DISSERTATION

Unravelling the Influence of Donor Age on Renal Allograft Survival
-
Entschlüsselung des Einflusses des Spenderalters auf das renale Allograftüberleben

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List of Abbreviations

Abbreviation	Definition
%	percent
°C	degree celsius
µl	microliter
2D	two-dimensional
3D	three-dimensional
AKI	acute kidney injury
APC	antigen-presenting cell
BCL	b-cell lymphoma
BSA	bovine serum albumin
CAD	coronary artery disease
CD	cluster of differentiation
CKD	chronic kidney disease
CM	central memory
CMV	cytomegalovirus
CNI	calcineurin inhibitors
CO_2	carbon dioxide
CREP2	creatinine plus ver.2
CTLA4	cytotoxic t-lymphocyte-associated protein 4
DAPI	4',6-diamidin-2-phenylindol
DC	dendritic cells
DGF	delayed graft function
DMEM	dulbecco's modified eagle medium
DMSO	dimethylsulfoxid
DN	double negative
DNA	deoxyribonucleic acid
EBV	Epstein Barr virus
ECM	extracellular matrix
eGFR	estimated glomerular filtration rate
EM	effector memory
EMRA	terminally differentiated effector cells

ESRD	end-stage renal disease
FCS	fetal calf serum
FoxP3	forkhead-box-protein p3
GvHD	graft-versus-host-disease
h	hour
HLA-A2	human leukocyte antigen serotype a2
HLA-DR	human leukocyte antigen-dr isotype
HPRT1	hypoxanthine phosphoribosyl transferase 1
HVCR1	hepatitis a virus cellular receptor 1
ICAM	intercellular adhesion molecule
IFNγ	Interferon γ
IL	interleukin
IL-12/15/18R	interleukin 12/15/18 receptor
IL-2/5/13/17	interleukin 2/5/13/17
ILC	innate lymphoid cell
IRI	ischemia-reperfusion injury
KIM-1	kidney injury molecule-1
KLRG1	killer cell lectin like receptor g1
L/D	live/dead
MAIT	mucosa-associated invariant t
MCP1	monocyte chemotactic protein 1
MELC	multi-epitope ligand cartography
MFI	mean fluorescent intensity
mg	milligram
MHC	major histocompatibility complex
min	minute
ml	millilitre
MNCs	mononuclear cells
MR1	major histocompatibility complex class I related gene protein
mTOR	mechanistic target of rapamycin
NaCl	sodium chloride
NAFLD	non-alcoholic fatty liver disease
NFAT	nuclear factor of activated T-cells

NK	natural killer cell
ns	not specified
OCT	optimal cutting temperature compound
P/S	penicillin/streptomycin
PBMCs	blood peripheral mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD-1	programmed cell death protein 1
PMA	phorbol myristal acetat
POD	post operative day
PTEC	proximal tubule epithelial cells
RANTES	regulated upon activation, normal t cell expressed and secreted
RCC	renal cell carcinoma
RNA	ribonucleic acid
ROI	region of interest
RORγt	rar-related orphan receptor gamma
RPMI	medium developed at “roswell park memorial institute”
RT	room temperature
SASP	senescence-associated secretory phenotype
SD	standard deviation
SOT	solid organ transplantation
TCR	t cell receptor
TGF-β	transforming growth factor-β
TNFa	tumour necrosis factor a
TR	tissue-resident
Treg	regulatory t cells
TRM	tissue-resident memory
tSNE	t-distributed stochastic neighbour embedding
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor

Abstract

Background: Kidney transplantation is a common treatment for end-stage renal disease (ESRD). However, various immunological factors influence allograft outcomes. This thesis aimed to explore the impact of donor age and the role of natural killer (NK) cells in kidney transplantation, with the objective of improving transplant outcomes.
Methods: Murine kidney transplantation models were used to investigate the underlying immunological mechanisms for allograft rejection. Comprehensive immunophenotyping was performed to assess the cellular composition of young and aged kidneys. The effects of the senolytic drug ABT-263 on recipient immune response were evaluated.
Results: Immunophenotyping revealed significant differences of immune cell populations between young and aged kidneys. Aged kidneys exhibit higher frequencies of effector memory T cells and senescent T and NK cells, with altered functional characteristics. In the transplantation model, recipient-derived T cells infiltrating aged grafts exhibited enhanced proinflammatory and cytotoxic responses. However, pretreatment of aged kidney donors with ABT-263 resulted in a modified recipient immune response characterised by reduced production of effector molecules and improved graft function.
Conclusion: The findings of this thesis highlight the importance of immunological considerations in kidney transplantation. Donor age influences the immunological profile of transplanted kidneys, leading to altered recipient-mediated immune responses. These insights provide a basis for future strategies aimed at enhancing graft survival and function in kidney transplantation.

Zusammenfassung

Hintergrund: Die Nierentransplantation ist eine gängige Behandlung für Nierenerkrankungen im Endstadium (ESRD). Allerdings beeinflussen verschiedene immunologische Faktoren die Ergebnisse der Allotransplantation. Ziel dieser Arbeit war es, den Einfluss des Spenderalters und die Rolle der natürlichen Killerzellen (NK) bei der Nierentransplantation zu untersuchen, um die Transplantationsergebnisse zu verbessern.
Methoden: Anhand von Nierentransplantationsmodellen der Maus wurden die immunologischen Mechanismen untersucht, die der Abstoßung von Allotransplantaten zugrunde

liegen. Zur Beurteilung der zellulären Zusammensetzung von jungen und gealterten Nieren wurde eine umfassende Immunphänotypisierung durchgeführt. Die Auswirkungen des Senolytikums ABT-263 auf die Immunantwort des Empfängers wurden untersucht.

Ergebnisse: Die Immunphänotypisierung ergab signifikante Unterschiede in der Population der Immunzellen zwischen jungen und gealterten Nieren. Gealterte Nieren wiesen eine höhere Häufigkeit von Effektor-Gedächtnis-T-Zellen und seneszenten T- und NK-Zellen mit veränderten funktionellen Eigenschaften auf. Im Transplantationsmodell zeigten vom Empfänger stammende T-Zellen, die in gealterte Transplantate eindringen, verstärkte proinflammatorische und zytotoxische Reaktionen. Die Vorbehandlung gealterter Nierenspender mit ABT-263 führte jedoch zu einer veränderten Immunantwort des Empfängers, die durch eine geringere Produktion von Effektormolekülen und eine verbesserte Transplantatfunktion gekennzeichnet war.

Schlussfolgerung: Die Ergebnisse dieser Arbeit zeigen, wie wichtig immunologische Überlegungen bei der Nierentransplantation sind. Das Alter des Spenders beeinflusst das immunologische Profil der transplantierten Niere, was zu veränderten, vom Empfänger vermittelten Immunreaktionen führt. Diese Erkenntnisse bilden eine Grundlage für künftige Strategien zur Verbesserung des Überlebens und der Funktion des Transplantats bei Nierentransplantationen

1 Introduction

1.1 Kidney Transplantation and Donor Age

The incidence of Chronic Kidney Disease (CKD) is soaring worldwide with an estimated global prevalence of 11-13% (1). Transplantation serves as a gold standard for meeting the aggravating medical cost associated with end-stage renal disease (ESRD). There is a huge and still growing discrepancy in organ demand and organ supply. According to Eurotransplant (2022), there were 10,373 individuals on the waiting list for organ transplants in 2022, of which 2,970 received a transplant (Eurotransplant, 2022). This gap is primarily met by accepting expanded criteria donors (ECD). Expanded criteria donors (ECD) are defined as either over the age of 60 or between 50-59 with two of the following condition: death from cerebrovascular accident, a history of hypertension, and terminal serum creatinine more than 1.5 mg/dL (2). Regardless of the recipient age, there is a 1.7 times higher risk of graft failure associated with ECD (3).

Donor age is one of the most critical determinants of kidney transplantation outcomes. In a study comprising 50,322 patients undergoing primary deceased-donor kidney transplantations, stratification of donors into several age groups showed that patient survival at 5 and 10 years, adjusted for various variables, began to decline in donors aged 36–40 years. The recipients were divided into age groups of 40, 41–54, and 55 years. The data suggested that over the age of 40 years, the impact of donor age on patient survival is a continuous variable, influencing the survival rates across all recipient age categories (4). Kidneys procured from older donors have a higher risk of complications, including ischaemia-reperfusion injury (IRI), delayed graft function (DFG), acute rejection rates, chronic allograft dysfunction (CAD), graft failure, and malignancies (5-12). DGF is a common complication of kidney transplantation, which occurs when the transplanted kidney does not function immediately after transplantation. DGF is associated with a higher risk of acute rejection, infection, and reduced long-term graft survival (13). CAD is characterised by a gradual decline in graft function over time. It is a significant contributor to long-term failure of kidney transplants, and its progression may be expedited in aged kidneys owing to age-related alterations in the immune system, inflammation, and oxidative stress (10, 14-18). Older kidney grafts from donors are more prone to injury and damage owing to a decrease in renal reserve, reduced capacity for repair and regeneration, and increased susceptibility to oxidative stress and inflammation (13, 19). Furthermore, aged kidney

grafts are more immunogenic and elicit a stronger immune response than young grafts (20, 21).

