathways described in transplant immunology, as reported in **Figure 2** (53, 68, 70, 71):

- Direct antigen presentation: In this setting, donor APCs interact with recipient T cells. Of note, donor DC-derived EVs contain high density of allogeneic peptides complexed with donor MHC (p-MHC) and can interact directly with $CD8^+$ and $CD4^+$ T cells.
- Indirect antigen presentation: In this pathway, recipient APCs interact with recipient T cells. Graft EVs are internalized into the recipient APC and transfer their peptides to MHC class II molecules. These complexes are then exposed to APC surface for indirect presentation to T lymphocytes.
- Indirect antigen presentation by "cross-dressing" APCs (semi-direct antigen presentation): Donor-derived EVs

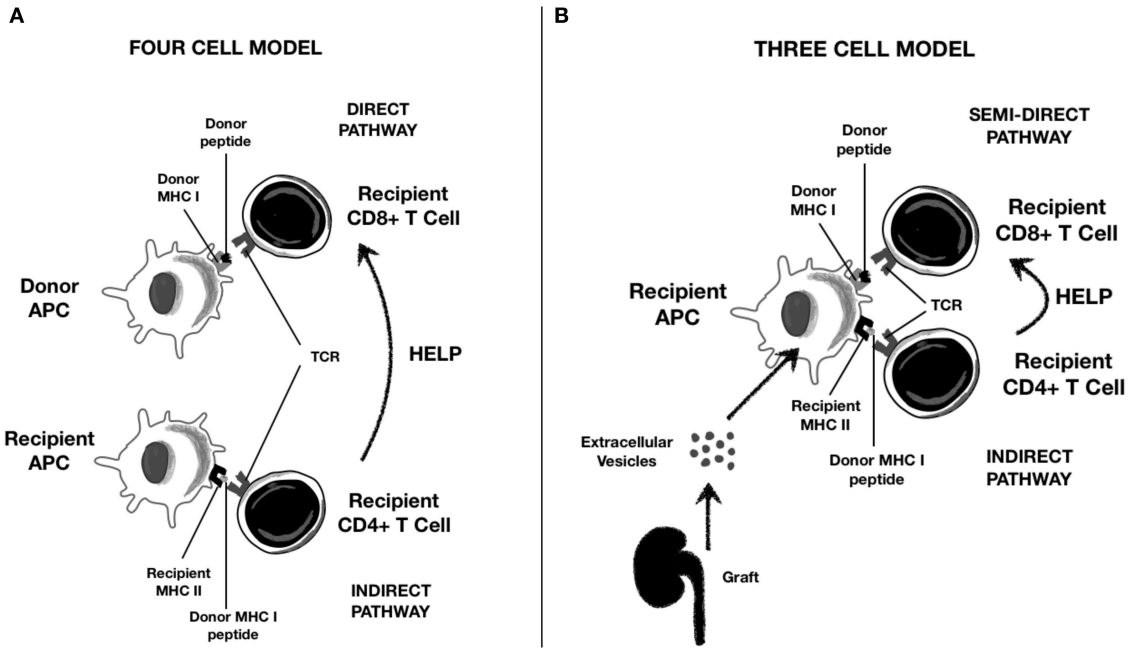

FIGURE 2 | Role of Extracellular Vescicles (EVs) in alloantigen presentation to T lymphocytes. **(A)** Classical direct and indirect presentation; **(B)** "semi-direct" presentation trough cross-dressing of recipient APC with graft-derived EVs.

containing p-MHC complexes are captured by recipient APC on their surface and then presented directly to T cells without any p-MHC reprocessing, a phenomenon referred to as "cross-dressing."

Recent evidence suggests that donor DC transplanted with the graft are *antigen transporting* rather than *antigen presenting* cells and that "cross-dressing" rather than "passenger leukocyte" is the main mechanism of alloantigen presentation from donor APC (70, 71). Although semi-direct modality rapidly initiates alloresponse and leads to acute rejection, indirect T-cell activation has been associated with chronic antibody-mediated rejection (72). "Cross-dressing" is also typical of follicular DCs, key players in germinal center reactions (54).

The effectiveness of DC-derived EVs in p-MHC presentation depends on the coexistence of other molecules in their cargo (MHC class II, CD86, and ICAM) and on parental cell maturation (20):

- Mature DC-derived EVs are characterized by higher expression of surface MHC, adhesion, and costimulatory molecules (55, 73) and present antigens to $CD4^+$ T lymphocytes through "cross-dressing," promoting Th1 phenotype (56, 74).
- Immature DC-derived EVs are efficiently internalized by mature APCs and transfer their antigens to the target cell MHC. Thus, the antigen is indirectly presented to $CD4^+$ T lymphocytes, skewing them toward a Th2 phenotype. Additionally, immature DC can release immunoregulatory EVs loaded with anti-inflammatory cytokines such as TGF-β1 (4) and can target other DCs to amplify a tolerogenic response (75).

Therefore, donor EVs target recipient cells and generate a chimerism that can determine either DC activation or DC inhibition depending on their content (76, 77). For example, EV-derived CD86, a costimulatory molecule, activates T cells through direct or semi-direct pathway, whereas the indirect pathway vehicles miRNAs upregulating PD-L1 and induces CD4 T cells anergy (78). Indeed, graft-infiltrating PD-L1hi cross-dressed DCs blunted T-cell response in a mouse model of liver transplantation (77).

Finally, the relationship between DCs and adaptive immunity is bidirectional: for example, DCs are targeted by Treg-derived EVs that induce a tolerogenic phenotype trough transfer of miRNAs (miR-150-5p and miR-142-3p) (79).

MAST CELL-DERIVED EXTRACELLULAR VESICLES

MC-derived EVs contain p-MHC complexes or endocytosis-derived antigens and can be released by both activated and resting BM-MCs. The main target of these particles is DCs and other professional APCs (51, 80, 81). Skokos et al. investigated the role of MCs in allo-antigen presentation; the authors observed that ovalbumin was more effectively recognized by T cell if taken up by MCs and then transferred to DCs rather than presented directly by DCs (57). Indeed, MCs and DCs form a highly structured immune synapsis devoted to antigen transfer through EVs (58).

Several molecules with immunomodulatory roles have been isolated in MC-derived EVs. Heat shock proteins (Hsp 60 and 70) are essential for antigen loading and EV uptake by DCs (57, 59) and are capable of inducing BM-DC maturation;

FceRI–IgE complexes contribute to horizontal antigen transfer among MCs; additionally, phospholipases (PLA-A2, C, and D2), neolipid antigens, and lysophospholipids (60, 61) inhibit DC functions (e.g., phosphatidic acid) and induce Th2 response [lysophosphatidylcholine (LPC)] (60, 82). MC-derived EVs also carry proteases that inactivate cytokines and also target T-cell proteins (34, 60). Finally, CD40L-positive EVs from BM-derived MCs generate IL-10 competent B cells (62, 83).

OTHER INNATE IMMUNE CELL-DERIVED EXTRACELLULAR VESICLES

There is a paucity of data about EVs generated by other innate immune cells.

Eosinophils can release EVs containing major basic protein (MBP) and eosinophil peroxidase (EPO) when stimulated with IFNγ; both promote DC maturation (63, 64, 84).

NK cells can release EVs loaded with several cytotoxic proteins (85, 86), including perforin and FasL, which can induce lysis of activated T lymphocyte and thus possibly blunt inflammation (65, 87).

The main immune-modulating effects of innate cell-derived EVs on other immune system cells or molecular targets are summarized in **Table 1**.

EXTRACELLULAR VESICLES AND THE COMPLEMENT SYSTEM

EVs can play a dual role in this setting, either activating or inhibiting the complement cascade (88).

This function is extremely relevant to the transplant setting, as EVs play a role in complement attack on ECs in both antibody-mediated rejection (89) and IRI (90).

a) Extracellular Vesicles as Complement Activators

T cell-derived EVs can activate complement through immunoglobulin binding, whereas other types of EVs do so directly, through interactions between C1q and their membrane lipids (91, 92). For instance, both PMN- and erythrocyte-derived EVs (93, 94) can provide a platform for C1q deposition, with consequent activation of classic pathway on their surface.

Activated endothelial cells can shed EVs under inflammatory conditions; this phenomenon has been observed after complement activation and membrane attack complex formation on endothelial cell surface (95). These endothelial-derived EVs express membrane attack complex and have a strong procoagulant phenotype, which further triggers complement activation through thrombin formation. This creates a vicious circle of endothelial complement-mediated damage and endothelial shedding of complement-enhancing EVs (88).

b) Extracellular Vesicles as Complement Inhibitors

On the other hand, EV shedding could also represent a mechanism to protect cells from complement attack: indeed, EVs remove complement molecules from cell surface acting as "scavengers" and allowing complement evasion (88). Complement-induced EVs shedding has been demonstrated in PMNs, erythrocytes, and glomerular endothelial and epithelial cells (96).

Consistently, complement-coated EVs from leukocytes can be rapidly phagocytosed by PMNs. Clearance of these opsonized EVs is also facilitated by complement receptor 1, expressed on erythrocytes; as such, red blood cells bind EVs and transport them to the liver and spleen (94).

EVs also carry several complement inhibitors that allow them to transport activated complement factors without being lysed: CR1, CD55, or decay-accelerating factor (modulation of C3 and C5 convertase), CD59 (direct MAC inhibitor), and membrane cofactor protein (MCP or CD46) (2, 97). Interestingly, endothelial EVs are also rich in complement inhibitor mRNA and prevent glomerular injury in experimental models of glomerulonephritis (98).

EXTRACELLULAR VESICLES AND THE COAGULATION SYSTEM

Complement and coagulation cascades are key components of innate immunity and are tightly connected to each other; their simultaneous activation has been extensively studied in transplant rejection and IRI (99–102). EVs released from endothelial cells and platelets (PLTs) are critical promoters of coagulation in renal disease (89); besides carrying inflammatory and chemotactic proteins, these vesicles release also a number of growth factors [e.g., PLT-derived growth factor (PDGF)] and promote tissue regeneration.

a) Endothelial Extracellular Vesicles

When shed after complement activation, endothelial cell EVs have procoagulant and PLT-activating effects (95, 103). They expose phosphatidylserine and binding sites for factor Va and tissue factor (TF) (104, 105); the latter triggers extrinsic pathway determining thrombin generation (106). Thrombin directly cleaves complement components C3 and C5 into C3/C5 convertase, further amplifying the cascade (107). Endothelial EVs can also transfer TF to monocytes and PLTs (108). On the other hand, these EVs preserve endothelial cell survival in physiological condition (caspase-3 removal and protein C receptor exposure) (109), and EVs derived from endothelial progenitor cells can promote angiogenesis (110).

b) Platelet-Derived Extracellular Vesicles

These play a key role in hemostasis and coagulation (111) through a variety of mechanisms summarized in **Table 2** (112–119). Of note, PLT-derived EVs have a 50- to 100-fold stronger procoagulant/prothrombotic effect than have PLTs (120). On

TABLE 2 | Platelet-derived EVs procoagulant and prothrombotic effects.

Molecule	Mechanism	References
Phosphatidylserine surface expression	Negative charged surface creates binding sites for factors II, Va, Xa (prothrombinase complex)	(112)
Tissue Factor surface expression	It binds factor VIIa on phosphatidylserine- containing surface and activates extrinsic pathway of coagulation	(113)
Protein disulfide isomerase (PDI)	Platelet aggregation	(114)
Receptors for factor VIII	Thrombin generation	(115)
Release of factor XIIa	Activation of intrinsic pathway	(116)
Thromboxane A2 synthesis and release	Platelet aggregation	(117)
IL 1-β release	Monocyte adhesion to endothelium, endothelial cell activation	(118)
RANTES deposition	Monocyte recruitment to endothelium	(119)

the other hand, they promote angiogenesis and endothelial cell regeneration after vascular injury (2, 121).

EXTRACELLULAR VESICLES IN ISCHEMIA–REPERFUSION INJURY AND IN THE AUTOIMMUNE COMPONENT OF REJECTION

IRI is the main cause of delayed graft function (DGF), which determines an increased risk of acute rejection and progression to chronic allograft dysfunction (122). IRI triggers a complex, alloantigen-independent immune response characterized by crosstalk between PMNs, macrophages, and DCs (123). All these cells release EVs with pro-inflammatory and anti-inflammatory effects (see above) (19).

Two other cell types release critical EVs in this condition: endothelial cells and renal tubular epithelial cells. Both release EV when exposed to hypoxia, oxidative stress, acidic pH, or inflammation. Hypoxia determines an accumulation of hypoxia-inducible factor (HIF)-α subunit, which dimerizes with HIF-β to form HIF, a transcription factor that can activate over 70 target genes. This results in changes in surface receptors and remodeling of plasma membrane, which triggers release of EVs (124). Furthermore, HIF increases Rab22, an essential element for EV biogenesis (125).

ENDOTHELIAL CELLS

IRI induces a complex vascular phenotype characterized by a progressive spectrum of functional and structural alterations: vasoconstriction, vascular inflammation, microvascular rarefaction of peritubular capillaries, chronic hypoxia, interstitial fibrosis, and tubular atrophy (126, 127). Microvascular lesions appear to be a key driver of fibrosis after IRI, with a predominant effect over tubular ones (128).

Transplant procedure itself is characterized by tissue damage and some degree of ischemia, resulting in activation of different cell death programs (apoptosis, necrosis, necroptosis, pyroptosis, and autophagy-associated cell death) with release of damage-associated molecular patterns. Bacterial and viral components can also be released during transplant surgery or in infections after KT (122, 123). Both damage-associated molecular patterns and pathogen-associated molecular patterns bind a wide range of innate pattern recognition receptors expressed on several cells, including macrophages, DCs, and endothelia (129). Pattern recognition receptor activation triggers inflammatory response and EV release (126).

Caspase-3 is a pivotal regulator of cell apoptosis (128); under physiological conditions, endothelial EVs protect parental cell by removing caspase-3 (130). During IRI, caspase-3 hyper-activation can overtake EV clearance and cause cell death. In this scenario, endothelial cell generate both "classical" apoptotic bodies and smaller exosome-like vesicles; both are overloaded with caspase-3 and can propagate cell death. Additionally, these exosome-like vesicles carry activated 20S proteasome; this complex recruits adaptive immune cells and induces the production of auto-antibodies toward perlecan/LG3, angiotensin-1 receptor, and dsDNA, further aggravating vascular inflammation (46, 127, 131). Reperfusion has also been associated with the occurrence of a broad range of IgM "natural antibodies," targeting "neo-epitopes" on ischemic tissues and activating complement (123). Thus, EVs shed by an activated or injured endothelium can trigger mechanisms of alloimmunity and autoimmunity.

The role of EVs in the autoimmune component of rejection has been the focus of recent studies. Tissue-specific self-antigens were found in circulating EVs released by apoptotic cells in the lung, heart, islet, and KT recipients while rejection is developing, whereas they were not detected in control grafts (132). For example, EVs from KT recipients with transplant glomerulopathy have an increased expression of fibronectin and type IV collagen than have EVs from stable KT recipients (133).

Innate immune response generates graft tissue damage, which can favor continuous release of sequestered self-antigens through EVs, with secondary activation of self-reactive T lymphocytes and development of a tissue-restricted form of autoimmunity (46, 72).

It must be emphasized that only in an inflammatory environment (e.g., IRI) can adaptive cells determine autoimmunity. Consistently, Sharma et al. showed that anti-cardiac myosin (CM) antibodies trigger graft rejection in syngeneic heart transplantation only when administered at time of surgery, but not 1 week after it (134).

RENAL TUBULAR EPITHELIAL CELLS

In general, whereas EVs from injured cells can promote tubule interstitial inflammation and fibrosis, those derived from cells with regenerative properties can promote cell proliferation and

tissue repair. However, this distinction is blurred, as injured renal tubular epithelial cells can also stimulate repair (as detailed below), whereas mesenchymal stromal cell (MSC)- or endothelial progenitor cell-derived EVs can have harmful effects (135).

With this caveat, we will now focus on actions mediated by EVs released by ischemic renal tubular epithelial cells, whereas EV potential to repair tissue damage will be dealt with in a specific paragraph.

Renal proximal tubular epithelial cells are especially prone to ischemic damage because they depend on mitochondrial metabolism for ATP production owing to their modest glycolytic capacity.

Under hypoxic conditions, HIF-1 mediates EV release by renal proximal tubular epithelial cells (136), which modulate severity of kidney injury by targeting neighboring cells (3).

Furthermore, renal proximal tubular epithelial cells express receptors for complement fractions C3a and C5a and toll-like receptors, making them responsive to innate immune activation (137). Damage-associated molecular patterns can be transferred into renal tubular epithelial cells trough EVs (138) and prevent tubular recovery (139, 140). Pathogen-associated molecular patterns, such as LPS, upregulate the expression of DC-SIGN and toll-like receptor 4, stimulating tubular secretion of IL-6 and TNFα (141).

In this early inflammatory phase, tubular EVs containing cytokines, growth factors, and complement fractions can recruit innate immune cells such as PMNs, M_1 macrophages, and NK cells (135). EVs released by hypoxic renal tubular epithelial cells are characterized by a decreased content of miR-7641-2-3p, a downregulator of chemoattractant CXCL1, resulting in increased PMN influx (142).

Injured hypoxic tubular cells can transfer TGF-β-containing EVs across disrupted basement membrane to interstitial fibroblasts, activating them and mediating progression to CKD (143). Furthermore, TGF-β itself stimulates renal tubular epithelial cells in an autocrine way to secrete EVs enriched for miR-21, which targets recipient tubules enhancing Akt-mTOR proliferative pathway and consequently exacerbating interstitial fibrosis (144); of note, miR-21 is also released by several other types of human cells through toll-like receptor 3 activation (145).

Also, miR-155 worsens tubular damage during IRI, as it promotes tubular pyroptosis by upregulating expression of caspase-1 and downregulating FoxO3a expression together with its downstream protein "apoptosis repressor with caspase recruitment domain" (146, 147).

In addition to hypoxia, also albuminuria triggers release of CCL2-containing EVs, activating interstitial macrophages and promoting tubule interstitial inflammation (148). Proteases and glycosidase on EV surface may contribute to interstitial fibrosis by degrading extracellular matrix (149).

Furthermore, besides tubular-interstitial diffusion, EVs from renal proximal epithelial cells can also move downstream through urinary tract to target distal tubule or collecting duct (3), although with largely unknown effects.

Tubule-derived EVs can mediate anti-inflammatory and pro-angiogenic actions, for example, secreting IL-10, which polarizes macrophages toward an M_2 phenotype, and galectin-1 and CD73, which promote Treg function (135, 150–152). Tubule-derived EVs can also directly interact with T lymphocytes through T-cell immunoglobulin- and mucin-containing molecules Tim-1 and Tim-4; interestingly, the same receptor Tim-1 (also called KIM-1) is expressed on renal tubular epithelial cells surface and mediates suppression of NF-κB (153).

EVs also mediate a less defined crosstalk between endothelia and renal tubular epithelial cells. On the one hand, tubule-derived EVs transport ApoA1, which inhibits ICAM-1 and P-selectin and alleviates ischemic damage and PMN retention (154); on the other hand, endothelial EVs can pass into urinary space upregulating HIFα/VEGFα signaling in renal tubular epithelial cells (155).

EXTRACELLULAR VESICLES AS A THERAPEUTIC TOOL IN RENAL TRANSPLANTATION

Most studies on EVs as a therapeutic tool in renal transplantation have employed MSC-derived EVs (MSC-EVs) and have focused especially on IRI.

MSCs themselves have drawn much interest in transplantation, mainly because of their capacity to stimulate tissue repair after ischemic injury and their immunomodulatory properties (156).

Injected MSCs can inhibit tubular cell apoptosis and interstitial fibrosis while stimulating proliferation of tissue-specific progenitor cells. Although MSCs can engraft in renal tubular and endothelial cells, regenerative actions are primarily mediated by EVs (157, 158).

Additionally, MSC modulate both innate (DCs, monocytes, and NK cells) and adaptive (T and B lymphocytes) immune cells, with predominantly anti-inflammatory and immunosuppressive effects, which may play a role in preventing or counteracting rejection. Also these effects are predominantly mediated by MSC-EVs (159).

Since side effects and practical challenges of MSC therapy have been reported (160), MSC-EVs have been proposed by several studies as a safer, cell-free alternative. Nevertheless, they have shown similar or even potentially additive regenerative and immunomodulatory properties (161).

Recent evidence suggests that innate immune EVs and MCS EVs play opposite roles in immune system regulation: whereas the former can carry and spread alloantigens, stimulating allorecognition and rejection, the latter can exert immunosuppressive and tolerogenic effects. In particular, MSC-EVs inhibit DC maturation and NK function and skew T lymphocytes toward a Treg phenotype. Of note, MSC-EV proteomic analysis has identified 938 proteins, which could be relevant to MSC-EV interaction with immune cells (159, 162).

MSC-EVs and immune cell EVs are phenotypically different, as they reflect profile of surface molecules of respective parental cell; in particular, MSCs are defined by the expression of CD73, D90, and CD105 and lack CD14, CD34, and CD45 markers (163). However, MSC-EV cargo is not merely a reflection of their parental cell, as it is characterized by a peculiar enrichment

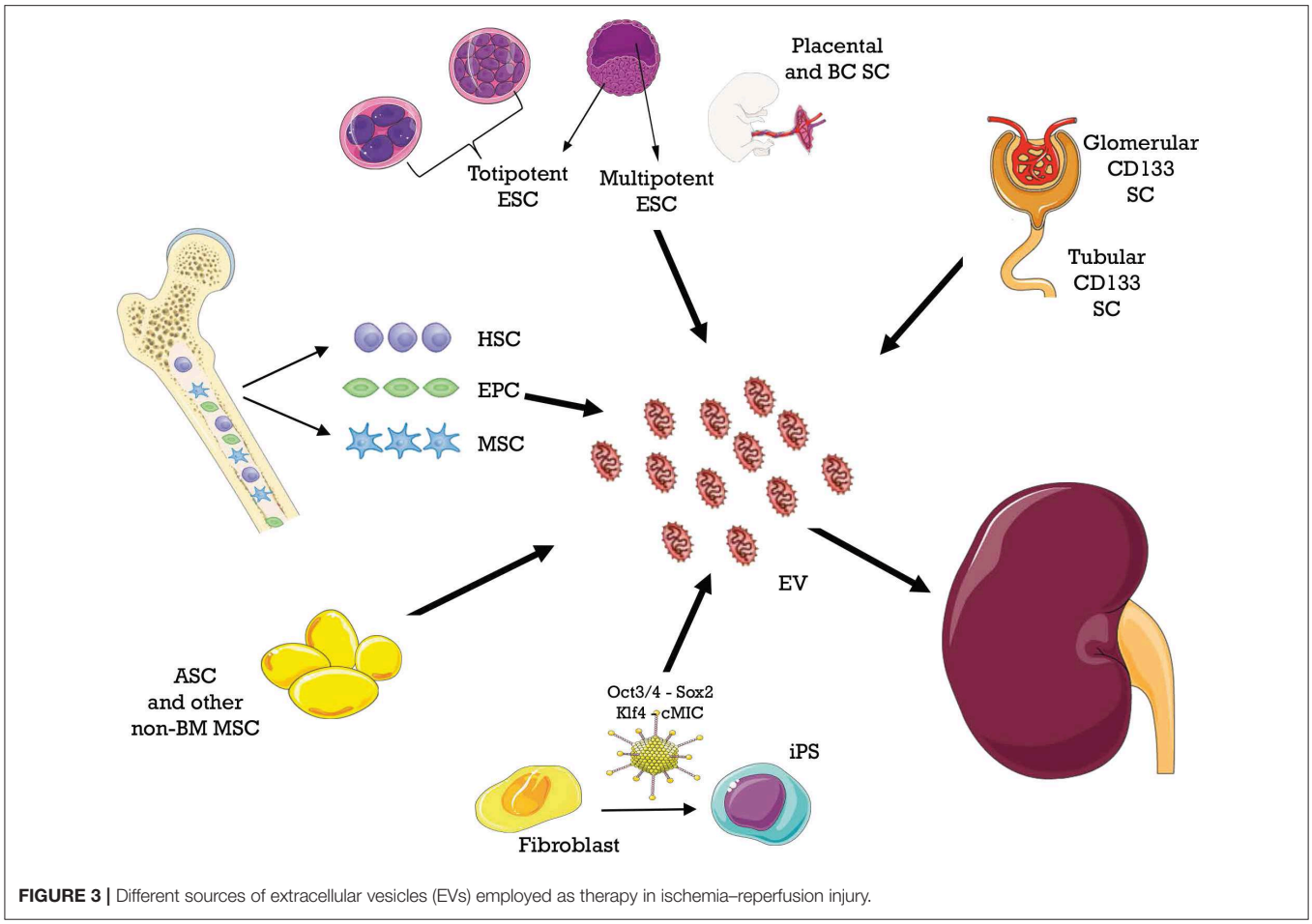

FIGURE 3 | Different sources of extracellular vesicles (EVs) employed as therapy in ischemia–reperfusion injury.

in mRNA, miRNAs, and proteins involved in key processes, such as cell cycle regulation, cell differentiation, and immune regulation (157).

a) Ischemia–Reperfusion Injury

Mesenchymal Stromal Cell-Derived Extracellular Vesicles

MSCs can be isolated from different tissues, as shown in **Figure 3**. MSC-EVs recapitulate beneficial properties of origin cells, which are mediated by a variety of mRNAs, miRNAs, and proteins. These molecules are horizontally shuttled into recipient cells and activate signaling pathways related to the following (164):

- renal protection: inhibition of apoptosis/necrosis, inflammation, oxidative stress, fibrogenesis, and promotion of autophagy (165); and
- renal regeneration: stimulation of cell proliferation, migration, tubular dedifferentiation, and angiogenesis.

Importantly, pretreatment with RNAase abolishes these effects, indicating that mRNAs and/or miRNAs account for them (166).

Ferguson et al. identified 23 top miRNAs, which account for over 79% of total miRNA load in MCS exosomes and seem to mediate the predominant effects, targeting 5,481 genes (167). Different miRNAs carried by MSC-EVs are extensively reviewed elsewhere (168–171).

The main miRNAs involved in renal protection from IRI, type of secreting cell, and mechanisms of action are outlined in **Table 3** (172–181).

Specific functions of these miRNAs are being defined: miR-125a can promote endothelial cell angiogenesis (172); miR-29b inhibits angiotensin II-induced epithelial-to-mesenchymal transition of rat RTECs (173) and blunts inflammation by inhibiting NF-κB; miR-21 prevents renal tubule epithelial cell apoptosis and inhibits DC maturation (174); and miR-199a-5p alleviates endoplasmic reticulum stress at very early reperfusion stages (8–16 h after reperfusion *in vivo*) (177).

Murine studies in which MSC-EVs were employed as a therapeutic tool for IRI are summarized in **Table 4** (166, 177, 182–194). In all of them, administration of MSC-EVs improved renal function and/or decreased tubular injury through multiple mechanisms (164). Most studies were performed with BM- and umbilical cord-derived EVs; however, other MSC have been used including kidney resident populations (189, 190) and adipose tissue (191). Of interest, i.v. administered human MSC-EVs were effective in alleviating renal damage in rats that had received KT

TABLE 3 | Main miRNAs involved in renal protection from IRI.

miRNA	Parental cell	Mechanism of action	References
miR-125a	Adipose tissue-MSC	Increases endothelial cell angiogenesis	(172)
miR-29b	MSC	Inhibits epithelial mesenchymal transition of rat renal tubule epithelial cells	(173)
miR-21	MSC	Inhibits renal tubule epithelial cells apoptosis and DC maturation	(174)
miR-let7c	MSC	Inhibits renal fibrosis	(175)
mi-R 30	Wharton Jelly-MSC	Inhibits renal tubule epithelial cells mitochondrial fission	(176)
miR-199a-5p	Bone marrow-MSC	Alleviate endoplasmic reticulum stress at reperfusion	(177)
miR-486-5p	Endothelial cell forming colonies	Inhibits endothelial cell apoptosis and endothelial-mesenchymal transition	(178, 179)
miR-218	Renal artery progenitor cell	Increases endothelial cell migration	(180)
miR-126 miR-296	Endothelial progenitor cell	Increases endothelial cell angiogenesis	(181)

from cardiac death donor, a procedure characterized by severe IRI (194) (**Table 3**).

Trophic factors carried in MSC-EVs depend on the parental cell and the surrounding milieu, such as inflammation and hypoxia (136, 164, 195).

Hypoxia has a profound impact on EV properties. In general, ischemic conditioning (preconditioning, postconditioning, and remote conditioning) provides positive results in the setting of myocardial infarction, and hypoxic EVs appear to mediate these effects (124, 125, 195).

Hypoxic EVs derived from BM MSCs can exert protective effects in experimental models of AKI through several mechanisms: inhibition of renal tubule and endothelial cell apoptosis, stimulation of endothelial cell proliferation, reduction of inflammation and PMN infiltration, and inhibition of renal fibrosis (124).

Of interest, hypoxia can stimulate the secretion of EVs by adipose tissue-derived MSCs and can enhance their regenerative properties; specific anti-apoptotic, anti-oxidative, anti-inflammatory and pro-angiogenic pathways are activated by hypoxic EVs, and a distinct proteomic pattern is determined by this type of EVs in renal proximal tubule epithelial cells (196).

In the study by Collino et al. (196), four effects were specifically enhanced in hypoxic EV and could blunt progression of ischemic AKI to CKD: downregulation of fibroblast growth factor receptor 1 (FGFR-1), which mediates TGF-β1-induced epithelial-to-mesenchymal transition, and inhibition of maladaptive repair and fibrogenesis (197); angiogenesis stimulation, alleviating renal microvasculature rarefaction under hypoxia (198); translocation of Nrf-2 into the nucleus, activating antioxidant genes such as HO-1 (199); and downregulation of IL-6, blunting macrophage infiltration and polarization toward a M2 phenotype (200).

Moreover, hypoxic EVs carry respiratory complexes, supporting a non-mitochondrial aerobic metabolism when mitochondrial respiratory capacity is impaired (201); they reestablish intracellular ATP levels and reverse pre-apoptotic changes like histone H2 and H2B upregulation (202); they favor cell proliferation through JNK pathway activation (203) and downregulate calnexin, a NADPH oxidase NOX4-interacting protein, reducing reactive oxygen radical formation (204–206).

However, remote ischemic preconditioning on KT recipients has not proven to be as clinically effective as in ischemic heart disease, and further studies are needed to implement these findings into clinical tools (207, 208).

Another therapeutic approach is MSC transfection with specific miRNA. These engineered EVs proved to be more effective than those derived from naïve MSCs (209).

Other Cell Type-Derived Extracellular Vesicles

Cell types other than mesenchymal stromal cells also release reno-protective extracellular vesicles.

Under hypoxic conditions, endothelial colony-forming cells inhibit endothelial cell apoptosis and endothelial mesenchymal transition through EV containing miR-486-5p (178, 179), whereas renal artery progenitor cells increase endothelial cell migration through EV containing miR-218 (180).

Endothelial progenitor cells inhibit capillary rarefaction and progression toward chronic lesions in ischemic AKI; this effect was lost after depletion of pro-angiogenic miR-126 and miR-296 by transfection with specific antagomirs (181).

EVs from renal tubule cells also are capable of accelerating recovery of established renal ischemic damage (210).

b) Acute Rejection

Mesenchymal Stromal Cell-Derived Extracellular Vesicles

Studies using EVs from stem cells and tumors have shown immunosuppressive effects of their transcription factors and miRNAs (159).

In an MHC-mismatched rat model of kidney transplant, injection of recipient MSC-EVs on day 7 after transplant has reduced NK infiltrates and almost completely abolished intra-graft TNFα expression. However, B- and T-lymphocyte infiltrates were higher in EV-treated rats, whereas there was no difference in macrophage populations. Importantly, no difference was observed in antibody response against the donor, which occurred in both groups. These data suggest that MSC-EVs mainly affect some type of innate immunity cells (NK cells and related cytokines, such as TNFα), whereas they do not suppress adaptive immunity and rejection in a strong alloreactive model (162, 211).

TABLE 4 | Studies on MSC-derived EVs as therapeutic tool in AKI from IRI.

MSC origin	Mechanism	References
Human bone marrow	Reduced apoptosis and increased proliferation of renal tubule epithelial cells	(166)
Rat bone marrow	Reduced inflammatory cytokines (IL1β; TNFα)	(182)
Human umbilical cord	Antioxidation through activation of Nrf2/antioxidant response elements (ARE) and decreased expression of NOX2	(183, 184)
Human umbilical cord	Decreased renal fibrosis (downregulation of CX3CL1, decrease of CD68+macrophages); increased angiogenesis (increased expression of renal VEGF)	(185–187)
Human umbilical cord	Tubular cell dedifferentiation and growth (increased ERK1/2 and HGE expression)	(188)
Human umbilical cord	Inhibition of mitochondrial fission (miR-30) and reduced apoptosis	(176)
Mouse kidney resident	Increased proliferation and reduced apoptosis; increased angiogenesis	(189)
Mouse kidney resident (glomeruli)	Increased proliferation of renal tubule epithelial cells	(190)
Rat adipose tissue	Inhibition of oxidative stress, apoptosis, renal fibrosis	(191)
Human umbilical cord	Increased proliferation and fibrosis (releasing from G2/M cell cycle arrest)	(192)
Human bone marrow	Inhibition of apoptosis (downregulation of Sema3A expression and activation of AKT/ERK pathways through miR-199a-3p); inhibition of NK	(193)
Human umbilical cord	Inhibition of apoptosis, increased proliferation of renal tubule epithelial cells; reduced CD68+macrophages infiltration; reduced fibrosis (decreased expression of aSMA and TGFβ; increased expression of HGF)	(194)
Human BM	Suppression of endoplasmic reticulum stress (miR-199a-5p)	(177)

Immune Cell-Derived Extracellular Vesicles

Immunosuppressive properties of EVs (75–79) could be exploited to inhibit innate component of rejection, for example, skewing DC function and maturation toward a tolerogenic profile (212–214). EVs released from Treg lymphocytes modulated DC maturation and prolonged kidney allograft survival in a rat model (215).

In a study on heart transplant rat model, DC-derived EVs were administered together with LF-15-0195, a DC maturation blocker. This approach determined a donor-specific tolerance with significantly blunted anti-donor proliferative response and chronic rejection, resulting in prolonged graft survival (2, 211).

EXTRACELLULAR VESICLES AS BIOMARKERS IN KIDNEY TRANSPLANTATION

EVs have also been investigated as possible biomarkers in KT. Plasma and urinary EVs have been studied in different transplant settings and will be discussed separately (216).

a) Acute Rejection

Plasma Extracellular Vesicles

Plasma EVs are one of the most promising biomarkers for solid organ transplantation, reducing or even obviating the need for renal biopsy (216–218).

In a recent study, Zhang et al. compared levels of mRNA transcripts carried by plasma EVs of patients with antibody-mediated rejection, T-cell mediated rejection, and no rejection and their related genes, identifying those that were significantly overexpressed in EVs from patients with antibody-mediated rejection. On this basis, they created a gene combination score elaborated from mRNA transcripts of four genes (gp130, SH2D1B, TNFα, and CCL4), which was able to predict imminent antibody-mediated rejection (219).

In a study on 231 KT patients, circulating endothelial microparticles were analyzed before and periodically after KT (up to 2 months); plasma levels increased during antibody-mediated rejection episodes and decreased after therapy, with a slower decline in patients with peritubular capillary C4d staining (220).

In another study, quantification was carried out of plasma $C4d^{+}CD144^{+}$ EVs released from endothelial cells associated with antibody-mediated rejection (11-fold increase in concentration compared with that in patients with no rejection), its severity, and response to treatment (over 70% decrease in concentration after successful anti-rejection therapy) (100).

Urinary Extracellular Vesicles

In one study (221), 11 proteins were significantly enriched in urinary EVs from patients with T cell-mediated rejection; of note, the association was lost when the whole urinary protein fraction was analyzed. This finding highlighted the impact of "background noise" from uromodulin and proteinuria, suggesting that urinary EVs are a more selective source of biomarkers. Despite this, little evidence has been produced on urinary EV RNAs so far, as most papers have focused on total, cell-derived, or cell-free urinary transcripts (222).

In a more recent study, increased expression of 17 urinary EV proteins was found in patients with T cell-mediated rejection and two proteins—tetraspanin-1 and hemopexin—were proposed as biomarkers (223).

Finally, a urine-based platform termed IKEA ("integrated kidney exosome analysis"), detecting EVs shed by T cells into urine, revealed high levels of CD3-positive EVs in patients with rejection, with an accuracy of over 90% for T cell-mediated rejection (224).

b) Delayed Graft Function and Other Settings

Plasma Extracellular Vesicles

In the already mentioned study by Qamri et al., circulating endothelial microparticles decreased within 2 months of KT, paralleling renal function recovery, only in patients with specific types of causal nephropathies such as diabetic nephropathy or glomerulonephritis secondary to autoimmune disorders (220).