1.2 Age-related Deterioration in Kidney

The kidney undergoes age-related changes associated with a decline in function. Aging is often characterised by the progressive loss of physiological integrity caused by the accumulation of damage, deterioration of protein function, and impaired organelle function (22). Aging results in the alteration of kidney morphology and the formation of renal cysts, which are more prevalent in individuals over the age of 40. Although these cysts were previously thought to be harmless, recent studies have linked them to hypertension (23), reduced kidney size and function (24, 25), and potential kidney damage (26). Another vital indicator of damage is the kidney volume, which declines with age. It is estimated that there is a 10% decrease in kidney volume per decade of life. The decrease in kidney volume is due to a reduction in parenchymal thickness and cortical volume, accompanied by an increase in medullary volume, until the age of 50 years. The aging process in individuals older than 50 years has led to a notable decline in overall kidney volume. This phenomenon is characterised by a substantial difference in kidney mass, ranging from 20% to 25%, when comparing individuals aged 30–80 years (27).
In addition, kidneys undergo various intricate changes during aging. These include nephrosclerosis, thickening of the glomerular basement membrane (28), broadening of the mesangium, and increased accumulation of the extracellular matrix (29). Nephrosclerosis, a primary pathological factor, involves the loss of nephrons, hypertrophy of the remaining nephrons, and arteriosclerosis. It significantly contributes to age-related structural and functional changes in the kidneys (29-31). Approximately 6,000-6,500 nephrons are lost each year after the age of 30 years due to nephrosclerosis, and glomerular sclerosis is estimated to exceed 10% (32). However, recent studies have indicated that the magnitude of nephron depletion may be considerably higher than previously estimated (33). For instance, one study revealed a 48% decline in nephron counts among individuals aged 70-75 years comparison to those aged 18-29. These changes can have significant implications for kidney function, which can affect the overall health and quality of life in older individuals (32).

The functional decline observed in older kidneys is associated with age-related morphological changes. To measure this decline, researchers typically use total glomerular filtration rate (GFR). Numerous studies have investigated the rate of renal function decline associated with aging and have reported annual mean GFR ranging from 0.4 2.6 mL/min/year (34). GFR usually decreases by approximately 5-10% per decade after the age of 35 years (35). Additionally, research suggests that about half of individuals aged 70 and above have an estimated GFR of less than 60 mL/min/1.73 m^2 (36). The decline in GFR with age is thought to be due to nephron loss, which increases the risk of kidney disease and mimics the pathobiology of a remnant kidney (37). This age-related decline in renal function has significant implications, including an increased risk of acute kidney injury (AKI) (38) and chronic kidney disease (CKD) (39). Furthermore, this decline in renal function is a critical factor in determining outcomes for recipients of kidney transplantation. To address the increasing organ demand, kidneys from extended-criteria donors, including older donors, are now accepted [10]. However, this approach introduces a new set of complexities.

1.3 Cellular Senescence and Renal-Inflammaging

Despite considerable progress in understanding the pathophysiology of renal aging, there remains a lack of comprehensive understanding of the precise cellular and molecular mechanisms responsible for age-related phenomena such as nephron depletion and extracellular matrix build-up. Nevertheless, accumulating evidence indicates that cellular senescence plays a pivotal role in the progression of renal aging and age-associated disorders (40). The significance of cellular senescence in renal aging has come to the forefront as several studies have showed the intricate connection between senescent cells and kidney function. Baker et al. (2016) and Valentijn et al. (2018) demonstrated that age-related increases in senescent cells within the kidney are correlated with the development of renal lesions (41, 42). Baker et al. (2016) found that eliminating senescent cells systemically can prevent age-related glomerulosclerosis, suggesting potential therapeutic value in targeting senescence (41). The relationship between senescence and renal aging presents a paradox, primarily affecting tubular cells, whereas lesions mainly appear in glomeruli, as observed by Melk et al. (2004) and Baker et al. (2016) (41, 43). This complex relationship emphasises the need for comprehensive examination of various cell types to understand the role of senescent cells in the aging kidney. Valentijn

et al. (2018) extended the spectrum of senescence involvement in pathological conditions characterized by glomerular lesions, such as diabetic nephropathy, focal segmental glomerular sclerosis, or glomerulonephritis. This study highlights the involvement of podocytes, mesangial cells, endothelial cells, and parietal epithelial cells in the senescence process, emphasising their significance in diverse renal pathologies (42). Moreover, findings by Omori et al. (2020) revealed that senescence influences endothelial cells in both the liver and kidneys under both physiological and pathological conditions. This broader understanding highlights the systemic nature of senescence and its pervasive effect on various cell types in the kidney (44). This aging process is characterised by an increase in senescent cells, reduced function, and development of interstitial fibrosis (IF) and tubular atrophy (TA). These effects have been observed in both human cases and model organisms, such as mice that have undergone IRI and transplantation (45, 46). These cells have been shown to affect renal anatomy and are largely responsible for renal cysts due to an increase in the size of the medulla and shrinkage of the cortex. In another study, these cells were linked to renal tubulointerstitial injury and tubular atrophy, and their deletion reduced inflammatory cell infiltration (47). Furthermore, they have been linked to poor survival and susceptibility to IRI in older organs (48).

1.4 Senolytic Therapies in Kidney Transplantation

Cellular senescence is a hallmark of aging that maintains genomic integrity and prevents mutations, thus evading cancer. Cellular senescence drives cells into a state of no return, which is characterised by permanent cell cycle arrest. It is maintained by the expression of mitochondrial anti-apoptotic factors known as B-cell lymphoma 2 (BCL-2) family proteins, specifically BCL-w, BCL-XL, BCL-2, A1, and MCL-1 (Figure 15). Typically, senescence stimuli (i.e., DNA damage, stress, and telomere shortening) induce the expression of pro-apoptotic proteins, which are antagonised by anti-apoptotic proteins to counteract the risk of cell loss. The balance between these two factors determines the final fate of the cell (49). Senescent cells are enlarged, vacuolated, and granulated in morphology with increased lysosomal content and activity (β-galactosidase) (50). The senescence state is maintained by p16 and p21 mediated pathway, which are used as the main markers for the identification of these cells. Senescent cells are resistant to apoptosis but remain active and secrete a highly conserved subset of signature molecules, termed SASP.

The composition of the senescence associated secretory phenotype (SASP) is heterogeneous in nature but mainly comprises proinflammatory factors (for example, IL-1, 6, 8, GM-CSF, and MCPs), growth factors (for example, GRO alpha, HGF), profibrotic factors (for example, TGF-β), migration (for example, MMPs), and connective tissue growth factors (51, 52). SASP is released both as soluble factors and vesicles and possesses the capacity to exert a far-reaching detrimental impact on neighboring cells. In addition, SASP contributes to age-related chronic low-grade sterile inflammation called “inflammaging”. Inflammaging is a highly complex and multifactorial phenomenon. It is a collective consequence of several factors, including mitochondrial dysfunction, cellular senescence, and the primary dysregulation of immune cells. Although the inflammaging conferred by these components differs mechanistically, their net effect seems to converge and intertwine, conceivably in a cause-and-consequence manner (Figure 15).

Renal senescent cells can accumulate in tissues with age and contribute to age-related decline in tissue function. Recent research suggests that they may also play a role in the pathogenesis of various diseases, including CKD (53). These cells may contribute to the increased immunogenicity of the graft as they can secrete proinflammatory molecules and activate the immune system. Additionally, they may impair the regenerative capacity of the graft by secreting factors that inhibit the growth and differentiation of neighbouring cells. These studies showed a positive correlation between these cells and renal pathologies, suggesting that targeting them has the therapeutic potential to ameliorate donor-age-related deterioration and improve graft outcomes.

Senolytic therapies are a relatively new class of drugs that target senescent cells, which are cells that have stopped dividing but remain metabolically active and can contribute to age-related diseases and inflammation. These therapies have been shown to improve outcomes in aging and age-related diseases in animal models (48, 54-69). There is growing interest in their potential use in aging and transplantation. In the context of aged organ transplantation, these therapies may be used to improve the quality of donated organs by removing senescent cells, which can contribute to inflammation and tissue damage. This may help reduce the risk of complications, such as DFG and chronic graft dysfunction. Additionally, these therapies may improve the immune response to the transplanted organ, reduce inflammation, and improve the overall health outcomes in older patients.

1.5 Mechanisms of Immunosuppression in Solid Organ Transplantation

Given the heightened susceptibility of aged organs to dysfunction and damage, as compared to their younger counterparts, the risk of rejection and the likelihood of inferior transplantation outcomes are amplified. Therefore, immunosuppression in aged kidneys may present a formidable challenge. That is why understanding immunosuppressive strategies for improvement are pivotal and important research focus along with the donor age. Immunosuppression is the process of suppressing the recipient's immune response to prevent rejection of a transplanted organ. Immunosuppressive regimens such as calcineurin inhibitors (CNIs), mammalian target of rapamycin (mTOR) inhibitors, glucocorticoids, and antibodies are commonly used to suppress the recipient's immune system and prevent allograft rejection after solid organ transplantation (SOT). CNIs both CyA and Tacrolimus (Tac) were first made available in clinics in the early 1980s and early 1990s, and they continue to be the most frequently used therapy after transplantation (71). Tac and CyA have different chemical structures: Tac is a macrocyclic lactone (70), whereas CyA is a cyclic endecapeptide (71). Inhibiting calcineurin, which suppresses T cell activation, a significant lymphocyte component of the adaptive immune response crucial for graft rejection, is a common pharmacodynamic property of immunosuppressants. When CyA penetrates T cells, it attaches to immunophilins, such as cyclophilins and FK-binding proteins, with high affinity. Calmodulin-dependent serine/threonine phosphatase, calcineurin, is bound by a combination of CNIs and immunophilins, which inhibit its activity. In the absence of this, nuclear factor of activated T cell (NFATc) members are dephosphorylated by active calcineurin, which activates them. The expression of numerous cytokines and costimulatory molecules necessary for the full activation of T cells, such as IL-2, IL-4, and CD40 ligands, is regulated by activated NFATc proteins, as they translocate into the nucleus and interact with other transcription factors such as activating protein-1 (AP-1). T cell-mediated immunological responses following dephosphorylation and activation of NFATc are prevented by CyA-mediated inhibition of calcineurin (72-74).

1.6 Limitations and Side Effects of Calcineurin Inhibitors

Commonly used immunosuppressive medications have successfully decreased the frequency of acute T cell-mediated rejection, leading to one-year graft survival rates for renal allografts between 88% and 95% (75). However, long-term graft survival and recurrence of acute rejection events continue to be the main barriers to successful transplantation.