Consistently, Al Massarani et al. found a progressive decrease in serum EV concentration and in their procoagulant activity after KT. This evolution was independent from the type of immunosuppression, whereas it seemed to be influenced by history of cardiovascular disease and CMV infection (225, 226). In a Brazilian cohort of 91 KT patients, PLT and endothelial EV size and concentration were significantly different depending on renal function and time from KT (227).

Taken together, these data suggest that decreased endothelial EVs after KT reflect not only antibody- or T cell-mediated rejection but also improvement of preexisting endothelial dysfunction and of cardiovascular risk factors, paralleling recovery of renal function after KT (227, 228).

Urinary Extracellular Vesicles

Urinary EVs have been proposed an enriched source of biomarkers of DGF. For example, neutrophil gelatinase-associated lipocalin expression in EVs was higher than in urinary cells and correlated with DGF (229).

Urinary $CD133^+$ EVs appear to be decreased in KT patients with slow graft function and vascular damage, suggesting possible damage to renal stem cell compartment (230).

Likewise, a reduction in urinary aquaporin-1- and aquaporin-2-containing EVs was observed in rat model of IRI, probably reflecting impaired trafficking and expression of these proteins in renal tubule epithelial cells (231), confirming previous finding of decreased abundance of aquaporin-1 in KT recipients in the immediate postoperative days (232).

LIMITS, PERSPECTIVES, AND CONCLUSIONS

Despite the large volume of literature, our knowledge of innate immunity EVs is still limited (233). Further studies are needed to widen our understanding in graft antigen spreading and processing by DCs (53, 70–72) and to clarify their tolerogenic potential (75–78). Little evidence has been produced on PMN or macrophage vesicles. Additionally, few studies identified the target genes of EV miRNAs.

Finally, a major limit of EV analysis is the lack of standardization and consistency (234): based on different techniques, diverse markers with almost no overlapping results have been proposed. Of note, most housekeeping genes used for cellular assay normalization (e.g., β-actin or GAPDH) are not consistently expressed in EVs. Normalization of urinary EV proteins with tetraspanins (CD9, CD63, or CD81) is not a validated approach, and mRNA analysis remains problematic (235). Finally, most urinary markers should be standardized for urinary creatinine, but not all studies have adopted this method.

Despite these barriers, EVs appear promising as both biomarkers and therapeutic agents in KT. Enhancement of MSC-EVs therapeutic potential through stimulation with biophysical or biochemical cues (i.e., LPS, hypoxia, inflammatory cytokines, growth factors, hormones such as erythropoietin, nitric oxide, and EVs from other cells such as endothelial cells) is an attractive perspective (236). Genetically engineered EVs overexpressing specific proteins or miRNAs acquire stronger therapeutic properties: for example, HIF-α-overexpressing MSCs have enhanced angiogenic activity and repaired more efficiently cardiac tissue in a mouse model compared with control EVs (237); miR-let7c-overexpressing MSCs selectively homed to damaged kidney, where they upregulated miR-let7c genes and downregulated expression of TGF-β, its receptor (TGF-β-R1), and other pro-fibrotic genes in a renal mouse model of unilateral ureteral obstruction (175).

Bioengineered EVs hold promise as targeted vehicle of drugs or miRNAs, as they naturally overcome biological barriers (209, 238). "Decoy EVs" have also been employed to antagonize inflammatory cytokines (239).

In conclusion, EVs finely tune the crosstalk among innate immune cells and graft tissue; in particular, they determine antigen spreading and "cross-dressing" in the early transplant phases, thus being a key trigger of either alloimmunity or graft tolerance. Systemically, they modulate complement and coagulation cascades during transplant related kidney injuries as antibody-mediated rejection and IRI.

Growing evidence support a potential application of EVs derived from MSCs and other cell types as therapeutic tools in different settings of renal transplantation. Finally, urinary and serum EVs are promising biomarkers of rejection and DGF, opening new paths toward a renal "liquid biopsy."

AUTHOR CONTRIBUTIONS

MQ and VC designed, wrote, and critically revised the Review. SD analyzed innate cells derived EVs. GG and GM analyzed EVs role in DGF and rejection. GC dealt with EVs and complement and coagulation system.

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Inflammaging and Complement System: A Link Between Acute Kidney Injury and Chronic Graft Damage

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The aberrant activation of complement system in several kidney diseases suggests that this pillar of innate immunity has a critical role in the pathophysiology of renal damage of different etiologies. A growing body of experimental evidence indicates that complement activation contributes to the pathogenesis of acute kidney injury (AKI) such as delayed graft function (DGF) in transplant patients. AKI is characterized by the rapid loss of the kidney's excretory function and is a complex syndrome currently lacking a specific medical treatment to arrest or attenuate progression in chronic kidney disease (CKD). Recent evidence suggests that independently from the initial trigger (i.e., sepsis or ischemia/reperfusions injury), an episode of AKI is strongly associated with an increased risk of subsequent CKD. The AKI-to-CKD transition may involve a wide range of mechanisms including scar-forming myofibroblasts generated from different sources, microvascular rarefaction, mitochondrial dysfunction, or cell cycle arrest by the involvement of epigenetic, gene, and protein alterations leading to common final signaling pathways [i.e., transforming growth factor beta (TGF-β), $p16^{ink4a}$, Wnt/β-catenin pathway] involved in renal aging. Research in recent years has revealed that several stressors or complications such as rejection after renal transplantation can lead to accelerated renal aging with detrimental effects with the establishment of chronic proinflammatory cellular phenotypes within the kidney. Despite a greater understanding of these mechanisms, the role of complement system in the context of the AKI-to-CKD transition and renal inflammaging is still poorly explored. The purpose of this review is to summarize recent findings describing the role of complement in AKI-to-CKD transition. We will also address how and when complement inhibitors might be used to prevent AKI and CKD progression, therefore improving graft function.

Keywords: renal aging, complement system, AKI-to-CKD transition, cellular senescence and SASP, complement inhibition therapy

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Abbreviations: ABMR, antibody-mediated rejection; AKI, acute kidney injury; AP, alternative pathway; C1-INH, C1 esterase inhibitor; CKD, chronic kidney injury; CP, classical pathway; DAMPs, damage-associated molecular patterns; DGF, delay graft function; EndMT, endothelial-to-mesenchymal transition; I/R, ischemia/reperfusion; IRI, ischemia/reperfusion injury; LP, lectin pathway; MAC, membrane attack complex; MASP, MBL-associated serine protease; MBL, mannose-binding lectin; PAI-1/*SERPINE1*, plasminogen activator inhibitor-1; PAMPs, pathogen-associated molecular patterns; PBMC, peripheral blood mononuclear cells; PMT, pericyte-to-myofibroblast transition; PRM, pattern recognition molecules; SASP, senescence-associated secretory phenotype.

OVERVIEW OF THE COMPLEMENT SYSTEM

Complement is an essential part of the innate immune system. Over a century ago, complement was first identified by Paul Ehrilch as a heat-labile component in serum that literally "complemented" the antibody- and cell-mediated immune responses against pathogens (1). Today, we do know that complement system consists of more than 40 blood-circulating, membrane-associated, and intracellular proteins. Complement can be activated in the serum, in local tissue, and at intracellular level (2) and exerts three major physiological functions. First, complement proteins are involved in host defense against infection (3). This activity is mediated by several events: (i) the pathogens opsonization (i.e., covalent C3b, C3d, C4b complement fragments deposition on microbial surfaces that boost phagocytosis), (ii) the leukocytes chemotaxis and activation that amplify the inflammatory process (i.e., the binding of complement anaphylatoxin to receptors on leukocytes), and (iii) the direct lysis of bacteria or infected cells. Second, complement can be considered as a connection between innate and adaptive immune response (4). Indeed, the C1q, the principal component of the classical pathway, can activate complement cascade after the binding to antibody–antigen complexes, which originated during the adaptive immune response. In addition, complement can also enhance the antibody response and consolidate the immunological memory since C3 receptors are expressed on B cells, antigen-presenting cells (APC), and follicular dendritic cells (5). Third, after the resolution of inflammatory injury, complement mediates the clearance of apoptotic/necrotic, ischemic, or damaged self-cells (i.e., by the binding of C1q or C3 fragments to host self-surfaces) (6).

In the serum and interstitial fluids, complement proteins circulate largely in an inactive form: however, in response to pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs), they become activated through a sequential cascade of reactions (6) (7). The recognition of these highly conserved molecular patterns is achieved via different types of pattern recognition molecules (PRMs) (8) (**Figure 1**). The activation of complement system occurs via three different pathways: the classical pathway (CP), the alternative pathway (AP), and the lectin pathway (LP) (4). Independently from the signaling initiated, all the pathways lead to the formation of a central enzyme, the C3 convertase, that cleaves C3 into C3a and C3b. In the CP, immune complexes of immunoglobulin M (IgM) or hexameric IgG are recognized by C1q together with the associated proteases C1r and C1s (9). The LP contains six PRM: mannose-binding lectin (MBL), Ficolin-1, Ficolin-2, Ficolin-3, Collectin-10, and Collectin-11, which recognize carbohydrate and acetylated structures on pathogens and form a complex with MBL-associated serine proteases (MASPs) (10, 11). The AP is continuously activated at low level by the spontaneous hydrolysis of C3 called the "tick-over." This mechanism generates C3b that can then covalently bind to various proteins, lipids, and carbohydrate structures on microbial surfaces (4). As examples of DAMP-mediated complement activation, we could mention the CP induction by C-reactive protein (CRP) or Pentraxin-3 (12) (8); in IgA nephropathy, LP can be triggered by IgA (13), and after ischemia/reperfusion injury (IRI), L-fucose induced LP on stressed cells (7). With regard to AP, the cleavage of C3 can be induced by neutrophil enzyme elastase or myeloperoxidase (MPO) (14). Progressive C3 activation results in the formation of the C5 convertase, which cleaves C5 into C5b and C5a. C5 is the initiator of the terminal step, and C5b merged together with the components C6 till C9 assembling the membrane attack complex (MAC) pores (**Figure 1**). In the last steps of complement activation, the MAC leads to the direct lysis of the pathogen or target cells. Interestingly, MAC can also trigger a range of non-lethal effects on cells as NLRP3 inflammasome activation in the cytosol (15). Complement activation also leads to the generation of other effector molecules such as opsonins (C4b, C4d, C3b, iC3b, C3dg, and C3d) and anaphylatoxins (C3a, C5a), which can interact with their respective receptors and recruits granulocytes, monocytes, and other inflammatory cells on site of infection (16). Anaphylatoxins can bind specific receptors expressed not only on PBMCs but also on parenchymal cells such as tubular epithelial cells within the kidney, initiating inflammation and chemotaxis (C3aR, C5aR1 and C5aR2) (17) (**Figure 1**).

However, complement functions have been implicated in the pathogenesis of disorders not necessarily related to infections such as cancer (18), neurodegenerative and age-related disorders [i.e., age-related macular degeneration (AMD)], metabolic diseases (2), the progression of chronic kidney disease (CKD) (19, 20), and more importantly renal aging (21, 22). Therefore, increasing efforts are necessary to evaluate the efficacy of targeting complement to arrest the progression of renal aging during CKD (20, 23).

LOCAL PRODUCTION OF COMPLEMENT FACTORS AT RENAL LEVEL

Complement factors are produced predominantly by the liver; however, some factors as C1q (24), properdin, and C7 (25) are released by leukocytes (26); in addition, adipocytes can synthesize factors B and D (also known as adipsin) (27). In the kidney, tubular epithelial cells can produce virtually all complement proteins (28). The percentage of tubular complement biosynthesis can increase significantly during inflammation (29–31). Following IRI, complement C3 can be expressed by proximal tubular epithelial cells (32), endothelial cells (33), glomerular epithelial and mesangial cells (34). The C3 messenger RNA (mRNA) upregulation and the subsequent biosynthesis has been demonstrated to play a central role in kidney transplantation (35, 36). Pratt et al. demonstrated that wild-type (WT) mice with intact serum complement activity do not reject allogenic C3-deficient kidneys, underlying that kidney-derived complement is a key mediator of renal injury (37, 38). Thus, complement can switch the immune system balance toward a persistent and proinflammatory response that,

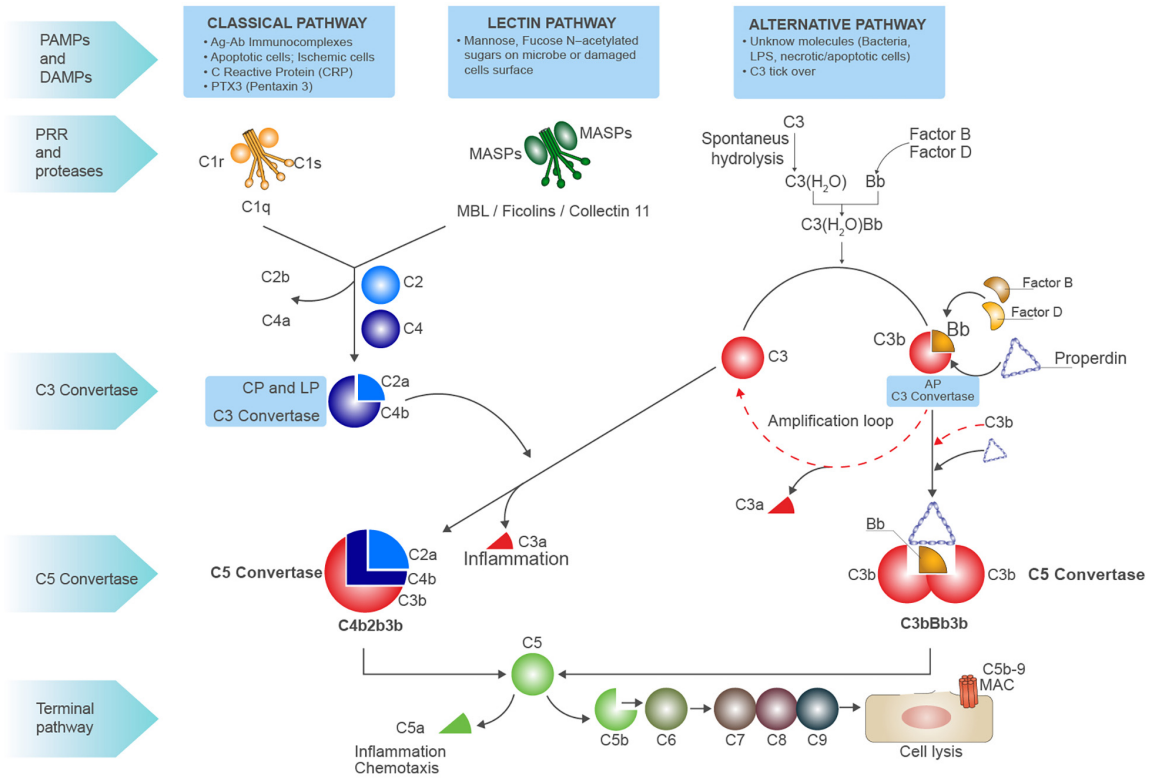

FIGURE 1 | Schematic overview of complement system. Complement system can be initiated by three different pathways: the classic pathway, the lectin pathway, and the alternative pathway, all converging to the formation of C3 convertases. The classic pathway is initiated by the binding of C1q globular domains to the Fc of immunoglobins bound to their antigen (immunocomplexes), apoptotic or ischemic cells, acute phase proteins [i.e., C-reactive protein (CRP) and Pentraxins]. When the binding of C1q to substrate occurs, a conformational change of C1q leads to activation of proteases C1r and C1s that are associated to C1q. It activates C1s, then cleaves C4 into C4b, subsequently C2 is cleaved which binds to C4b forming the CP (membrane-attached) C3 convertase, the C4b2a complex. This classical C3 convertase activates and cleaves C3 molecules to C3b and C3a. The pattern recognition receptor (PRR) of lectin pathway involves several molecules as MBL, Ficolins, and Collectin-11 that, after binding to mannose, fucose, or *N*-acetylated residues on microbial surfaces or damaged cells, can activate the serine proteases MASP1 and MASP2 leading to C3 convertase formation as for CP. At low level, the activation of alternative pathway (AP) can be induced by spontaneous hydrolysis of C3 into C3(H_2O), an event called C3 tick-over. The hydrolysis changes the structure of C3 by the translocation of the thiol ester domain that allows the new formed structure to form covalent bonds with -OH or -NH2 residues on the target surfaces. The C3(H_2O) can bind factor B (FB), resulting in the cleavage of FB by factor D (FD) and generating Ba and Bb and the formation of the AP C3 convertase C3(H_2O)Bb. The C3(H_2O)Bb complex is the initial C3 convertase of the AP (fluid phase C3 convertase) and can cleave C3 to C3a and C3b. The C3b fragment can bind to FB, and after the cleavage of FB by FD, the C3 convertase C3bBb (high level) is formed. This C3 convertase cleaves more C3 to C3b to generate even more C3 convertase in an amplification loop. The protein properdin stabilizes C3bBb. After formation of the classical C3 convertase C4b2a or the alternative C3 convertase C3bBb, the final pathway (common to all three pathways) may be initiated. An additional C3b molecule is incorporated in both the C3 convertases leading to the formation of the C5 convertase. Properdin stabilization occurs in AP C5 convertase formation (C3bBb3b). The C5 convertase cleavages C5 into C5a (the anaphylatoxin) and C5b, C5b then binds to C6, and this allow the binding of C7, C8, and C9 and results in the formation of the C5b-9 terminal membrane attack complex (MAC). The latter forms pores in the membrane of pathogens and damaged self-cells, thus promoting cell lysis. C3a and C5a are powerful anaphylatoxins able to induce chemotaxis and inflammation.

if directed against self-antigens, might promote the induction of autoimmunity or, if directed against donor antigens, might lead to rejection (23).

INTRACELLULAR COMPLEMENT ACTIVATION AND EVs CARRIED COMPLEMENT

Recent studies have revealed that complement activation is not confined in the serum or produced locally by resident and infiltrating cells into interstitial fluids. Complement cascade can also be initiated intracellularly. The intracellular complement activation, the Complosome, has been investigated mainly in human CD4 + T cells (2); however, it has also been described in adipocytes, monocytes, fibroblasts, B cell, and epithelial and endothelial cells. In resting T cells, the function of C3 and C5 intracellular activation has been associated to the homeostatic cell survival by keeping low level of mTOR signaling (2). Nevertheless, after T-cell receptor (TCR) activation, intracellularly cleaved C3 can induce the Th1 differentiation, the NLRP3 inflammasome activation, and the T cell metabolism reprogramming by regulation of glycolysis and mitochondrial oxidative phosphorylation (39). Interestingly, aging is also a process strongly integrated with chronic inflammation and metabolism; therefore, the recently discovered connection

between the Complosome and cellular metabolome might add a new layer of complexity in the impact of complement intracellular activation in several aging-related diseases (as obesity) and in the acceleration of renal aging during CKD.

Lastly, complement components can be also identified in circulating extracellular vesicles (EVs), particularly in microvesicles (MVs) with a size ranging from 0.1 to 1 μM. EVs can carry and modulate complement system in several age-related disease, such as AMD (40), providing a new, extracellular way to deliver complement in different body compartments.

COMPLEMENT IN KIDNEY DISEASE

The complement system is considered a crucial pathogenic mediator in the development of several renal diseases. The kidney is particularly susceptible to complement-mediated injury, mainly due to the ultrafiltration function, the low expression of complement regulators, and the local complement production (23). Complement aberrant activation, acquired or inherited dysregulation, and ineffective clearance have been observed in a wide spectrum of glomerulonephritis [lupus nephritis (41), C3 glomerulopathy, IgAN, antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis], in thrombotic microangiopathy [atypical hemolytic uremic syndrome (aHUS)], in renal transplantation, and in the progression to CKD (20, 38). A predominant role for glomerular immunocomplex deposition has been observed in lupus nephritis with the involvement of CP, LP, and also AP. Moreover, the impairment of AP predominantly characterizes the aHUS and the C3 glomerulopathy. These findings have led to the clinical use of complement blocking therapeutics as Eculizumab in aHUS (42).

In the progression to CKD, the role of all the three pathways has been assessed, and promising results are coming from clinical trials. However, we are still far from the clinical use of complement inhibitors to delay the progression of renal fibrosis.

AKI-TO-CKD TRANSITION: THE ROLE OF COMPLEMENT

Acute kidney injury (AKI) characterized by a rapid loss of renal function and is still associated to a high morbidity and mortality (43). The most common causes of AKI include renal IRI, sepsis, or several exogenous nephrotoxins such as drugs. Currently, it is well known that AKI predisposes to the future development of CKD and subsequently to end-stage chronic renal disease (ESRD) (43). However, the cellular and molecular mechanisms underlying the progression from AKI to CKD remains incompletely understood.

Complement system was traditionally related to the early development of AKI (44); nonetheless, several evidence indicated that complement is a pivotal mediator of tubular senescence (21, 22) and interstitial fibrosis, the common hallmark of premature aging that characterizes the CKD (45). The major complement components involved in the AKI-to-CKD transition seems to be the anaphylatoxins C3a and C5a and the terminal C5b-9 that contribute to the damage during CKD progression through various mechanisms. After binding to C5aR and C3aR, these anaphylatoxins exert a proinflammatory and fibrogenic activity on tubular and endothelial cells (46, 47), pericytes (31, 48), and resident fibroblasts; moreover, they can mediate renal fibrosis by stimulating transforming growth factor beta 1 (TGF-β1) production in cultured murine tubular cells. As a consequence, activated endothelium, monocytes, and injured tubular epithelium (49) have all been shown to secrete profibrogenic factors such as TGF-β and platelet-derived growth factor (PDGF), able to activate resident fibroblasts promoting collagen deposition. In addition, we recently demonstrated that the complement anaphylatoxin C5a contribute to fibrosis inducing the pericytes to myofibroblast transdifferentiation (PMT) through pERK activation (48).

Other mechanisms of complement-mediated transition to CKD are the chemotactic effect on different infiltrating leukocytes (50) with the inhibition of the polarization of T-helper cells to Th1 cells (51) (52). The subsequent shift of T-helper cells to Th2 cells, together with their cytokines release, such as TGF-β, has been shown to act in a profibrotic manner (53). The predominant profibrotic effect of TGF-β signaling in AKI-to-CKD transition, in tubular cell cycle arrest, and myofibroblast transdifferentiation has been reviewed elsewhere (54).

Finally, the terminal C5b-9 complex is a powerful inducer of profibrotic and proinflammatory cytokines by a variety of renal cells. Incubation of human glomerular epithelial cells with sublytic doses of C5b-9 significantly increased the collagen synthesis (55) and the release of TGF-β1 and interleukin IL-6 (56). In addition, endothelial cells exposed to sublytic concentration of C5b-9 released profibrotic factors including fibroblast growth factor (FGF) and PDGF (57). Similar effects were observed in tubular epithelial cells; stimulating proximal tubular epithelial cells with C5b-9 led to increased expression of collagen type IV (58). Collectively, these *in vitro* evidence supported that C5b-9 can increase the profibrotic process associated with progressive renal injury. Uncontrolled complement activation may ultimately result in maladaptive tissue repair with irreversible development of fibrosis and renal aging.

THE ROLE OF COMPLEMENT IN IRI

Recent improvements in immunosuppressive therapy have made kidney transplantation the treatment of choice for ESRD patients (59). Complement system might have a detrimental role in different phases of renal transplantation from brain (DBD)/cardiac death (DCD) in deceased donors, to organ procurement, to IRI, allograft rejection, until the chronic graft deterioration (60). Increased systemic levels of sC5b-9 were observed in DBD and DCD but not in living donors, which correlate with increased acute rejection in the recipients (61). Furthermore, a strong association between chronic graft injury and overexpression of complement components has been found by proteomic analysis in kidney donor biopsies (62). These results indicated that shorter periods of ischemia are clearly

associated with less complement activation; in addition, the protein profiles of preservation solutions in which kidney from deceased donors had been stored revealed intense activity of complement effectors (as C3, factor B) during organ storage preceding transplantation (63).

Following organ procurement, the role of complement in renal IRI has been extensively investigated by several studies (64, 65). Importantly, renal IRI is the pivotal contributor in the development of delay graft function (DGF), traditionally defined as the requirement for dialysis during the first week after transplantation. IRI is initiated by the occlusion of blood flow that is necessary for organ collection and during hypothermic ischemia for the storage; in this conditions, renal cells are permanently damaged due to hypoxia, ATP depletion, and accumulation of metabolic waste, resulting in the production of reactive oxygen species (ROS) and DAMPs (i.e., histones, heat-shock proteins). Reperfusion leads to a more detrimental inflammatory response, resulting in further tissue damage characterized by early release of inflammatory cytokines such as IL-6, tumor necrosis factor alpha (TNFα), and IL-1α that represent a powerful inflammatory milieu capable to induce a cellular senescence-associated secretory phenotype (SASP).

A large body of evidence from both experimental (66–68) and clinical (20) studies has identified in complement activation a crucial mediator of chronic tubulointerstitial fibrosis following renal IRI (69). In the past years, using complement-deficient animals, the terminal C5b-9 was identified as principal inducer of tubular injury after IRI (70). In particular, Zhou et al. demonstrated that $C3^{-}$-, $C5^{-}$-, and $C6^{-}$-deficient mice were protected against ischemic damage, whereas $C4^{-}$-deficient mice were not (59). These initial findings underlined the importance of tubular (and not endothelial) injury in the I/R physiopathology. Next, we suggested a more significant role for the MAC and the AP pathway. The involvement of AP was also elegantly confirmed by Thruman et al. in transgenic mouse models (68, 71). More recent reports have focused on pattern recognition receptors of lectin pathway (LP-PRRs) (MBL, Collectin-11, Ficolin-3), CP-C1q, and C5aR1/C5aR2, indicating that all these complement components were able to trigger the IRI and fuel the progression to CKD (**Figure 2**). Hence, renal function in MBL-deficient mice was significantly preserved after IRI (67).

Furthermore, Collectin-11, a PRR that binds a ligand (L-fucose) (72) expressed on stressed tubular cells, was demonstrated capable to activate complement LP in C4-independent manner. This mechanism, called C2/C4 bypass, has been proposed by Yaseen et al. (73) and depends on the unique capacity of MASP2 to directly activate C3, leading to C3b and C3a fragment formation without the involvement of C4 or C2. These findings finally explained previous and contradictory results that showed protection from IRI in MASP2-deficient (74) but not in C4-deficient mice (70, 75, 76). More importantly, compared with wild-type, Collectin-11-deficient mice showed significantly reduced renal functional impairment and leukocyte infiltration, less chronic inflammation, and tubulointerstitial fibrosis after renal IRI (77). The analysis of other LP factors in patients showed that high pretransplant level of Ficolin-3 was strongly associated with poor allograft survival and age after kidney transplantation (78). In accordance, Ficolin-2 gene rs7851696 polymorphism influenced kidney allograft functions, with specific allele increasing the risk of DGF and rejection (79). These results revealed a central role of LP in the development of renal fibrosis after IRI, with strong clinical implication in the transition from AKI to CKD (77).

Even if the contribution of CP has been controversial (80), the CP and LP are effectively involved in development of early fibrosis. In a pig model of renal IRI injury, we demonstrated the deposition of C1q and MBL on peritubular capillaries colocalization with C4d after 15 min from reperfusion. The treatment with recombinant human C1-INH (C1 esterase inhibitor) (81), an inhibitor of CP and LP pathways, conferred protection not only reducing infiltrating cells but also modulating the generation of myofibroblasts by reducing endothelial-to-mesenchymal (EndMT) (82) and the pericytes-to-myofibroblast (PMT) transitions (48). Consistent with these results, Delpech et al. demonstrated that treatment by C1-INH appeared to be protective also after 3 months from IRI, reducing the development of chronic graft fibrosis (83). These data have been translated to humans, and the use of C1-INH in patients receiving deceased donor kidney transplants with high risk for DGF has been investigated in a recent clinical trial (84) and will be further discussed in the paragraphs below.

Next to C1 blockage, the C5aR1 and C5aR2 inhibition could offer promising results. C5aR receptors are expressed both on peripheral and infiltrating leukocytes (such as dendritic and T cells) and on renal parenchymal cells such as tubular epithelial cells, mediating the recruitment of leukocytes. Additionally, these receptors mediated allograft injury before (85) and after kidney transplantation (86) (**Figure 2**). Consistently, pathogenic roles for C5aR1 in renal tubulo-interstitial fibrosis have been reported in different models of renal IRI (66, 87–91), murine model of unilateral ureteral obstruction (UUO) (92), and chronic pyelonephritis (93). Remarkably, in clinical settings, donor urinary C5a concentrations before transplantation has been shown to be higher in the recipients with risk of DGF (92, 94).

Considering the C5aR1 profibrotic and complement-independent detrimental effect, the use of C5aR inhibitors should be taken into consideration. Recently, successful evidence are coming from trials using the Avacopan (CCX168), an orally administered, selective C5a receptor inhibitor in aHUS (NCT02464891), ANCA-associated vasculitis (NCT01363388, NCT02222155) (95), C3 glomerulonephitis (NCT03301467), and IgA nephropathy (13) (NCT02384317). The potential beneficial role of C5aR1 inhibition in the context of renal IRI should be encouraged.

FROM ANTIBODY-MEDIATED REJECTION TO CKD: THE ROLE OF COMPLEMENT

After kidney transplantation, antibody-mediated rejection (ABMR) is one of the leading cause of long-term graft failure and CKD in which complement system plays a

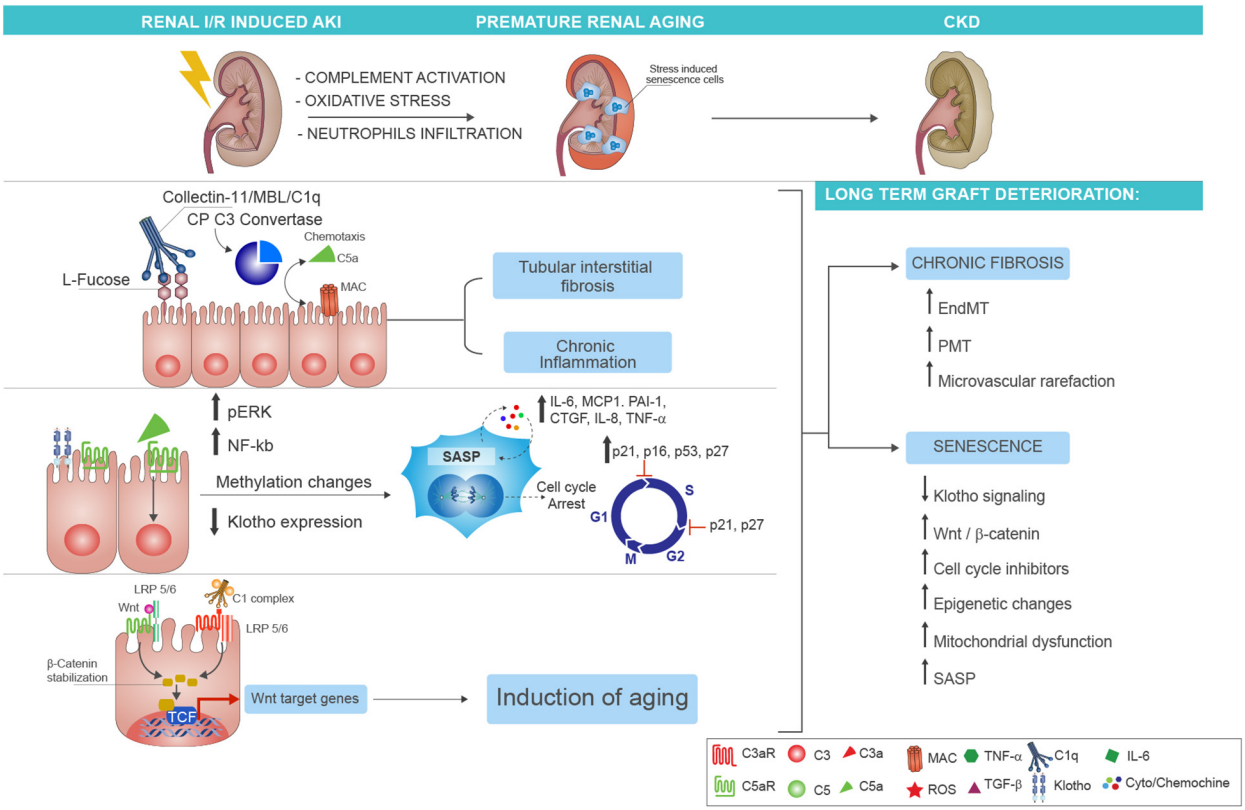

FIGURE 2 | Complement-driven accelerated renal senescence after IRI-AKI leading to CKD progression. During renal ischemia/reperfusion injury (IRI), activation of complement may lead to reactive oxygen species (ROS) generation and neutrophils infiltration, thereby establishing a prosenescence microenvironment that promotes accelerated renal aging. Several molecular mechanisms can be responsible for the establishment of tubular senescence after complement activation. First, renal tubular epithelial cells expressed fucosylated glucose patterns upon IRI, which can be recognized by the lectin pathway pattern recognition receptor (PRR) (as Collectin-11), therefore inducing complement activation and tubular interstitial fibrosis with persistent chronic inflammation (upper part). Second, the release of C5a anaphylatoxin, through methylation changes, can induce cellular senescence characterized by growth arrest, inhibition of apoptosis, and acquirement of a senescence-associated secretory phenotype (SASP). The molecular mediators of the growth arrest are mainly G1-S cell cycle inhibitors as p16INK4a, p21 WAF/CIP1, p53, and p27. The p21 and p27 proteins can mediate the cell cycle arrest also during the transition from phase S to phase G2 of cellular cycle. The SASP is maintained and amplified by the increased expression of proinflammatory [such as interleukin (IL)-6, monocyte chemoattractant protein-1 (MCP-1), IL-8, PAI-1, tumor necrosis factor alpha (TNFα)] and profibrotic cytokines [as connective tissue growth factor (CTGF)] (in the middle). Finally, another molecular mechanism of complement-induced renal inflammaging is mediated by Wnt/β-catenin signaling. The C1 complex (that is composed by the association of C1q with C1s and C1r serine proteases), after binding the serpentine Frizzled receptor (indicated in red), can cleave the N-terminal domain of LRP5/6 and stabilize the β-catenin protein (in the left). The function to bind Frizzled receptors and to cleave the LPR5/6 extracellular domain is normally exerted by the Wnt protein (indicated in green). The β-catenin stabilization allows the nuclear translocation, the interaction with transcription factors, and the augmented gene expression of proaging Wnt target genes. The final and common event of all these complement-mediated pathways is represented by the generation of stress-induced senescence cells, indicated in the figure as enlarged cells, indicated as blue cells to highlight the positivity to SA-βGal enzymatic assay. The persistence of these cells and the higher increase in SASP chemokines result in chronic fibrosis by the induction of endothelial-to-mesenchymal transition (EndMT) and pericyte-to-myofibroblast transition (PMT) that can amplify microvascular rarefaction together with generation of new myofibroblast and proliferation of resident fibroblast. Lastly, several mechanisms are involved in the maintenance of renal inflammaging such as the downregulation of Klotho, the increased Wnt signaling, mitochondrial dysfunction, the epigenetic changes, and the increased and stable expression of cell cycle inhibitors.

key role (96, 97). ABMR is characterized by glomerulitis, peritubular capillaritis, acute thrombotic microangiopathy tubular injury, C4d deposition in the peritubular capillaries, and microvascular inflammation (98, 99). As a consequence of these immunological attack to the graft, there is a significant increased incidence of late graft loss after ABMR (100–103). Even in presence of early acute rejection, several pathogenic mechanisms will progressively contribute to the later development of tubulo-interstitial fibrosis and progression to CKD (104, 105).

In the recipients, the presence of donor-specific antibodies (DSAs), i.e., natural IgM and IgG directed against donor endothelial human leukocyte antigen (HLA) or ABO antigens is referred as sensitization and represents the principal risk factor for ABMR. The immune complexes generated will activate CP by C1q-r-s complexes, therefore leading to covalent C4d deposition on peritubular capillaries. Consistently, for several years, the C4d deposition has been considered the gold standard for ABMR diagnosis; however, today, it is recognized that up to 55% of patients can develop ABMR without detectable capillary C4d

deposits. Indeed, a C4d-negative ABMR phenotype has been included in Banff 2013 classification (106–109).