This may be because of the insufficient influence of CNIs on other immune subpopulations. Furthermore, chronic CNI use is linked to nephrotoxicity and other negative consequences such as hypertension, hyperplasia, diabetes mellitus, and neurotoxicity (76-79). However, the primary goal is to develop regimens that are both safe and efficient, reducing the need for immunosuppressants while maintaining patient and graft survival. Therefore, it is critical to identify effector immune cells that threaten transplant survival but are not currently targeted by CNIs.

1.7 NK Cells and CNIs

Although the effects of CNIs on T cells have been extensively investigated, their effects on other immune cells, including NK cells, remain unclear. The latter can be partly ascribed to NK cell subpopulations, investigated effector functions, CNI dosing, and different model systems examined (80-82). For example, in vitro stimulation of human NK cells with IL-2 and IL-15 demonstrated that CD56dim NK cell proliferation was selectively inhibited by CyA, whereas CD56bright NK cell proliferation was unaffected. On the other hand, when stimulated with IL-12 and IL-18, NK cells that had been exposed to CyA released a larger percentage of interferon (IFN) than the controls (80). Notably, most of the data are derived from in vitro stimulation of PBMCs isolated from healthy donors or transplant recipients who were receiving CyA or Tac immunosuppression (83) and are conflicting.

Similarly, it has been demonstrated that cyclosporine and tacrolimus, significantly inhibit NK cell function in vitro. Both the drugs reduced NK cell degranulation and IFN-γ production in a dose-dependent manner. Even at therapeutic trough levels, there was a substantial reduction in NK cell activity, with greater effects at peak drug levels. This suggests that CNIs used in transplant immunosuppression, particularly cyclosporine and tacrolimus, have a notable suppressive effect on NK cell function, which may have implications for the immune response in transplant recipients, especially in the early post-transplant period (82). Additionally, Neudorfl et al. showed that the type of CNI immunosuppression utilized had an impact on the NK cell repertoire in the peripheral blood of kidney transplant recipients, which was different from that of healthy persons (83). Wai et al., on the other hand, showed that CyA and Tac have no influence on the proliferation and cytotoxicity of NK cells utilizing rat NK cell lines, primary NK cells, and peripheral blood cells from liver transplant patients (84). It is important to note that most of the data were derived from in

vitro stimulation of PBMCs isolated from healthy donors or transplant recipients receiving CyA or tacrolimus immunosuppression. The lack of information regarding the distinct effects of CyA and Tac on the NK cell phenotype and function is problematic. Therefore, we aimed to understand the effects of immunosuppression on NK cells following acute and chronic transplantation *in vivo*.

2 Aim of the Study

Donor age is a major determinant of transplantation outcome (10, 89, 90). Aging organs exhibit a heightened vulnerability to ischaemic damage, delayed graft function, and diminished graft survival rates. Hence, a comprehensive understanding of the age-related immunogenic aspects inherent in aged kidneys is imperative to alleviate organ damage after transplantation. Moreover, an understanding of age-specific changes will pave the way for pioneering strategies in pretransplant organ management by addressing the pressing issue of organ shortage and optimising the utilisation of organs procured from older donors. The aging process results in the accumulation of cells undergoing senescence, a state of permanent cell cycle arrest characterised by the release of various growth hormones and pro-inflammatory cytokines, collectively referred to as the SASP. This accumulation contributes to a chronic low-grade inflammatory milieu known as inflammaging, which has detrimental effects on the tissue microenvironment. Chronic secretion of SASP disrupts healthy tissue regeneration and function, thereby contributing to age-related deterioration of organ function. Senescent cells have been implicated in age-associated diseases, such as glomerular disease, chronic allograft nephropathy, osteoarthritis, and atherosclerosis. Additionally, elevated expression of the senescence-associated marker p16INK4a in donor organs is correlated with poor transplantation outcomes (85, 86). Given these considerations, the present study aimed to target senescent cells to improve the long-term quality and outcomes of grafts.

In addition, the unfavourable prognosis associated with older organs necessitates the development of more effective post-transplant management strategies to enhance long-term survival. Therefore, the secondary objective of this thesis was to gain a comprehensive understanding of the effects of the calcineurin inhibitor CyA on NK cells. Extensive research has demonstrated a critical role of NK cells in allograft rejection (87-90). However, the specific effects of various immunosuppressants, including calcineurin inhibitors, on NK cells *in vivo* remain to be elucidated. Our hypothesis posits that CNIs inadequately target NK cells, thereby affecting allograft outcomes after solid organ transplantation. The principal objective of this study was to investigate the potential influence of CyA on NK cell migration, phenotype, and function in a murine model of kidney transplant.

Consequently, the aim of this study was to:

1. Characterise cellular and immunosenescent aspects associated with inflammaging in aged organs.

2. Perform a comparative analysis of the alloimmune response elicited by old and young grafts employing a murine model of kidney transplantation.
3. Elucidating the role of inflammaging in immunogenicity linked to donor age.
4. Comprehensive phenotypic and functional analyses of NK cells after kidney transplantation in the presence of CyA and Tac.
5. Investigate the synergistic effect of NK cell depletion in conjunction with CyA or tacrolimus to preclude acute allograft rejection and improve long-term graft outcomes.
6. Exploring the potential of NK cell-neutralising antibodies to reduce the necessity for daily immunosuppression.

By addressing these objectives, this study aimed to advance our understanding of the complex interplay among donor age, inflammaging, NK cells, and immunosuppressive treatments, ultimately contributing to the development of improved strategies for transplantation management and long-term graft survival.

3 Materials and Methods

3.1 Materials

3.1.1 Reagents

Reagents utilized in the experiments are enlisted in the table below:

Table 1: List of Reagents Used in Experiments. (Source: He, A., Sarwar, A., et al. AJT (2022)).

Reagents	Manufacturer
Albumin Fraktion V	Carl Roth, Karlsruhe, Germany
Bovine serum albumin (BSA)	Carl Roth, Karlsruhe, Germany
Brefeldin A	Cayman, Michigan, USA
CD45 sort Beads	Miltenyi, Bergisch Gladbach, Germany
Collagenase IV	Gibco, Thermo Fisher, Darmstadt, Germany
Collagenase II	Gibco, Thermo Fisher, Darmstadt, Germany
Dimethylsulfoxide (DMSO)	Honeywell, Seelze, Germany
DNAse I	Sigma Aldrich, Merck, Darmstadt, Germany
FC Block	Miltenyi, Bergisch Gladbach, Germany
Fetal calf serum (FCS)	Gibco, Thermo Fisher, Darmstadt, Germany
Fix/Perm kit	BD, Heidelberg, Germany
Ionomycin	Sigma Aldrich, Darmstadt, Germany
Isoflurane	FEM Apotheke
Histopaque 1083	Sigma Aldrich, Darmstadt, Germany
L-Glutamine (200mM)	Thermo Fisher Scientific, Schwerte, Germany
M-MLV Reverse Transcriptase (Enzyme)	Promega
M-MLV Reverse Transcriptase (Buffer)	Promega
Monensin	Biolegend, San Diego, USA
oligo(dT) primer	Thermo Fisher Scientific, Schwerte, Germany
Paraformaldehyde 2% (methanol- and RNAse-free)	Electron Microscopy Sciences, Hatfield, PA, USA
Penicillin/Streptomycin (P/S)	Sigma Aldrich, Merck, Darmstadt, Germany
Phorbol myristat acetat (PMA)	Sigma Aldrich, Darmstadt, Germany
Phosphate buffered saline (PBS)	Biochrom, Berlin, Germany
RevertAidTM H Minus M-MuLV Reverse Transcriptase	Promega, Heidelberg, Germany
RPMI-1640 medium	Corning, Falcon, Kaiserslautern, Germany
RNeasy Mini Kit	Qiagen, Hilden, Germany
Taqman® gene expression assays (ADD assays)	Life Technologies, Carlsbad, CA, USA
Senescence Detection Kit	Abcam
Sodium chloride (NaCl) 0.9 % injection solution	B. Braun Melsungen AG, Melsungen, Germany

3.1.2 Equipment

Table 2: List of Equipment and Manufacturer. (Source: He, A., Sarwar, A., et al. AJT (2022)).

Equipment	Manufacturer
ABI 7500 Fast Real-Time PCR System	Life Technologies, Carlsbad, CA, USA
Bench EnvairEco Safe basic Plus	Envair, Emmendingen, Germany
Bench HeraSafe	Kendro, Langenselbold, Germany
CASY Cell counter & analyzer	OLS, Bremen, Germany
CASY Cups	OLS, Bremen, Germany
Centrifuge 5415 R	Eppendorf, Hamburg, Germany
Centrifuge Multifuge 3 S-R	Thermo Fisher Scientific, Schwerte, Germany
Centrifuge Universal 320 R	Hettich, Tuttlingen, Germany
Cover slides (APES-coated)	Menzel-Gläser, Braunschweig, Germany
Cryomold	Sakura, Alphen aan den Rijn, Netherlands
Cryotome NX80	ThermoFisher, Waltham, MA, USA
FACS LSR-Fortessa X-20	BD, Heidelberg, Germany
FACS tubes	Corning, Falcon, Kaiserslautern, Germany
FlowJo Version 10	BD, Heidelberg, Germany
Freezer -80°C	Heraeus, Hanau, Germany
Fridge Freezer Combination	Liebherr Medline, Bulle, Switzerland
GraphPad Prism 7	GraphPad Software, Inc., San Diego, USA
Incubator	Eppendorf, Hamburg, Germany
Liquid nitrogen tank Cyrosystem 2000	MVE, Jüchen, Germany
LS Column	Miltenyi, Bergisch Gladbach, Germany
Lithium Heparin blood selection tubes	Greiner Bio-One, Frickenhausen, Germany
MACS MultiStand	Miltenyi, Bergisch Gladbach, Germany
PCR Eppis	Carl Roth
Petri dishes	Corning, Falcon, Kaiserslautern, Germany
Pipetboy	Neolab, Heidelberg, Germany
Pipette/Diluter CAVRO XL3000	Tecan GmbH, Crailsheim, Germany
Pipets	Eppendorf, Hamburg, Germany
QuadroMACS Separator	Miltenyi, Bergisch Gladbach, Germany
Roche/Hitachi Cobas C 701/702 system	Roche Diagnostics, Mannheim, Germany
Silicone sheets	Life Technologies, Carlsbad, CA, USA
Strainer 100 µl	Corning, Falcon, Kaiserslautern, Germany
Syringes 2 ml	B. Braun Melsungen AG, Melsungen, Germany
Vacuum pump	Neolab, Heidelberg, Germany
Vortex Shaker	Neolab, Heidelberg, Germany
Waterbath SW22	Julabo, Seelbach, Germany

3.2 Methods

The sources of methods are:
Ashraf, M. I., Sarwar, A., et al. (2019). Natural killer cells promote kidney graft rejection independently of cyclosporine a therapy. Frontiers in immunology, 10, 2279.
He, A., Sarwar, A., et al. (2022). Renal inflamm-aging provokes intra-graft inflammation following experimental kidney transplantation. American Journal of Transplantation, *22*(11), 2529-2547.

3.2.1 Animals

Male C57BL/6J and BALB/c mice were purchased from Charles River Laboratories International (UK). C57BL/6J mice, aged 3 months (weighing 24 to 30 g) and 18 months (weighing 30 to 40 g) were used. Animals were housed and cared for according to the ethical guidelines outlined in the National Academy of Sciences "Principles of Laboratory Animal Care" (NIH Publication No. 86–23, revised 1985). The experimental protocols involving animal subjects were approved by Landesamt für Gesundheit und Soziales Berlin (approval no. G0089/16) (91, 92).

3.2.2 Kidney Transplantation

Kidney transplantation was performed according to previously published methods (93). Briefly, the left donor kidney was retrieved and rinsed in situ with histidine-tryptophane-ketoglutarate solution. End-to-side anastomoses between the recipient's abdominal aorta, inferior vena cava, and donor renal arteries were performed using the knotless technique. The ureter and bladder were anastomosed directly for urinary tract repair. Cold and warm ischaemia for 30 min each was applied to the allografts. On postoperative day

(POD), the contralateral native kidney was removed 24 h before the animals were sacrificed. In the long-term survival group, the opposing kidney was removed on POD 7. Animals with postsurgical complications were excluded from the study (91, 92).

3.2.3 Assessment of Renal Function

Serum samples were stored at −20°C for the evaluation of renal function. Urea and creatinine levels were measured using a urea/BUN assay and CREP2 Creatinine Plus version 2, respectively (91, 92).

Table 3: List of Antibodies Used for Extracellular Staining. (Source: He, A., Sarwar, A., et al. AJT (2022)).

Antibody	Clone	Fluorochrome	Manufacturer
CD3	REA641	PerCP-Vio700	Miltenyi, Bergisch Gladbach, Germany
CD4	RM4-5	BV711	Biolegend, San Diego, USA
CD8	53-6.7	BV605	Biolegend, San Diego, USA
NKp46	29A1.4	FITC	ThermoFisher, Darmstadt, Germany
NKp46	29A1.4	APC	Biolegend, San Diego, USA
DX5	DX5	APC-Cy7	Biolegend, San Diego, USA
CD11b	M1/70	BV785	Biolegend, San Diego, USA
CD27	LG.3A10	Pe-Cy7	Biolegend, San Diego, USA
CD49a	Ha31/8	BV711	BD, Heidelberg, Germany
NKG2D	C7	FITC	Biolegend, San Diego, USA
MHCII	M5/114.15.2	BV650	Biolegend, San Diego, USA
CXCR6	SA051D1	PE-Dazzle594	Biolegend, San Diego, USA
B220	RA3-6B2	BV510	Biolegend, San Diego, USA
CD103	2E7	PE	Biolegend, San Diego, USA
CD69	H1.2F3	BV421	Biolegend, San Diego, USA
CD45	30-F11	UV395	BD, Heidelberg, Germany
Viability dye	none	BV510	Biolegend, San Diego, USA
CD44	IM7	APC-eFluor 780	ThermoFisher,Darmstadt, Germany
CD62L	MEL-14	BV421	Biolegend, San Diego, USA
KLRG1	2F1/KLRG1	PE-Dazzle594	Biolegend, San Diego, USA
CD69	H1.2F3	PE-Cy7	Biolegend, San Diego, USA
CD25	PC61	PE	Biolegend, San Diego, USA

3.2.4 Cell Isolation and *Ex vivo* Phenotypic Analysis

To isolate mononuclear cells (MNCs), kidney and liver tissues were treated with collagenases IV and II, respectively, along with DNase X in RPMI medium for 45 min at 37°C (91, 92). Isolated leukocytes from the liver were subjected to Histopaque density gradient centrifugation, whereas $CD45^+$ microbeads were used to purify leukocytes from the kidney. Leukocytes from the lymph nodes and spleen were separated using density gradient centrifugation. Bone marrow leukocytes were obtained by flushing murine bones with RPMI medium, followed by centrifugation. Flow cytometry analysis was performed on 1×10^6 leukocytes treated with antibodies for extracellular and intracellular staining. Using FACS LSR Fortessa X-20, 100.000 events from a live gate were recorded to determine the number of cells. The data were analysed using FlowJo software (version 10.0). The gating strategy is illustrated in Figure 1 and 2 (A and B). Dendritic cells (DCs) were isolated from young and old BALB/c kidneys using the MojoSort panDC kit (BioLegend) and MACS LD-type columns (Miltenyi Biotec). Splenic $CD3^+$ T cells were enriched in C57BL/6 mice sensitised to BALB/c hearts. Co-culture of labelled cells with DCs was performed, and T cell proliferation was assessed by flow cytometry after 4 days (91, 92).

Table 4: List of Antibodies Used for Intracellular Staining. (Source: He, A., Sarwar, A., et al. (2022). Renal inflamm-aging provokes intra-graft inflammation following experimental kidney transplantation. American Journal of Transplantation, *22*(11), 2529-2547).

Antibody	Clone	Fluorochrome	Manufacturer
FoxP3	MF-14	AF 647	Biolegend, San Diego, USA
Ki67	SolA15	Pe-Cy7	eBioscience
CD122	TM-β1	FITC	eBioscience
CD107a	1D4B	PE	Biolegend, San Diego, USA
Granzyme B	NGZB	PE-Cy7	eBioscience
IFNγ	XMG1.2	BV650	Biolegend, San Diego, USA
IL-17	TC11-18H10.1	BV421	Biolegend, San Diego, USA
IL-10	JES5-16E3	PE	Biolegend, San Diego, USA
Perforin	S16009B	APC	Biolegend, San Diego, USA

3.2.5 Ex vivo Functional Analysis

To assess the functional profile, NK and T cells isolated from murine organs were stimulated *ex vivo* with the following reagents:50 ng phorbol 12-myristate 13-acetate (PMA), 1

µg ionomycin calcium salt, 5 µg/ml brefeldin A, and 2 µM monensin. The cells were stimulated for 4 h at 37°C and 5% CO2. After stimulation, cells were washed and stained with the antibodies listed in Table 4. Lysosome-associated membrane protein-1 (LAMP-1) expression in CD107a cells was used as a measure of degranulation. For intracellular staining, cells were fixed and permeabilised using a transcription factor staining buffer. The staining was recorded using a flow cytometer (LSR Fortessa X-20). Figure 2 (A and B) illustrates the gating strategy for the major cell populations analysed in this study (91, 92).

3.2.6 Histology

For histological analysis, kidney sections of 1-2 µm thickness were cut and dewaxed. The sections were then stained using various methods, including periodic acid-Schiff (PAS), haematoxylin and eosin (H&E), Elastica van Gieson staining kit (Merck) for connective tissue, monoclonal rabbit anti-CD3 antibody (clone SP7, Thermo Scientific, Waltham, MA, USA), and polyclonal goat anti-mouse C3d antibody. The samples were examined for necrosis, acute tubular damage, and glomerulitis. Semi-quantitative scores based on Banff criteria were assigned to each lesion as follows: acute tubular damage (0 = none, 1 = mild, 2 = moderate, 3 = severe), necrosis (0 = none, 1 = 1-10% of tissue, 2 = 11-20% of tissue, 3 = more than 20% of tissue), interstitial inflammation in the non-fibrotic cortex (0 = <10%, 1 = 11-25%, 2 = 26-50%, 2 = more than 50%), and glomerulitis (0 = none, 1 = present in at least one glomerulus). Scoring was performed in a blinded manner, based on the entire slide of each sample.

3.2.7 Immunofluorescence Microscopy

Immunofluorescence (IF) microscopy was performed as previously described (28). Briefly, the transplanted kidneys were removed, and a quarter of each kidney was fixed with PLP fixative overnight at 4°C. After washing with PBS, the kidneys were dehydrated in 30% sucrose in PBS overnight at 4°C, snap-frozen in OCT, and stored at −80°C. Kidney cryosections of 16 µm thickness were mounted on Superfrost Plus microscope slides (Thermo Fisher) and allowed to dry overnight. Blocking and staining were performed overnight at room temperature using purified or directly conjugated antibodies in a humidified box (Table S3). The following morning, after washing with PBS, the sections were stained with the appropriate secondary antibody for one hour at room temperature. Hoechst was

used for counterstaining, and the sections were mounted using Fluoromount-G (eBiosciences). The samples were scanned using a Zeiss LSM780 confocal microscope with a dry 20× 0.8 N.A. objective. Tiled z-stacks were acquired at 1024 × 1024 pixels with a line averaging of four and a pinhole size of 1. The acquired images were processed, and maximum intensity projection snapshots were generated using Fuji software (version 1.53r) (91, 92).

3.2.8 *In vivo* ABT-263 Treatment

C57BL/6J mice at 18 months of age were randomly divided into treatment and control groups. ABT-263 (navitoclax) was administered orally at a dosage of 50 mg/kg/day in two cycles, with a one-week interval between cycles. Corn oil was used as the vehicle (91, 92).