Interestingly, all complement pathways are involved in ABMR (110, 111), leading to recruitment of leukocytes such as natural killer cell, monocyte/macrophage-mediated damage, endothelial injury, and increased intragraft coagulation (112, 113). Besides HLA matching and alloimmune response, other factors can influence the development of ABMR as donor and patient ages, cardiovascular complications, time on dialysis, glomerular disease recurrence, or more commonly hypertension, dyslipidemia, proteinuria, anemia, and diabetes. Interestingly, a significant complement activation has been observed also in these conditions. Bobka et al. demonstrated an increased complement activation in pretransplant biopsies from diabetic, hypertensive, or smoking donors (97). The authors showed a predictive value of complement activation in donor biopsies for later outcome; effective analyses of these deposits in the donor were characterized by C1q, factor D, C3c, and C5b-9 and tubular MASP2 and Collectin-11 in kidney that would have developed ABMR. Interestingly, at the diagnosis of ABMR, the expressions of these complement component were associated with higher serum creatinine and morphological changes.

Although is not clear whether the complement deposition occurred already in the donor or during the following IRI, a role of intragraft complement release has been hypothesized. We do know from the animal model of acute renal transplant rejection that early complement deposition can be associated by local synthesis of complement. By performing renal transplantation of a donor $C3^{-/-}$ kidney in a wild-type recipient mice (donor, $C3^{-/-}$; recipient: wild type), Pratt et al. demonstrated a significant increased long-term survival and less rejection incidence compared to wild-type mice recipients transplanted with allogenic wild-type kidney (Wtype/Wtype). Therefore, locally synthesized C3 is the most important trigger of rejection than circulating C3 and a powerful inducer of chronic damage (92). Other experimental evidence to support the role of complement in rejection were provided by Wang et al. (114). In a mouse model of ABMR induced after heart transplantation, Wang et al. showed that C5 blocking prevented ABMR and allowed long-term renal function. In conclusion, all these data support the use of complement inhibitors as therapeutic strategy to prevent the long-term complications of ABMR.

COMPLEMENT AND RENAL INFLAMMAGING: AN UNEXPLORED FIELD

Complement in Aging Diseases

Complement activation has been investigated in diseases of aging such as Alzheimer's and Parkinson's disease, amyotrophic lateral sclerosis, and multiple sclerosis or AMD (115). For instance, polymorphisms in factor H are known to increase several folds the risk of AMD, the most common cause of irreversible blindness. In addition, C3 gene expression is upregulated with aging in humans (116). Furthermore, C1q levels, which mediate synapse elimination in CNS, are dramatically increased in aged brains (117).

Interestingly, systemic protein C1q level increases with aging and can activate the Wnt/β-catenin signaling that is primarily involved in mammalian skeletal muscle aging (118, 119). The canonical Wnt signaling is activated by two kinds of receptors: the Frizzled family of serpentine proteins and the single-transmembrane protein low-density lipoprotein receptor-related protein 5/6 (LRP5/6) (120, 121) (**Figure 2**). Recently, Naito et al. demonstrated that C1q-r-s complex, after binding to Frizzled receptors, could induce the N-terminal cleavage of the ectodomain of LRP6, thereby activating Wnt pathway (119). In renal tubular epithelial cells, we found that C5a induced aberrant methylation changes in Wnt signaling related genes and in particular in Frizzled 6 (*FZD6*) receptor gene (22). This unexpected role of complement C1q in inducing an impaired regenerative capacity of skeletal muscle in aged animals has been further confirmed by several studies (122) showing that C1q secretion led to muscle fibrosis (122) and induced an increased proliferation of vascular smooth muscle cells via β-catenin signaling. From these observations (123), a role of C1q in the development of arteriosclerosis and arterial stiffening that occurs in advancing aging has been hypothesized. By the analysis of the circulating C1q and other cytokines associated with cardiovascular diseases (as TNF-α and IL-6), there emerged a significant correlation between C1q and aging-induced arterial stiffness. Regarding the role of LP in aging, evidence from Tomaiuolo et al. (124) showed that the specific MBL2 gene haplotypes (in particular, the high-activity-associated haplotypes as HYPA and LYQA) were significantly lower in centenarians than in the general population. The investigators identified also a role of MBL in the clearance of senescent cells. However, the mechanism underlying this peculiar connection between reduced MBL levels and longevity deserves more investigations.

In the healthy subjects, the correlation between complement and aging has its roots in earlier studies (125). In 1978, Yonemasu (126) demonstrated that, in a cohort of healthy volunteers (from birth up to 75 years), C1q and C3 levels independently oscillated with age. C1q increased gradually from birth to 60 years, whereas C3 reached higher level at 1 year, decreased until puberty, and augmented steadily after this age. Accordingly, in another cohort, Nagaki et al. detected an increased levels of CH50 activity, C1q, and C3 and decrease in factor B in older healthy subjects (127).

From these studies emerged a predominant role of C1q-CP in physiological aging. However, more recently, the findings from Gaya da Costa et al. provided strong evidence that also the AP was significantly activated in the elderly (76). In addition, authors also revealed increased terminal pathway components with age: these results are in line with the capability of complement to contribute to the clearance of senescent cells by MAC deposition (128).

The link between complement activation and physiological aging has been clarified in several experimental knockout models. Qiaoqiao Shi et al. (129) demonstrate that C3-deficient mice were protected from the synapse, neuron loss, and cognitive decline typically observed in older mice, suggesting an important role of C3 in the aging brain. Accordingly, in a model of AMD, $CD59a^{-/-}$ mice showed an age-dependent increased expression

of activators of the alternative complement pathway (C3, FB, FB) in the retinal pigment epithelium (RPE) choroid (130).

Furthermore, an age-related increase in complement C1q, C4, C3, and factor B expression was found in wild-type mouse brain (116).

All together, these studies demonstrate that aging is linked to a dysregulation of complement system, in particular of CP and AP, therefore to a progressive impairment of immune response.

Moreover, aging is associated to the establishment of a proinflammatory milieu generated by the hypersecretion of several cytokines [TNFα, IL-6, monocyte chemoattractant protein-1 (MCP-1), PAI-1] associated to higher risk for cardiovascular morbidity and mortality (131, 132).

More importantly, premature renal aging immediately after kidney transplantation could be modulated by soluble and circulating factors and, virtually, also by complement system. Liu et al. (133) showed that blood from young mouse was able to reduce IRI-induced AKI in older mouse (134). Using an experimental model of parabiosis, a surgical procedure that allowed a shared circulation between older and younger mice, Liu et al. demonstrated that a youthful systemic milieu was able to attenuate inflammation, oxidative stress, and apoptosis after renal IRI (133). These results are in line with previous findings demonstrating that bone marrow from young donor mice alleviated renal aging (135) and with recent data indicating that transplantation of young bone marrow can rejuvenate the hematopoietic system and preserved cognitive function in old recipient mice (136, 137).

Mechanisms of Renal Inflammaging

The term *renal senescence* reflects the complex interplay between genetics, immunological, and hormonal factors able to lead to structural and functional changes observed in aged kidneys (138).

During physiological aging that occurs in the elderly, a low-grade of systemic inflammation and the dysregulation of innate and acquired immune responses are normally observed. This systemic, chronic proinflammatory status has been defined for the first time by Claudio Franceschi as inflammaging, and the associated immunological impairment has been named immunosenescence. [all reviewed in more detail by Franceschi et al. (139)]. Inflammaging is a risk factor for multiple chronic diseases, such as CKD, cardiovascular diseases, cancer, depression, dementia, osteoporosis, sarcopenia, and anemia. Besides physiological aging, several mechanisms can induce inflammaging such as oxidative stress, mitochondrial dysfunction, complement activation, DNA damage, changes to microbiota composition, NLRP3 inflammasome activation, visceral obesity, and cellular senescence. In the kidney, inflammaging has been strongly connected to tubular senescence, characterized by cell cycle arrest and the acquirement of a SASP. The common features of renal aging have been observed in a wide range of kidney disorders as pretransplant cold storage preservation, IRI, ABMR, diabetic nephropathy, and IgA nephropathy (114). Histological features of kidney aging include glomerulosclerosis, interstitial fibrosis, glomerular basement membrane thickness, microvascular rarefaction, and tubular atrophy. Interestingly, similar changes are also observed in transplant injured kidney, suggesting that maladaptive repair after acute insults can be considered as the fuel for kidney inflammaging (140).

The SASP cell secretome involves the increased release of a large spectrum of proinflammatory [IL-6, IL-1α, IL-1β, IL-8, MCP-1, C–X–C motif chemokine ligand 1 (CXCL-1)], profibrotic [TGF-β, connective tissue growth factor (CTGF)] cytokines, growth factors (fibroblast growth factor 2 and hepatocyte growth factor), and matrix metalloproteinases (MMPs) (141). These factors acting on neighboring health cells and in the circulation exacerbate the progression of the inflammation, lately of the fibrosis and then progression to CKD (138, 142) (**Figure 2**). Healthy aging must rely on the ability to maintain a balanced immunological response between pro- and anti-inflammatory factors, allowing the inflammation resolution in a timely effective manner (143). In senescent cells, the persistent, chronic inflammaging is maintained by controlled downregulation or unchanged stable levels of anti-inflammatory cytokines as IL-10, IL-4, IL-2, IL-11, IL-12 or Fractalkine (CX3CL-1). For that reason, another well-described consequence of the SASP secretome is the tumor initiation and progression in cells residing in proximity of senescent cells (141). The list of molecular processes involved in premature kidney aging is complex; below, we will focus on the main processes that have been shown to link inflammaging with renal transplantation and complement system such as Klotho signaling, Wnt/β-catenin pathway, increased expression of cell cycle inhibitors, epigenetic changes, and mitochondrial dysfunction (144).

Klotho and the Aging Kidney

The Klotho protein, expressed predominantly in epithelial distal convolute (DCT) and proximal tubules, is an antisenescence factor. Although the transmembrane form of Klotho functions as a coreceptor for FGF23 signaling, the extracellular domain is cleaved and released into the blood, urine, and the cerebrospinal fluid acting as an endocrine factor on several distant organs, such as the heart (145).

Klotho gene is strongly involved in human aging and longevity. For instance, Klotho-deficient mice exhibit a shortened life span, skin and muscle atrophy, cognitive impairment, osteoporosis, and hearing loss, resembling an accelerated aging phenotype (146). In contrast, overexpression in Klotho gene in transgenic mice has been associated to increased life span (147, 148). In human, serum levels of Klotho decrease with age and are downregulated in several forms of AKI and chronic kidney injury (149–153). The principle function of Klotho, which acts in the FGF 23 signaling, is mainly implicated with calcium, phosphate, and Vitamin D metabolism, explaining the central involvement in aging-related-vascular calcification and osteoporosis (154).

A huge body of literature describes the reduced Klotho expression in the kidney, blood, and urine after IRI in mouse (155, 156), rat (155, 157, 158), and swine (21) models. Hu et al. (155) induced IRI in mice with different genetic background that led to various endogenous Klotho levels ranged from heterozygous Klotho haploinsufficient (with low/absent Klotho expression), to wild-type (WT, normal Klotho expression), to transgenic mice overexpressing Klotho. Compared with WT

mice, after I/R, Klotho levels were lower in haploinsufficient and higher in transgenic. In addition, the haploinsufficient mice had more deleterious functional and histological damage compared with WT mice, whereas these changes were milder in overexpressing transgenic mice. These results support the concept that reduced Klotho levels predispose the kidney to injury, accelerating renal fibrosis, and senescence, therefore promoting to transition from AKI to CKD (159).

In accordance, the restoring of Klotho level by exogeneous supplementation has been demonstrated to be renoprotective from fibrosis, senescence, and apoptosis (157). Although the Klotho expression was spontaneously restored with recovery in the WT [after 7 days from IRI (155)], preventing the early Klotho drop is crucial to avoid or to delay the AKI-to-CKD progression, together with cardiovascular complications (156). Different methods of Klotho supplementation have been evaluated, from exogenous administration of recombinant α-Klotho (155, 156) to forced expression by adenoviral vectors (157, 160), to minicircle vectors that allowed self-production of Klotho protein in the cells (161).

Other therapeutic strategies to reduce the Klotho loss with significant limitation of chronic damage could arise from complement inhibition. Our group recently demonstrated in a pig model of IRI significant downregulation of Klotho by 24 h from injury; importantly, Klotho was efficiently preserved after treatment with C1-INH, which efficiently modulated nuclear factor kappa B (NF-kB) signaling (21). Furthermore, the C5a anaphylatoxin led to a significant Klotho protein and gene expression decrease through a mechanism mediated by NF-kB (21). In addition, tubular cells exposed to C5a acquired a senescent phenotype as demonstrated by increased SA-βgal positivity, cell cycle arrest induced by increased p53, p21, and p16, and the acquirement of a SASP as detected by *IL-6, MCP-1*, *CTGF*, SERPINE 1 (*PAI-1*) gene expression. Interestingly, C5aR1 inhibition by monoclonal antibody protected the tubular cells from senescence (22).

Between all the cytokine involved in the SASP development, PAI-1 is also an essential mediator of cellular senescence (162) and could offer a target to counteract renal inflammaging. PAI-1 is expressed in senescent cells and tissue and is particularly highly increased in Klotho-deficient (kl/kl) mice. Furthermore, PAI-1 can be induced by C5a in human macrophages (163) and renal tubular cells (22). Using Klotho- and PAI-1 deficient mice (kl/kl$^{-/-}$pai-1$^{-/-}$) (164), it was demonstrated that PAI-1 deficiency in kl/kl$^{-/-}$ led to reduced senescence, preserved organ structure, and function with a fourfold increase in lifespan. Therefore, PAI-1 could be considered as a downstream effector of the IRI-induced Klotho loss; both the PAI-1 inhibition, by the development of selective PAI-1 antagonists (such as TM5441), together with the C5a blocking, could offer a new possibility to modulate the impairment in Klotho expression (165).

Wnt/β-Catenin Pathway in Renal Aging

Wnt/β-catenin signaling, a pathway involved in organ development, normally is kept silent in normal adult kidneys (166) but reactivated during aging (118), renal tubulointerstitial fibrosis (167), vascular calcification, and progression to CKD (121, 168, 169). Wnt signaling is antagonized by the protein Klotho that can bind to multiple Wnt ligands and inhibit the signal transduction mediated by Frizzled receptors (118, 170).

Recently, Luo et al. (171) identified a predominant role for component Wnt9 in promoting renal fibrosis by accelerating tubular senescence both in human and in experimental model of renal IRI and CKD (171). Interestingly, Wnt9a expression level correlated with the extent of tubular senescence and interstitial fibrosis and, functionally, with decline of estimated glomerular filtration rate (eGFR). The Wnt/β-catenin signaling constitutive activation has already been demonstrated to induce myofibroblast activation in the absence of other type of injury (172), with Wnt4 playing a pivotal role in chronic fibrosis (173). We have already discussed the capacity of C1q to activate Wnt signaling, leading to mammalian aging. Our *in vivo* studies confirmed that renal IRI activated Wnt4/β-catenin signaling, whereas the C1-INH treatment, blocking CP and LP, abrogated Wnt4/β-catenin activation preventing renal senescence and inflammaging (22). Lastly, in renal tubular cells, mitochondria are essential for energy production and are dysfunctional in AKI and CKD, leading to fibrosis and accelerated aging. Recent evidence indicated that Wnt/β-catenin signaling mediates age-related renal fibrosis and is associated with mitochondrial dysfunction (174) (**Figure 2**).

Cell Cycle Arrest and Renal Senescence

Tubular epithelial cells have a great regenerative potential after an ischemic or toxic injury (175) (176). Early after an episode of AKI, in damaged tubular cells, cell cycle is arrested by specific inhibitors in order to provide time for DNA repair, avoiding exaggerate progression to apoptosis. However, after IRI induced AKI, the prolonged injury can lead to a permanently arrested cell cycle maintained by a persistent increase in cell cycle inhibitors. Cell cycle arrest is a common marker of cellular senescence and is regulated by three major proteins belonging to cyclin-dependent kinase (CDK) inhibitors: p16^{ink4a}, p21$^{waf1/cip}$, and p53 (177) (**Figure 2**). p16^{ink4a}, encoded by the Ink4a/Arf locus, also known as CDKN2A, binds the kinases CDK4 and CDK6 that are necessary for cyclin D activation, therefore arresting cell cycle in G1 phase (178); the pivotal role of p16^{ink4a} in multiorgan aging has been revealed by Baker et al. (179). Interestingly, the elimination of naturally occurring p16^{ink4a}-positive cells during physiological aging attenuated glomerulosclerosis and tubular senescence, extending lifespan. In rodents models of renal I/R, several evidence have been provided for p16 ^{ink4a} involvement in long-term graft deterioration (180–182). In particular, Braun et al. (180) demonstrated that after IRI, p16ink4a-deficient mice showed less interstitial fibrosis and tubular atrophy. Furthermore, p16^{ink4a}(-/-) mice were associated with improved renal function, preservations of nephron mass, and transplant survival compared with wild-type controls. Consistently, mice that received kidney transplants from p16^{Ink4a} (-/-) donors had significantly better survival and developed a reduced amount of tubulointerstitial fibrosis (180). Similar results were obtained by other groups (182) even if some discrepancies exists in term of timing of p16 increased expression (181) or in correlation to the type of injury (183).

These results, describing the crucial role of p16 in mice model of aging, were confirmed in human kidney biopsies. In a seminal paper, Melk et al. (184) provided evidences that in normal human renal biopsies, nuclear p16INK4a staining was increased with aging. However, transplanted kidney with interstitial fibrosis and tubular atrophy or transplanted biopsies with chronic allograft dysfunction, exhibited a strongest nuclear and cytoplasmic staining, beyond the level expected from physiological aging. From this initial study, the hypothesis that the assessment of senescence by p16 measurement in time zero kidney biopsies could have a value for the prediction of chronic renal dysfunction in the recipient was investigated by other groups (185–187).

Another cell cycle inhibitor is $p21^{WAF1/Cip1}$, a protein that after binding to CDK2, can block the CDK2-cyclin E complex, therefore arresting cell cycle in G1/S checkpoint. Megyesi et al. (188) demonstrated the role of p21 in tubular interstitial fibrosis and CKD progression in proximal tubular cells. In large experimental models, using an *ex vivo* hemoperfusion of pig kidneys after I/R, cold preservation, and machine perfusion, Chktoua et al. (189) found an increased p16 and p21 expression at tubular level after 180 min of reperfusion. In contrast with these results, in our swine model of renal I/R, p16 increased expression was not detectable before 24 h from reperfusion, and interestingly, the p16 and p21 protein level appeared to be modulated by C1-INH treatment (22). In accordance with these findings, C5a stimulated renal proximal tubular cells and exhibited a higher increase in p21 protein after both short time (3 h) and longer time (24 h) of C5a exposure. However, p21 seemed to be downregulated after 24 h of C5a exposition, followed by 24 h of normal culture, indicating a potential recovery of tubular cells. These *in vitro* results are in line with findings that indicated that p21 could transiently increase after injury (190), describing that p21 is essential for the beneficial effects of renal ischemic preconditioning. Temporary cell cycle arrest induced by a p21-dependent pathway could be important for subsequent tubular cell proliferation after I/R (190, 191). To confirm the establishment of cellular senescence, we also assessed the p16INK4a protein level. Stimulation with C5a significantly induced a constant augment in protein expression of p16INK4a compared to untreated condition (22).

Complement and Epigenetic Changes in Aging

Epigenetic modifications are stable, heritable, and reversible genome changes that occur without the presence of alterations in the original DNA sequence (192). These modifications include DNA methylation, histone, phosphorylation, acetylation, methylation ubiquitylation, sumoylation, and miRNA pattern variations (193). There is an emerging evidence that epigenetics is crucial in healthy and accelerated renal aging (194). Not only physiological environmental factors (i.e., diet, exercise, education, and lifestyle factors) (195) but also acute inflammation, oxidative stress, or uremic toxins can contribute to susceptibility to CKD progression by epigenome changes (196, 197).

During transplantation, several stressors such as IRI, cold ischemia, and acute rejection can induce aberrant DNA methylation changes with serious implications for graft outcomes and acceleration of renal aging (198) (**Figure 2**). A great body of evidence recently provided the epigenomic, transcriptomic, and proteomic signature that characterize the biological older allografts (199–202) and the CKD methylation patterns (203, 204). Comparable results showing the importance of epigenetic modifications in AKI-to-CKD progression were obtained by rat and mice model of IRI, CKD, and premature renal aging.

Shasha Yin et al., in a mouse model of UUO, demonstrated that TGF-β can inhibit Klotho expression by epigenetic mechanisms leading to progression to renal fibrosis; TGF-β induces aberrant expression of DNMT1 and DNMT3a through inhibiting miR-152 and miR-30a, subsequently leading to Klotho promoter hypermethylation and Klotho protein suppression (205). In a rat model of IRI, Pratt et al. (206) found aberrant methylation in the C3 promoter gene in response to 24 h of cold ischemia and a subsequent 2 h of reperfusion, indicating an increased C3 release, therefore an amplification of local complement activation following the oxidative stress. However, these studies neither demonstrate a correlation between C3 aberrant methylation and increased gene expression (207) nor provided clinical translation data (208).

Recently, Denisenko et al. (209), in rat old kidneys, found an abnormal epigenetic pattern of extracellular matrix laminins that are involved in the development of glomerulosclerosis and tubulointerstitial fibrosis. *In vitro*, a predominant role for DNA methylation changes was identified by Bechtel et al. (210), who correlated the hypermethylation of *RASAL1*, a gene encoding an inhibitor of the RAS oncoprotein, with the fibrogenesis in the kidney. In our studies, we demonstrated that complement component C5a can induce a global tubular epithelial cell DNA hypomethylation (22), as observed in premature and accelerated renal aging (195, 211, 212). Furthermore, we found that C5a induced methylation modification-regulated genes involved in the prosenescence Wnt/β-catenin pathway and induced a SASP phenotype and cell cycle arrest (22) (**Figure 2**).

CELL-SPECIFIC EFFECTS OF COMPLEMENT IN AKI-TO-CKD TRANSITION

Renal Tubular Epithelial Cells and Complement

The impairment of tubular function is considered a critical step in many cases of AKI (213). During tubular injury, tubular cells dedifferentiate to replace the lost epithelial cells, but some of them fail in the recovery process and continue to produce factors that stimulate inflammation leading to fibrosis. This maladaptive response contributes to the development of CKD (214).

Activation of complement factors on tubular epithelium (215) is considered a key factor in tubulointerstitial inflammation and in the progression of renal dysfunction (216). Proteinuria is a common feature of kidney transplantation, and the

association between proteinuria, complement activation, and tubulointerstitial fibrosis is well established (217). Indeed, the proteinuric condition provides a source of complement proteins to renal tubuli with amplification of the cascade (20).

The increase in albumin, which is associated to a higher risk of adverse transplant outcomes (218), compromised the balance between complement activation and inhibition, reducing factor H binding at tubular level (219). Several data also showed that urinary pH or ammonia released from stressed epithelial cells directly activated C3 (20) (**Figure 3**).

Recent studies indicated a key role of properdin in complement activation and in progression of proteinuria-induced tubulointerstitial injury. Properdin binds the glycosaminoglycans of the apical surface of tubular epithelium and stabilizes the AP convertase, enhancing AP activation. Then, interfering with properdin binding to tubular cells may provide a therapeutic option for the treatment of renal disease and prevention of CKD progression (20) (**Figure 3**).

Complement-cleavage products, C5a and C3a, are important mediators of renal inflammation and injury (20). These mediators bind their receptors C3aR and C5aR expressed on renal tubular, endothelial, and innate immune cells. When tubular cells were exposed to C3a and/or C5a, they synthesized collagen I and acquired a mesenchymal profibrotic phenotype contributing to renal fibrosis (47). The effects of C5a and C3a on tubular cells were mediated by TGF-β synthesis that consequently promoted epithelial-mesenchymal transition (EMT) (220). In accordance, studies in rodent knockout showed that the absence of C3aR and C5aR on renal tubular epithelial cells or circulating leukocytes attenuated renal IRI. Treatment *in vivo* using antagonist for C3aR and C5aR and for factor B could improve graft survival, reducing the decrease in renal injury, tubular apoptosis, and inflammation (216, 220, 221).

In addition, there are evidence that complement might contribute to renal injury in diabetic nephropathy. Complement activation and subsequent deposition of MAC on tubular epithelial cells induced a significant production of proinflammatory cytokines, as IL-6 and TNF-α, ROS, and components of matrix that contributed to amplify renal injury and fibrosis process. In this setting, tubular cells increased the expression of histocompatibility antigens stimulating T-cell response and autoimmunity process (20).

Therefore, complement has to be considered one of the principal actor in the progression from AKI to CKD, and its modulation could prevent tubular dysfunction. In our previous studies, we demonstrated the pathogenic role of the complement cascade in a swine model of IRI (222). We showed the link between oxidative stress/NOX activity, complement activation, and EMT process at tubular level (223). We also demonstrated the ability of C1-INH to reduce tubular dysfunction with prevention of I/R-induced renal injury (82) (**Figure 3**).

Endothelial Cells and Complement System

Several studies highlighted the interactions between complement and the endothelium in pathogenesis of different renal diseases, including IRI, hemolytic uremic syndrome, and renal allograft injury (224). During inflammation, endothelium is continuously exposed to autologous complement (225) generated by the local or systemic activation of all complement pathways. Complement components such as C1q, C3a, C5a, and C5b-9 have direct effects on endothelial cells impairing their function. It is well known that C5b-9 not only induces cell lysis but also stimulates endothelial cells to acquire a prothrombotic cell surface. Furthermore, C5b-9 also contributes to platelet clumping as well as increased leukocyte adhesion and subsequent proinflammatory cytokine release (226) (**Figure 3**).

Accordingly, our group demonstrated that complement was primarily activated on peritubular and glomerular capillaries in a swine model of renal IRI, suggesting that endothelial cells are the primary target of injury (222). We also investigated an intriguing pathogenic process named EndMT in a swine model of renal IRI (82). EndMT has been shown to play a significant role in cardiac fibrosis, in arteriovenous fistula stenosis (227), and also in the recruitment of carcinoma-associated fibroblasts (228–230). In this model, a relevant portion of activated fibroblasts coexpress the endothelial marker CD31, indicating that these fibroblasts likely carry an endothelial imprint. This observation was also supported in renal diseases such as diabetic nephropathy (231–233) by colabeling the tissue with the endothelial marker CD31 and the fibroblast markers α-smooth muscle actin (α-SMA) and fibroblast-specific protein 1 (FSP1). In our study, we found that complement played a central role in this pathogenic process regulating fibrosis development within the graft (82). We also showed the effects of C1-INH in preventing C5b-9 deposition along peritubular capillaries, decreasing endothelial dysfunction and subsequent fibrosis (47, 222) (**Figure 3**).

A number of recent studies have shown that diseases of the vasculature and kidneys, including CKD, are associated with increased numbers of circulating endothelial microparticles and complement activation (224). EVs are actively shed from cells in response to injury. In particular, the microparticles found in the plasma of CKD patients presented increased levels of factor D that contributes to alternative pathway activation and systemic inflammation. Interfering with complement activation and microparticle release may be a potential therapeutic strategy to ameliorate kidney dysfunction in these patients (224).

In aHUS, complement-mediated injury is particularly active in renal glomerular capillaries and arterioles (234). Circulating complement fragments and local renal complement production lead to uncontrolled complement activation that induced platelet, leukocyte, and endothelial cell activation and systemic thrombotic microangiopathy with end organ damage or failure (235).

Pericytes Dysfunction Upon Complement Activation

The tubular interstitial fibrosis and glomerulosclerosis are considered the principal responsible for progression of renal disease. The principal source of interstitial fibrosis in

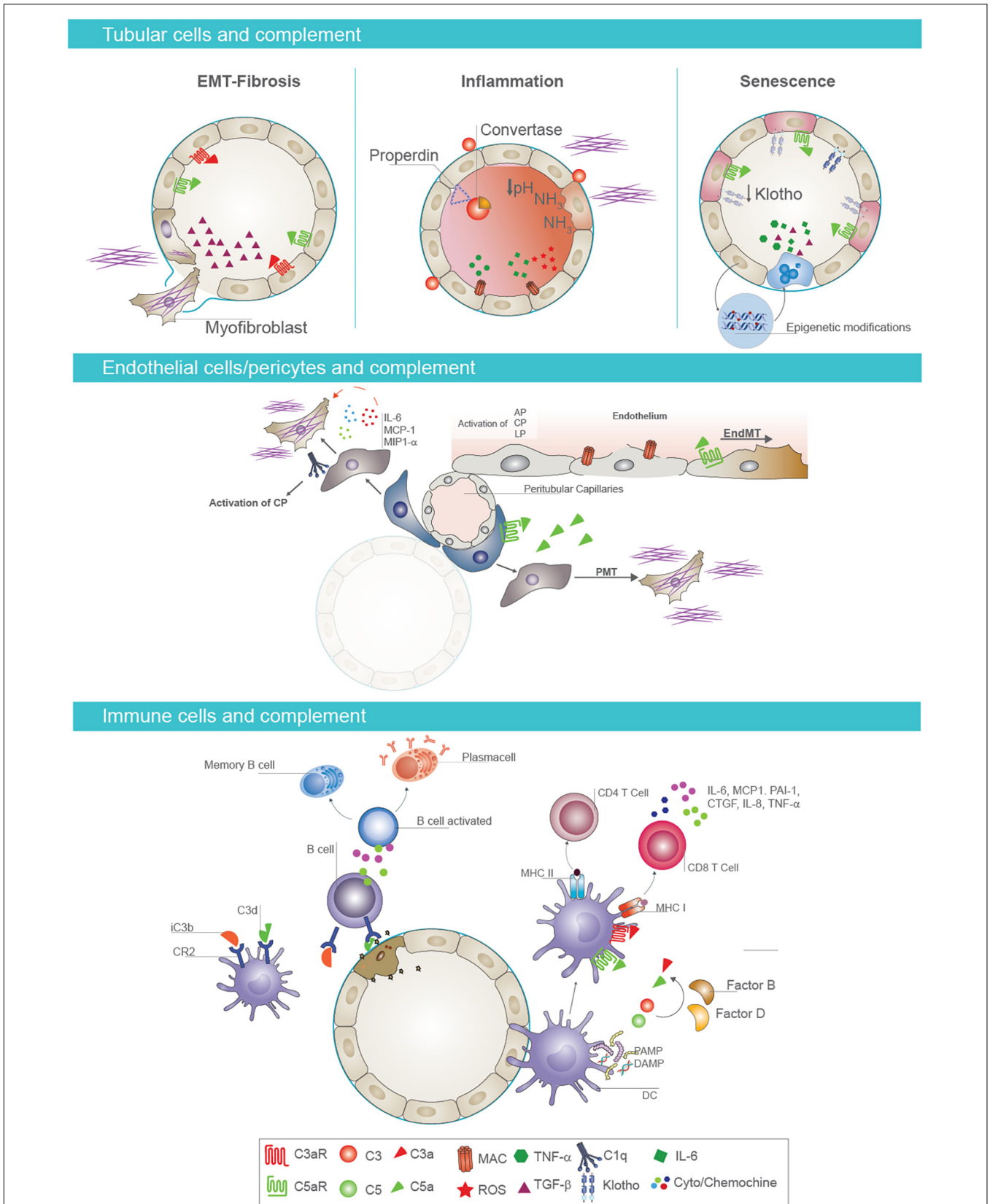

FIGURE 3 | Continued

FIGURE 3 | Cell-specific effects of complement in AKI-to-CKD transition. Tubular epithelial cells and complement activation (first panel). Activation of complement mediators on tubular epithelium is considered a key factor in renal fibrosis, inflammation, and senescence. Proximal tubular epithelial cells synthesize most components of the activation cascade as C4, C2, C3, factor B, and factor H. Reperfusion of the kidney following ischemia induces endothelial activation and release of nitrous oxide, leading to vasodilatation and leakage of complement components into the interstitial space In addition, complement proteins can be abnormally filtered across the altered glomerular barrier, leading to intratubular deposition of C3 and formation of membrane attack complex (MAC). When tubular cells are exposed to C3a and/or C5a, they synthesize transforming growth factor beta (TGF-β) that consequently promotes the EMT and fibrotic processes (on the left). Properdin, a key regulator of the complement system, enhances alternative pathway activation on the apical surface of tubular epithelium. Urinary pH and ammonia released from stressed tubular cells directly activate complement factor C3. This local complement activation and subsequent deposition of MAC on tubular cells induces a significant production of proinflammatory cytokines, contributing to renal inflammation (in the middle). Complement activation also induces a decrease in tubular expression of Klotho protein, an important antiaging factor. Complement promotes the acquirement of senescent tubular phenotype through epigenetic mechanisms, as DNA methylation (on the right). Endothelial cell/pericytes axis and complement activation (second panel). Complement also primes fibrotic process by inducing endothelial-to-mesenchymal transition (EndMT) and pericyte-to-mesenchymal transition (PMT) processes. In particular, C5a enhances EndMT process, causing phenotypic changes, with a decrease in endothelial markers and gain of fibroblast markers. In addition, pericytes, after C5a stimulation, acquire myofibroblast phenotype contributing to kidney fibrosis. Immune cells and complement (third panel). Complement components influence immune response in renal parenchyma. The binding of C3 fragments, iC3b and C3dg, to CR2 on B cells modulates B-cell response, increasing their activation and the development of memory B cells. Follicular DC also expressed CR2 and bind C3 fragments. After renal injury, PAMP and DAMP induce an increased expression of C3aR, C5aR1, and MHC class II on the surface of follicular DC and the synthesis and secretion of complement components C3 and C5 and factors B and D with local generation of C3a and C5a. These anaphylatoxins are strongly required for T-cell stimulation and activation in renal parenchyma.

kidney disease is represented by activated fibroblasts, named myofibroblasts (236). These cells derive from different precursors such as renal resident fibroblasts, endothelial cells, tubular cells, circulating bone-marrow-derived cells and pericytes (237). Recent studies highlighted the role of pericytes in the pathogenesis of renal fibrosis (238). Numerous secreted factors are involved in the generation and persistence of fibrotic process such as TGF-β, VEGF, CTGF, MMP, WNT ligands, and PDGF (31) (**Figure 3**).

Recent advances demonstrated that complement system not only contributed to local renal inflammation and adaptive immune response but also primed fibrotic process (239). Specifically, Xavier et al. demonstrated a local synthesis and secretion of C1q, C1r, and C1s by PDGFRβ-positive pericytes in two different animal model of CKD (31). Moreover, they showed that the C1q released by UUO-mice pericytes was associated to increased expression of extracellular matrix components, collagens, and augmented Wnt/β-catenin signaling, all common hallmark of myofibroblast activation. Finally, the C1q local synthesis amplified interstitial inflammation by the release of IL-6, MCP-1, and macrophage inflammatory protein 1-alpha (MIP1-α) that in turn contributed to fibrosis by macrophages recruitment (31, 240).

In addition to C1q, we recently demonstrated for the first time that also complement component C5a promoted the PMT, amplifying tubulo-interstitial fibrosis (48). *In vitro*, C5a-exposed pericytes downregulated the constitutive marker PDGFR-β and upregulated $aSMA^{+}$ stress fibers, the collagen I production, and the CTGF expression by TGF-β signaling. The C5aR blocking counteracted the PMT, reduced the C5a-induced collagen production, and more importantly inhibited the TGF-β pathway. In a swine model of I/R injury, we observed that C1-INH, acting upstream of C5 activation, indirectly reduced the release of C5a, preventing PMT process and ameliorating progressive kidney disease (48). Furthermore, also $C5aR1^{-/-}$ were spared from PMT in a mouse model of bilateral I/R.

These data indicate that pericytes are an important source of complement components at renal level and expressed receptors for complement anaphylatoxins. Therefore, pericytes are pivotal target for complement inhibition therapy to delay progression from AKI to CKD.

Immune Cells and Complement System

Next to direct effects on renal resident cells, complement components can influence the priming of alloantigen-specific immunity, modulating the interaction between dendritic cells (DCs) and T lymphocytes.

DCs are able to initiate an immune response by stimulating naive T cells, regulating the balance between Th1 and Th2 responses (241, 242). Moreover, complement components cooperate with DC to modulate T-cell response, and DCs themselves express complement factors, receptors, and regulators (243). Accordingly, we have demonstrated that C1q impaired DC activation leading to a limited T-cell response and preventing the overall immune response (244) (**Figure 3**).

Since the renal microenvironment has a strong influence on DC behavior, recent studies demonstrated the impact of local complement C3 on the differentiation and activation of DC. DC are considered the principal constituent of the tubulointerstitial compartment (245), and they produced C1q (41) and C3 in quantities similar to macrophages (246–248). Therefore, the contribution of C3 produced by these APC is strongly required for T-cell stimulation and activation in renal parenchyma. As a consequence, in C3-knockout organ, DCs have a reduced surface expression of major histocompatibility complex (MHC) class II and CD86, and they produce less IL-12 leading to a decrease in T-cell responsiveness (240, 249). Then, T-cell stimulation was reduced, and there was a shift for the generation of regulatory T cells (**Figure 3**).