3.2.9 *In vivo* NK1.1 Treatment for NK Cell Depletion

On postoperative days (POD) 2 and POD+2, NK cells were depleted *in vivo* by intraperitoneal injection of an anti-mouse NK1.1 monoclonal antibody (200 µg; PK136, BioXCell, Lebanon, NH, USA). C57BL/6 recipients received subcutaneous injections of CyA starting from the day of kidney transplantation (POD0) until POD7 at a dosage of 10 mg/kg body weight. In the long-term survival group, CyA was administered daily for 14 d to prevent acute rejection. NK cell depletion was performed by intraperitoneal injection of the anti-mouse NK1.1 monoclonal antibody against POD-2 and POD+2 (91, 92).

3.2.10 β-Galactosidase Assay for Senescence Detection

Senescent cells were detected using a senescence detection kit following the manufacturer's instructions, with slight modifications. Briefly, tissue samples were embedded in OCT and stored at -80°C until staining. Tissue sections were fixed with the fixative solution provided in the kit for 10-15 minutes at room temperature. Subsequently, cells were incubated at 37°C for 1 h in a staining solution containing X-gal. Hematoxylin staining was used as a counterstain to enhance the contrast. The tissue samples were examined under a microscope at 200X total magnification to observe the development of blue colour (91, 92).

3.2.11 Real-Time and RT-PCR for Gene Expression Analysis

Total RNA was extracted from snap-frozen samples for gene expression analysis using the RNeasy Mini Kit according to the manufacturer's instructions. RNA integrity was assessed using NanoDrop 2000c spectrophotometre. Reverse transcription was performed using 2 µg of total RNA, oligo (dT) 18 primer, and M-MLV reverse transcriptase in a 40 µL reaction volume. Real-time Polymerase Chain Reaction (RT-PCR) for gene expression analysis was performed using Taqman® gene expression assays and an ABI 7500 Fast Real-Time PCR System. Samples with genomic DNA contamination were excluded from the analysis. Gene expression was normalised to that of the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) using the 2-ΔCt formula. For calculations involving both the housekeeping gene and gene of interest, the mean CT value from duplicates was used (91, 92).

Table 5: List of Gene Expression Assays Used in Experiments. (Source: He, A., Sarwar, A., et al. (2022). Renal inflamm-aging provokes intra-graft inflammation following experimental kidney transplantation. American Journal of Transplantation, 22(11), 2529-2547).

Gene	Clone	Manufacturer
MCP-1	Mm00441242_m1	ThermoFisher, Darmstadt, Germany
KIM-1	Mm00506686_m1	ThermoFisher, Darmstadt, Germany
P16^{INK4a}	Mm00494449_m1	ThermoFisher, Darmstadt, Germany

3.2.12 Flow Cytometry Analysis and Statistics

Briefly, 1 × 10^6 cells were stained with antibodies listed in Table 1. The stained cells were analysed using a FACS Fortessa X20 flow cytometer (BD Biosciences). Data analysis was performed using the FlowJo software (version 10.0; Tree Star Inc.). A gating strategy, as shown in Figures 1 and 2 (A and B), was employed to identify different lymphocyte subsets. The polyfunctionality was evaluated using Boolean gating. t-distributed stochastic neighbour embedding (t-SNE) plots, FlowSOM analysis, and heatmaps were generated using the Cytobank software (Beckman Coulter). Graphs were created and statistical analyses were conducted using GraphPad Prism version 8. Graft survival was analyzed using Kaplan-Meier plots, and the statistical significance of differences between survival curves were assessed using the log-rank test. For group comparisons, significant differences were calculated using either the Student’s t-test (for two groups) or the Kruskal-Wallis test (for multiple groups) after assessing the normality of the distribution using the D'Agostino and Pearson test (91, 92).

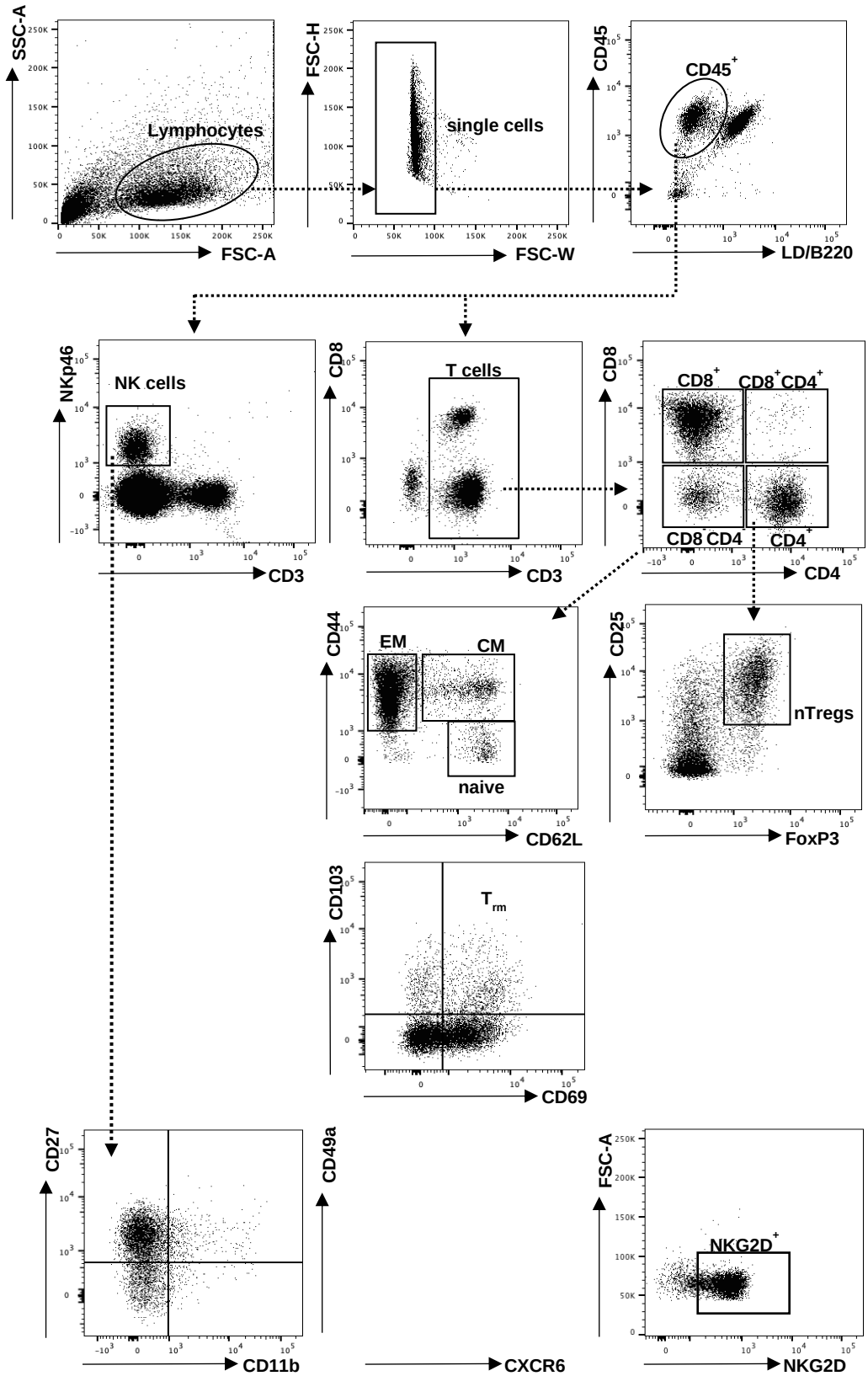

Figure 1: Gating Strategy for Identifying T and NK Cells. Lymphocyte subsets were examined using specific markers. The analysis involved the gating of live CD45⁺ B220 single lymphocytes, followed by further classification into different subtypes. The classification was based on parameters such as forward scatter (FSC-A), side scatter (SSC-A), effector memory (EM), central memory (CM), and tissue resident

memory (Trm) (Source: He, A., Sarwar, A., et al. (2022). Renal inflamm-aging provokes intra-graft inflammation following experimental kidney transplantation. American Journal of Transplantation, 22(11), 2529-2547).