This observation was confirmed *in vivo* in a skin allograft model. In this setting, infusion of mice with C3-knockout DC resulted in a less vigorous rejection of the skin allograft compared to mice infused with wild-type DC (248). Production of C3 has also been demonstrated for human monocyte-derived DC (240). The development of human monocyte-derived DC in either normal or C3-deficient

human serum resulted in a reduced expression of HLA-DR, CD1a, CD80, and CD86 in the absence of C3, leading to a reduced responsiveness upon lipopolysaccharide (LPS) activation (240).

Finally, other studies clearly demonstrated that generation of C3a and signaling through C3a receptors was a very important event at the interface between DC and T lymphocytes interaction and had a major role in immune activation (240, 249).

Moreover, it is well known that complement receptor type 2 (CR2, also known as CD21) is expressed on B cells and follicular DC, and it binds C3 fragments iC3b and C3d,g when associated to antigens (250). This binding modulates B-cell response, and the blockade of CR2 may be a potential strategy to reduce immune response in renal transplantation. Interestingly, the increase in C3 plasma levels also controls the development of memory B cells in kidney diseases (251).

Follicular DC can arise anywhere in the body, during chronic inflammatory reactions. Krautler et al. showed in a murine model of chronic inflammation that mature follicular DC localized in renal tissue and generated from tissue intrinsic precursors (252). Therefore, this finding proved that follicular DC may be ubiquitous and could regulate renal local immune response. These observations point toward an important role of complement activation at the immunological synapse and the contribution of complement regulators in this process.

Notably, C3aR and C5aR1 are extra- and intracellularly expressed in human CD4 + T cells and regulate the activation of mTOR pathway and NLRP3 inflammasome (109). In a mouse model of renal transplantation, the expression of these two receptors have been reported in regulatory T (Treg) cells, and they are shown to drive Th1 cells maturation and activation.

Moreover, PAMP or DAMP induced an increased expression of C3aR, C5aR1, and MHC class II on the surface of DC (109, 253), and the synthesis and secretion of complement components C3, C5, and factors B and D can locally generate C3a and C5a. Several data showed that both C3a and C5a stimulated CD4 + T cells to release interferon gamma (IFNγ) and IL-2 and induced TH1 and TH17 cell responses. Moreover, CD4 + T cells secreted IFNγ also upon C5aR1 activation (253). Therefore, therapeutic blockade of either C3aR or C5aR1 signaling could induce human tolerance to alloantigens and may prolong allogenic graft survival. Altogether, these data strongly suggest that the inhibition of complement acting on immune cells may represent a potential target for preventing rejection and progression of kidney diseases.

COMPLEMENT TARGETS STRATEGIES IN KIDNEY TRANSPLANTATION TO PREVENT AKI AND PROGRESSION TO CHRONIC DISFUNCTION

The involvement of complement in a broad range of disease processes renders this system an interesting and promising target for therapeutic interventions (254). Several clinical trials evaluating dozen of candidate drugs targeting specific complement's components are ongoing to date; most of them act as protein-protein interaction inhibitors, while others are physiological regulators or act on the genetic level, impairing the production of complement components (42). Eculizumab, the humanized monoclonal IgG2/4-antibody targeting C5, was the first complement drug available in the clinic, approved by the Food and Drug Administration (FDA) for the treatment of paroxysmal nocturnal hemoglobinuria (PNH) in 2007. PNH is a life-threatening disease characterized by an intravascular hemolytic anemia due to the destruction of red blood cells mediated by the complement system. In 2011, Eculizumab was also approved in the treatment of aHUS, where an uncontrolled activation of AP of the complement system (mutations in the complement regulatory proteins or acquired neutralizing autoantibodies against these regulatory factors) leads to a systemic thrombotic microangiopathy (255).

More than 10 years from its approval, the off-label usage of Eculizumab has been impressive, and several clinical trials are still assessing the potential indications, such as in kidney transplantation (42). As previously described, complement plays a major role in the IRI, such as DGF after kidney transplantation, and may be involved in the maladaptive repair leading to the progression to renal fibrosis and CKD (256). The role of Eculizumab in preventing and treating aHUS recurrence (255, 257) or *de novo* aHUS after kidney transplantation is well established to date (258). Recent evidence suggested its efficacy in the treatment of severe, progressive ABMR, or preventing ABMR in recipients with positive crossmatch against their living donors (rate of ABMR within 3 months after transplantation is 7.7% compared to 41.2% in patients receiving only plasma exchange) (259). However, the authors showed no differences between the treated and control groups in the incidence of chronic ABMR and death-censored graft survival, suggesting that the blockage of more proximal elements of the complement system may be pivotal in preventing the progression to chronic allograft disfunction (259).

Complement inhibition with Eculizumab to prevent IRI and DGF is still under investigation. In a single-center randomized controlled trial (RCT) of 57 children receiving a single dose of Eculizumab (700 mg/m^2) prior to transplantation, Eculizumab-treated patients had a significantly better early graft function, less arteriolar hyalinosis, and chronic glomerulopathy on protocol biopsies taken on day 30, 1 year, and 3 years after transplantation; however, an increased number of early graft losses due to flu-like infection has been documented (260).

Other complement-blocking agents have been used in kidney transplantation, and there are ongoing clinical trials evaluating the efficacy of recombinant C1-INH in preventing the development of IRI and DGF, as well as in the prevention and treatment of ABMR (258). C1-INH inhibits both the CP and LP of complement activation during IRI, reducing the release of renal microvesicles by inhibiting the

kallikrein-kinin system, and inhibits the coagulation pathway and, consequently, the formation of microthrombi in renal vessels (84, 254, 261). C1-INH is already licensed in many countries for the prevention and treatment of relapse of hereditary angioedema with important results and safety. However, its usage has been extended in other settings in order to prevent the acute development of organ disease and progression to chronic condition, particularly in the setting of kidney transplantation. Vo et al. conducted a phase I/II RCT evaluating the role of C1-INH in preventing ABMR in 20 highly HLA-sensitized recipients (262). After desensibilization, patients randomly received C1-INH 20 IU/kg or placebo intraoperatively and then another seven additional doses in the first month: DGF developed only in one patient in the treatment group, while four patients in the placebo group developed DGF (262). Moreover, no C1-INH-treated patients developed ABMR within 1 month; serum C4 levels recovered more quickly in the study group, and C3 and C4 levels were significantly higher, suggesting that C1-INH treatment may be effective in reducing antigen presentation and DSA production in this setting (262). The same investigators conducted a double-blind RCT where 70 high-risk and/or DCD donor kidney recipients were randomized to receive C1-INH 50 IU/kg intraoperatively and 24 h later versus placebo (263). The development of DGF (need of dialysis in the first post-transplantation week) was reduced in the study group but not statistically significant (42.9 versus 60% in the placebo group) (264); however, dialysis requirement and the mean number of dialysis sessions were reduced in C1-INH-treated group, particularly among recipients of grafts with KDPI. Furthermore, eGFR at 1 year was significantly higher in the treated group compared to the control group (p = 0.006), suggesting that C1-INH treatment safely reduces the need for dialysis and prevents progression to chronic graft disfunction (263).

The potential beneficial effect of C1-INH has been recently investigated in two recent studies. In a double-blind RCT performed in 18 DSA-positive recipients with an episode of biopsy-proven ABMR, randomized to placebo or C1-INH treatment in addition to alternate day plasmapheresis and IVIG, Montgomery et al. showed that there was no difference between groups with respect to the primary end points of 20-day graft survival or histological findings, but the C1-INH group showed a sustained improvement in renal function (264). Moreover, transplant glomerulopathy in 14 patients with available allograft biopsies at 6 months was significantly higher in the placebo group (3 of 7 patients) compared to none who received C1-INH (264). Viglietti et al. investigated the usage of C1-INH in six patients with acute ABMR and allograft disfunction that was refractory to standard therapy (steroids, plasmapheresis, high-dose IVIg, and rituximab). The authors showed a significant improvement in renal function (eGFR at 6 months) and, interestingly, a decrease in C1q binding anti-HLA DSA and the proportion of patients with C4d staining in peritubular capillaries in the treated patients: however, no differences in the typical histological findings of ABMR were described (265).

Overall, the main limitation in these studies using complement blockers is the small sample size; moreover, there is no direct competing trial with the use of eculizumab versus C1-INH; therefore, comparison of efficacy of different inhibitors of the complement system in clinical settings is not yet available. Several clinical trials evaluating C1-INH are currently ongoing and will guarantee a better understanding of the opportunities for the use of these agents in clinical transplantation.

In addition to eculizumab and C1-INH, other complement inhibitors have been studied in the transplantation setting, mostly in preclinical studies. These included engineered forms of complement receptor type 1 (CR1) (14), like TP-10 and Mirococept, and synthetic inhibitors of complement convertases (Compstatin) (266). TP-10 has been evaluated to reduce IRI in lung transplantation, showing reduced time of extubation, ventilatory days, and intensive care unit stay compared to patients in the placebo group (267). Furthermore, in a humanized mouse model of islet allograft, pretreatment with Mirococept reduced significantly intraislet inflammation, preserving insulin production by beta cells (268). The EMPIRIKAL trial is ongoing to evaluate the efficacy of an *ex vivo* administered complement inhibitor (Mirococept) in preventing DGF in cadaveric human renal transplantation (269). Pegcetacoplan (APL-2) is a pegylated Compstatin analog that acts as a cyclic peptide inhibitor of C3 and prevents both intravascular and extravascular hemolysis in patients with PHN. In a phase II clinical trial, APL-2 showed significant reduction in lactate dehydrogenas (LDH), total bilirubin, and absolute reticulocyte count with a sustained increase in hemoglobin (270). A phase III study (NCT03500549) comparing eculizumab and APL-2 in patients with PHN is ongoing. Finally, CCX168 (Avacopan), a selective C5a receptor inhibitor, has been investigated in preclinical and clinical studies in patients with ANCA-associated vasculitis. The important advantage of Avacopan is the preservation of the final common pathway of complement activation (MAC); thus, the innate immune response toward microbial agents remains fully active. Results from two phase II clinical trial CLEAR (NCT01363388) and CLASSIC (NCT0222155) showed that Avacopan is safe and effective in patients with ANCA-associated vasculitis allowing a safe reduction or suspension of corticosteroids (95, 271). Preliminary reports from the pivotal phase III ADVOCATE clinical trial (NCT02994927) showed the superiority of Avacopan in terms of sustained remission at 52 weeks and improvement in renal function in patients with ANCA-associated vasculitis in order to replace oral glucocorticoids (95). In this scenario, this therapeutic approach could represent an interesting alternative option also in other complement-based settings, such as IRI in transplantation.

The role of complement in heath and disease as an important component of the antimicrobial defense system raises questions about the safety and feasibility of complement inhibitors. The clinical experience with extended use of these drugs showed that they are considered safe and effective options with limited risk for complications (as infusion-related

effects, developing immunogenicity or severe infections): in this scenario, prophylactic measures (meningococcal vaccination before the use of eculizumab or vaccines for other bacteria such as pneumococci) and the prompt antibiotic treatment upon initial signs of infection may minimize the onset of severe adverse effects (272).

CONCLUSION AND FUTURE PROSPECTIVE

In summary, a growing body of experimental evidence indicates that complement activation contributes to the pathogenesis of renal inflammaging, particularly in the context of AKI-to-CKD transition. Complement components may regulate a wide range of molecular mechanisms both on infiltrating cells and renal parenchymal cells including scar-forming myofibroblasts, pericytes endothelial, and smooth muscle cells. We provided evidence supporting the pathogenic role of the complement system in promoting tubular epithelial cells senescence by genetic, epigenetic, and protein changes. Cellular senescence and the development of a SASP are involved in the progression from AKI to CKD, leading to common final signaling pathways involved in renal aging and fibrosis.

Currently, there are no validated therapeutic strategies to prevent renal inflammaging. However, promising results in clinical trials using new complement inhibitors suggest that interfering with this pivotal pathway of innate immune system may preserve the kidney from detrimental effect of AKI, reducing the progression of renal fibrosis and the accelerated renal aging.

AUTHOR CONTRIBUTIONS

RF and GC mainly contributed to the conception, the design and the writing of the manuscript. AS and MF contributed to writing of the parts relative to: cell-specific effects (for AS) and Complement therapeutics (for MF) and to literature bibliography search. GS and VC supported the final draft editing and revised the manuscript critically for final acceptance for publication. LG and GC supported and supervised the overall design of the article. RF and AS conceived of all figures. RF took the lead in writing the manuscript,reviewers revisions and figure changes. All authors gave final approval for the present version to be submitted.

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REVIEW
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Metabolic Flexibility and Innate Immunity in Renal Ischemia Reperfusion Injury: The Fine Balance Between Adaptive Repair and Tissue Degeneration

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Renal ischemia reperfusion injury (IRI), a common event after renal transplantation, causes acute kidney injury (AKI), increases the risk of delayed graft function (DGF), primes the donor kidney for rejection, and contributes to the long-term risk of graft loss. In the last decade, epidemiological studies have linked even mild episodes of AKI to chronic kidney disease (CKD) progression, and innate immunity seems to play a crucial role. The ischemic insult triggers an acute inflammatory reaction that is elicited by Pattern Recognition Receptors (PRRs), expressed on both infiltrating immune cells as well as tubular epithelial cells (TECs). Among the PRRs, Toll-like receptors (TLRs), their synergistic receptors, Nod-like receptors (NLRs), and the inflammasomes, play a pivotal role in shaping inflammation and TEC repair, in response to renal IRI. These receptors represent promising targets to modulate the extent of inflammation, but also function as gatekeepers of tissue repair, protecting against AKI-to-CKD progression. Despite the important considerations on timely use of therapeutics, in the context of IRI, treatment options are limited by a lack of understanding of the intra- and intercellular mechanisms associated with the activation of innate immune receptors and their impact on adaptive tubular repair. Accumulating evidence suggests that TEC-associated innate immunity shapes the tubular response to stress through the regulation of immunometabolism. Engagement of innate immune receptors provides TECs with the metabolic flexibility necessary for their plasticity during injury and repair. This could significantly affect pathogenic processes within TECs, such as cell death, mitochondrial damage, senescence, and pro-fibrotic cytokine secretion, well-known to exacerbate inflammation and fibrosis. This article provides an overview of the past 5 years of research on the role of innate immunity in experimental and human IRI, with a focus on the cascade of events activated by hypoxic damage in TECs: from programmed cell death (PCD) and

mitochondrial dysfunction-mediated metabolic rewiring of TECs to maladaptive repair and progression to fibrosis. Finally, we will discuss the important crosstalk between metabolism and innate immunity observed in TECs and their therapeutic potential in both experimental and clinical research.

Keywords: kidney transplantation, tubular repair, innate immunity, cell death, mitochondria, senescence

INTRODUCTION

Kidney diseases are a growing health problem, considered to have a direct and indirect impact on morbidity and mortality worldwide, by increasing the risks associated with the major killers, such as cardiovascular diseases, diabetes, hypertension, and infection (1). The timely identification and management of kidney diseases represent the most effective strategy to sustainably address the growing global burden and prevent the progression to end-stage renal disease (ESRD).

In 2010, 2.62 million people received dialysis worldwide, and the need for dialysis was projected to double by 2030. Despite being a life-saving treatment, dialysis is merely a supportive measure. Indeed, the life expectancy and the quality of life in dialysis patients is much lower compared to the general population (2). Basic research studies have identified many targets to delay the progression of kidney disease; however, only few of these promising results can be recapitulated in clinical studies, and yet do not represent a great alternative to dialysis or transplantation.

The life-sustaining job of the kidneys comprises filtering and reabsorbing about 180 liters of fluid from the bloodstream every 24 h. As a consequence, it is the organ with the highest metabolic rate as determined by tubular epithelial cell (TEC) metabolism (3). TECs, the most abundant cell type in the kidney, are densely packed with mitochondria. These cells combust fatty acids to generate adenosine triphosphate (ATP) through oxidative phosphorylation (4). Proper mitochondrial function and metabolism of these cells is crucial for the high transport and reabsorption activities. Given the high metabolic demand, the kidneys are sensitive to decreased blood oxygenation and perfusion. Whenever the kidneys experience extremely low oxygen exposure, or metabolic substrates become inadequate, acute kidney injury (AKI) occurs.

AKI is defined as an abrupt reduction in kidney function that results in disturbances in the milieu intérieur and the retention of uremic toxins that especially influence the cardiovascular, immune, and nervous system. With an incidence of 9–15% of hospital admissions and up to 40% in critically ill patients, AKI is a major cause of morbidity, mortality (40–70% for the critically ill needing dialysis), increased health-care costs, and chronic kidney disease (CKD) (5, 6). Although research has made great efforts to understand the pathogenesis of AKI, the only available therapy is still supportive (dialysis) and has not changed for decades. Additionally, AKI patients have a greater risk to develop chronic complications later in life (7). A systematic review and meta-analysis comprising estimates from more than 2,000,000 individuals identified AKI as a risk factor for new or progressive CKD (hazard ratio [HR] = 2.67), ESRD (HR = 4.81), and mortality (HR = 1.80) (7). These important clinical findings have resulted in a shift toward studies that investigate the link between experimental AKI and progressive kidney fibrosis and failure. Pattern recognition receptors (PRRs), the sensors of the innate immune system, are one of the major players determining short-term outcomes after experimental AKI, a topic of many good reviews (8, 9). In this review, we will discuss recent data showing that danger signals and PRRs are involved in cell fate decisions, metabolism, and mitochondrial function in TECs, thereby determining not only whether a pro- or anti-inflammatory phenotype will emerge, but also the success of regeneration and repair. We specifically intend to highlight the link between metabolism and innate immunity in TECs and the altered cell phenotype that occurs during experimental and human ischemia reperfusion injury (IRI)-induced AKI.

AKI AND EXPERIMENTAL ISCHEMIA-REPERFUSION INJURY

The pathophysiology of AKI is complex. One of the major causes of AKI is IRI (10, 11). IRI is an inevitable event during renal transplantation and is responsible for delayed graft function (DGF), resulting in loss of vital kidney parenchyma and priming of adaptive immune responses that initiate rejection, which altogether lead to graft loss (10, 12). DGF is a clinical syndrome defined as the need for renal replacement therapy in the early phase after transplantation in order to support the function of the newly acquired renal transplant (12). Besides renal transplantation, IRI can also develop in the context of other diseases with low perfusion and/or oxygenation states, including thrombotic diseases, sepsis, trauma, and cardiac surgery (13). Experimental IRI in the mouse is the most widely used preclinical model to mimic human AKI. Despite having many limitations, particularly regarding the immune response (14) it is still the most valuable tool for understanding AKI pathophysiology.

The Early Phase of Experimental IRI Is Characterized by Programmed Cell Death That Activates a Pro-inflammatory Innate Immune Response

In experimental IRI, following the hypoxic event, TECs, especially the proximal TECs located in the S3 segment of the nephron at the cortico-medullary area, are unable to maintain adequate intracellular ATP levels for the essential processes mentioned earlier. Additionally, restoration of blood

perfusion, followed by re-oxygenation of the kidney, provokes the production of reactive oxygen species (ROS), eliciting mitochondrial dysfunction. This, together with the ATP-depletion, leads to cell activation and injury, and if severe enough, can lead to programmed cell death (PCD) and secondary necrosis, the hallmark of early IRI (15).

PCD is essential, not only for the maintenance of cellular homeostasis but also in response to irreparable damage caused by injury or disease (15–17). The pathways of cell death execution can present as a diverse morphological pallet in the spectrum from apoptotic to necrotic, with a corresponding degree of danger-associated molecular pattern (DAMP) release and inflammatory potential (18–20). DAMPs are a heterogeneous group of ligands, which are constitutively expressed in different biological compartments of cells hidden from the innate immune system. DAMPs are recognized by a broad spectrum of PRRs, in order to elicit an innate inflammatory response (19).

It has been observed that the severity of the injury determines the path by which cell death is realized, indicating a possible role for PRRs in guiding cell fate decisions by integrating signals from the microenvironment (21). Various currently recognized forms of PCD (18, 22), including necroptosis, pyroptosis, ferroptosis, mitochondrial permeabilization transition (MPT)-mediated regulated necrosis, and parthanatos are initiated as a response to hypoxic injury, either directly or indirectly, and blocking their crucial pathways during the early phase of IRI generally leads to reduced necrotic (tubular) damage, reduced inflammation, preservation of renal function, and reduced mortality (15). PRRs are known inducers of PCD, particularly necroptosis, pyroptosis, and apoptosis, which is why these modalities are discussed in more detail below.

Pattern Recognition Receptors Are Involved in Cell Death Signaling During IRI

In necroptosis, receptor-interacting protein (RIP) kinase-1 and 3 interaction via their RIP–homotypic interacting motif (RHIM) (23) leads to phosphorylation and activation of RIPK3. In turn, RIPK3 catalyzes the phosphorylation and oligomerization of mixed lineage kinase domain–like (MLKL) (24), thereby inducing a molecular switch that leads to plasma membrane rupture and cell death (23–26). Blocking components of the necroptosis pathway in a similar renal phenotype of reduced tubular necrosis, reduced inflammation, better preservation of renal function, and reduced mortality in the first period after reperfusion (27). A recent report by Chen and colleagues showed, in a chimeric bone marrow transplantation model, that in the early phase of IRI, RIPK3 and MLKL in kidney parenchymal cells (including proximal TECs, as shown by *in vitro* studies) are important for initiation of the vicious inflammatory circle, but that pyroptosis in macrophages is more important in the later stage after reperfusion, suggesting temporal variation in cell death modalities during the course of IRI (28). Pyroptosis is a necrotic form of cell death most often observed in immune cells, such as macrophages and dendritic cells (DCs) (18). During pyroptosis, the presence of DAMPs initiates inflammasome formation, which activates both caspase-1 and caspase-11 (29–31). An effector function of these caspases is to process the inactive precursors of IL-18 and IL-1beta, leading to an intracellular accumulation of pro-inflammatory cytokines (31). These caspases also induce plasma membrane rupture, and essentially cell death, through the cleavage of gasdermin D (GSDMD) (32). The inevitable release of IL-18 and IL-1beta makes this form of cell death highly inflammatory (33). There is some debate as to whether pyroptosis occurs in renal cells as well, however, Yang et al. suggest the occurrence of pyroptosis in TECs based on a significant increase in pyroptosis-related proteins following IRI (34). A recent report by Miao et al. suggests the direct involvement of pyroptosis in IRI and cisplatin toxicity based on *Casp11* KO mice (35). In additional experiments they showed that in *Gsdmd* KO mice, renal tubular damage was less severe, and urinary IL-18 levels were reduced upon cisplatin toxicity (35). Although very suggestive, we do not know whether *Gsdmd* KO mice have the same phenotype in IRI compared to cisplatin toxicitiy *in vivo*. Apoptosis is considered a more quiescent form of regulated cell death due to the swift clearance of apoptotic bodies by phagocytes. Apoptosis is initiated through an intrinsic route via mitochondrial outer membrane permeabilization (MOMP) or an extrinsic route via death or dependence receptors (36, 37). Death receptors can initiate apoptosis via cognate ligand binding (e.g., FAS or TNFR1 signaling) whereas dependence receptors initiate apoptosis when there is a lack of ligand binding (i.e., reduced homeostatic survival signaling via e.g., the netrin 1 receptors) (38). After initiation, executioner caspases-3 and−7 are responsible for neat cellular and nuclear fragmentation during apoptosis, releasing "find-me" signals, and flagging apoptotic bodies to be phagocytosed via "eat-me" signals (39). Apoptosis is believed to play a minor role in the early pro-inflammatory phase after reperfusion. Multiple reports have shown that pharmacologically inhibited apoptosis by a pan-caspase inhibitor zVAD or genetic KO of executioner caspase-3 did not reduce but rather exaggerated renal tubular damage and failure (40, 41). In the long-term, *Casp3* KO mice appeared to have less peritubular capillary rarefaction, less activated interstitial fibroblasts, less interstitial fibrosis, and evidence of less tubular hypoxia after reperfusion, suggesting a potentially interesting link between late peritubular capillary apoptosis and endothelial-mesenchymal transition and/or pericyte-fibroblast transdifferentiation (41). PRRs can initiate regulated cell death in multiple ways. Toll-like receptor (TLR) signaling via MYD88 results in activation of NFkB, transcriptionally regulating multiple cytokines that can subsequently induce regulated cell death via para- and autocrine signaling to death receptors. However, a more direct route of cell death initiation by TLRs is via Toll/IL-1R domain-containing adaptor-inducing interferon (IFN)-beta (TRIF). TRIF can initiate apoptosis via FADD- and caspase-8-dependent pathways. TRIF also contains a RHIM domain, and could therefore function as a docking site for the RIPK3-MLKL complex during necroptosis initiation (42), as was shown for TLR3 (43). TLR-TRIF-induced active caspase-8 was able to cleave Gasdermin D in macrophages inducing pyroptosis (44), suggesting the bypassing of the inflammasome in these cells.

(MAL)ADAPTIVE REPAIR RESPONSES AS A MODEL FOR AKI-TO-CKD PROGRESSION

Tubular regeneration and successful renal repair after an episode of AKI can be observed in the majority of surviving patients, especially in cases of mild injury (45–47). Adaptive tubular repair depends on the presence of an appropriate microenvironment, in which inflammation and tubular response to damage are balanced. In the adaptive repair, surviving TECs undergo dedifferentiation and proliferation in order to restore a functional epithelium. However, in case of severe or repetitive injuries or aged kidneys, maladaptive repair of proximal tubules can occur, which can contribute to progressive renal fibrosis (47). Maladaptive repair of kidney tissue after AKI is characterized by rarefaction of peritubular capillaries, interstitial fibrosis and tubular atrophy, glomerulosclerosis, and vascular remodeling, which interfere with repair and eventually lead to a decline in renal function. Therefore, AKI-to-CKD should be regarded as accelerated renal aging (47, 48). As the determinants of renal aging and CKD overlap (49), identifying patients with premature renal aging could be a strategy to identify AKI survivors at risk for CKD.

Among the culprits in the AKI-to-CKD progression, is the persistence of a senescent state in TECs (45). Senescence describes a proliferative arrest with changes in chromatin organization, gene transcription, and protein secretion, which can occur as a response to cell stress and aging (50). Given the high degree of pro-inflammatory molecules released by senescent TECs, it remains elusive whether progressive accumulation of senescent TECs is causally related to an aberrant innate immune response. Recent results from our group and others (51–53) point toward a role for TEC-mediated inflammation, innate immunity, and mitochondrial metabolism in senescence and fibrosis. Given that these mechanisms fall into the new discipline of immunometabolism, and TECs can be regarded as part of the renal innate immune system, further studies are required to characterize the role of immunometabolism in AKI-to-CKD progression.

Innate Immune Receptors as Gatekeeper of Damage and Repair

The role of the innate immune system was originally to combat infections. However, we now know that its role extends beyond that to include the surveillance of tissue homeostasis, by detecting distinct DAMPs released during tissue injury. Our group pioneered the discovery of TEC-associated innate immune sensors having a crucial role in the initiation of the injury response during IRI, shedding a novel light on the role of TECs as innate immune cells of the kidney (54). Experimental data suggests that the DAMPs released by necrotic cells activate the inflammatory signal initiated through TLRs, and their synergistic receptors, the Nod-like receptors (NLRs) and the NLRP3 inflammasome (55, 56). Initial considerations suggested that pharmaceutical intervention to block TEC-induced innate immune cell signaling could lead to novel therapeutics against renal tissue inflammation and injury after IRI (54, 57). However, in the last decade, seminal studies on the role of innate immune sensors and their ligands in renal IRI have provided an additional prospective: the innate immune sensors translate kidney injury into an immune response, essential for shaping adaptive tubular repair and kidney regeneration. Thus, the tubular innate immune response to IRI seems to be a very well-orchestrated phenomenon (58). After the early inflammatory phase, macrophage populations assume a reparative phenotype that is characterized by the production of numerous growth factors, including Platelet-derived growth factor (PDGF), Transforming growth factor beta 1 (TGF-beta1), Insulin-like Growth Factor I (IGF-1), and Vascular endothelial growth factor A (VEGF-A) that promote tubular regeneration (59–61). Long-term sustained inflammation is detrimental, but the absence of the inflammatory response can also predispose to the development and progression of CKD (8). Whether this is dependent on a faulty inflammatory response or the inability of TECs to regenerate due to extensive damage is still not completely understood. Improving our understanding of the role of innate immune receptors, not only in the early pro-inflammatory phase during IRI, but also in (mal)adaptive tubular repair, is therefore crucial for the development of specific therapies or prevention of AKI and its detrimental sequelae.

Mitochondrial Dysfunction and Metabolic Reprogramming

Mitochondrial injury, fragmentation, and ROS generation induce aberrant tubular inflammation and are central mediators of AKI, as well as the AKI-to-CKD progression (62–66). Oxygen tension in TECs is crucial for proper mitochondrial function. When this becomes inadequate, mitochondrial respiration is inhibited and the kidney undergoes a metabolic rewiring toward glycolysis, thereby decreasing ATP production (67). Mitochondrial number and integrity (through fission and fusion processes) (65, 66) are associated with accelerated tubular repair and improved survival after IRI, suggesting that in order to achieve adaptive tubular repair, restoration of mitochondrial homeostasis is an indispensable event (64). Thus, metabolic flexibility seems to be a crucial ability of the tubular epithelium to quickly adapt to the hypoxic environment. However, in order to promote repair, TECs should be able to return to their primary fuel source, and when this flexibility is impaired and metabolic rewiring persists during repair, this leads to a failed re-differentiation and mesenchymal arrest (68).

Hypoxia is likely not the only cause of reduced oxygen availability in the kidney. The widening of interstitial spaces by edema and inflammation and the regression of capillaries during fibrosis could also be involved. Indeed, pathologic hypoxia persists as fibrosis develops and could thus prevent epithelial recovery through feedback effects, ultimately leading to tubular atrophy (69). Consequent to hypoxia, tubular repair after AKI could be impaired by oxidative stress and growth arrest or senescence, which represents the known adverse effects of hypoxia (51, 52, 70, 71). TECs rely mostly on fatty acid oxidation (FAO) for their functions (72), which requires mitochondria and

oxygen for efficient ATP generation. All the enzymes required for FAO reside in the mitochondrial matrix, and proper functioning of the mitochondrial cristae is necessary to provide substrates for the respiratory complex. Recently, several groups have reported that mitochondrial function and energy metabolism are involved in the progression from AKI-to-CKD.

Within mitochondria, the coenzyme Nicotinamide adenine dinucleotide (NAD^+) carries high-energy electrons from FAO to the electron transport chain. NAD^+ is, therefore, a rate-limiting catalyst for FAO (67). In other terms, decreased NAD^+ availability results in impaired energy metabolism in these cells (73). Hypoxia and aging (74, 75) have been known to induce NAD^+-consuming enzymes, which lowers NAD^+ availability. Tran and colleagues recently showed that renal tubular cell NAD^+ levels are suppressed in IRI-induced AKI, and this reduction in NAD^+ may impede FAO, reduce ATP generation, and elevate susceptibility to AKI stressors (76). They identified a novel function of the mitochondrial biogenesis regulator PPAR-gamma-coactivator-1alpha (PGC1-alpha) to induce the enzymes that sequentially convert the amino acid tryptophan to NAD^+, after IRI. Tubular PGC1-alpha expression protects against hypoxia-related stress and enhancing NAD^+ could effectively mimic PGC1-alpha's effects in the tubule (76). Interestingly, biopsies of human AKI showed reduced PGC1-alpha expression (77).

The same group, through a metabolomics study, has demonstrated the elevation of urinary quinolinic acid (uQuin) in murine AKI. Quin becomes NAD^+ through the action of quinolinate phosphoribosyltransferase (QPRT) and subsequent enzymes. The elevation of uQuin suggested suppression of QPRT during AKI. Genetic targeting of *Qprt* showed enhanced susceptibility to IRI and recapitulated the majority of the urinary metabolic changes measured in experimental AKI (78). Additionally, few other studies described that either *de novo* NAD^+ biosynthetic pathway activation (79) or replenishment by means of NAD^+ precursors (80) are able to protect the kidney from ischemic damage. This is of great importance given that in mammals, only the kidney and the liver exhibit appreciable *de novo* NAD^+ biosynthesis (79, 81). Taken together, these encouraging studies points toward a therapeutic potential of NAD^+ enhancers in AKI and its long-term sequelae.

Apart from PGC1-alpha, the AMP-activated protein kinase (AMPK) is a promising component of a signaling cascade that may modulate the severity of ischemic injury (82). AMPK is a ubiquitously expressed serine-threonine kinase that serves as an important intracellular energy sensor. It is activated by conditions that deplete ATP and alter the AMP:ATP ratio, including ischemia and glucose deprivation. AMPK stimulates FAO, glucose uptake, and glycolysis, while downregulating ATP-utilizing systems. Other targets of AMPK include pathways modulating inflammation, apoptosis, angiogenesis, blood flow, and maintenance of cell polarity, with or without energetic stress (83). Metformin, a widely used drug for the treatment of type II diabetes mellitus, enhances AMPK activity (84). AMPK pre-activation partially ameliorates renal IRI *in vivo* but also long-term sequelae after IRI (84).

Defective FAO in TECs plays a pivotal role in renal aging (85) and fibrosis (72), but studies directly linking defective FAO to renal IRI are currently missing. The Susztak group showed that the dramatic repression of FAO is induced by TGF-beta signaling (72), which seems to play a role in the early events of renal IRI, therefore, it would be interesting to investigate whether failure to fully return to FAO fuel after IRI might be underlying the AKI-to-CKD transition, or whether defective FAO is associated with a senescent tubular phenotype. Although fatty acid accumulation has been shown to be associated with lipid deposition, this is not *per se* enough to drive fibrosis.

Interestingly, TGF-beta1 stimulates the Warburg-like metabolic reprogramming in kidney cells, which is relevant because it mirrors the metabolic state during AKI (72). Metabolic reprogramming, toward glycolysis, rapidly generates ATP and involves the enzyme Pyruvate Kinase M2 (PKM2), which is involved in the last step of glycolysis (86). Zhou and colleagues found that disabling PKM2 resulted in a significant increase in cell-repair and a concomitant decrease in energy generation, leading to significant protection against kidney injury in mice (87). A key molecule in this process is nitric oxide (NO), which can be transported to different proteins through Co-enzyme A, thereby, switching off their activity. PKM2 is one such protein. Indeed, adding NO to PKM2 activates repair, suggesting it as an important mechanism that can be used to determine whether kidney cells are using their pathways for energy or repair. The same team found that a protein called AKR1A1 could remove NO from PKM2, thereby switching it on and re-activating a robust energy-generating process. Disabling AKR1A1 protected the kidney from disease by stimulating repair (87).

It has been well-established that mitochondrial dysfunction is a principal mediator of AKI through decreased ATP production, oxidative stress, mitochondrial DNA (mtDNA) release, and cell death. Mitochondria also play a crucial role in maintaining organelle function in cells. Organelle stress and crosstalk in the AKI-to-CKD transition has been recently reviewed (64). The ROS generated upon re-oxygenation and the inability to maintain endogenous antioxidant levels, results in mitochondrial oxidative stress and promotes AKI (88). Enhancement of the antioxidant defense via mitochondria-targeted approaches has been successful in ameliorating IRI-induced AKI. These have been summarized in **Table 1**. Additionally, a visual representation of the phenotypic changes occurring in TECs after IRI and the majority of the mechanisms described in this chapter can be found in **Figures 1**, **2**.

Senescence-Mediated (Mal)adaptive Repair

Kidney regeneration after IRI is accomplished by active tissue repair, in which the role of macrophage phenotype is crucial, as described in recent work (96). A balanced inflammatory response, together with a moderate level of damage, establishes an optimal microenvironment in which surviving TECs fully repair (3, 97). In this scenario of adaptive repair, the kidney regenerates over the course of a few days without scarring. The progression

TABLE 1 | Mitochondria-targeted approaches in renal IRI.