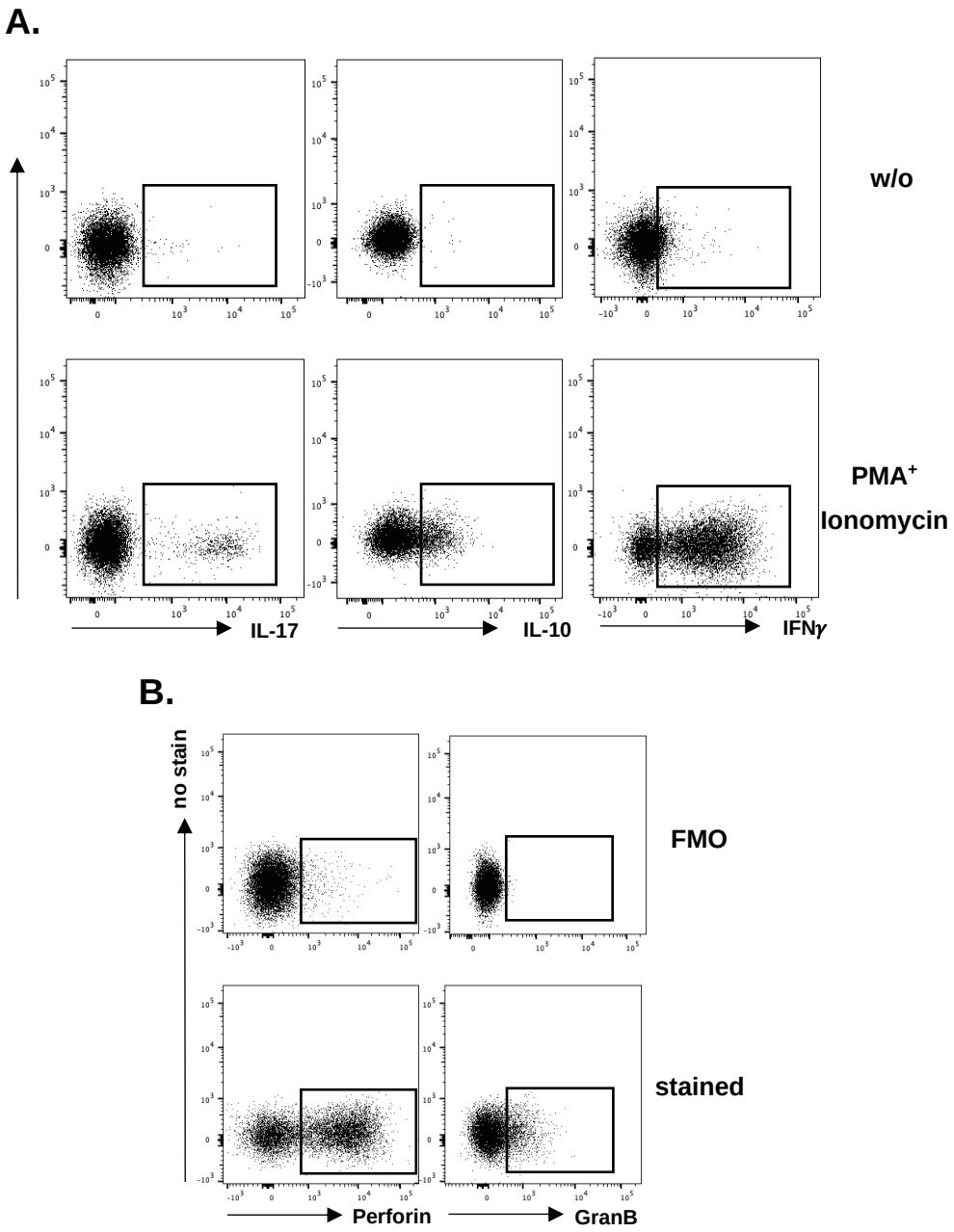

Figure 2: Gating Strategy for Assessing T and NK Cells Functional Profile. (A) The analysis of cytokines IL-17, IL-10, and IFNγ was performed by the following gating strategy. The gates were determined based on the respective unstimulated controls. (B) The analysis of cytotoxic mediators, perforin, and granzyme also employed a gating strategy, using the respective unstimulated controls as reference (Source: He, A., Sarwar, A., et al. (2022). Renal inflamm-aging provokes intra-graft inflammation following experimental kidney transplantation. American Journal of Transplantation, 22(11), 2529-2547).

4 Results

4.1 Aging Alters the Lymphocyte Composition in Organs

We employed the viSNE algorithm to analyse T cell diversity. By overlaying the generated viSNE maps with FlowSOM metaclusters, we identified various T-cell subpopulations based on the presence of their memory markers in both young and old kidneys which is consistent with the manual gating results (Figure 3A). To investigate how aging affects the distribution and surface phenotype of T and NK cell subsets in potential donor organs, a comprehensive phenotypic analysis of T and NK cells was conducted in non-lymphoid organs (kidney) as well as lymphoid organs (spleen and lymph nodes) Our results revealed significant alterations in the T cell compartment with age, characterized by a dominance of $CD3^+$ $CD8^+$ cells. This was evident through increased frequencies of $CD3^+$ $CD8^+$ cells and decreased frequencies of $CD3^+$ $CD4^+$ cells in all organs (91).

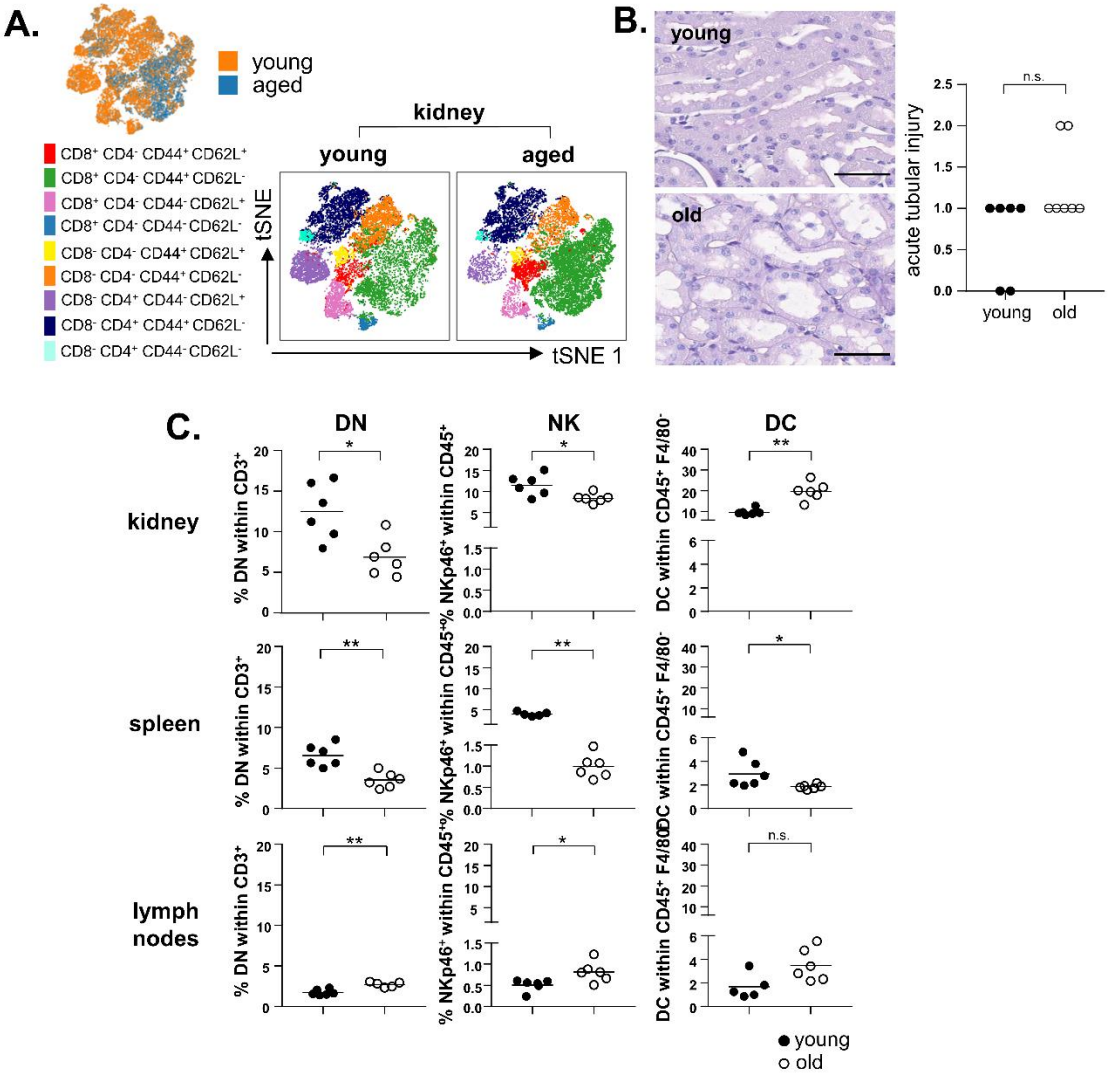

Figure 3: Impact of Aging on T, NK and DCs Composition in Organs. (A) Visualization of t-distributed stochastic neighbour embedding (viSNE) analysis was used to assess the clustering of viable $CD3^+$ T cell subsets in C57BL/6 kidneys based on the markers CD4, CD8, CD44, CD62L, CD103, CD69, and KLRG1, indicating age-related differences. The viSNE plots were generated by combining FCS files (n = 5-6) with an equal number of 17,674 $CD3^+$ events per file, using default parameters (1000 iterations, perplexity of 30, 0.5 Theta) in Cytobank. FlowSOM analysis of T cell subsets (right panel) was performed on the viSNE map, utilizing CD4, CD8, CD44, and CD62L for hierarchical clustering (B) Representative images of kidney histology from young and old mice (not transplanted) showing slightly more acute tubular injury in the kidneys of aged mice. The scale bar represents 50 µm. (C) $CD8^-$ $CD4^-$ T cells, NK and DC isolated from the kidney, spleen, and lymph nodes of young (3 months) and aged (20 months) C57BL/6 mice (5-6 animals per group). Statistical significance was determined using the Mann–Whitney U test, and the results are presented as means. *$p < .05$; **$p < .01$; n.s. = not significant (Source: He, A., Sarwar, A., et al. (2022). Renal inflamm-aging provokes intra-graft inflammation following experimental kidney transplantation. American Journal of Transplantation, 22(11), 2529-2547). For a detailed author contribution refer to the "Declaration of Own Contribution to the Publications" section in this thesis).

It is worth noting that elderly kidneys did exhibit a slightly more acute tubular injury (Figure 3B). Furthermore, the frequencies of double negative ($CD3^+$ $CD8^-$ $CD4^-$) T cells and $NKp46^+$ cells showed a significant decline with age, except in the lymph nodes, where both subsets exhibited a notable increase. Moreover, older kidneys exhibited a significant increase in $CD11c^+$ $MHCII^+$ DCs (Figure 3C). Using CD62L and CD44 expression, $CD8^+$ and $CD4^+$ T cells in mice were classified into memory and naïve phenotypes: the $CD44^-$ $CD62L^+$ population was identified as naïve, the $CD44^+$ $CD62L^+$ population was identified as central memory (CM), and the $CD44^+$ $CD62L^-$ population was identified as effector and/or effector memory (EM). It is well known that CD4 and CD8 T cells have different distributions in lymphoid and peripheral organs. In aged mice, the proportion of $CD4^+$ and $CD8^+$ T cells in the kidney was significantly skewed towards the effector memory (EM) phenotype, while the number of naive cells was reduced. However, we observed no significant differences in $CD8^+$ and $CD4^+$ CM between the young and old kidneys (91). Furthermore,

4.2 Ageing Affects the Treg Composition and Proliferation

We observed that old kidneys had a significantly smaller number of $CD4^+$ $CD25^+$ $FoxP3^+$ and $CD8^+$ $CD122^+$ Tregs compared to young kidneys (Figure 4A). While there was no difference in the frequencies of old and young splenic $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs, a significantly higher percentage of $CD8^+$ $CD122^+$ Tregs was observed in the old spleens compared with the young ones. However, both $CD4^+$ $CD25^+$ $FoxP3^+$ and $CD8^+$ $CD122^+$ Treg frequencies were significantly higher in old lymph nodes (Figure 4B and C). Overall, there was a significant reduction in the proliferation capacity of both classes of Tregs with age in all organs (Figure 4A-C).

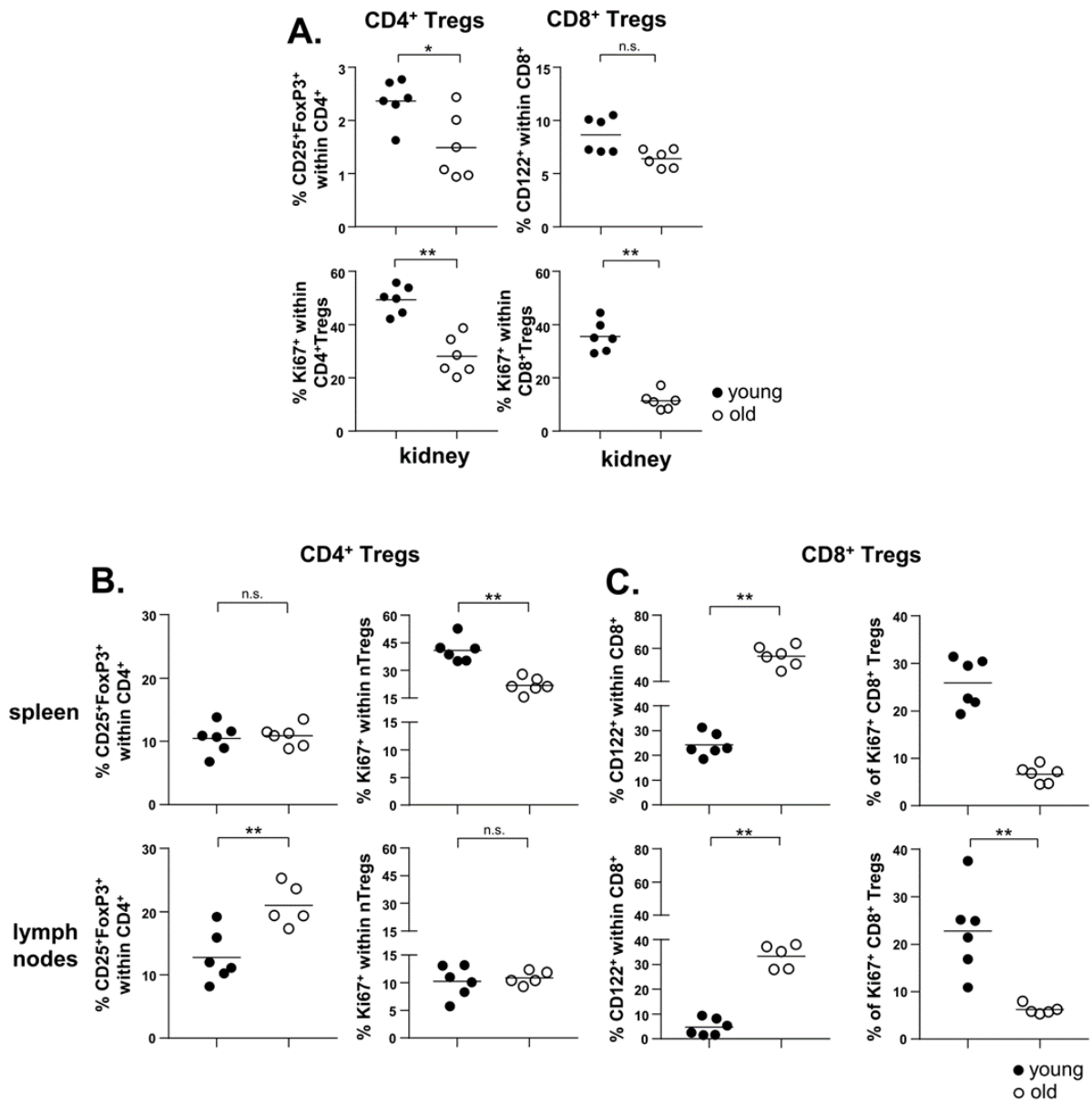

Figure 4: Impact of Aging on Treg Composition and Proliferation. (A) Frequencies of manually gated CD4⁺ CD25⁺ FoxP3⁺ Tregs CD8⁺ CD122⁺ nTregs, isolated from young (3 months) and aged (20 months) C57BL/6 mice kidney (B and C) and spleen and lymph node (5–6 animals/group). Proliferation of the Tregs was analysed based on Ki67 marker. Statistically significant differences were tested with Mann–Whitney U test and presented as means, *p < .05; **p < .01; n.s. = not significant. *p < .05; **p < .01; n.s. = not significant (Source: He, A., Sarwar, A., et al. AJT (2022). For a detailed author contribution refer to the "Declaration of Own Contribution to the Publications" section in this thesis).

4.3 Aging Shapes the NK Cell Repertoire, Both Phenotypically and Functionally

To determine whether the phenotype and function of NK cells change with age, we conducted a comprehensive characterization of NK cell subsets in both young and old kidneys.

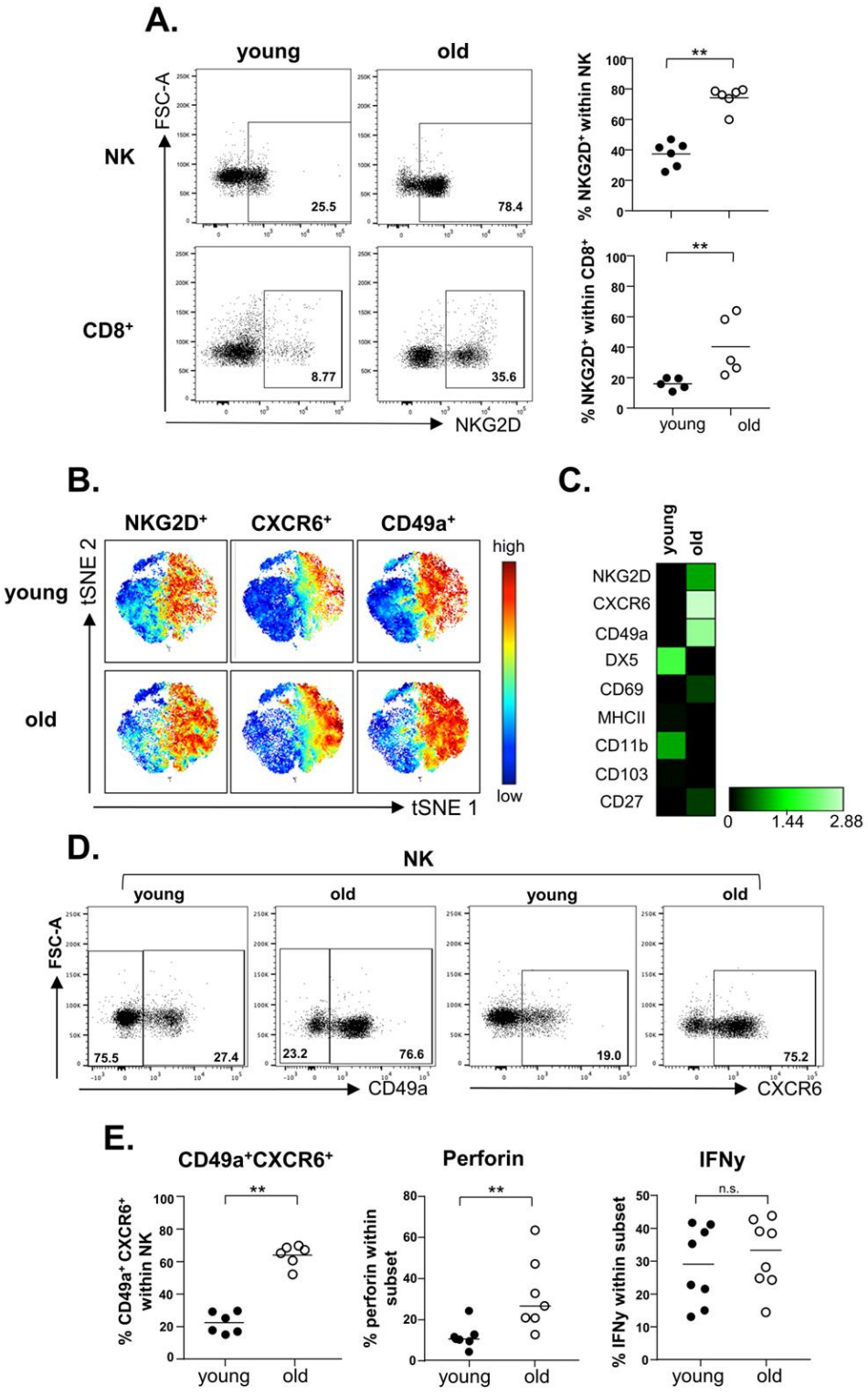

Figure 5: Accumulation of NKp46⁺ NK Cells with Upregulated NKG2D, CD49a, and CXCR6 in Senescent Murine Kidneys. (A) The figure shows representative FACS dot plots (left panel) and frequencies (right panel) of NKG2D⁺ NK cells and CD8⁺ T cells derived from young and aged C57BL/6 kidneys. (B) viSNE plots of viable NKp46⁺ CD3⁻ NK cells demonstrate increased expression of NKG2D⁺, CXCR6⁺, and CD49a⁺ in NK cells isolated from aged kidneys compared to young kidneys. Expression is color-coded based on relative intensity. The viSNE plots were generated using concatenated FCS files (n = 5-6) and considered markers such as NKG2D, CXCR6, CD49a, DX5, CD69, MHC II, CD11b, CD103, and CD27. A total of 25,111 events were sampled per file. (C) The heatmap plot presents the flow cytometric analysis of NK cell populations, illustrating the log2-transformed median of relative expression levels of the measured markers. (D) Exemplary dot plots display the expression of CD49a and CXCR6 on NK cells derived from

C57BL/6 kidneys. (E) The figure also provides the frequencies of CD49a$^+$ CXCR6$^+$ senescent NK cells isolated from kidney samples and their expression of perforin and IFNγ (Source: He, A., Sarwar, A., et al. AJT (2022). For detailed author contribution refer to the “Declaration of Own Contribution to the Publications” section in this thesis).