Experimental model	Species	Compound/approach	Function	Findings
Unilateral IR, 30 min clamping	Rat	**Fasting** for 3 days before IRI	Fasting promotes mitochondrial biogenesis through the AMPK pathway	Decrease kidney damage, oxidative stress, and ameliorates mitochondrial function and has long-term beneficial effects (89).
Bilateral IR, 45 min clamping	mouse	**MitoQ** 4 mg/kg injected into the tail vein 15 min before ischemia	Mitochondria- targeted antioxidants	Decreases oxidative damage and improves renal function (90).
Unilateral IR, 40 min clamping	Rat	**SkQR1** 100 nmol/kg injected ip 3 h before IR, 1 h after IR, and subsequent injections at 13, 25, and 37 h; for a total of 500 nmol/kg.	Mitochondria- targeted antioxidants	Decreases oxidative damage and improves renal function (91).
Bilateral IR, 30–45 min clamping	rat	**SS-31** (0.5, 2.0, or 5.0 mg/kg) injected 30 min before IR, at the onset of reperfusion and 2 h later.	Mitochondria-targeted antioxidants	Decreases mitochondrial swelling, improves mitochondrial pathology and protect cristae membranes. Protects rats from kidney dysfunction, inflammation, and fibrosis (92, 93).
Unilateral IR, 15 min clamping	rat	Bolus injection of **glycine** before induction of renal IR but also a diet containing 5% glycine postoperatively for 2 weeks.	Amino acid with an important role in metabolic regulation and anti-oxidative reactions.	Improved renal function, decreased tubular injury, and oxidative stress. Prevents initial damage and chronic hypoxia (94).
Unilateral IR, 60 min clamping	Rat	**Resveratrol** (0.23 μg/kg body weight) via intragastrical administration (1.5 mL) 30 min before IR	Antioxidant	Decreased renal dysfunction, oxidative stress and inflammation. Ameliorated renal histologic damage (95).

of AKI-to-CKD is the result of maladaptive tubular repair, which can be mediated by TEC senescence.

The hypoxic injury activates a DNA damage response (DDR) in TECs [Ataxia-Telangiectasia, Mutated(ATM)/ATM and Rad3-related(ATR) pathway] which results in cell cycle arrest (71, 98–100). This allows TECs to repair the damage and avoid further amplification of the vicious cycle of injury-induced cell death. ATR, an enzyme involved in DDR activation has been shown to protect against maladaptive tubular repair (100), further demonstrating a crucial role for DDR activation in TEC repair.

One of the consequences of irreparable DNA damage and mitochondrial dysfunction, especially in cells with a high energy demand, is a proliferation arrest termed "senescence" (101). Senescence is generally regarded as an irreversible event (102); however, there are reports that describe senescent cells being able to re-enter the cell cycle, suggesting that more research is needed to understand which scenario determines the fate of these cells (103). Senescent TECs, identified by different markers, with Senescence-associated beta-galactosidase (SA-β-gal) and the anti-proliferative proteins p16 and p21 being the most common, seem to accumulate acutely following experimental and human IRI (53, 104), as a consequence of oxidative stress, the so-called stress-induced senescence. *In vivo* studies using the renal IRI model showed that elimination of these cells might hinder regeneration (104, 105). Indeed, senescence also plays a key role in the healing of wounds, tissue repair, and during embryonic development (106). Acute and transient senescence, where damaged cells are eliminated, clearly has beneficial effects for an organism and in the adaptive tubular repair after IRI. Paradoxically, subtle accumulation of senescent cells (chronic senescence), instead, impairs the kidney's regenerative capacity, leading to AKI-to-CKD transition (52, 107, 108).

In this scenario, the use of senolytics (aimed at clearing senescent cells) seems a promising therapeutic strategy (49). However, given the non-specificity of current senescence markers and the existence of different senescence programs, elegantly reviewed by the De Maria group, more research is needed for this novel potential therapeutic field (109). The intervention strategies aimed at senescent cell removal or modulation, which seem to be effective in limiting the progression of IRI, have been reviewed previously (104). Nonetheless, having a specific biomarker for tubular senescence burden could be an effective way to stratify patients that could benefit from the use of senolytics.

Despite being arrested in the cell cycle, senescent cells remain metabolically very active and become fibrogenic due to their innate ability to create a pro-inflammatory environment and secrete pro-fibrotic molecules, components of the senescence-associated secretory phenotype (SASP) (110). Activation of SASP in TECs leads to activation and proliferation of fibroblasts and perivascular pericytes, which in turn induce extracellular matrix production and tubulointerstitial inflammation, with impaired M2 macrophage conversion (47, 71, 96). Perhaps redirecting the metabolism of senescent cells could be an effective way to turn their detrimental phenotype and fate. Recent studies have indeed highlighted the plasticity of these cells, being able to re-enter the cell cycle, posing a new challenge to the postulated theory, that cells can instead be re-programmed to change their cell fate (111).

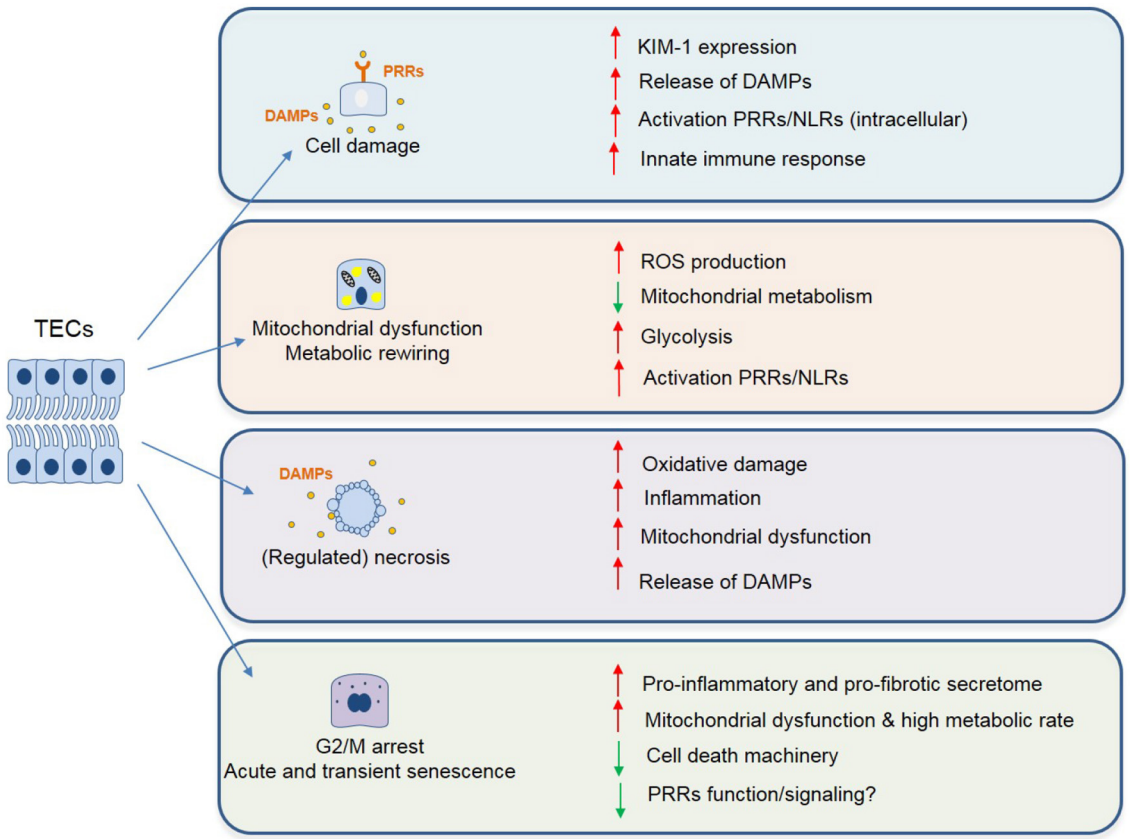

FIGURE 1 | Phenotypic changes described in TECs following IRI associated with maladaptive tubular repair and progressive renal interstitial fibrosis. These changes include but are not limited to: cell damage (chronic inflammation with persistent cytokine production and immune cell infiltrate), mitochondrial dysfunction and cell death (enhanced ROS signaling, metabolic reprogramming, and release of mitochondrial DNA/ROS acting as danger molecules, ultimately leading to cell death). Lastly, as a result of incomplete repair or severe damage, TECs can undergo transient cell cycle arrest, as a protective mechanism to ensure genome stability. However, if persistent, this leads to a pro-inflammatory and profibrotic secretome, ultimately leading to fibrosis.

In addition to the common components of the SASP, senescent cells produce exacerbated levels of mitochondrial-derived ROS and might also release mtDNA, which can act as a DAMP, further fueling PRR activation (101, 112). Collectively, they amplify the vicious cycle of inflammation, mitochondrial dysfunction, and senescence, ultimately leading to maladaptive tubular repair. Our group has recently described that the innate immune receptor TREM-1 links mitochondrial dysfunction, tubular senescence and maladaptive repair after AKI. This is the first study linking epithelial immunometabolism to cellular senescence in the context of IRI (52).

Epithelial Immunometabolism

TECs possess an incredible plasticity. By tightly controlling their metabolism, they are able to anticipate and adapt to constantly changing environments in both health and disease. Activation of the innate immune system and metabolic reprogramming are tightly linked (113). The theory of immunometabolism has been increasingly appreciated to drive effector functions in immune cells. Despite TECs being regarded as innate immune cells of the kidney, research on epithelial immunometabolism is still in its infancy, particularly in the context of renal IRI (113). In the last 2 years, our group has found that the metabolic choice of TECs is driven by either membrane-bound or intracellular receptors of the innate immune system, proposing the involvement of epithelial immunometabolism in the pathogenesis of IRI and its detrimental consequences (51, 52).

The effect of renal injury on cellular metabolism has been well-established in various AKI models. The induction of AKI with mercuric chloride results in increased glycolytic activity (114), while FAO has been reported to be reduced in folic acid nephropathy (72). The general consensus is that such metabolic shifts are likely necessary to facilitate the initial recovery process; however, their effects may become deleterious when the altered metabolic state persists. Specifically, numerous studies have reported the damaging effects of increased glycolytic activity in TEC repair following IRI. Increased levels of glycolysis and glycolytic enzymes have been observed in TECs that failed to re-differentiate and underwent atrophy (68). As mentioned earlier, Zhou et al. reported the renoprotective effect of PKM2 inhibition by the S-nitroso-CoA reductase system following bilateral IRI.

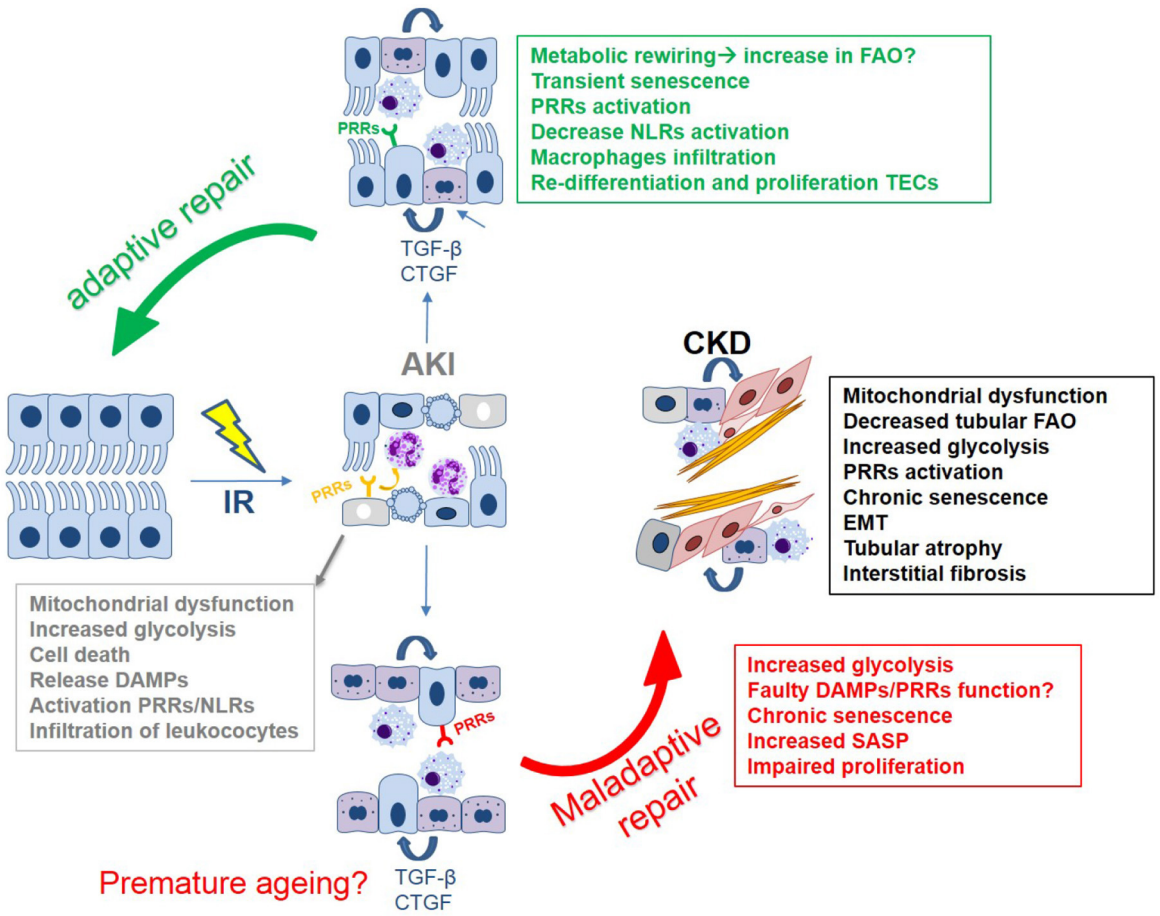

FIGURE 2 | The tubular epithelial response to IR: a simplified overview of the main mechanisms driving (mal)adaptive repair leading to kidney fibrosis. Key mechanisms include: epithelial innate immune response, cell death, mitochondrial dysfunction, metabolic reprogramming, and cell cycle arrest/cellular senescence.

This obstruction of the glycolytic pathway was accompanied by a shift toward the pentose phosphate pathway (PPP), which generates Nicotinamide adenine dinucleotide phosphate (NADPH) and aids in the supplementation of antioxidant reservoirs, thereby offering protection against ROS-induced injury (87). Similarly, Kim et al. report the renoprotective effect of TP53 Induced Glycolysis Regulatory Phosphatase (TIGAR) activation in IRI, which leads to a metabolic shift away from glycolysis and employs the redox protection of the PPP (115). These studies emphasize the need for 'metabolic fluidity' while simultaneously displaying the need for strict metabolic control during IRI. Results from our group and that of others have shown the effect of TEC-specific expression of PRRs during IRI (8), while our recent reports demonstrate the direct involvement of innate immune signaling (via NLRX1 and TREM1) in regulating TEC metabolism following IRI (51, 52). Altogether these data show the inextricable connection between innate immune signaling and cellular metabolism in TECs, which ultimately defines their fate following an acute insult. It is for these reasons that immunometabolism should be recognized as a crucial mechanism through which TECs respond to, and recover from, IRI.

PRRs, THE INFLAMMASOME AND THEIR LIGANDS IN EXPERIMENTAL AKI

Toll-Like Receptors

In addition to their function as gatekeeper, protecting against micro-organism invasion, TLRs can be activated by a wide range of DAMPs released upon tissue injury. Upon IRI, tubular necrosis occurs together with the release of potential DAMPs, including High mobility group box 1 (HMGB1), histones, heat shock proteins, S100A8/A9, hyaluronic acid, biglycan, mtDNA, ROS, and ATP, which can function as ligands for PRRs. The type of ligands recognized by a specific TLR is partially dependent on their intracellular localization. One group can be found on the cellular surface and includes TLR2 and TLR4 and the other group, including TLR3, TLR7, TLR8, and TLR9, is expressed in intracellular vesicles such as endosomes, lysosomes, and the endoplasmic reticulum. Upon ligand recognition, the intracellular TIR-domain of a TLR functions as a scaffold for the recruitment of specific adaptor proteins via homotypic interactions between their TIR-domains.

Signaling pathways activated downstream of these adaptor molecules promote the expression of pro-inflammatory

cytokines, chemokines, and type I and type III IFNs. Although TLRs provide protection against a wide variety of pathogens, inappropriate or unregulated activation of TLR signaling can lead to chronic inflammatory and autoimmune disorders (116, 117).

TLR2/4

Tlr2 and *Tlr4* mRNA is constitutively expressed by TECs and is upregulated upon IRI in mice (118). Both *Tlr2* and *Tlr4* overexpression in TECs after IRI induced an exaggerated inflammatory response, resulting in impaired renal function (8, 57, 119). Antisense oligonucleotides targeting *Tlr2* protected renal tissue and function after IRI in mice. Compared to the single KO mice, double *Tlr2* and *Tlr4* KO animals showed no additional protective effects (120). Likewise, mice deficient for *Myd88* or *Trif* were not significantly protected against IRI compared to WT animals (119). This might be the consequence of adaptive mechanisms in these genetically modified animals.

Inhibition of TLRs must take place in the acute phase of injury and cannot be longstanding. Indeed, it has been shown that TLR4 blockade during the recovery phase after IRI slows down the process of tubular repair after IRI in rodents (58). In light of these data and in the context of our review, we believe that inhibiting the endogenous ligands, rather than the receptor itself, might be a safer approach.

TLR2/4 Ligands

One such potential ligand is HMGB1. HMGB1 is a nuclear factor that is highly and ubiquitously expressed in nearly all cell types. Upon renal IRI, HMGB1 is overexpressed, and secondary to cell injury, HMGB1 can be leaked into the circulation and activate TLR2, TLR4, and TLR9 (see below). We and others showed that treatment with anti-HMGB1 antibodies reduced renal injury, inflammation, and dysfunction in a murine model of IRI (121, 122). This protective effect of anti-HMGB1 treatment was confirmed in a model of cold and warm renal IRI in miniature swine (123). Another potential agonist for TLR4 is uromodulin. Uromodulin, or Tamm-Horsfall protein, is a highly glycosylated protein, normally secreted by epithelial cells of the thick ascending limb of Henle's loop in the intraluminal compartment. The function of uromodulin remains elusive, but data suggest that uromodulin might prevent the formation of kidney stones and urinary tract infection (124). *In vitro*, uromodulin has been shown to activate TLR4 signaling in myeloid DCs and bone marrow-derived macrophages (125). Upon kidney injury, uromodulin can leak into the renal interstitial space, potentially leading to the activation of intrarenal resident macrophages and DCs. In contrast with our expectations, uromodulin-deficient mice have markedly fewer resident macrophages. Upon IRI, these uromodulin-deficient mice exhibit aggravated renal injury and impaired polarization of macrophages toward an M2 healing phenotype (126). The discrepancy between *in vivo* and *in vitro* data might eventually be due to the different forms of uromodulin, i.e., full-length or truncated, monomeric or aggregating form.

Histones released from dying cells were also found to exacerbate renal tissue injury in AKI in a TLR2/TLR4 dependent manner, and administration of anti-histone antibody suppressed renal inflammation and injury and improved renal function (127).

Among other DAMPs, the calcium binding proteins (S100A8/A9), ligands of TLR4 and the RAGE receptor, are released following renal IRI and seem to play a pivotal role in orchestrating the repair response after hypoxic damage. During the initial injury phase, immune cells, particularly macrophages, are polarized toward an M1-like phenotype and produce pro-inflammatory cytokines, such as TNF-alpha and IL-6, which exacerbate inflammation. However, in a later phase, they switch their phenotype to a more reparative one, the M2-like phenotype, which secretes anti-inflammatory factors, determining the resolution of inflammation and stimulating tubular regeneration (128). Our group showed that S100A8/A9 proteins play a role in this process of macrophage polarization. By controlling excessive M2 polarization, S100A8/A9 fine-tunes the adaptive response of the kidney to IRI-induced AKI (56).

TLR3

TLR3 is activated upon the binding of single or double-stranded RNA and induces an antiviral immune response, characterized by the production of type I IFNs. TLR3 is constitutively expressed in mouse and human TECs and is activated earlier (few minutes) than TLR2 and TLR4 after IRI. In a model of bilateral IRI, *Tlr3*-deficient mice were significantly protected against kidney injury as indicated by less inflammation, less tubular apoptosis and necrosis, and preserved renal function 24 h after reperfusion (129). How TLR3 is involved in IRI remains speculative. Since *Tlr3* KO mice had diminished TRIF protein expression, reduction of necroptosis might be one explanation. Also, type 1 IFN-mediated auto- and paracrine activation of IFN receptors might contribute, for instance, to pyroptosis via transactivation of caspase-11 (130, 131). The effects of TLR3 in the later phases of reperfusion were not studied in this paper.

TLR3 Ligands

Double-stranded RNA released upon viral replication is the major ligand of TLR3, but TLR3 can also recognize mRNA and mRNA-protein complexes released by necrotic cells (132). Therefore, it is tempting to speculate that both mRNA and mRNA-protein complexes might amplify the pro-inflammatory loop via their interaction with TLR3 upon renal IRI. However, scientific evidences are still lacking.

TLR9

TLR9 is a cytosolic DNA sensing receptor and has evolved to detect unmethylated CpG DNA, commonly found in microbial DNA and DNA viruses, and initiate the production of type I IFN and proinflammatory cytokines. TLR9 is highly expressed in professional innate immune cells, such as plasmacytoid DCs and macrophages but also in the kidney. In a model of moderate renal IRI, TLR9 was not involved in renal dysfunction (133). In contrast, in a model of severe IRI, we showed that *Tlr9* deficiency resulted in improved survival in mice but not in the improvement of renal function and kidney damage (134). Improved survival was associated with reduced plasma mtDNA

content and a subsequent decrease in hepatic injury. Surprisingly, and somewhat in contrast with our study, a recent study by Han et al. showed that selective intestinal TLR9 deficiency led to increased ischemic AKI and was associated with remote intestinal and hepatic injury. Intestinal *Tlr9* deficiency was associated with enlarged Paneth cell granules and increased IL-17A expression (135). We would expect that *Tlr9* KO animals would also present with this phenotype, but caution must be applied when interpreting scientific results generated by genetically-modified animals.

The mitochondrial dysfunction, cellular stress, and cell death involved in renal IRI result in the liberation of mtDNA, both in murine models of IRI and after renal transplantation in humans. Recently, our group reported a correlation between urinary mtDNA levels and the occurrence of DGF following renal transplantation (136). Outside of the mitochondrial matrix, mtDNA acts as a DAMP that can elicit neutrophil-mediated injury through TLR9, and other receptors (137). Indeed, the cyclic GMP–AMP synthase (cGAS)-stimulator of interferon genes (STING) pathway is also able to recognize cytosolic DNA. Cytosolic cGAS binds double-stranded DNA and catalyzes the production of the novel second messenger 2′-3′-cyclic AMP-GMP (2′3′-cGAMP) from ATP and GTP. The binding of cGAMP to the ER-resident protein STING releases DNA-triggered signals and activates the innate immune system (138, 139). Recently, Maekawa et al. showed that cytosolic translocation of mtDNA leads to tubular inflammation via the cGAS-STING pathway, linking mitochondrial dysfunction to enhanced inflammation (112). cGAS-STING is activated in the cortex of animals 24 h after renal IRI and *Sting* KO animals display mild tubular injury and inflammation. Therefore, we cannot exclude the possibility that the absence of reprotection in *Tlr9* KO animals, described in (134) after renal IRI, can be explained by an additional contribution of cGAS-STING. As cGAS-STING is activated in other pathogenic processes such as renal fibrosis (140) and senescence (141), further study into its effect on tubular repair will shed more light on the role of immunometabolism in AKI-to-CKD progression. Altogether, these studies strongly suggest that mitochondrial protection and a decrease in oxidative stress can be an elegant way to prevent AKI.

TREM-1

Triggering receptor expressed on myeloid cells-1 (TREM-1) is an activating receptor located primarily on cells of the innate immune system and some parenchymal cells (142). TREM-1 signals through its adapter protein, DNAX-activating protein (DAP12), to activate transcription factors capable of inducing the expression of pro-inflammatory cytokines and chemokines. TREM-1 can initiate inflammation, but can also work synergistically with TLRs to enhance an inflammatory response. Although initially studied in the context of infectious diseases, TREM-1 is also active in sterile inflammation (142). TREM-1 seems to be a hypoxia-inducible gene in myeloid DCs and TECs (52, 143) and might be involved in regulated cell death through amplification of inflammatory signals leading to necroptosis and pyroptosis, as has been shown in brain microglia (144). Interestingly, however, TREM-1 was also shown to mediate an inhibitory effect on necroptosis and pyroptosis in neonatal lung tissue, as suggested in previous work by Syed et al. (145). In our hands, in the early phase of renal IRI, TREM-1 modulation did not affect tubular damage or renal function (146). However, we did find that mice lacking TREM-1 displayed maladaptive repair characterized by persistent tubular damage, inflammation, fibrosis, and mitochondrial dysfunction-induced TEC senescence when exposed to renal IRI (52).

Inflammasomes

NOD1/2

Nucleotide-binding oligomerization domain (NOD) 1 and 2 are part of the NOD-like receptor (NLR) family of cytoplasmic PRRs. NOD1/2 can either initiate immune responses to pathogenic invasion through the recognition of PAMPs or sterile inflammation in response to DAMPs released upon cell stress (8). Both NOD1 and NOD2 contain 3 basic structural units: a NOD region, a caspase recruitment domain (CARD), and a ligand-binding domain. Once activated through ligand binding, the resulting oligomerization leads to the binding of signaling molecules to CARD. NOD1/2 activation can lead to apoptotic signaling through RIP-like interacting caspase-like apoptosis-regulatory protein kinase (RIP2) or the release of inflammatory chemokines and cytokines (147). NOD1/2 are expressed on TECs in both mice and humans (148), and due to their structural resemblance to other NLRs and TLRs that are known to play a role in kidney disease, it is plausible that NOD1/2 may play a role in renal injury. Indeed, a study by Shigeoka et al. found that *Nod1/2* double KO led to a reduction in apoptosis following IRI (148). Expression of the pro-inflammatory cytokines IL-6, KC, and TNF-alpha were also reduced in mice deficient for NOD1/2, leading to the general suppression of the inflammatory response to IRI. Interestingly, NOD2-deficient mice were better protected against renal injury than NOD1-deficient mice, indicating that although both NOD proteins are involved in renal injury, the underlying mechanisms may be different (148). Endogenous ligands for NOD1/2 remain unknown, therefore, their discovery would certainly aid in the development of a therapeutic intervention.

NLRP3

NLRP3 (NOD-, LRR-, and pyrin domain-containing 3) is by far the best-characterized inflammasome-forming protein in the kidney. Once activated, the cytosolic innate immune receptor NLRP3 initiates the assembly of an inflammasome, leading to an inflammatory form of cell death (pyroptosis) and the proteolytic activation of the IL-1beta family of pro-inflammatory cytokines. The NLRP3 inflammasome can trigger inflammation by sensing a wide range of stimuli, but the specific mechanisms are still unclear. Among other factors, K^+ efflux, ATP released from damaged mitochondria, and ROS production promote NLRP3 inflammasome activation (149).

Nlrp3 gene expression in murine kidneys increased after IRI and peaked 5 days after reperfusion, corresponding to the repair phase. Although the *Nlrp3* gene was primarily expressed by leukocytes, TECs also expressed *Nlrp3* after hypoxia or

LPS stimulation (150). In a murine model of bilateral IRI, we showed that *Nlrp3*-deficient animals were protected against mortality, renal dysfunction, and displayed a reduced influx of neutrophils into the kidneys despite similar degrees of tubular necrosis. In this study, activation of NLRP3 was triggered, in part, through ATP produced by mitochondria released from necrotic cells (151).

Uromodulin has also been shown to activate the NLRP3 inflammasome in human peripheral blood mononuclear cells, leading to the secretion of IL-1beta. However, as previously mentioned, uromodulin-deficient mice displayed aggravated renal injury upon IRI. This illustrates the complex role of uromodulin in regulating inflammation.

The role of leukocyte- vs. renal-associated *Nlrp3* expression has been studied in chimeric mice. In the early phase following IRI (day 1) only renal *Nlrp3* contributed to renal dysfunction based on serum creatinine. In contrast, 5 days after reperfusion (repair phase) both renal- and leukocyte-associated *Nlrp3* mediated loss of renal function. Interestingly, *Nlrp3*-deficient TECs showed increased proliferation and a superior repair response both *in vivo* and *in vitro* when compared to wildtype TECs (150).

More recently, it was shown that NLRP3 relocalizes from the cytosol to the mitochondria in TECs during hypoxia. The deletion of NLRP3 in TECs resulted in less mitochondrial ROS production, less mitochondrial damage, and less apoptosis in a model of *in vitro* hypoxia (152). Understanding the mechanisms of NLRP3 inflammasome activation will boost the development of small-molecule inhibitors for the treatment of NLRP3-related diseases (153).

NLRC5

NLR family CARD domain containing 5 (NLRC5) protein is a recently identified member of the NLR family that interferes with the assembly and activity of the NALP3 inflammasome complex by competing with ASC for pro-caspase-1 binding. Although NLRC5 activity leads to caspase-1 activation, induction of pyroptotic cell death dependent on NLRC5 has not yet been shown (154). *Nlrc5* is significantly upregulated in the kidney 24 and 48 h after IRI. *Nlrc5* deficiency significantly ameliorated renal function, injury, and inflammation 24 and 48 h after IRI. This was associated with less apoptosis in TECs and reduced inflammation in the kidneys (155).

NLRX1

In contrast to the hereinabove studied innate immune receptors, NLR family member X1 (NLRX1) exerts inflammasome independent anti-inflammatory effects by interfering with the canonical NF-kB signaling via inhibition of TRAF6 binding to IkB kinase (156). A unique feature of NLRX1 is its localization in the mitochondria. In 2017, we reported that NLRX1 protects against mortality and renal dysfunction after IRI by preventing excessive oxidative stress. We found that NLRX1 may act as an inhibitor of mitochondrial activity and prevents excessive oxidative stress, thereby preventing apoptosis of TECs during IRI (51).

Contrary to the studies by Zhou and Kim, we discovered renoprotective effects of glycolysis in IRI through our studies of NLRX1 (87, 152). Genetic deletion of *Nlrx1* potentiates mitochondrial oxidative phosphorylation in TECs, while glycolysis results in enhanced oxidative stress after ischemia and ultimately increases cell death. Using a KO mouse model for acute renal IRI, we found that NLRX1 deficiency enhanced oxidative stress, thereby profoundly enhancing tubular apoptosis, renal dysfunction, and mortality, in the early days after IRI (51). However, the consequences of this metabolic rewiring toward glycolysis on epithelial repair have not yet been investigated. A proper investigation into our hypothesis on the renoprotective effects of NLRX1 in IRI would require NLRX1 activation. *In silico* studies have postulated polyunsaturated fatty acids as NLRX1 ligands, although further validation is required (157). As these are naturally occurring lipids, we propose that NLRX1 activation through nutritional supplementation may be a possible therapeutic approach to impede AKI-induced oxidative stress.

INNATE IMMUNITY IN HUMAN IRI AND AKI

Pattern Recognition Receptor Expression and Variation During Human IRI

Little is known about the direct *in vivo* role for PRRs in human AKI and specific data on the spatiotemporal dynamics of activation are lacking. Instead, most of the studies conducted in humans that investigated PRRs tried to identify single nucleotide variants related to certain outcomes as a weak proxy for a human knock-out or knock-in model, depending on the (often estimated) consequence of the genetic variant. We previously conducted a comprehensive screening of TLR single nucleotide variants (*TLR1-8 + SIGIRR*) that were of interest because of their estimated effect on protein function. In a cohort of over 1,000 matched donor and recipient DNA samples, neither donor (renal) nor recipient (inflammatory cells) genetic variants were associated with DGF and the calculated effect size was low for individual variants, even after stratification for deceased donor type or analyzing the effect of all *TLR* gene variants in bulk, as compared to a baseline prediction model of cold ischemia time, donor age, and recipient age (158). A smaller study from Germany was able to find a positive association between a *TLR3* gene variant and DGF, although for the other *TLR* genes they were unable to find a relation with DGF (159). Furthermore, in a Brazilian study by Nogueira et al., again, no association between several *TLR4* gene variants and DGF were found (160). In the same cohort, with a similar experimental setup, we were also unable to find *NLRP3* and *TREM1* gene variants associated with DGF (146, 161). These data altogether suggest that the potential impact of these PRR genetic variants do not determine whether a patient will develop acute tubular necrosis and DGF, even though they were estimated to result in a PRR protein anomaly. Redundancy among these relatively common PRR genetic variants might partly explain this lack of association, since complete knockout of the gene of interest is a very rare event and not covered in the variants tested. At the expression

level, polymerase chain reaction on implantation (time = 0) renal transplant biopsies showed a higher expression of *TLR4* and *MYD88* mRNA in deceased vs. living donor kidneys, but the authors could not find an association with the development of DGF (162). In an interesting study by McGuinness and colleagues from Glasgow, multi-omics analysis on pre- and postperfusion biopies (RNA sequencing, DNA methylation by whole genome bisulphite sequencing, and western blotting) identified a panel of expression markers associated with the development of DGF. They identified a transcriptional panel for DGF that was associated with innate immune signaling, TREM1 signaling, PRRs, and B cell development, with top-ranked networks related to immune system activation, cell death, and survival and cellular fitness. It seemed that cellular stress and restoration of physiological homeostasis (i.e., regeneration) was exacerbated in patients who developed DGF. At the protein level, DGF and perfusion status were associated with a state of cellular senescence (163). Altogether, the data from this study put PRR signaling, cell death and survival signaling, cellular stress and fitness, an exacerbated regenerative response, and cellular senescence at the center stage in the development of human IRI, and the authors suggest that these events probably occur independently of (minor) genetic differences in PRR. These conceptual findings seem to be in line with animal models.

The MABSOT Project and the Human TLR2 Opsona OPN-305 Trial

As mentioned before, within the context of the EU FP7 MABSOT project, the humanized anti-TLR2 monoclonal antibody (OPN-305) was tested for the treatment of DGF after transplantation. In 2016, a phase I/II multicenter, randomized, double-blind placebo-controlled trial (NCT01794663) (164) in selected individuals at high risk for DGF showed that a preferred dose of 0.5 mg/kg (lowest dose tested) resulted in a lower percentage of DGF (primary endpoint; 26.5% in the treatment arm vs. 29.4% in the placebo arm) and functional DGF (failure of serum creatinine to decrease at least 10% daily; 38.2 vs. 52.9%, respectively) (165). Doses of 1.5 and 5 mg/kg appeared to be associated with a higher percentage of functional DGF, possibly in part due to the low number of DGF cases in the placebo group, according to the writers of the preliminary report. Data on long-term follow-up of the enrolled patients is pending, and a follow-up randomized controlled trial specifically looking at the clinical impact of the 0.5 mg/kg in recipients of an extended criteria donor kidney is planned according to the preliminary report. What can we learn thus far from this efficacy MABSOT trial as a first proof-of-concept for PRR blockade during human IRI? An interesting finding from the trial is the

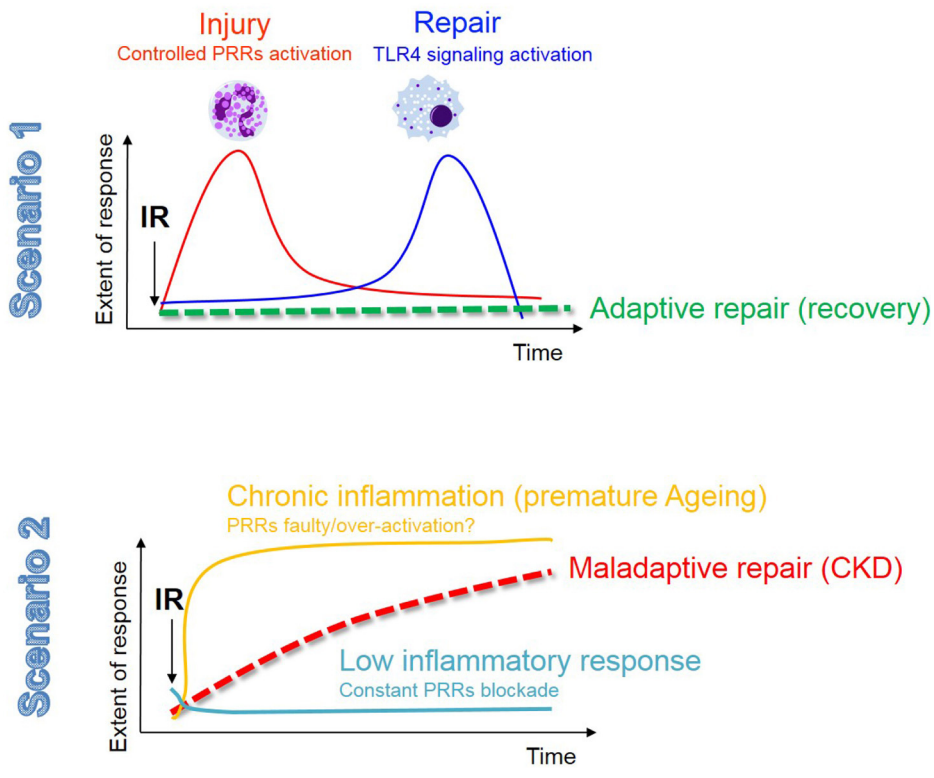

FIGURE 3 | The innate immune response in renal IR: the fine balance between adaptive repair and chronic degeneration. In this figure we describe the 2 possible scenarios that may occur in the kidney following AKI. The fist scenario involves a moderate injury resolved by a balanced and timely activation of the innate immune response, most likely associated with a low and transient senescence burden, resulting in adaptive tubular repair and kidney regeneration. In the second scenario we envision that either a low-grade chronic inflammation (possibly related to an aging phenotype and the associated high senescent burden) or a constant PRRs blockade (to avoid excessive inflammation), contribute to phenotypic changes in TECs (as described in **Figure 1**) and maladaptive tubular repair.

efficacy of the lowest antibody dose tested. Mean duration of 100% TLR2 receptor occupancy for this dose was 1 day, whereas for 1.5 mg/kg this was 6 days, and for the highest dose this was even 13 days. The use of OPN-305 in the prevention of IRI is limited by the fact that it does not act specifically on TECs, and the systemic blocking of TLR2 in all cells might result in an increased risk for (severe) infections. It might be the case that TLR blockade in the reparative phase after IRI in humans results in delayed tubular regeneration and even maladaptive repair, increasing the risk of renal fibrosis and infections. Long-term follow-up, with respect to rejection rate and renal function, would be of great interest for further investigation. Efficacy came at the cost of serious infectious and renal side effects in a select number of patients, but these results are the first to suggest the therapeutic potential for early pharmaceutical manipulation of PRRs in the context of human renal IRI.

CONCLUDING REMARKS AND FUTURE OUTLOOK

This review described the main phenotypic changes occurring during renal IRI in TECs and the role of innate immunity in dictating inflammation and repair. The central dogma on the role of innate immunity in IRI still remains; excessive inflammation is just as detrimental as a faulty response, as both scenarios predispose to maladaptive repair and chronic progression (see **Figure 3**). We summarized the current evidence that the activation of innate immune receptors, in a very delicate balance (intensity, timing, cell types), is also necessary for adaptive repair. Moreover, here we have provided an additional and novel perspective about the role of epithelial metabolism and cell fate in IRI, which may be controlled by innate immunity.

As TECs have an incredible phenotypic plasticity and are among the most metabolically active cells, they are crucial for kidney function and homeostasis. Consequently, targeting epithelial immunometabolism holds great potential to alter the initiation of acute renal failure, but also progression towards CKD. As a final consideration, we are in a time where longevity is increasing and research on cellular senescence in the kidney, as a strategy to limit the AKI-to-CKD transition, is a fascinating area to explore for therapeutic potential. Unraveling differences between drivers of acute and chronic senescence will be essential to further investigate whether these cells could be trained to change their cell fate and function, in order to slow down the progression of kidney remodeling. Changes in mitochondrial homeostasis, metabolism, and function of innate immune receptors are known pathological mechanisms associated with aging, and thus could also take place in senescent TECs. Detailed studies on the hallmarks of senescent TECs in the different phases of renal IRI may shed light on novel mechanisms that can be targeted to redirect their phenotype and overcome the issue of repair by elimination. We envision that determining the therapeutic window of opportunity to regulate renal homeostasis by targeting innate immunity, immunometabolism, and cellular senescence should be among the future research goals in the field of nephrology and kidney transplantation.

AUTHOR CONTRIBUTIONS

AT, JK, and SF designed and directed the project. AS contributed to the study. All authors contributed to the article and approved the submitted version.

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REVIEW
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The Role of Natural Killer Cells in the Immune Response in Kidney Transplantation

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Natural killer cells (NK) represent a population of lymphocytes involved in innate immune response. In addition to their role in anti-viral and anti-tumor defense, they also regulate several aspects of the allo-immune response in kidney transplant recipients. Growing evidence suggests a key role of NK cells in the pathogenesis of immune-mediated graft damage in kidney transplantation. Specific NK cell subsets are associated with operational tolerance in kidney transplant patients. On the other side, allo-reactive NK cells are associated with chronic antibody-mediated rejection and graft loss. Moreover, NK cells can prime the adaptive immune system and promote the migration of other immune cells, such as dendritic cells, into the graft leading to an increased allo-immune response and, eventually, to chronic graft rejection. Finally, activated NK cells can infiltrate the transplanted kidney and cause a direct graft damage. Interestingly, immunosuppression can influence NK cell numbers and function, thus causing an increased risk of post-transplant neoplasia or infection. In this review, we will describe how these cells can influence the innate and the adaptive immune response in kidney transplantation and how immunosuppression can modulate NK behavior.

Keywords: natural killer cells, innate and adaptive immune response, kidney graft rejection, tolerance, immunosuppression

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PHENOTYPE, MATURATION, CYTOTOXIC ACTIVITY, AND DISTRIBUTION OF NATURAL KILLER (NK) CELLS

Natural killer (NK) cells are effector lymphocytes deriving from common lymphoid progenitors and represent 5–10% of circulating lymphocytes. NK cells are natural cytotoxic cells, but, unlike cytotoxic T lymphocytes, they do not require antigen exposure to mediate their effect (1). NK cells represent one of the main cellular components of innate immunity along with mast cells, eosinophils, basophils, macrophages, neutrophils, and dendritic cells. They mediate immune responses against intracellular pathogens representing key mediators of the anti-viral and anti-neoplastic defense, but they also play a key role, through the production and release of several cytokines, in many inflammatory diseases, including acute and chronic kidney diseases (2–4). Interestingly, the role of this lymphocyte subset in the progression of kidney injury is starting to be uncovered (5).

The NK cells accomplish their cytolytic effector activity through two main mechanisms of action (6):

1. **Direct lysis**. The recognition of HLA class I molecules by inhibitory receptors (KIRs: Killer cell immunoglobulin-like receptors) on NK cells inhibits their cytotoxic activity and maintains the recognition of self. In the case of "missing self" instead, the absence of class I HLA molecules on target cells (e.g., cancer cells) prevents inhibitory signals from switching off the cytotoxicity of NK cells.
2. **Antibody-dependent cellular cytotoxicity (ADCC)**. The interaction between the Fc receptor FcγRIII (CD16) expressed on NK cells and the Fc fragment of an antibody recognizing foreign antigens on target cells (e.g., infected cells) induces the lysis of these cells.

In both cases, the lytic function of NK cells depends upon cytolytic molecules, mainly granzyme and perforin, and their activation leads to the production of several inflammatory cytokines (7). Granzyme and perforin are included into cytoplasmic lytic granules, characterized by several lysosomal-associated membrane glycoproteins (LAMPs) into the lipid bilayer. These proteins appear on cell surface after cytotoxic granules exocytosis (8). Among the different LAMPs, CD107a/LAMP-1 has been widely used as a functional marker to identify NK cell activity, since its expression is significantly higher on the surface of NK cells after MHC stimulation and correlates with both cytokine secretion and NK cell-mediated lysis of target cells (9, 10). Interestingly, Conehn et al. demonstrated that CD107a/LAMP-1 protects NK cells from self-destruction upon target cell killing, since CD107a/LAMP-1 deficiency, both in human and in mice NK cells, increased NK cell apoptosis after degranulation (11).

The interaction of NK cells with the target cell can occur through distinct inhibitory or stimulatory receptors and, therefore, defines the fate of the target cell (12). Normal cells are protected from NK cell killing since stimulatory receptors signals are balanced by inhibitory receptors signals coming from the interaction with the self-molecules of the MHC class I complex. Neoplastic transformation or cellular infection can induce the expression of stimulatory ligands that overcome the inhibition induced by inhibitory receptors. In this case, an induced-cell recognition occurs (12). In many contexts both missing-self and induced-self recognition are likely to operate simultaneously to provide to NK cells the maximum ability to discriminate normal cells from transformed or infected ones.

NK cells can express on their surface different receptors able to recognize several polymorphic variants of MHC I molecules. Indeed, the human NK cell receptor repertoire is highly complex in each individual (13). In addition, NK cells express specific stimulatory and inhibitory receptors for various other ligands present on the surface of the target cells and the balance of inhibitory and stimulatory signals received by a NK cell determines the outcome of its interactions with target cells (14). These signals involve immunoreceptor tyrosine-based activation motif (ITAM)-bearing molecules and inhibitory receptors, other stimulatory receptors and adhesion molecules, such as KIR, immunoglobulin-like transcript (LIR), leukocyte-associated immunoglobulin-like receptor (LAIR), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule (ICAM) (14). Thus, the activation program of NK cells derives from the integration of activator and inhibitor signals, which varies according to the nature of the interacting cells. In addition, NK cells also express cytokines and chemokines receptors that are crucial for the regulation of NK cell functions and Toll like receptors that mediate the production of IFN-g and increase cytotoxicity (15).

NK cell maturation can occur in the medulla of secondary lymphoid organs, lymph nodes, tonsils, and spleen (16). The hematopoietic stem cells Lin–CD34+ can differentiate into multipotent lymphoid progenitors (LMPP) CD45RA+, expressing the stem marker CD34. The expression by multipotent lymphoid progenitors of CD38, CD7, CD10, and the cytokine receptor CD127 (IL-7 receptor-alpha) drives the transition to common lymphoid progenitors that, in turn, may generate progenitors of T cells, B cells, NK cells, and other innate lymphoid cells (ILCs). An ILC restricted progenitor can origin two main ILC lineages, Killer ILC and helper-like ILC (17). Helper-like ILCs express IL-7R-alpha, require GATA-3 for differentiation, and are composed of various cytokine-producing ILC subsets: ILC1, ILC2, and ILC3. ILC1 expresses and requires the transcription factor T-bet for lineage specification and produces large amounts of IFN-g (18). ILC2 produces type 2 cytokines and amphiregulin (19), thus driving type 2 adaptive immune responses through activation of Th2 cells, ILC3 express type 17 cytokines such as IL-22 and IL-17A (20). Similarities between helper-like ILC and T cell subsets led us to propose ILC as the innate counterparts of T cell subsets (21). The expression of CD122 (IL-2 receptor beta) marks the irreversible commitment of common lymphoid progenitors to give rise to conventional NK cells. Conventional NK cells and helper-like ILC1 can be distinguished by the expression of some transcription factors such as the T-box protein in T cells (Tbet) and Eomesodermin (Eomes) since mature NK are Tbet+ Eomes+ while ILC1 are Tbet+ Eomes-; however, the distinction between NK cells and other ILC populations also concerns their cytotoxic properties and other molecules (such as CD200r1, Eomes, CD49b) expressed in different organs and different activation states (22) that can drive different response during homeostasis and viral-induced inflammation (23). Finally, the appearance of the adhesion molecule CD56 (NCAM: neural cell adhesion molecule) designates the final transition of immature NK cells toward a mature phenotype (16).

The NK cell maturation gives rise to a small population of $CD56^{bright}$ (5%) cells, expressing high levels of this adhesion molecule, and a $CD56^{dim}$ population, expressing lower levels of this adhesion molecule, that represent about 90% of mature NK cells (16). Thus, phenotypically, human NK cells are defined as $CD3^{-}/CD56^{+}/CD335\ (NKp46)^{+}$ mononuclear cells that can be further divided into low density $CD56^{dim}$ and high-density $CD56^{bright}$ subsets (24). The NKp46 is a member of the highly conserved family of natural cytotoxicity receptor (NCR), a family of NK-activating receptors, also expressed by a small subset of

cytotoxic T lymphocytes (25). The two NK cell subpopulations present specific localization, phenotype, and function. $CD56^{dim}$ NK cells are the dominant subset in peripheral blood and are cytotoxic effector cells, which express high levels of FcγRIII (CD16), an immunoglobulin superfamily member, and CD57 (HNK-1, Leu-7). In addition, they present on their cell surface CXCR1 and the Chemerin receptor that play a role in NK cell recruitment into peripheral inflammatory sites. This subset, however, has a lower ability to produce cytokines in response to activation. On the other side, $CD56^{bright}$ NK cells are present in secondary lymphoid organs and peripheral tissues. They express CCR7 that regulate their homing to lymphonodes, lack perforin, presenting little or no ability to spontaneously kill tumor cell targets, are $CD16^{-}$, and mediate immune response by secreting pro-inflammatory cytokines, such as interferon-γ and tumor necrosis factor (TNF) alfa (26, 27).

The production of pro-inflammatory or immunosuppressive cytokines is another essential feature of NK cell, distinct from the secretion of cytotoxic granules, and NK cells use diverse activation signals to regulate these two functions in a differential way (16, 28).

As CD4+ T cells, also NK cells can be distinct into Natural killer type-1 (NK1), NK type-2 (NK2) (29), and NK regulatory cells (NKreg) (30). The different functional NK subsets are characterized by different expression of cell surface proteins and cytokines. NK1 cells are NK cells with activating signals, are mainly $CD56^{dim}$ $CD11b^{+}$ $CD27^{-}$ NK cells, and produce IFN-g. NK2 cells are characterized by inhibitory signals, are mainly $CD56^{bright}$ $CD27^{-}$ $CD11b^{-}$ NK cells, and produce type-2 cytokines, including IL-5 and IL-13; NKreg cells are mainly $CD56^{bright}CD27^{+}$ NK cells and play their immune regulatory effect by cytokines secretion or cell-to-cell contact (29, 31). The differentiation of NK cell subsets depends on the specific microenvironment in physiological or pathological conditions other than intrinsic regulation by various transcription factors.

In response to tumor ligands or intracellular pathogens, NK cells mainly produce Th1 type cytokines including IFN-g, TNF, and GMCSF which facilitate the activation of T cells, dendritic cells, macrophages, and neutrophils (32, 33). NK cells also produce cytokines with chemotactic action, including CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES) which attract effector lymphocytes and myeloid cells toward inflamed tissues (34).

Similarly, different cells can produce inflammatory mediators that act on NK cells influencing their behavior. Dendritic cells play a pivotal role in this setting. These antigen-presenting cells, through the production of critical cytokines such as IL15, 12, 23, 27, and 18 (35–37), can enforce NK cell cytolytic activity. On the other hand, type 1 interferons, IL-12, IL-18, IL-27 released by dendritic cells are powerful activators of NK cell effector functions (38). IL2, produced by T cells, promotes the proliferation, cytotoxicity, and secretion of cytokines by NK cells (16). The NK cell functions can also be regulated by TGF beta released by regulatory T cells (Treg) (39, 40).

NK cells are key regulators of the immune response and of the cross-talk between innate and adaptive immunity, since they not only play a protective role from pathogen infections but also from excessive immune response to these agents (14). In fact, NK stimulation by various soluble factors (such as IL-15, type I IFN, IL-12, IL-18) can increase the maturation and activation of dendritic cells, macrophages, and T cells (41) but simultaneously NK can also present a cytotoxic action on immature dendritic cells, on activated T cells, and on hyper-reactive macrophages (42, 43).

All the described NK cell functions play an important role not only in physiological but also in pathological conditions, where NK cells can locally modulate several mechanisms of injury (16). Although many studies on human NK cells focused on peripheral blood, it is now clear that both $CD56^{dim}$ and $CD56^{bright}$ populate also healthy lymphoid and non-lymphoid organs including liver and kidneys. The kidney resident NK cells display a specific surface marker profile in different pathological conditions (44, 45). A significantly increased NK cell number is present in kidney biopsies from patients with chronic kidney disease (CKD). In this setting both NK $CD56^{dim}$ and $CD56^{bright}$ cells were increased in fibrotic renal tissue, but only $CD56^{bright}$ correlated significantly with loss of kidney function (46), thus indicating that these cells, through the production of pro-inflammatory cytokines and IFN-g, can play an important role in fibrotic process and in the progression of kidney injury (46).

NK CELLS IN KIDNEY TRANSPLANTATION

NK cells in the transplanted kidneys are a heterogeneous population of innate lymphocytes with subset-specific functional roles and with complex functions during homeostatic and pathological conditions. In fact, their role in immune reactivity to solid-organ transplant is still controversial. It is well-known that NK cells might promote allograft injury. However, some evidences indicate that NK cells may play a significant role in the priming of allograft tolerance (47). Post-transplantation NK cell subsets can change also at the peripheral level when compared to pre-transplant cells, and these variations affect both the number and the phenotype (48), thus suggesting that NK cell immunoregulatory characteristics can largely influence the graft outcome.

NK Cell Involvement in Acute and Chronic Allograft Rejection

Kidney graft rejection is classified pathologically into two types: T cell-mediated rejection (TCMR) and antibody-mediated rejection (ABMR) (49–51). Through various interactions with different cell types involved in the immunological response activated by organ transplantation, NK cells can contribute in different ways to the pathogenesis of both acute and chronic T cell-mediated and antibody-mediated rejection (47). Yagisawa et al. recently demonstrated, in a mice model of kidney transplantation, that acute kidney allograft rejection is induced by the presence of both NK cells and donor specific antibodies (DSA), whereas in the absence of NK cell activation the presence of DSA alone cannot induce acute antibody-mediated rejection, although it can still lead to late graft failure (52).

NK cells can also influence maturation of dendritic cells and the subsequent activation of T cells (53). Moreover, NK cells are an early source of IFN-g, which drive a Th1-type immune response. NK cells can interact directly with CD4+ T lymphocytes (54), increasing their reactivity, and these activities can induce acute rejection mechanisms.

Turner et al. (2) proposed a mechanism describing the pathogenic role of NK cells in human antibody-mediated rejection through the expression of CD16. This function can be triggered by anti-HLA antibodies, in particular by DSAs that represent a major risk factor for graft loss. NK cells have been identified in the peri-tubular capillaries of the biopsies of patients ABMR (55) where DSA bind the graft endothelial cells. Once bound to endothelial cells DSA can interact with FcγRIII present on NK cells inducing an ADCC against the graft (2). This model specifically involves the NK $CD56^{dim}$ subset, expressing CD16, and recruited at the graft level. Thus, $CD56^{dim}$/CD16 NK cells could represent the main NK subset involved in the pathogenesis of antibody-mediated rejection and responsible of ADCC on target cells into the graft (**Figure 1A**). Patients with DSA present, indeed, a reduced number of circulating $CD56^{dim}$ NK cells compared to patients without anti-HLA antibodies or with non-DSA anti-HLA antibodies (56), and this observation might be the result of NK cytotoxic subset homing within the rejecting graft. Sablik et al. recently reported that there are no significant differences in the total percentage and distribution of NK cells, B cells, and T cells between patients with chronic active antibody-mediated rejection and control transplant recipients. However, antibody-mediated graft rejection is characterized by differences in the activation status of circulating monocytes, NK cells, and γδ T cells, mainly regarding the CD16 expression (57).

NK cells may play also a role in the pathogenesis of T cell-mediated rejection. Immunohistochemical characterization of graft infiltrating cells demonstrated that patients with acute T cell-mediated rejection are characterized by a higher number of $CD56^+$ and $CD57^+$ cells within the interstitial compartment, associated with interstitial inflammation and tubulitis, both characteristics of T cell-mediated rejection (58, 59). Authors also established a cut-off of 0.56 cells/mm^2 both in the interstitial infiltrate and at the glomerular level, which was significantly associated with a worse graft survival (59). However, the main limits of these studies were the identification of NK cells based only on the expression of a single marker, CD56 or CD16, and the impossibility to identify the NK cell subpopulations involved in this context.

These findings suggest that NK cells need to be carefully evaluated, because variations in NK cell marker expression might be associated with the activation of different immune pathways in graft rejection. A recently published study, where authors isolated lymphomonocytes directly from graft biopsies and used a multi-color flow cytometry to define NK cell subsets involved in different graft rejection, confirmed this hypothesis (60). Biopsies from patients with T cell-mediated rejection showed an increased absolute number of $CD56^{bright}$ NK cells while in the biopsies of patients with antibody-mediated rejection both $CD56^{bright}$ and $CD56^{dim}$ NK cells were increased. Only $CD56^{dim}$ showed the expression of activation markers such as CD69 and high levels of cytotoxic effector molecules (perforin, granzyme A, and granulysin) in supernatants obtained from ABMR biopsies (60). Once again, these data highlight the importance of $CD56^{dim}$ cells activation by the micro-environment featuring ABMR, where they can guide vascular damage. $CD56^{bright}$ NK cells can instead play a specific role in TCMR through the secretion of pro-inflammatory molecules such as IFN-g (**Figure 1A**), which increase the recruitment of alloreactive T cells and up-regulate HLA alloantigens (MHC I and II) on graft target cells, making them more susceptible to cytotoxic killing (60).

Hidalgo et al. analyzed the transcriptional profiles of graft biopsies of patients with antibody-mediated rejection in the presence and absence of DSA (61). They demonstrated that the presence of DSAs was associated with 132 different transcripts, some in common with the T cell mediated rejection. By eliminating these shared transcripts, the authors identified 23 selective associated transcripts. Six of these 23 transcripts showed a high expression in NK cells, while the rest were mainly expressed at the endothelial level (61).

The different role of NK cells in T cell-and B cell-mediated rejections was also confirmed by the analysis of transcriptomic profiles of 403 kidney graft biopsies (62). Gene expression profiling of human kidney allografts identified high levels of NK cell transcripts in early T cell-mediated rejection, thus suggesting a distinct role for NK cells in this tubule-interstitial disease, while late biopsies showed increased number of NK cell transcripts in patients with antibody-mediated rejection, microvascular inflammation, and DSA. These data support the different role of NK cells in ABMR compared to the TCMR. Shin et al. also reported a positive correlation between the number of $CD56^+$ cells and the severity of T cell-mediated rejection (63). In addition, it has been recently demonstrated that transcripts from activated NK cells are the only among those from leukocyte types that differentiate antibody- and T cell-mediated rejections and correlate with transplant outcome (55).

Several immune cells subtypes such as $CD4^+$ and $CD8^+$ T lymphocytes, monocytes/macrophages, dendritic cells, and NK cells infiltrate the kidney during graft rejection; however, kidney biopsies from ABMR patients are specifically characterized by a significant enrichment of NK cell transcripts, and activated NK cell infiltration can discriminate ABMR from TCMR and can predict graft failure after kidney transplantation (55). NK-cell depletion in mice models can, indeed, significantly attenuate the frequency and severity of antibody-mediated chronic rejection and the presence of NK cells is important in the pathogenesis of antibody-mediated graft lesions (64).

Although different cell types and different cell subsets are involved in ABMR and TCMR, respectively, NK cells and CD8 T cells, shared transcripts are expressed in the graft in both antibody- and T cell-mediated rejections, such as CD160, XCL1, TNFRSF9, and IFN-g, thus indicating possible similar effector systems, important in rejection mechanisms (65).

Our group has recently identified specific transcripts from NK cells also on peripheral blood mononuclear cells isolated from patients with antibody-mediated rejection compared to control transplant recipients with normal graft function and histology (Pontrelli P, personal communication). In particular, we observed

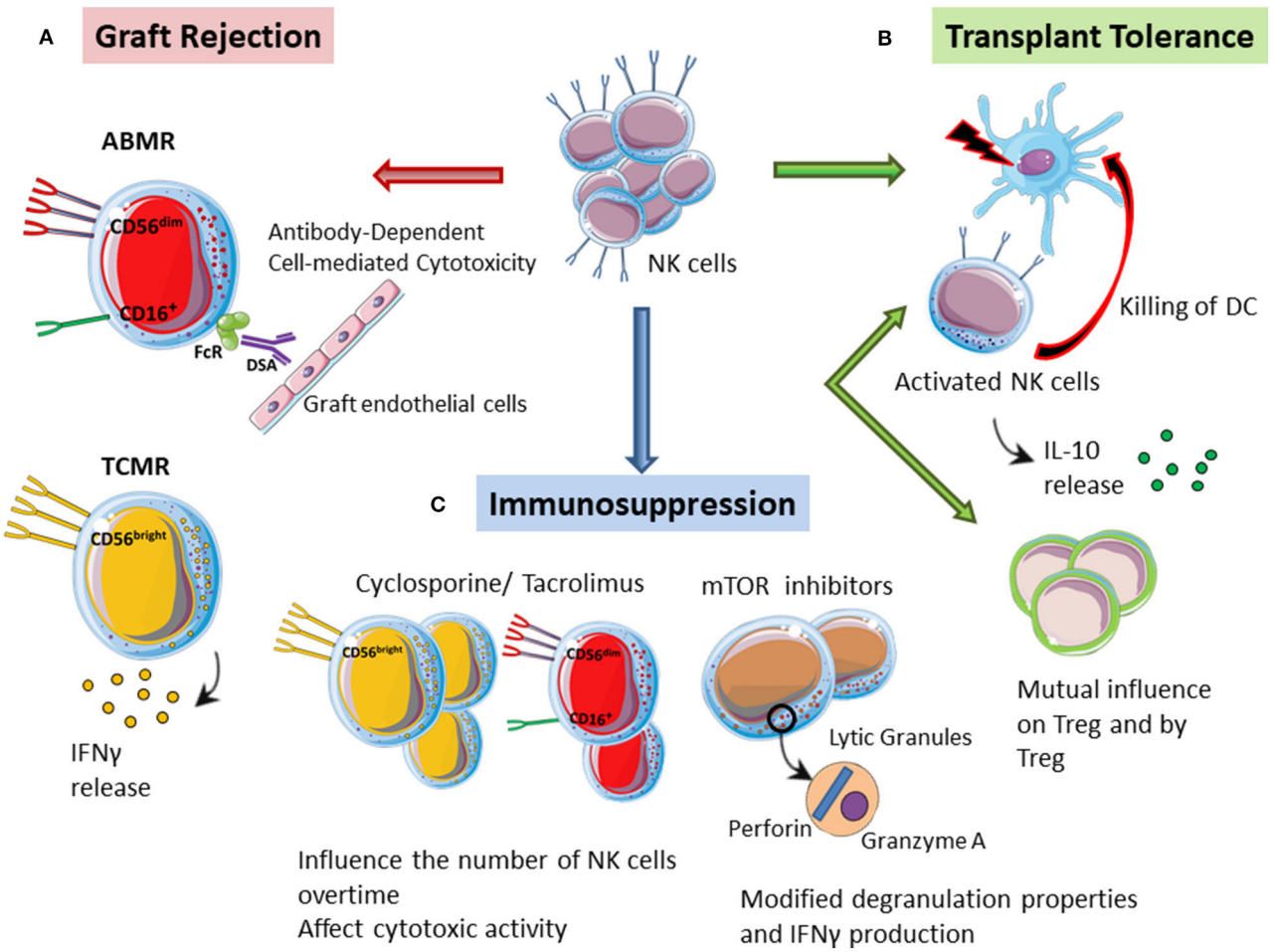

FIGURE 1 | The multiple role of NK cells in kidney transplantation. **(A)** Graft rejection. $CD56^{dim}$/CD16 NK cells can promote ADCC against the graft by interacting with DSA bound to the graft endothelial cells, thus driving antibody-mediated rejection (ABMR). $CD56^{bright}$ NK cells can instead play a specific role in T cell-mediated rejection (TCMR) through the secretion of pro-inflammatory molecules such as IFN-g. **(B)** Transplant tolerance. Activated NK cells can directly kill donor-derived dendritic cells, thus promoting transplant tolerance. In mice tolerant models, NK cells can also produce high levels of IL10 thus showing tolerogenic ability. NK cells and Tregs might also influence each other by a mutual antagonism or by a temporary definition of their contribution in the induction of transplant tolerance. **(C)** Immunosuppression. Immunosuppressive drugs might modulate the phenotype of NK cells that can retain their ability to respond to stimulation. Moreover, immunosuppression can reduce the number of NK cells after kidney transplantation. Monitoring NK cell numbers and functions in transplanted patients under specific immunosuppressive regiments is important to control and predict the onset of infections and neoplasia.

that patients with antibody-mediated rejection are characterized by the increased presence of specific NK cell receptors that are essential for NK cell behavior. The key mediators of NK cell alloreactivity, in fact, are multiple receptors, KIRs, that predominantly recognize HLA Class I molecules (66). The different KIR expression on NK cells allows the ability of these cells to evaluate minute changes in MHC class I expression (67). Inhibitory KIRs expressed by NK cells of solid organ transplant recipients with donor mismatched for HLA KIR ligands may not recognize HLA class I molecules of donor and may dramatically induce NK cell alloreactivity against the graft (68, 69). Indeed, Littera et al. demonstrated, in a retrospective study including 174 donor/recipient pairs, a significantly higher risk of chronic rejection when recipient and donor pairs completely lacked the two KIR-HLA ligand combinations rKIR2DL1/dHLA-C2 and rKIR3DL1/dHLA-Bw4 corresponding to a low level of NK cell inhibition (70). Van Bergen et al. in a retrospective cohort study of 397 HLA-DR-compatible kidney transplantations demonstrated that KIR-ligand mismatching contributes to the rejection of human solid allografts as an independent risk factor in HLA-A-B-DR-compatible transplantations, indicating that suppression of NK-cell activity can improve the kidney graft survival (71).

NK (mis)matching between KIR receptors and HLA molecules can largely influence a transplant outcome and many studies confirmed the importance of NK KIR mismatching in graft-vs.-host disease (72). Recently, it has been demonstrated that in hematopoietic stem cell transplantation, activation of donor NK cells, in the absence of appropriate inhibitory ligands, can largely influence the outcome of transplantation. The specific

analysis on KIRs genotype and HLA-A/B genotypes on a cohort of 100 patients with acute leukemia who received hematopoietic stem cell transplantation from their HLA-matched siblings suggested that an appropriate selection based on donor-recipient KIR genotypes and recipient HLA class I molecules can modulate the risk of host's disease and the efficacy of transplantation (73).

How Can NK Cells Influence Transplant Tolerance?

Despite their essential role in allograft rejection, NK cells might also promote allograft tolerance (74). Immunological tolerance to a set of antigens is the absence of an immune response against those antigens, while normal responses to other antigens are preserved. Therefore, tolerance is an active antigen-specific process, is achieved under conditions that suppress the immune reaction, and is not just the absence of an immune response (75).

In specific settings, NK cells have potent immunoregulatory properties that promote tolerance induction. In a skin transplant model in mice it has been demonstrated that recipient's NK cells can contribute to the induction of graft tolerance by killing allogeneic antigen presenting cells (76). Donor antigen-presenting cells, in the absence of host NK cells, can survive and directly induce the activation of alloreactive T cells that are resistant to co-stimulatory blockade treatment (76). Thus, in those models in which NK cells have an altered function or are reduced, it will be difficult to obtain tolerance toward a MHC mismatched graft. After transplantation, in fact, both antigen presenting cells and T cells may represent potential targets of NK cell regulation (77). In this scenario, NK cells can be activated through different mechanisms: detection of the missing MHC I-self on the target cells, recognition of the Fc portion of the IgG, recognition of altered molecules on cells under stress conditions, inflammatory environment mediated by the cytokines produced by dendritic cells and T cells (78, 79). Activated NK cells can kill donor-derived dendritic cells through direct lysis (77), thus dampening the immune response and promoting a tolerogenic environment (**Figure 1B**). It is not yet clear which factors move NK cells toward immature donor-derived dendritic cells. Moreover, NK cells regulation could also affect recipient dendritic cells, thus influencing allograft antigen presentation (77). It is conceivable that NK cells might be able to integrate stimulating and inhibiting signals influenced also by T cell behavior, thus defining the final immune response (47).

The maintenance of transplant tolerance could be also associated with the production of IL-10 by NK cells (**Figure 1B**). Upon stimulation with glycolipids, such as galactosyl ceramide, NK cells produce high levels of IL-10 that can promote the development of regulatory dendritic cells (80). Moreover, NK cells from tolerant mice show high IL10 levels and can influence the immune response mediating heart transplant tolerance (81).

Lozano et al. described the presence of genes transcripts associated with NK cell-mediated cytotoxicity in the expression profiles of peripheral lymphocytes obtained from tolerant recipients, especially in liver transplant recipients (82). Even in kidney transplantation, the NK cell signature characterizes a tolerogenic action in recipients and kidney transplant patients with spontaneous operational tolerance are characterized by specific transcriptional profiles (82, 83). Dugast et al. showed that, although the frequency of circulating NK cells was normal in these spontaneously tolerant patients, these cells showed a reduced activation profile with a reduced expression of activating *KIR2DS5* gene, NKp46, and CD16 with a subsequent reduction in the effector functions of these cells including cytotoxicity and the release of cytokines such as IFN-g (84).

In the induction of tolerance by suppressing the immune response, Tregs play a leading role. Tregs are typically $CD4^+CD25^+$ and express the foxp3 transcription factor, which is the main inducer and regulator of Treg development and functions (85). $CD4^+CD25^+$T cells suppress the proliferation of $CD4^+$ and $CD8^+$ T lymphocytes. Thus, their major role is to shut down an immune reaction mediated by T cells and to suppress auto-reactive T lymphocytes that escaped the negative selection in the thymus (86). Tregs can influence the NK cell function in different ways, and this interaction can be positive in physiological conditions, such as pregnancy, or negative in some pathological conditions, such as autoimmune diseases or neoplasms, where Tregs suppress NK cells and inhibit their effector functions (87). On the other hand, NK cells maintain a complex crosstalk with different cells of the immune system (monocytes, B and T cells) (88–92) through direct contact or secretion of cytokines including TGF-beta. In correlation with higher TGF-beta level in inflammatory response, NK cells are able to induce Tregs (87, 93). However, how NK cells and Treg cells can influence each other in physiological and pathological conditions is still largely unknown.

A direct correlation between NK cells and Tregs in inducing tolerance is currently controversial (94). To date, most published evidences support the possibility of a mutual antagonism between NK cells and Tregs (94). An alternative proposal is that the reactivity of NK cells and Tregs are temporally distinct during the induction of tolerance (47). NK cells would induce tolerance in the first 3 weeks after transplantation by blocking dendritic cells and/or T cells that could start rejecting the graft, while Tregs, by maturing later, would maintain the long-term tolerance toward the graft (74). It is therefore possible that NK cells *per se* do not induce tolerance but simply allow the survival of the graft while the recipient develop a regulatory response (47) (**Figure 1B**).

HOW DOES IMMUNOSUPPRESSION INFLUENCE NK CELL BEHAVIOR?

Information regarding the influence of immunosuppressive drugs on the activity of NK cells in transplant recipients is rather limited compared to T cells, which represent the main target of immunosuppressive therapies.

It has been demonstrated that certain KIR genotypes and their specific HLA class I ligands could affect kidney transplantation outcome by interfering with the efficacy of immunosuppressive drugs (70). The interference of KIR with therapy effectiveness has been already explored in allogenic transplantation of hematopoietic stem cells in chronic myeloid leukemia (95–97).

Immunosuppressive drugs might modulate the phenotype of NK cells after kidney transplantation, thus suggesting that NK cells can serve as sensors for immunosuppression and can be considered for personalized immunosuppression therapy adjustment (98). In fact, among kidney transplant recipients with a reduced expression of CD16 and CD56 on NK cells compared to healthy controls, patients in immunosuppressive therapy with tacrolimus showed more significant phenotypic changes on the expression of these markers than patients treated with cyclosporine or tacrolimus in combination with mTOR inhibitors (98). In addition, the presence of mTOR inhibitors *in vitro* also had functional consequences regarding de-granulation and IFN-g production (98) (**Figure 1C**). However, it is unclear whether these phenotypic changes of NK cells, induced by immunosuppressive drugs, may represent an activation signal of NK cells rather than functional exhaustion.

Hoffmann et al. demonstrated that NK cells of kidney transplant recipients under immunosuppression retain their ability to respond to stimulation since they produce equal amounts of IFN-g, perforin, and granzyme compared to NK cells from healthy individuals in response to strong, non-specific stimulation by PMA/Ionomycin (3). Thus, the inability of current immunosuppressive regimens to down-regulate the function of NK cells represents an opportunity from a therapeutic point of view, and new treatments targeted to activated NK cells and/or their effector functions should be explored.

However, immunosuppression may influence the number of NK cells over time. In patients treated with cyclosporine compared to patients treated with tacrolimus, the number of NK cells as well as the ratio $CD56^{dim}/CD56^{bright}$ is lower and the cytotoxic activity is reduced 1 year after transplantation (99). It will be useful in the future to routinely monitor and evaluate NK cell function in the context of specific algorithms to personalize immunosuppressive regimens (**Figure 1C**).

Monitoring NK cell number and especially NK cell function in transplanted patients is also important to control and predict the onset of infections and neoplasia (100). NK cells, indeed, play an important role in cancer defense, and the incidence of cancer is deeply increased after transplantation (101). Peraldi et al., in a cross-sectional multi-center case control study, demonstrated that kidney transplant recipients with cancer had a lower frequency of the cytokine-enriched $CD56^{bright}$ NK cell subset compared to normal kidney graft recipients. The percentage of $NKp46^{+}$NK cells in these patients was significantly reduced (45 vs. 53%, $P = 0.001$) along with a significant reduction in the ability of NK cells to degranulate CD107a+ cytolytic vesicles and to secrete IFN-g (102). In addition, Dendle et al. recently demonstrated that NK cell cytotoxic functions predict the appearance of severe infections in kidney graft recipients 2 years after transplantation better than NK number (103). Activated KIR genes have been associated with the protection from human Cytomegalovirus infection in renal transplantation (85), an infection associated with graft loss and reduced survival. In particular the presence after Cytomegalovirus infection of a specific subset of mature NK cells expressing the CD94/NKG2C-activating receptor can control the viral infection in kidney transplant recipients (104, 105).

CONCLUDING REMARKS

The phenotype of NK cells in peripheral blood of kidney transplant recipients might be informative of the immune status after transplantation in terms of rejection vs. tolerance induced by immunosuppressive drugs.

A more careful evaluation of the number and function of these cells will allow us to balance the activation of mechanisms underlying graft rejection, favoring the immunological tolerance of the graft. This will achieve an equilibrium condition that allows the best survival of the graft and a reduction in the risk of developing malignancies or infections.

AUTHOR CONTRIBUTIONS

PP, GG, LG, and GS designed, wrote, and critically revised the review. FR and GC analyzed NK cell involvement in acute and chronic allograft rejection. All authors contributed to the article and approved the submitted version.

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Ischemia Reperfusion Injury Triggers CXCL13 Release and B-Cell Recruitment After Allogenic Kidney Transplantation

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Ischemia reperfusion injury (IRI) is linked with inflammation in kidney transplantation (ktx). The chemokine CXCL13, also known as B lymphocyte chemoattractant, mediates recruitment of B cells within follicles of lymphoid tissues and has recently been identified as a biomarker for acute kidney allograft rejection. The goal of this study was to explore whether IRI contributes to the up-regulation of CXCL13 levels in ktx. It is demonstrated that systemic levels of CXCL13 were increased in mouse models of uni- and bilateral renal IRI, which correlated with the duration of IRI. Moreover, in unilateral renal IRI CXCL13 expression in ischemic kidneys was up-regulated. Immunohistochemical studies revealed infiltration of CD22+ B-cells and, single-cell RNA sequencing analysis a higher number of cells expressing the CXCL13 receptor CXCR5, in ischemic kidneys 7 days post IRI, respectively. The potential relevance of these findings was also evaluated in a mouse model of ktx. Increased levels of serum CXCL13 correlated with the lengths of cold ischemia times and were further enhanced in allogenic compared to isogenic kidney transplants. Taken together, these findings indicate that IRI is associated with increased systemic levels of CXCL13 in renal IRI and ktx.

Keywords: ischemia reperfusion injury, delayed graft function, kidney transplantation, B-cell activation, CXCL13

INTRODUCTION

Ischemia reperfusion injury (IRI) in kidney transplantation (ktx) is linked with inflammation and leukocyte recruitment (1). The extent of IRI depends on donor-related factors and duration of the ischemia time. Short ischemia times (2–3 h) that are encountered in living donor ktx have been associated with better long-term graft survival as compared to deceased donor ktx. In deceased donor renal transplants, median cold ischemia time (CIT) in Germany can be up to 14 to 16 h. Extended CITs (longer than 25 h) have been reported in other countries (2). Prolonged CIT and old donor age increase the risk of developing delayed graft function (DGF) (3). Immunosuppressive agents—typically, a combination of prednisolone, mycophenolatmofetil (MMF), and calcineurin inhibitors (CNI)—are used to inhibit rejection. B-cell inhibitors are only used for pretreatment of

recipients in ABO-incompatible living donor ktx to reduce or eliminate blood group antibodies prior to transplantation. Despite these interventions, acute rejection occurs shortly after ktx in 6–12% of deceased allograft recipients and some of these patients have signs of antibody-mediated rejection although donor-specific antibodies are not detectable. Thus, activation of a humoral response may be triggered by IRI itself and immediate production of the chemokine CXCL13 after surgery could be an important mediator of B-cell activation and subsequent antibody- mediated transplant rejection. Recently, we showed that CXCL13 is a biomarker for acute mixed allograft rejection in kidney transplant recipients (4). The major function of CXCL13 is recruitment of CXCR5+ cells (mainly naïve B cells) into lymphoid follicles (5, 6). Moreover, CXCL13 causes resident kidney cells to produce pro-inflammatory cytokines and chemokines (4, 7–9). In liver transplantation, increased numbers of circulating CXCR5+CXCR3-CD4+ T-cells have been shown to correlate with acute transplant rejection (10) and CXCR5+ follicular T-helper cells (Tfh) have been linked to humoral immunity (11). Furthermore, it has been shown that CXCR5+CD8+ T-cells localize to B-cell follicles, express costimulatory proteins, and promote B-cell differentiation along with antibody isotype class switching (12). Here, we demonstrate that renal IRI causes increased levels of systemic CXCL13 with subsequent infiltration of CXCR5+ leukocytes in kidneys not only in models of IRI, but also in that of allogenic ktx.

MATERIALS AND METHODS

Animals

Male C57BL/6 and BalbC mice (11–15 weeks of age, 22–28 g in weight) were housed in a 14/10 h light/dark cycle under conventional conditions. Mice had free access to food (Altromin 1324) and tap water, and were monitored daily for behavior and well-being. All experiments were approved by the local authorities (Lower Saxony State office for Consumer Protection and Food Safety, LAVES: 33.12-42502-04-14/1569 and 33.9-42502-04-07/1396; 09/1637).

Models of Uni- and Bilateral Kidney IRI

Mice were anesthetized with isoflurane (3–5% induction and 1.5% maintenance). Prior to surgery butorphanol 1 mg/kg s.c was injected for analgesia. Depending on the model, bi- or unilateral renal IRI was induced after their abdomens were opened by renal pedicle clamping using a micro-aneurysm clip for 15–45 min to induce subclinical, transient or progressive acute kidney injury (AKI) (13, 14). Sham surgery was performed in the same manner, but the renal pedicle was not clamped. After surgery mice received metamizol (p.o. 200 mg/kg) in drinking water over 3 consecutive days for postoperative pain control. The animals were monitored daily. At the designated endpoints they were deeply anesthetized and euthanized by total body perfusion with ice cold PBS via the left ventricle. Sham kidneys as well as contralateral kidneys from the unilateral experiments served as control tissues.

Kidney Transplantation Model

C57BL/6 ($H2^b$) mice served as donors and fully mismatched BalbC ($H2^d$) mice as recipients for allogenic ktx. For isogenic ktx C57BL/6 mice served as donors as well as recipients. Ktx was performed by a vascular surgeon with >20 year experience in small animal microsurgery. Surgeries were performed after the animals were anesthetized with isoflurane inhalation (35% induction 1.5–2% maintenance) following i.p. injection of butorphanol (2 mg/kg) for analgesia. After surgery mice received metamizol (p.o. 200 mg/kg) in drinking water over 3 consecutive days for postoperative pain control. For graft retrieval the donor's left kidney including the renal vein, renal artery, and ureter were removed en bloc. The recipient was prepared for transplantation by removal of its left kidney. The kidney graft was then transplanted into the lower abdomen of the recipient by end-to-side anastomosis of the renal artery to the aorta and the renal vein to the inferior vena cava (15). The ureter was implanted into the bladder dome (16). Normal renal function was ensured by the remaining native, unaffected right kidney of the recipient. After surgery mice were monitored daily for general health, activity, and well-being. Reasons for study termination included behavioral changes (reduced activity, no food intake) or surgical complications (graft thrombosis, hind limb paralysis, bleeding, urinary leakage, or significant urinary congestion).

Single Cell RNA sequencing (sc-RNAseq) in Control and IRI kidneys

Kidneys from three male C57BL/6 control mice (no surgery) and three male C57BL/6 mice 7 days after unilateral IRI for 27 min were processed for sc-RNAseq to determine whether CXCR5+ cells were infiltrating the kidney. After kidney retrieval, tissue was sliced and digested by collagenase as described (17). Following red blood cell lysis and dead cell removal using a Miltenyi Dead Cell Removal Kit according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany) 10,000 cells per sample were subjected to single cell mRNA-Seq analysis (Chromium Single Cell 3 Reagent Kits v3 User Guide, Document Number CG000183, Rev A; 10x Genomics). Equimolar amounts of libraries were pooled, denatured with NaOH, and finally diluted to 1.8 pM according to the Denature and Dilute Libraries Guide (Document # 15048776 v02; Illumina). 1.3 ml of the denatured pool was sequenced on an Illumina NextSeq 550 sequencer using one third of a High Output Flowcell for 75 cycles per sample (#20024906; Illumina). The proprietary 10x Genomics CellRanger pipeline (v3.0.2) was employed with default parameters. CellRanger was used to build a "pre-mRNA" reference package from reference genome provided by 10x Genomics (Mouse reference dataset 3.0.0; November 19, 2018; mm 10) as described in https://support.10xgenomics.com/single-cell-gene-expression/software/pipelines/latest/advanced/references. Read data were then aligned to the "premRNA" reference package with CellRanger using the aligner STAR to count aligned reads per gene and calculate clustering and summary statistics. Finally, the Loupe Cell Browser from 10x was used to view

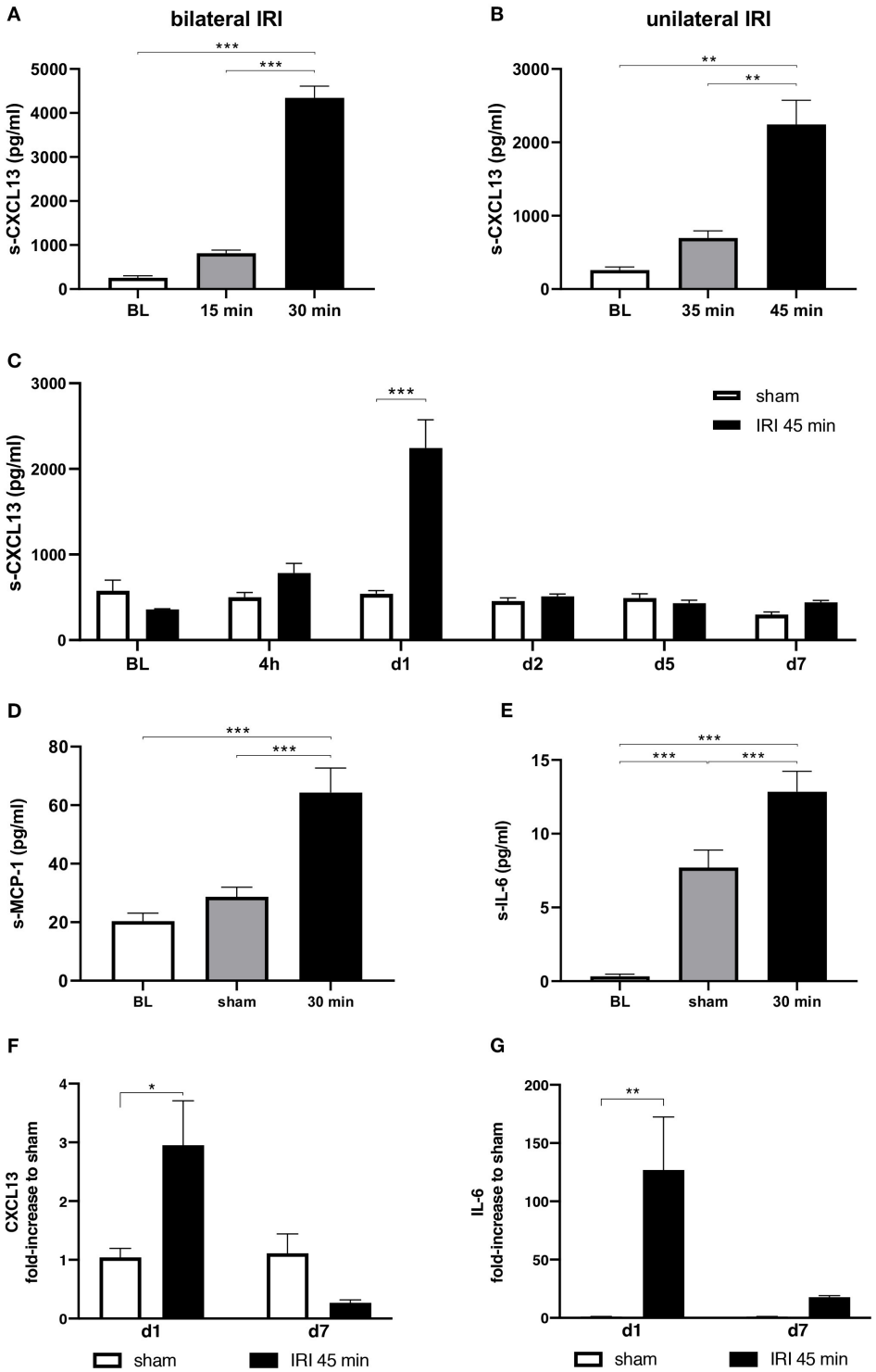

FIGURE 1 | Cytokine levels in serum samples and kidney tissue. Serum levels of pro-inflammatory cytokines were measured in models of uni- and bilateral renal IRI. CXCL13 levels significantly increased within 24 h in 30 min bilateral IRI as compared to 15 min IRI or baseline (BL) levels prior to IRI **(A)**. In unilateral IRI for 35 and 45 min a time-dependent increase of serum CXCL13 levels were observed 24 h after IRI **(B)**. To longitudinally determine the kinetics of CXCL13 release in 45 min unilateral IRI blood samples were taken at the indicated times. A maximum was measured 24 h after IRI **(C)**. MCP-1 **(D)** and IL-6 **(E)** were measured in comparison to sham surgery prior to IRI and 24 h after bilateral IRI. Both markers were significantly increased in comparison to baseline. 24 h after unilateral IRI a significant increase of CXCL13 **(F)** and of IL-6 **(G)** mRNA expression in renal tissue was observed in the 45 min unilateral IRI model ($n = 6$–10 mice per group, one-way ANOVA, $^{*}p < 0.05$; $^{**}p < 0.01$, $^{***}p < 0.001$). BL, baseline.

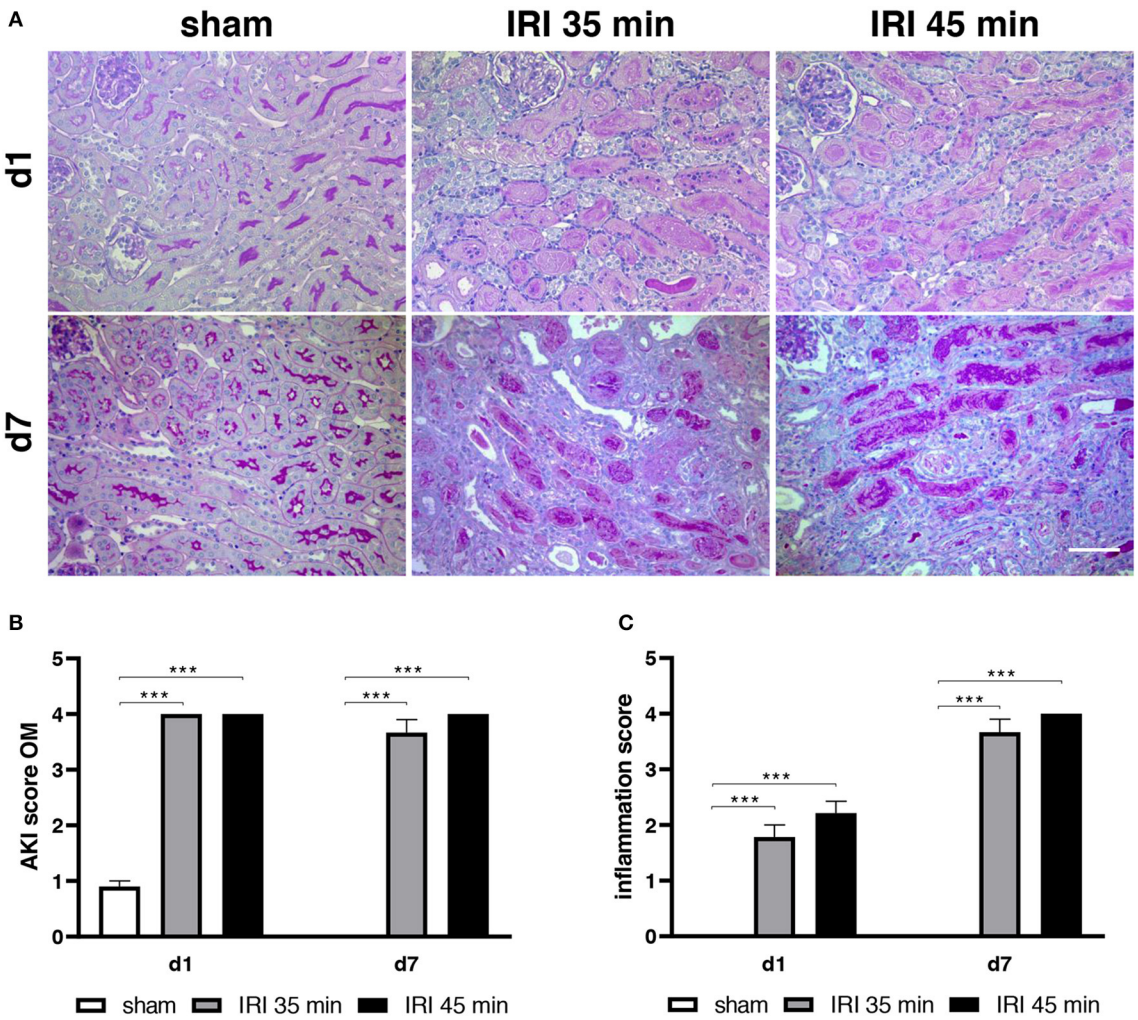

FIGURE 2 | Morphology of kidney tissue. PAS staining revealed significant acute kidney injury (AKI) after 35 and 45 min IRI and almost normal morphology after sham surgery. In **(A)** representative images from the outer medulla are depicted (bar: 100 μm). The respective AKI scoring is shown in **(B)**. The extent of inflammation increased from day 1 until day 7 **(C)**. No differences in the extent of AKI and inflammation between 35 and 45 min IRI were observed by an investigator blinded to animal group assignment ($n = 6$–8 mice per group, two-way ANOVA $^{***}p < 0.001$).

and revise annotated clusters, based on the implemented tSNE algorithm.

RNA Extraction and qPCR

Gene-specific primers for CXCL13 (Primer-sequence: fwd-TCT GGA CCA AGA rev-TGA AGA AAG TT), monocyte chemoattractant protein-1 (MCP-1; Mm_Ccl2_1_SG QuantiTect Primer Assay QT00167832), IL_6 (Mm_IL6_1_SG QuantiTect Primer Assay QT00098875), and TNF-α (Mm_TNF_1_SG QuantiTect Primer Assay QT00104006) were used. qPCR was performed as described previously (18). After fixation of renal tissue sections in RNAlater (Ambion) overnight, total RNA was isolated using RNeasy Mini Kit (Qiagen). For quantitative real time PCR (qPCR), 1 μg of DNase-treated total RNA was reverse transcribed using PrimeScript Reverse Transcriptase reagent Kit (Takara) and qPCR was performed with a LightCycler 96 (Roche Diagnostics). For each reaction 10 μl TB Green premix Ex Taq II (Takara), 3 μl DEPC-treated water (Ambion), and 10 pmol of forward and reverse primer were used in each well of a 96-well plate. The PCR reaction was initiated at 95°C (30 s), then 40 cycles followed: 5 s 95°C and 1 min at 60°C. HPRT (Mm_HPRT_1_SG QuantiTect Primer Assay QT00166768) was used as house keeping gene for normalization.

Cytokine Assays

CXCL13 serum levels were analyzed by ELISA (R&D Systems, MCX130) as described previously (19). Color development was measured by a Tecan spectra ELISA reader (Tecan, Crailsheim, Germany). In addition, a CBA bead assay was used to measure proinflammatory cytokines (IL-6, TNF-a, MCP-1) (BD Biosciences) in blood samples.

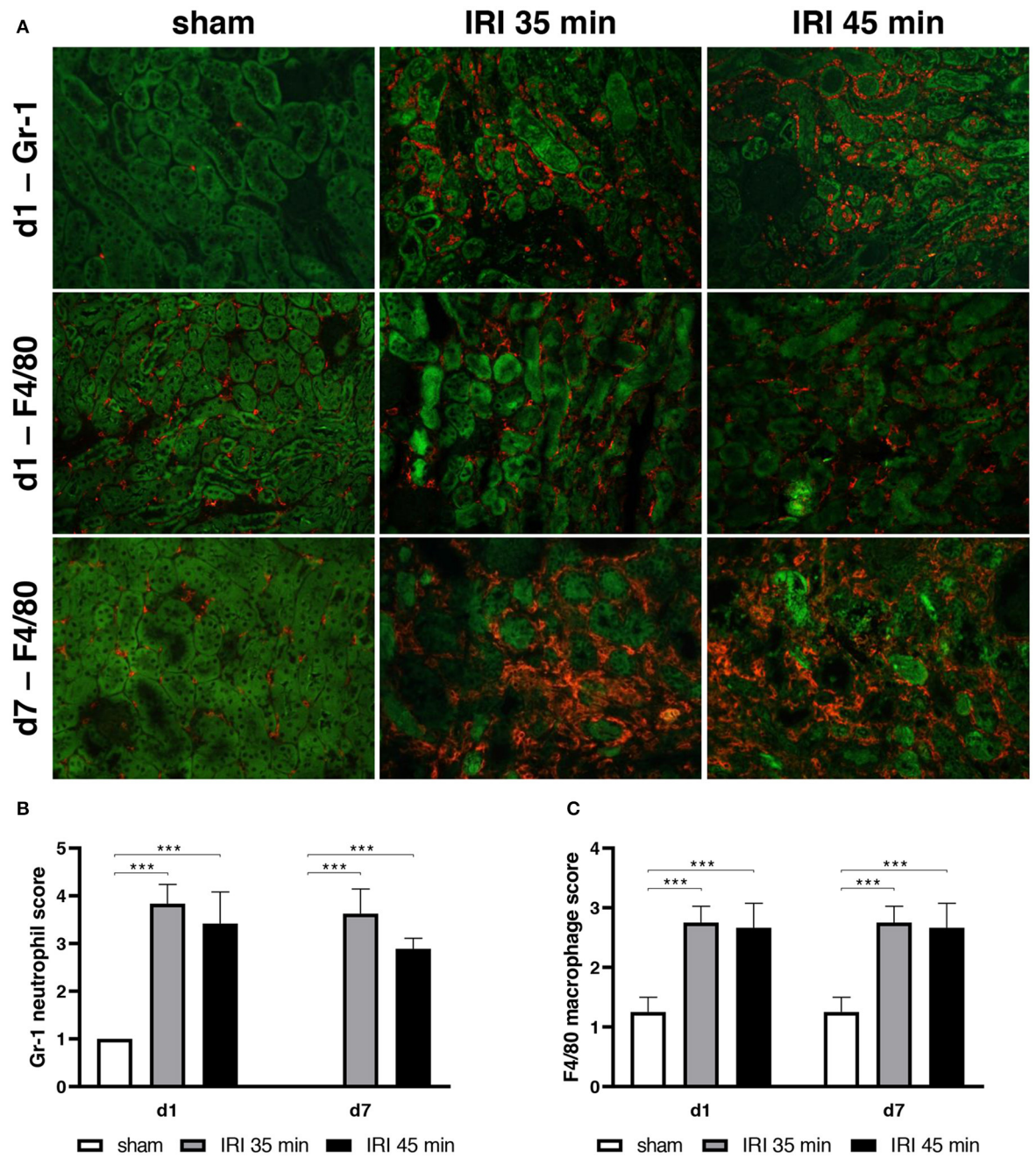

FIGURE 3 | Neutrophil and macrophage infiltration in kidney tissue. Myeloid cell infiltration was determined after unilateral IRI for 35 and 45 min and compared to sham surgery. 24 h after IRI the majority of infiltrating cells were Gr-1+ granulocytes which were mainly detected in the interstitium of the outer medulla (upper row, GR-1 in red, auto-fluorescence of the tubuli in green, **(A,B)**. At day 7 the most prominent cells were F4/80+ **(A,C)**. No differences between 35 and 45 min IRI were observed (bar: 100 μm, $n = 6$–8 mice per group, one-way ANOVA, ***$p < 0.001$).

Histology of Renal Tissue After IRI and ktx

Paraffin-embedded renal tissue was serially sectioned (2 μm) and stained with periodic acid Schiff (PAS) reaction. Signs of AKI varied from loss of brush border, an early lesion, to cell detachment from the basement membrane and accumulation of cell debris in the tubular lumen. AKI was characterized using a semi-quantitative grading system: 0 = no acute tubular injury (ATI), 1 = focal ATI with <10% of tubuli in the cortex affected, 2 = moderate ATI with 10–25% of tubuli affected, 3 = severe ATI with 25–50% of tubuli affected, 4 = very severe with >50% of the tubuli affected. Scores for interstitial inflammation and leukocyte infiltration ranged from

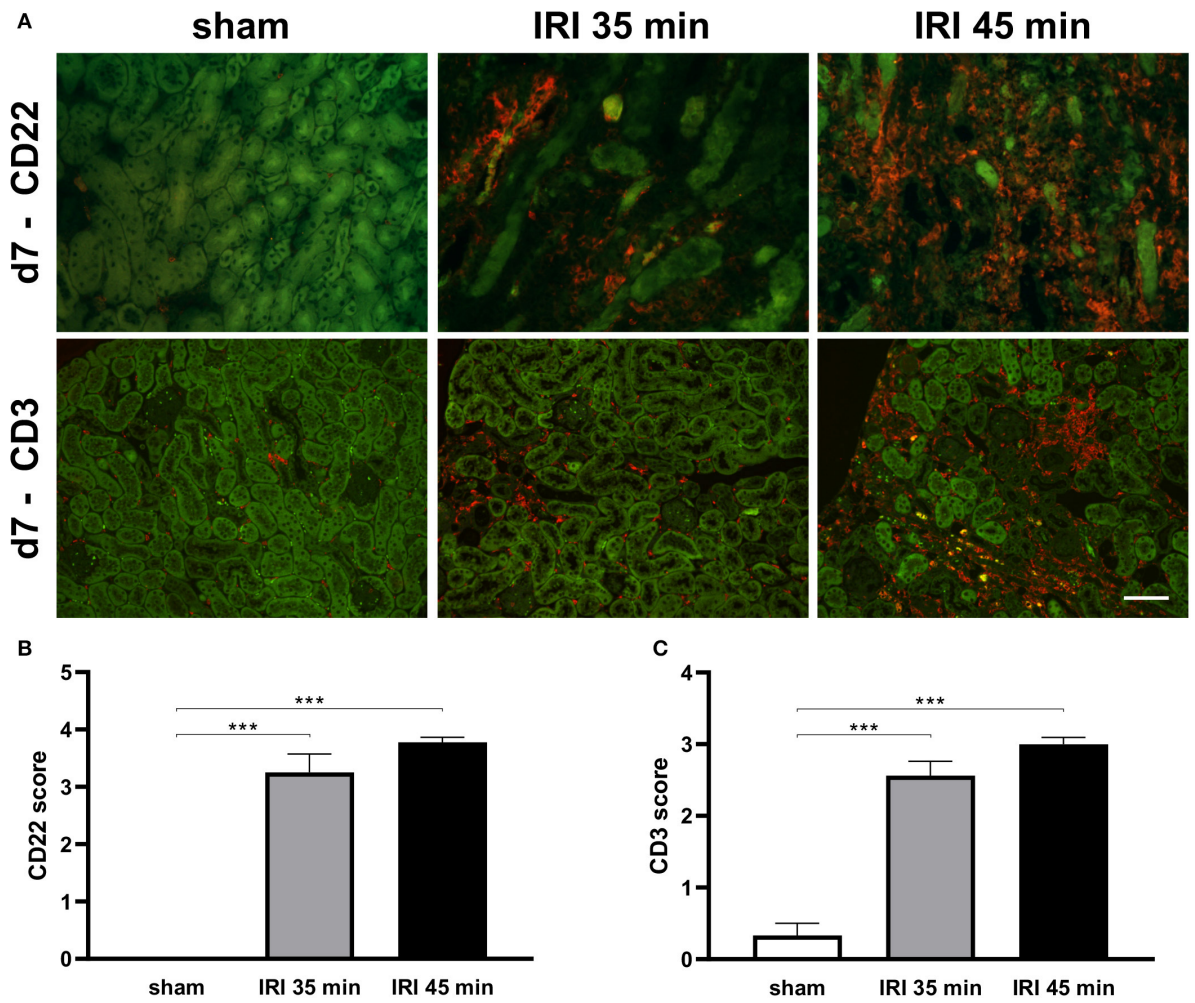

FIGURE 4 | B and T cell infiltration in kidney on day 7 after injury. Infiltration of B and T cells was determined on day 7 after injury. Representative images are shown in **(A)**. Both renal IRI models led to significant increase levels of both cell types as determined by semi-quantitative scoring **(B,C)**. No significant changes between different ischemia times were observed (bar: 100 μm, $n = 6$–8 mice per group, one-way ANOVA, $^{***}p < 0.001$).

0 to 4 correlating with mild, moderate, marked, and severe cell infiltration. Kidney grafts were graded according to the updated Banff classification (20). Analysis was performed without knowledge of the animal group identity by a nephropathologist with >20 years of experience. For further characterization of infiltrating leukocytes, immunostaining was performed with antibodies against Gr-1 (neutrophils) (Biorad, MCA 771G), F4/80 (monocytes/macrophages) (Acris Antibodies, BM4007), CD22 (Southern Biotech, 1580-01), and CD45R (B cells) (eBioscience, 14-0452-82), CD3 (T cells) (Dako, A0452), respectively.

RESULTS

IRI Causes Increased Serum Levels and Renal Expression of CXCL13

Severity of AKI is dependent on duration of renal ischemia times. Here, we compared systemic serum levels of CXCL13 in various models of renal IRI 24 h after surgery. A 15 min ischemia time causes subclinical IRI without elevation of creatinine and blood urea nitrogen (BUN), whereas a 30 min IRI induced these clinical parameters of AKI (**Supplementary Figure 1**). A pronounced increase of CXCL13 levels in serum was observed in animals that received 30 min compared to 15 min of bilateral IRI (**Figure 1A**). For comparison, the pro-inflammatory mediators MCP-1 and IL-6 were also significantly elevated after 24 h in the 30 min bilateral IRI model (**Figures 1D,E**). To allow for longitudinal follow-up without mortality after IRI, a model of unilateral IRI was used, in which only one kidney is clipped with unaltered overall renal function (21). In the unilateral model, 35 min of IRI caused a minor increase, but 45 min of IRI caused a marked increase in the levels of CXCL13 24 h after surgery (**Figure 1B**). Further time course studies in the 45 min unilateral IRI model revealed that the maximum increase in CXCL13 occurred at day 1 (24 h after IRI), which rapidly returned to basal levels at day 2 and did not increase further

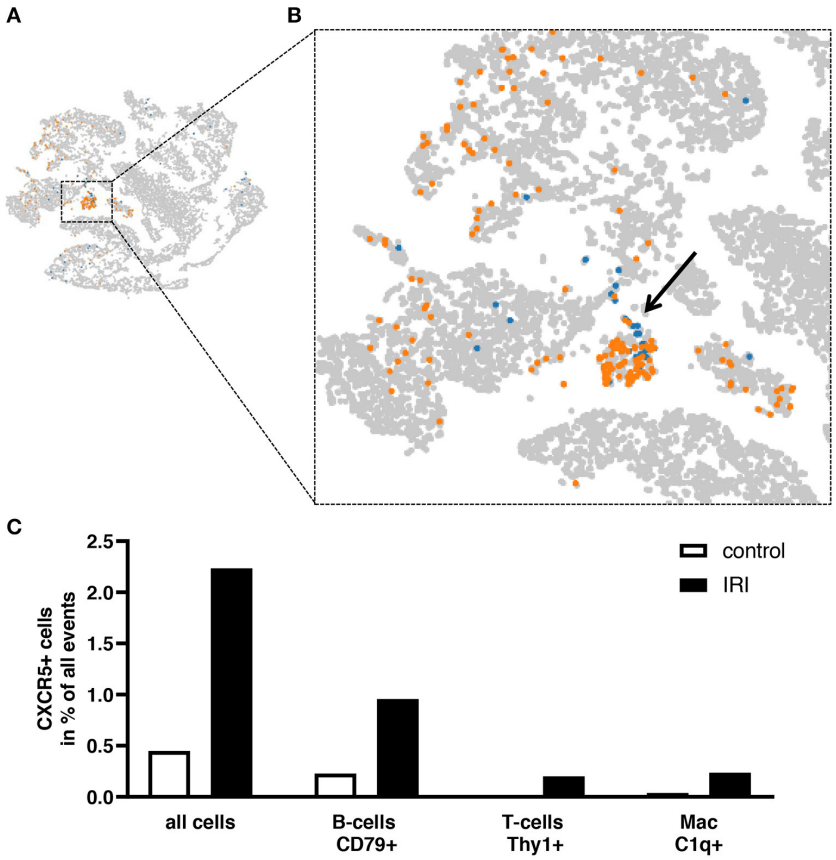

FIGURE 5 | Detection of CXCR5+ cells in kidney tissue by sc-RNAseq. Sc-RNAseq data from three pooled control kidneys were compared to those from three pooled IRI kidneys 7 days after IRI. Expression of CXCR5 was increased after IRI (orange) compared to control (blue). Clusters of CXCR5 positive cells and scattered single cells were identified (**B**, enlarged view of **A**). The highest proportion of CXCR5+ cells were identified amongst CD79+ B-cells, followed by Thy1+ T-cells and C1qc+ macrophages **(C)**.

until day 7 (**Figure 1C**). In addition, qPCR analysis of renal samples showed an increase in CXCL13 mRNA expression in ischemic kidneys at day 1 after IRI, but not at day 7 in comparison to renal samples from animals with sham surgery (**Figure 1F**). Although, a similar time-dependent up-regulation and decline was noted for the pro-inflammatory cytokine IL-6 in ischemic kidneys, the expression levels were still significantly elevated in comparison to sham controls at day 7 after IRI (**Figure 1G**).

In summary, renal IRI leads to an early increase in systemic serum CXCL13 levels and CXCL13 mRNA expression in ischemic kidneys.

IRI Causes AKI, Inflammation and Renal Infiltration of B-cells

Histomorphological changes were determined in animals after sham surgery or after 35 min and 45 min of unilateral IRI. In both groups of IRI typical alterations of severe AKI including the presence of apoptotic and necrotic cells at day 1 after surgery were observed and were still detectable at day 7 after IRI. Leukocyte infiltration increased from day 1 to day 7 after 35 and 45 min IRI (**Figures 2A–C**). As determined by immunohistochemistry the majority of infiltrating cells was GR-1 positive at day 1 (**Figures 3A,B**), whereas the more prominent cell type was F4/80+ myeloid cells at day 7 (**Figures 3A,C**). More importantly, at day 7 after IRI an infiltration of CD22+ B-cells was detected in ischemic kidneys, which was almost absent in animals that received sham surgery (**Figures 4A,B**). In addition, CD3+ T lymphocytes were found in the outer medulla at day 7 (**Figures 4A,C**). Since CXCL13 recruits cells expressing CXCR5, we performed sc-RNA seq analysis with three pooled control kidneys in comparison to three pooled IRI kidneys to identify CXCR5+ cells (**Figure 5**). In tSNE analysis IRI kidneys showed a cluster of CXCR5 expressing cells and some scattered single cells (**Figures 5A,B**). The majority of CXCR5+ cells were of B-cell origin (CD79+) followed by a subset of macrophages (C1qc+) and some T-cells (Thy1+). In control kidneys only few CXCR5+ cells were detected (**Figure 5C**).

Taken together, the data indicate that renal IRI causes recruitment of B cells to the inflamed tissue.

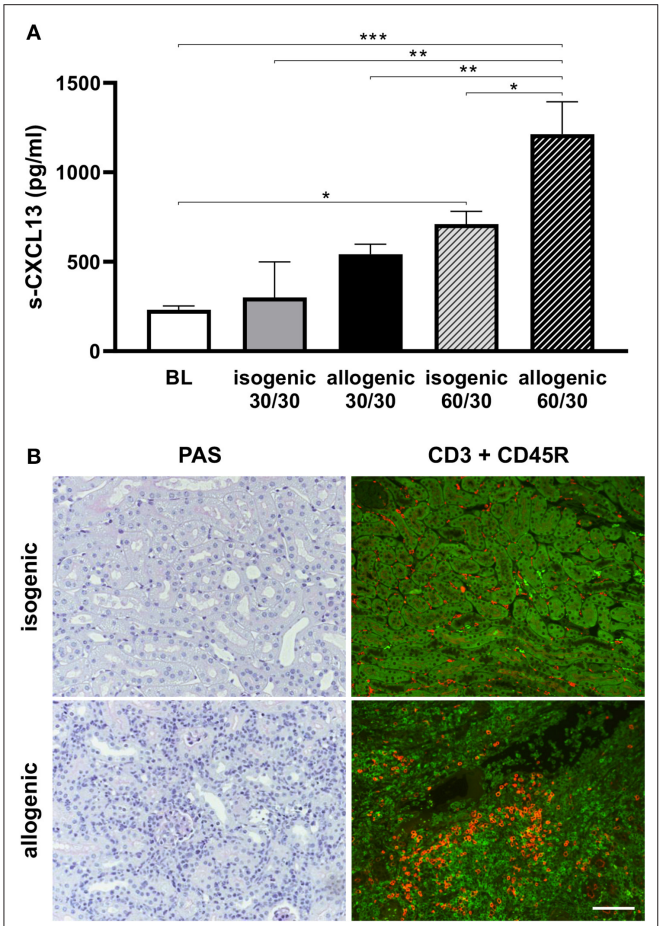

FIGURE 6 | Serum CXCL13 levels in mouse ktx. Post ktx levels of serum CXCL13 at day 1 were significantly increased compared to baseline. A higher increase was observed in longer cold ischemia time (30 vs. 60 min cold ischemia time). Isogenic ktx with prolonged cold ischemia time of 60 min had significantly lower CXCL13 levels compared to allogenic ktx **(A)**. PAS stain at day 7 revealed enhanced cell infiltration in allogenic compared to isogenic ktx **(B)**. Double staining for CD3+ T-lymphocytes (green) and CD45R+ B-cells (red) was performed at day 7. More interstitial CD3+ T-lymphocytes were observed in allografts compared to isografts. Allografts exhibited scattered B-cells in interstitial tissue, but also clusters of CD45R+ cells. Isografts showed only few B cells in the interstitium at day 7 **(B)** (bar: 100 μm, $n = 6$ per group, one-way ANOVA $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). BL, baseline.

CXCL13 Release Depends on Duration of Ischemia Time After Kidney Transplantation

In the following, allogenic ktx [C57BL/6 ($H2^b$) on BalbC ($H2^d$)] or isogenic ktx was performed with either 30 or 60 min of CIT. Serum levels of CXCL13 were significantly higher in animals that received kidneys with longer CITs. In addition, the increase in levels of systemic serum CXCL13 was amplified in the allogenic as compared to the isogenic transplant setting (**Figure 6A**). Histopathology revealed inflammation (i.e., Banff 1a rejection) in allografts with enhanced CD45R+ B-cell and CD3+ T lymphocyte infiltration 7 days after transplantation (**Figure 6B**). To summarize, increased levels of serum CXCL13 levels after ktx are dependent on the duration of CIT. Moreover, systemic levels of CXCL13 and B-cell infiltration into renal grafts post transplantation were higher in the allogenic setting.

DISCUSSION

This study provides evidence that the chemokine CXCL13, also termed B-lymphocyte chemoattractant, is up-regulated rapidly after renal IRI. Duration of IRI correlated with increased systemic serum CXCL13 levels in both models of uni- and bilateral clamping. Increased levels of CXCL13 in response to IRI were transient and reached a maximum after 24 h of IRI (**Figure 1**). B-cell infiltrates were detected in the kidney 1 week after IRI (**Figure 4**). The majority of cells expressing CXCR5 appeared to be of B-cell origin, but some T-cells and macrophages were also CXCR5 positive (**Figure 5**). Furthermore, in a mouse model of allogenic ktx systemic CXCL13 serum levels correlated with the length of graft ischemia time duration (**Figure 6**).

It is noteworthy that allogenic ktx caused higher levels of serum CXCL13 levels than isogenic ktx (**Figure 6**). This finding raises the question of whether the host inflammatory response, which is markedly higher in allogenic ktx may contribute to CXCL13 secretion. A limitation of the current study is that the source of CXCL13 was not identified. A very early event in IRI is rapid complement activation orchestrating the inflammation that follows. Complement activation might also influence the secretion of immune cell-mediated CXCL13 release. Accordingly, recent experimental evidences indicates that activated peritoneal macrophages release CXCL13 via a mechanism that involves the complement anaphylatoxin C5a. Macrophages that are deficient for the C5a receptor C5aR1 showed markedly reduced release of CXCL13. Alternatively, CXCL13 release may also be regulated via Toll-like receptor (TLR)2- and IL-10-dependent mechanisms (22). Since myeloid cells are early drivers of inflammation and the extent of macrophage infiltration is directly linked to the duration of ischemia times and the severity of IRI (21) it is conceivable that infiltrating macrophages after IRI may contribute to CXCL13 production and release. The observation that the mRNA levels of CXCL13 in ischemic kidneys were induced after 24 h also indicates that the source of CXCL13 production might be the damaged kidney. Although Tfhs are considered to be the major source of CXCL13, it has previously been shown that stromal cells can also produce CXCL13 in response to IL-17 in inflamed lung tissue in mice (23). Moreover, peripheral T helper cells, macrophages and damaged tubuli are potential candidate cells for CXCL13 production in kidney. Further studies are warranted to elucidate the underlying mechanisms of how CXCL13 expression is up-regulated in IRI and transplantation.

sc-RNAseq has emerged as a powerful technique for determination of different cell types (24, 25) and availability of new transcriptomic data expands the understanding of molecular mechanisms in various disease states. Accordingly, immune cell landscapes in particular renal disorders have been demonstrated (i.e., diabetic nephropathy, lupus nephritis) (26, 27). In the current study sc-RNAseq analysis was applied to identify cells that express the CXCL13 receptor CXCR5. It is shown that

IRI alone caused substantial B-cell infiltration into the kidney within 7 days of IRI and a subset of B-cells was positive for CXCR5. The expression of CXCR5 was not limited to B-cells as a subset of renal T-cells and macrophages were also identified to be positive for CXCR5. Several lines of existing evidence points to a detrimental role for CXCR5+ cells in transplant rejection. Notably, in a recent study, the presence of CXCR5+ CD4+ cells correlated with acute rejection after liver transplantation (10). In ABMR, CXCR5 containing exosomes from Tfhs were significantly higher compared to controls and in co-culture experiments these Tfh derived exosomes were able to promote B-cell proliferation and maturation (28). Tfhs play a critical role in germinal center reactions and development of tertiary lymphoid structures which have been reported in chronic allograft rejection of kidney (29, 30), lung (31), and heart transplants (32). These germinal centers are the primary sites of B-cell expansion and maturation directing the production of antibodies (33). However, it needs to be pointed out that the findings in this study are correlational with regard to CXCL13 and CXCR5. Further studies using strategies to block CXCL13 are required to validate the hypothesis that infiltration of CXCR5 positive cells in ischemic kidneys is a direct result of CXCL13 secretion. Alternatively, it is feasible that the recruitment of these cells is due to ongoing renal inflammation, independent of CXCL13 secretion. Due to necrotic cell death in ischemic tissue a variety of damage-associated molecular patterns (DAMPs) are released which can interact with TLR2 and−4 expressed on myeloid, dendritic, tubular epithelial and endothelial cells. TLR activation mediates downstream production of TNF-α, IL-1ß, and IL-6 promoting activation of the adaptive immune response (34, 35). Based on the findings we propose that systemic expression of CXCL13 after IRI and DGF might be a potential clinical candidate for early detection of interstitial inflammation and B-cell activation.

Further clinical studies are needed to determine the role of CXCL13 as a predictive biomarker for AKI, DGF and rejection in ktx.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Lower Saxony State Office for Consumer Protection and Food Safety.

AUTHOR CONTRIBUTIONS

FG designed and supervised the experimental studies. FG and SI drafted the manuscript. KK, M-SJ, SR, VV, and LW conducted the experimental studies. SR performed IRI and ktx surgeries. FG and JB analyzed the histology and immunohistochemistry. WG, CK, LS, and HH discussed the results and edited the manuscript. SV, RS, and OD-B performed single cell sequencing experiments. All authors participated in the interpretation of data, editing, and approval of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.01204/full#supplementary-material

Supplementary Figure 1 | Clinical parameters in bilateral ischemia model. Fifteen minutes bilateral IRI did not cause relevant increase of s-creatinine **(A)** or BUN **(B)**. Bilateral IRI for 30 min caused significant deterioration of renal function with highly significant s-creatinine and BUN elevation ($n = 5$ sham mice, $n = 6$ mice per IRI group, one-way ANOVA, $^{***}p < 0.001$). BL, baseline.

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Coagulation and Fibrinolysis in Kidney Graft Rejection

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Coagulation system is currently considered an integrated part of innate immunity. Clotting activation in response to bacterial surface along with complement cascade priming represents the first line of defense against pathogens. In the last three decades, we learned that several coagulation factors, including factor II or thrombin and factor X, can interact with specific cell surface receptors activated by an unusual proteolytic mechanism and belonging to a novel class of G-protein-coupled receptors known as protease-activated receptors (PARs). PARs are expressed by a variety of cells, including monocytes, dendritic cells, and endothelial cells and may play a key role in the modulation of innate immunity and in the regulation of its interaction with the adaptive branch of the immune system. Also, the fibrinolytic system, in which activation is controlled by coagulation, can interact with innate immunity, and it is a key modulator of extracellular matrix deposition eventually leading to scarring and fibrosis. In the setting of kidney transplantation, coagulation and fibrinolytic systems have been shown to play key roles in the ischemia/reperfusion injury featuring delayed graft function and in the pathogenesis of tissue damage following acute and chronic rejection. In the present review, we aim to describe the mechanisms leading to coagulation and fibrinolysis activation in this setting and their interaction with the priming of the innate immune response and their role in kidney graft rejection.

Keywords: coagulation, fibrinolysis, protease-activated receptors, innate immunity, graft function

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INTRODUCTION

Kidney transplantation is the treatment of choice for most patients with end-stage renal disease because kidney graft recipients live longer than dialysis patients and have a markedly higher quality of life. The improvement in the immunosuppressive drugs and protocols dramatically reduced the clinical impact of acute rejection and significantly enhanced graft survival. However, graft half-life is still far from being ideal. The quality of the grafts is significantly worsening, and they are more exposed to damage in immediately before and after transplantation, as a consequence of brain death and cold/warm ischemia (1). These peri-transplant events are often mediated by innate immunity and, therefore, are poorly influenced by current immunosuppressive approaches (2). In addition, we are now realizing that they are significantly associated with long-term transplant outcome and with the occurrence of rejection. It is conceivable, then, that the priming of innate immunity early

posttransplantation may significantly amplify the adaptive response to the graft (3, 4). Thus, the need to improve our therapeutic approaches to limit the effects of the innate arm of host immune system is now clear. The innate response has cellular and soluble components. Among the latter, complement cascade received in the last decade a considerable attention, and several drugs able to influence the activation of this proteolytic system have been introduced in the clinical scenario (5). However, complement is not the only enzymatic cascade activated in the innate immune response. Indeed, there is an increasing body of evidence that also the coagulation system may play a key role in this setting.

COAGULATION CASCADE AND INNATE IMMUNITY

The coagulation system is a proteolytic cascade that plays a key role in blood clotting. In the last decades, several intersection points between coagulation and immunity have been delineated, and the coagulation system is currently considered an integrated part of innate immune immunity.

Traditionally, the coagulation system has been classified into extrinsic and intrinsic pathways. These two pathways converge, forming the common pathway, which results in the activation of factor X to Xa. In the extrinsic pathway, tissue factor exposed by vascular injury interacts with plasma factor VIIa and activates factor IX and X, with results in the formation of small amounts of the serine protease thrombin. Thrombin not only acts on fibrinogen to form the fibrin clot but also stimulates platelet and vascular endothelial cells and activates plasma coagulation factors, priming the intrinsic pathway (6). Blood-circulating FXII is autoactivated by contact with negatively charged surfaces, such as nucleic acids, platelet, and microbial polyphosphate and thus converted to the serine protease FXIIa (7) finally promoting the sequential activation of coagulation factors XI and IX (**Figure 1**). Regulation of coagulation activation occurs by three distinct physiological anticoagulant pathways: antithrombin (AT) (which blocks factor Xa and thrombin), tissue factor pathway inhibitor (TFPI) (which inhibits the tissue factor–factor VIIa complex), and activated protein C (aPC) (which proteolytically degrades factor Va and factor VIIIa).

The coagulation cascade can be directly activated by different pathogens, including bacteria and viruses (8–12), and the formation of fibrin clots can trap bacteria and limit the invasiveness of pathogens. However, several other pathological conditions are characterized by the activation of the coagulation system, and studies performed in the past 15 years have provided novel insights into the role of coagulation proteases in kidney disease beyond their function in normal hemostasis and thrombosis (13).

CROSS TALK BETWEEN COAGULATION AND COMPLEMENT SYSTEM

Activation of the coagulation proteolytic cascade can influence innate immunity in different ways by interacting with diverse molecular mechanisms involved in the immune response. One of the main columns of innate immunity is represented by the complement system, and several possible interactions between the two cascades have been proposed (14).

The complement system is an important component of the innate immunity and functions primarily as a first-line host defense against pathogenetic infections and in the removal of immune complexes and apoptotic cells (15). The complement system can be activated by three main pathways—classical, lectin, and alternative—that include several components and regulators, produced by different cells under diverse conditions finally leading to a proteolytic cascade, which terminate in opsonization and lysis of pathogens as well as in the generation of proinflammatory molecules (16). The classical pathway uses C1 and is triggered by antigen–antibody immune complexes. It consists in the activation of the serine proteases C1r and C1s, the subsequent cleavage of C4 and C2, and the generation of the classic C3 convertase (C4bC2a), which cleaves C3 into the anaphylatoxin C3a and C3b. The lectin pathway activation also leads to the formation of C4bC2a C3 convertase complex but is activated by opsonin, mannose-binding lectin (MBL), and ficolins, instead of C1q. Finally, the alternative pathway is constitutively active at low levels in the normal host as a result of spontaneous C3 hydrolysis. It is regulated by factor H and factor I and need factor B and factor D to generate the alternative pathway C3 convertase (17). The terminal phase is similar for the classical, lectin, and alternative pathways. The incorporation of C3b in the C3 convertases results in the formation of the C5 convertases that cleave C5 into C5a and C5b, leading to the formation of the multimeric terminal membrane attack complex (C5b-9) (18).

Other factors of the coagulation and fibrinolytic pathway including thrombin; human factors XIa, Xa, and IXa; and plasmin can cleave C5 without the involvement of other complement factors, leading to the so-called extrinsic complement pathway (19).

Proteases of the lectin pathway induce thrombin and fibrin generation, stabilize the fibrin clot, and impair fibrinolysis (20). Takahashi K et al. demonstrated that the MBL and MBL-associated serine protease (MASP)-1/3 together can function as thrombin (21). In particular, they observed both *in vitro* and *in vivo* in MBL null mice that MBL deficiency may lead to disseminated intravascular coagulation and organ failure during infectious diseases (21). MASP-1 is a serine protease able to cleave several proteins, both complement and non-complement substrates, in the human blood. MASP-1 shares many characteristics with thrombin; for example, it can cleave several members of protease-activated receptors (PARs)—PAR-1, PAR-2, and PAR-4—thus leading to cytokine production leading to chemotaxis of neutrophils (22). MASP-1 is also involved in coagulation and thrombus formation by the activation of endothelial cells and generation of thrombin (22).

Several studies also reported that the coagulation system can activate the lectin pathway in turn (23, 24). These interactions can play an important role in clinical conditions because they can influence hypercoagulability and increase thrombosis risk.

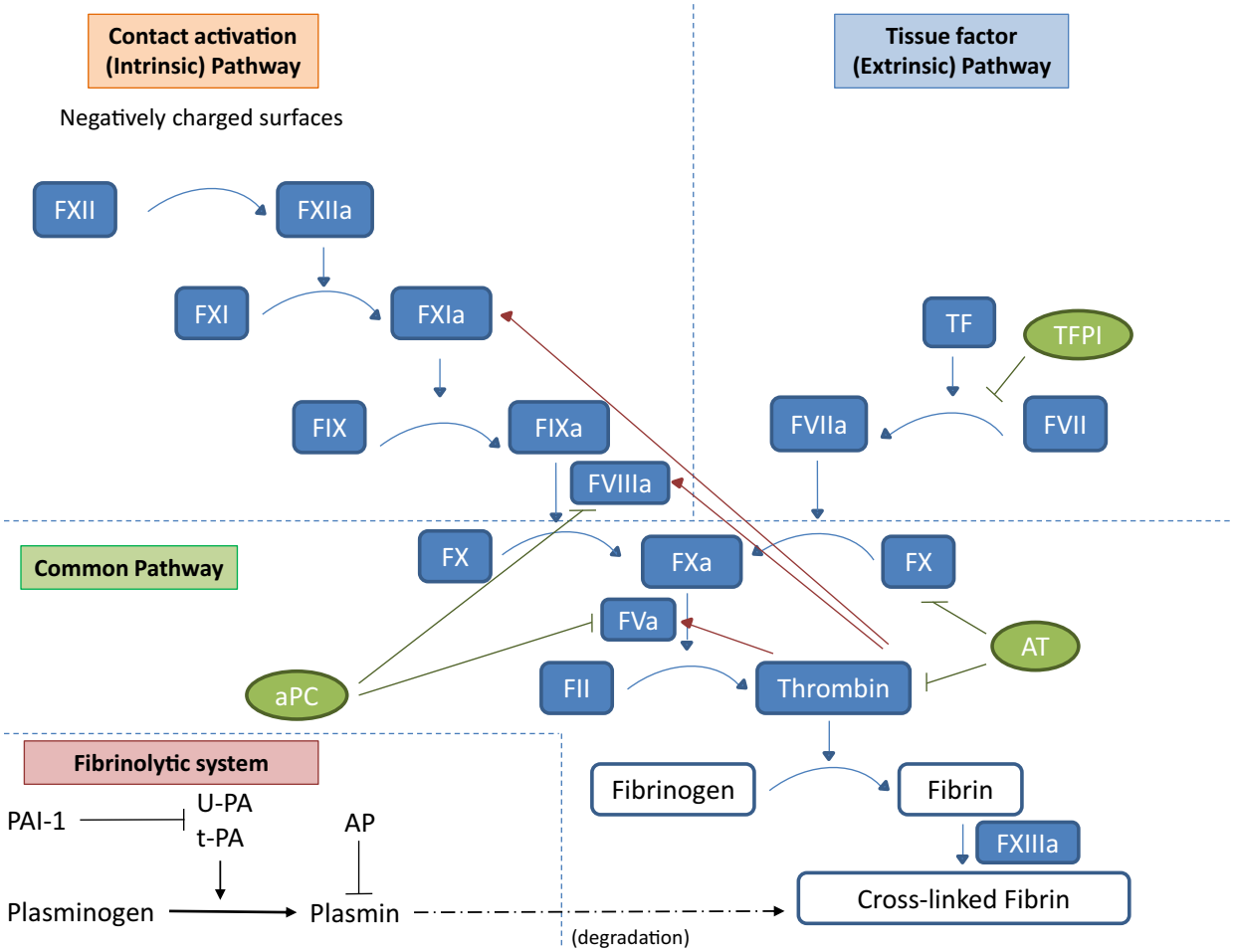

FIGURE 1 | Schematic representation of the coagulation cascade and the fibrinolytic system. In the coagulation cascade, the *extrinsic pathway* starts with tissue factor (TF) and activated factor VII (FVIIa). Tissue factor in physiological conditions is not in contact with the blood; however, damage of blood vessel structure can expose this membrane-associated glycoprotein to the blood, thus activating the extrinsic pathway of the coagulation cascade. TF can also be expressed by mononuclear cells or endothelial cells in response to inflammatory mediators. The TF/FVIIa complex promotes the activation of factor X (FXa). In the *intrinsic pathway*, negatively charged surfaces (such as phospholipids and polyphosphates from activated platelets) activate FXII, initiating a cascade leading to FXa. The *common pathway* FXa, along with the cofactor FVa, converts prothrombin (FII) to thrombin (FIIa). Finally, thrombin activates fibrinogen into fibrin and FXIIIa acts on fibrin strands to form a fibrin mesh. Regulation of coagulation activation occurs by three distinct natural anticoagulant pathways: AT (which blocks FXa and thrombin), TFPI (which inhibits the tissue factor–factor VIIa complex), and aPC (which proteolytically degrades factor Va and factor VIIIa). Natural regulators of the coagulation cascade are indicated in green: TFPI, AT, and aPC. Once the first thrombin is produced, it induces the propagation of the coagulation cascade (red arrows) but also a feedback regulation through aPC. In the fibrinolytic system, uPA and tPA catalyze the proteolysis of plasminogen into plasmin, which, in turn, degrades fibrin. Inhibition of the plasminogen system occurs by specific PAIs and by α2-AP at the level of plasmin.

Coagulation factor II or pro-thrombin, once activated into thrombin, can prime the complement system through a direct interaction with C5 (25). On the other hand, it is now clear that complement activation through C5 splits products and that C5b-9 can trigger the coagulation cascade promoting tissue factor expression by several cell types, including monocytes and endothelial cells (26). This positive feedback loop represents a powerful amplification mechanism of innate immune system activation.

Tissue factor is emerging as an important player not only in hemostasis and thrombo-inflammatory diseases but also in non-coagulant signaling pathways mainly through the family of G-protein-coupled receptors PARs (27).

Thrombin, other than having a key role in coagulation cascade, modulates immune and non-immune cell functions interacting with PARs (28). These seven transmembrane domains proteins are activated by a peculiar proteolytic mechanism. The protease cleaves the N terminal extracellular domain of the receptor, leading to the exposure of a tethered ligand that interact with the extracellular loop 2 domain and initiate receptor signaling. Thrombin can activate PAR-1, PAR-3, and PAR-4 (29). Interestingly, thrombin is not the only coagulation factor that

can interact with PARs. Indeed, coagulation factors VIIa and Xa are known activators of PAR-2 (28). PARs are expressed by several immune cells, thus representing the main link between coagulation and innate immunity (30), and their expression levels have been described as implicated in the pathogenesis of several kidney diseases (31).

Through their activation, coagulation factors may play a central role in promoting the release of pro-inflammatory cytokines and chemokines from different cell types including dendritic cells, monocytes, lymphocytes (32–34), endothelial cells (35, 36), glomerular mesangial cells, pericytes, and epithelial cells (37) participating in the modulation of the innate and adaptive arms of immune response. It has been recently demonstrated that interleukin (IL)-1α is directly activated by thrombin pointing the importance of this pathway not only for normal physiology but also in the pathogenesis of inflammatory and thrombotic diseases (38). Similarly, several factors in the coagulation and fibrinolytic pathways can trigger either proinflammatory or anti-inflammatory host responses.

Coagulation and intravascular thrombus growth *in vivo* can be induced by neutrophil-derived serine proteases and nucleosomes (39). Neutrophil serine proteases play an important role as regulators of cell signaling and immune response, in particular against microbial threats (40). Interestingly, neutrophils produce a pool of FXII, functionally distinct from hepatic-derived FXII, specifically involved into neutrophil trafficking at sites of inflammation, through urokinase plasminogen activator (uPA) receptor (uPAR) and Akt signaling (41). FXIIa-induced neutrophil activation is also involved in macrophage polarization and induces T-cell differentiation, all contributing to host defense against pathogens (42).

COAGULATION CASCADE AND ISCHEMIA/REPERFUSION INJURY

The coagulation system activation has been suggested to play a significant role in several pathologic processes involving the kidney graft such as the early posttransplant period (43). In this first phase, the main issue is represented by the exposure of the graft to ischemia and a subsequent reperfusion. It is well known that ischemia/reperfusion injury is a leading cause of acute kidney injury (AKI) (44). In the setting of kidney transplantation, the graft experiences a prolonged cold ischemia after harvesting and brief warm ischemia followed by reperfusion during the transplantation procedure. The tissue damage featuring ischemia/reperfusion in renal transplant recipient is represented by tubular cell apoptosis and interstitial inflammation, a pathogenic event underlying an early posttransplant form of AKI known as delayed graft function (DGF) (45). This early posttransplant event, complicating between 15 and 30% of kidney transplantations, does not only represent a delay in graft function recovery but dramatically influences the long-term outcome of the transplanted organs. Indeed, there are several evidences that grafts experiencing DGF present a significantly lower survival (46); thus, prevention of DGF might significantly improve the long-term outcome of kidney grafts.

The key role of innate immunity in transplant-associated ischemia/reperfusion damage leading to DGF is clearly suggested by the strong expression of pro-inflammatory mediators, cytokines, and chemokines and by the priming of the coagulation cascade leading to monocyte–macrophage recruitment and interstitial infiltration (47–49). The coagulation cascade is strongly activated at the time of transplantation mainly owing to the induced vascular expression of tissue factor after ischemia/reperfusion (50). Damage of blood vessels induces the formation of tissue factor-activated factor VII, which, in turn, activates factor X and ultimately activates factor II (prothrombin) to IIa (thrombin).

There are several direct and indirect evidences that suggest the activation of coagulation at this stage as a key potential mediator in determining graft quality and outcome (37, 51).

Thrombin, locally released, may contribute to inflammation by the stimulation of PARs expressed by several resident cells including endothelial, tubular epithelial, and mesangial cells with the subsequent activation of signaling pathways leading to the production of cytokines and growth factors (52). Akt-mammalian target of rapamycin-S6k and NF-kB-inducing kinase (NIK)-NF-kB axis are two signaling pathways regulating cell survival and inflammation. The activation of both Akt and NIK-NF-kB signaling was detected in graft biopsies from DGF patients and were both triggered by thrombin in cultured proximal tubular cells, inducing a nuclear translocation of the active form of Akt and a time-dependent increase of NIK phosphorylation (53). Activated Akt is able to phosphorylate several substrates in the cytoplasm and nucleus. In transplantation, Akt-mTOR-p70S6k pathway is inhibited by rapamycin. Indeed, the use of this treatment is associated with prolonged period of DGF (54). On the other hand, NF-kB induces several genes involved in inflammatory response (55). In addition, the simultaneous activation of these two keys signaling pathways in a pig model of ischemia/reperfusion injury and in human biopsies from DGF correlated with tubulointerstitial and glomerular fibrin deposition in both cases (53). Besides participating in the activation of innate immunity featuring ischemia/reperfusion injury underlying DGF, the activation of the coagulation system in this setting might represent a pivotal element in the priming of the adaptive alloimmune response. Indeed, infiltrating dendritic cells during DGF express PAR-1 and are localized in the proximity of fibrin and C3 deposits (56). *In vitro* PAR-1 activation on dendritic cells caused a significant upregulation of C3 and complement receptor expression. In addition, thrombin caused a marked reduction of IL-10 mRNA abundance and an increase of IL-12/IL-17 p40 gene expression and promoted C3a ability to increase IL-12/IL17 mRNA abundance. These changes can influence dendritic cells ability to induce interferon-γ production by T cells, thus suggesting the activation of a T helper-1 bias (56).

Another factor associated with renal damage after kidney transplantation is increased tissue factor expression. Tissue factor is one of the main activators of the coagulation system; it is a membrane component of many cells and is released in blood plasma after a cell damage. It has been recently demonstrated that recipients with postoperative complications had much higher concentrations of tissue factor in renal vein in the first

minute during reperfusion, which may be associated with kidney damage (57).

Altogether, these data strongly support the strict correlation between the signaling pathways activated by coagulation factors in resident and infiltrating cells and the ischemia/reperfusion damage. Thus, the coagulation cascade might represent a potential therapeutic target to reduce ischemia/reperfusion-induced injury in the attempt to lessen the incidence of DGF.

COAGULATION CASCADE AND CHRONIC REJECTION

Interstitial activation of the coagulation cascade is also a common feature of tubular-interstitial nephritis and in particular of renal graft rejection. Chronic allograft rejection is the final common pathway of progressive graft injury and is characterized by interstitial fibrosis and tubular atrophy along with a variable degree of chronic glomerular changes, and this condition is associated with both glomerular and interstitial fibrin deposition (58). Fibrin is known to induce migration and proliferation of the major cell types involved in interstitial fibrosis, such as macrophages and vascular smooth muscle cells (59, 60). In addition, PAR-1 expression is significantly increased at both the glomerular and tubular levels in chronic graft rejection, clearly suggesting that thrombin may significantly modulate resident cell activation in this setting (58).

Following kidney transplantation, microvascular thrombi along with endothelial dysfunction and fibrin deposition in the kidney graft were recognized as a challenging cause of graft dysfunction, particularly for highly sensitized kidney transplant recipients. Indeed, in these patients, the activation of endothelial cells by the involvement of both complement and coagulation pathways is a response to human leukocyte antigen (HLA) antibodies (61).

In vitro study suggests that incubation with specific anti-HLA antibody against a line of human umbilical endothelial cells induces upregulation of tissue factor expression and activity, defining a role in direct initiation of coagulation by HLA antibody binding (62). Yamakuchi et al. have shown that, both *in vitro* and *in vivo*, anti-HLA antibody induces endothelial cell exocytosis and, in turn, an increased expression of von Willebrand factor (vWF) (63).

Increasing evidence underlie that in response to HLA antibodies, both complement and coagulation cascades can be activated (20, 61); however, traditional views of HLA antibodies have focused on the initiation of complement, and studies relating to the initiation of coagulation by HLA antibody activation are still limited. Understanding the complexities of these interactions remains a challenge especially within the scope of intervention.

THE FIBRINOLYTIC SYSTEM IN INNATE IMMUNITY

The fibrinolytic system removes fibrin from the vascular system, preventing clots from occluding the vessel. The activation of the fibrinolytic system is controlled by coagulation itself. Activation of the fibrinolytic system depends mainly upon uPA and tissue-type plasminogen activator (tPA) that catalyze the proteolysis of plasminogen into plasmin, which, in turn, degrades fibrin, preventing its extracellular deposition (**Figure 1**). uPA and tPA, with the glycosylphosphatidylinositol (GPI)-linked uPAR, are expressed by a variety of cells of hematopoietic origin and are upregulated during infections and inflammation (64). The interaction between the plasminogen and complement systems at sites of tissue injury represents an important bridge between innate and adaptive immunity (65). Indeed, the activating effect of plasmin on complement cascade both *in vitro* and *in vivo* is well known (66).

The plasminogen system is also important for tissue remodeling, in particular through its specific endogenous inhibitor plasminogen activator inhibitor-type 1, PAI-1. Inhibition of the plasminogen system occurs by specific PAIs and by α2-antiplasmin (AP) at the level of plasmin (**Figure 1**). PAI-1 is not expressed in the healthy kidney (67). In contrast, its expression is significantly increased in several primary and secondary glomerulonephritis, and several evidences suggest a key role for this protease inhibitor in the progression of renal damage in this setting (68). In particular, PAI-1 may play an inhibitor effect also on different proteases involved in the degradation of extracellular matrix, including metalloproteases, thus promoting tissue fibrosis.

FIBRINOLYSIS ACTIVATION IN KIDNEY TRANSPLANTATION

The key role of fibrinolytic system is to dissolve blood clots. Plasmin is converted in the active form from plasminogen by the action of tPA. Fibrinolysis deficiency often occurs not only in hemodialyzed patients (69) but also in kidney transplant recipients. The association between fibrinolytic decrease and impaired renal function would be supported by the observation that successful renal transplantation is characterized by an improved fibrinolysis (70), although transplant patients continue to present hypofibrinolysis, which, however, unlike in dialysis patients, is secondary to a rise in PAI-1 (71). A role in the increase of the plasma levels of PAI-I in transplant patients is, indeed, played by the immunosuppressive therapy and in particular by corticosteroids and cyclosporine, in addition to the effects of metabolic disorders featuring transplant recipients, such as insulin resistance or dyslipoproteinemia (71).

Fibrinolysis activation can be influenced by the cause of cadaveric kidney donors' death. Zietek et al. demonstrated that organ donors who had injured death, such as road traffic injury, were characterized by an intensive activation of fibrinolytic process when compared with non-injured donors, which showed instead intensive activation of blood coagulation (72). This observation suggests that injured donors have a lower risk of microthrombi and fibrin deposits than non-injured donors; however, the clinical importance of this observation and the effect on the outcome of the graft are still unknown.

Renal ischemia/reperfusion injury leading to DGF is characterized by an increased expression of uPA and uPAR (73). uPAR plasma concentrations in kidney allograft recipients have been demonstrated to be significantly and negatively correlated with graft function in deceased-donor renal transplant recipients (74). uPAR plays an important role in the structure and function of the renal filtration barrier (75); thus, its upregulation is responsible of proteinuria (72). It has also been demonstrated that tPA regulates renal neutrophil influx in kidney ischemia and reperfusion injury (76), and recent evidences propose a proinflammatory role for postischemic tPA release (77). However, further and larger clinical investigations are needed.

Glomerular and interstitial fibrin deposition observed in chronic renal allograft failure is caused by, other than the pro-fibrotic effect of thrombin, a dysregulation of the plasminogen/plasmin system that results in a subsequent inhibition of fibrinolysis (78). Corticosteroids may induce PAI-1 expression in tubular cells *in vitro* and *in vivo* in chronic renal graft failure (58). Thrombin itself can induce at the tubular and endothelial levels PAI-1 gene and protein expression (13). Immunosuppressive therapy can significantly influence these events. Indeed, rapamycin, an mTOR inhibitor, has a beneficial effect on chronic graft injury progression because patients converted from calcineurin inhibitors to rapamycin present a significant reduction in the progression of interstitial fibrosis and glomerulosclerosis (79). Interestingly, in these patients, the introduction of rapamycin significantly reduced glomerular and tubulointerstitial expression of PAI-1. *In vitro* data demonstrate that rapamycin reduced PAI-1 expression induced by both thrombin and CD40L in proximal tubular cells (79).

TARGETING COAGULATION FACTORS TO LIMIT THE ACTIVATION OF INNATE IMMUNITY

A potential limit in the use of activated coagulation factors as therapeutic targets to limit the activation of the innate immunity is represented by their persistence within the fibrin clots where they are usually protected from the majority of their soluble natural or synthetic inhibitors. The fibrin deposition due to the activation of the coagulation system is in fact under the tight control of fibrinolysis, a highly regulated enzymatic process that prevents accumulation of intravascular fibrin (80).

Several animal models have been used to demonstrate the specific effect of coagulation inhibition on the graft outcome. In a porcine preclinical model of renal auto-transplantation, peri-transplantation treatment with an anti-factor Xa compound protected kidney grafts, improving functional recovery and reducing chronic lesions (81). The same authors demonstrated in this animal model the benefits of a preservation anticoagulation therapy using a specific and effective dual-molecule anti-coagulation factors Xa/IIa, which was able to protect the kidney by reducing thrombin generation with subsequent early functional recovery and decreased chronic lesions (82). These results opened the way to the potential therapeutic benefits of the use of anticoagulation in the reperfusion solutions, an approach that may significantly limit the potential side effects due to inhibition of the coagulation system in a surgical setting. Although phase I human studies have been conducted on these synthetic anticoagulants with a dual mechanism of action (83), their use in the clinical practice needs to be further investigated. One approach to possibly improve outcomes after transplantation is the use of C1 inhibitor, a serum protease inhibitor (serpin) that binds covalently and inactivates C1r, C1s, and mannan-binding protein blocks. Other than the classical and lectin pathways of complement activation, C1 inhibitor also inhibits the contact, coagulation, and kinin systems (84). It has been demonstrated in some trials and studies in kidney transplant recipients that C1 inhibitor treatment may reduce ischemia/reperfusion injury and DGF (85); however, small patient numbers are included in these studies, most results are from single centers, and differences in study design preclude meta-analyses.

CONCLUDING REMARKS

There is an increasing body of evidence that suggests that the activation of the coagulation system is associated with several pathological conditions of the graft and that it is invariably associated with a significant activation of the innate branch of the immune system also through a complex interaction with the complement proteolytic cascade. This activation plays a key part in the pathogenesis of the progression of graft injuries independently of their etiology. It is conceivable that in this setting coagulation cascade activation might mediate the activation of the innate immune system and might be involved, through its effects on dendritic cells, in the amplification of alloantigen-specific adaptive response. Thus, coagulation might be considered a potential therapeutic target to modulate innate immunity and to prevent progressive graft damage. Ideally, the inhibition of coagulation activation or of its downstream cellular effects should be associated with the inhibition of the harmful effects of the complement cascade priming. Targeting the common checkpoints shared by the two proteolytic systems might represent a promising therapeutic approach to reduce the activation of the innate immune system in kidney transplantation and reduce its deleterious consequences on graft function and survival.

AUTHOR CONTRIBUTIONS

GS, PP, LG, and GG designed, wrote, and critically revised the review. FR analyzed fibrinolysis activation in kidney transplantation. GC analyzed the cross talk between coagulation, fibrinolysis, and complement system. All authors contributed to the article and approved the submitted version.

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