We observed that the frequencies of NKG2D$^+$ NK cells were significantly higher in the old kidneys, and a similar result was observed for CD8$^+$ cells (Figure 5A). Additionally, we analysed NK cells for CD49a$^+$ CXCR6$^+$ subsets. Previously, it has been reported that the CD49a$^+$ CXCR6$^+$ NK cell subset is highly enriched in the liver and referred to as tissue-resident NK (trNK) cells (97). We detected increased frequencies of the CD49a$^+$ CXCR6$^+$ NK cell subset in the aged kidney and found that these cells had significantly elevated perforin production compared to their counterparts in young kidneys. Furthermore, we observed that IFNγ producing CD49a$^+$ CXCR6$^+$ NK cells remained unchanged (Figure 5D-E). Heatmap analysis provides a detailed illustration of the phenotype of NK cells obtained from old kidneys, which confirms the accumulation of the NK cell phenotype with age, as well as a decrease in DX5$^+$ and CD11b$^+$ NK cells (Figure 5B).

4.4 Aging Modifies NK Cell Maturation

We characterized the maturation level of natural killer (NK) cells with respect to age using a previously described methodology **(94)** based on the expression of CD27 and CD11b. Briefly, NK cell maturation begins with the expression of CD27, with CD27$^-$ CD11b$^-$ cells representing an immature stage. The maturation process then progresses through CD27$^+$ CD11b$^-$ and CD27$^+$ CD11b$^+$ as intermediate stages, culminating in a highly mature phenotype such as CD27$^-$ CD11b$^+$. Each maturation stage is associated with a distinct functional characteristic. CD27$^-$ CD11b$^-$ NK cells have been reported to exhibit maturation potential, whereas CD27$^+$ CD11b$^-$ and CD27$^+$ CD11b$^+$ NK cells display the best cytokine production ability. NK cells exhibit high cytotoxicity at the CD27$^-$ CD11b$^+$ stage. Our results revealed no significant difference between young and old individuals in terms of the frequencies of CD27$^+$ CD11b$^+$ and CD27$^-$ CD11b$^-$ NK cells. However, the frequencies of CD27$^-$ CD11b$^-$ NK cells were significantly lower in older kidneys. Interestingly, a greater percentage of NK cells in old kidneys exhibited a CD27$^+$ CD11b$^-$ phenotype than in their young counterparts (Figure 6).

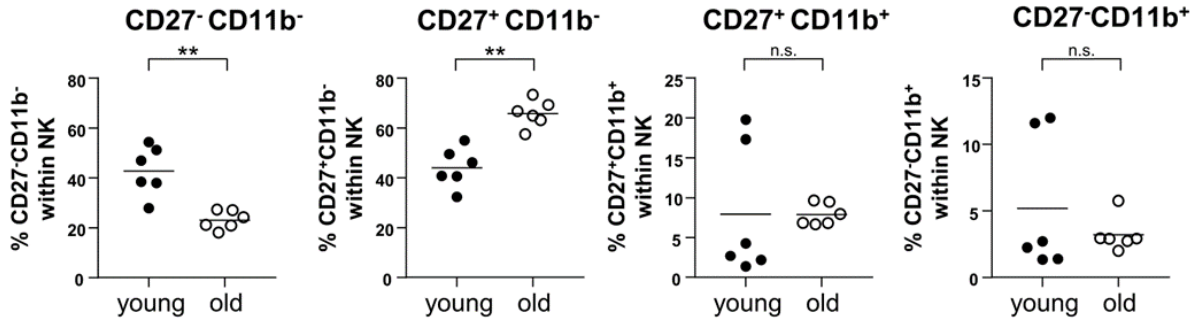

Figure 6: The Impact of Aging on NK Cell Maturation. This figure illustrates the frequencies of different NK cell subsets in young and aged C57BL/6 kidneys based on the expression of $CD27^+$ and $CD11b^+$. The data, obtained from 5-8 animals per group, were subjected to statistical analysis using the Mann-Whitney U test or unpaired t-test and presented as means. The figure indicates significant differences using **p < .01 and denotes non-significant differences as n.s. ((Source: He, A., Sarwar, A., et al. AJT (2022). For detailed author contribution refer to the "Declaration of Own Contribution to the Publications" section in this thesis).

4.5 Aging Alters T and NK Cells Effector Function

We analysed the functional profiles of T and NK cell subsets in young and old kidneys through ex vivo PMA/ionomycin activation. We found that T and NK cells from older organs tend to exhibit altered effector function compared to those from younger organs. This is characterized by higher cytotoxicity and a pro-inflammatory phenotype. Among the T cell subsets, $CD8^+$ and $CD4^+$ T cells in old kidneys displayed a significant pro-inflammatory status, as evidenced by higher production of IFNγ compared to their young counterparts. However, their degranulation capacity was significantly lower, as measured by CD107a. Additionally, granzyme B (GranB) capacity was significantly reduced, while perforin levels remained similar to the young kidneys.

We observed that $CD8^+$ cells isolated from the older kidney exhibited increased IL-10 production upon *ex vivo* stimulation (91). Moreover, NK cells from the older kidney displayed diminished effector function, demonstrated by reduced levels of granzyme B, perforin, and IFN-γ production. However, NK cells from the older kidney produced higher levels of IL-17 and IL-10 compared to those from the younger kidney. Furthermore, $CD8^-$ $CD4^-$ T cells from the older kidney exhibited elevated IL-17 production (Figure 7A).

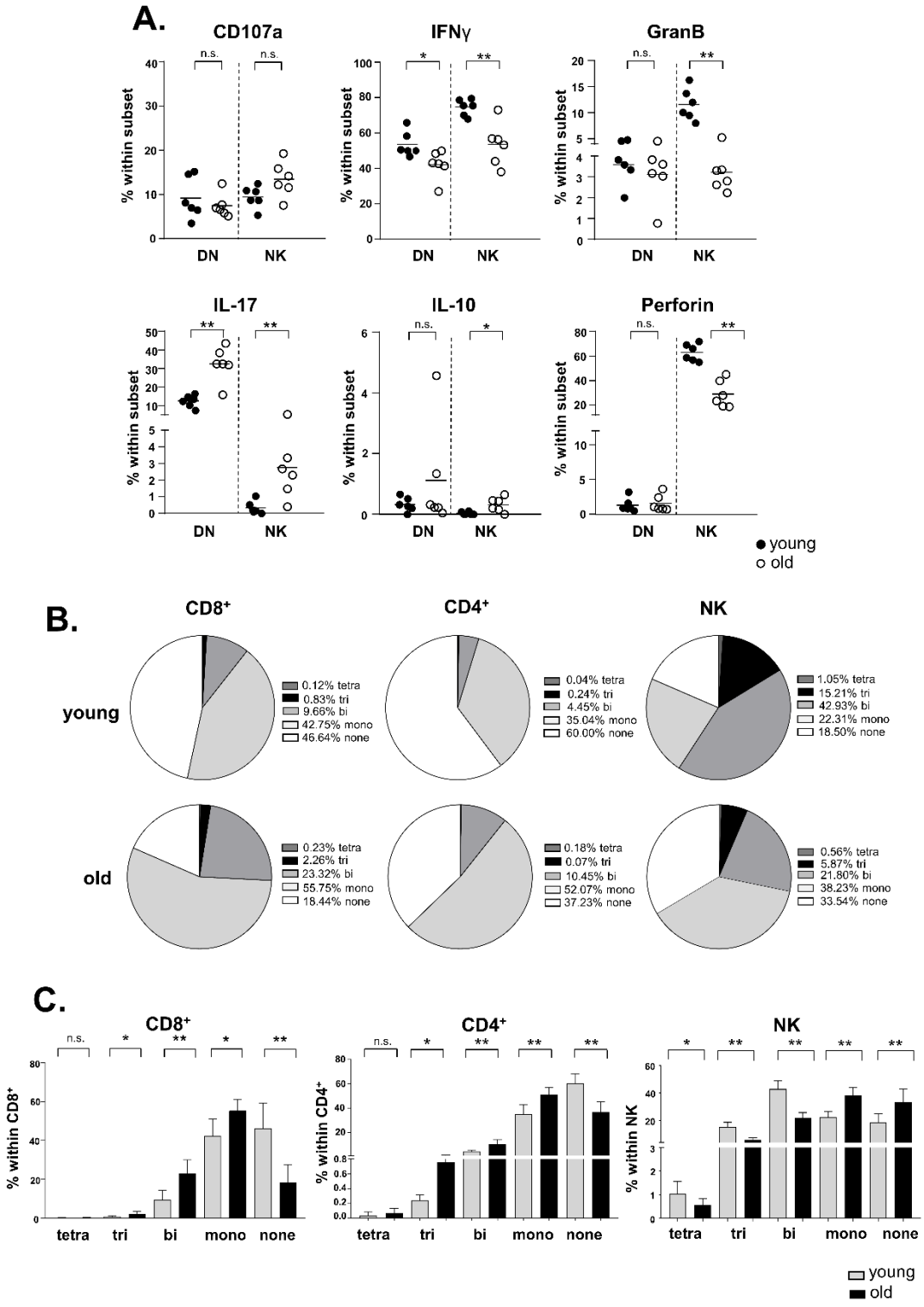

Figure 7: Effector Profile of Kidney-Resident Lymphocytes in Young and Aged Kidneys. (A) The expression levels of effector molecules, including CD107a, IFNγ, granzyme B, IL-17, IL-10, and perforin of $CD8^-$ $CD4^-$ T cells and NK cells. Statistical analysis was performed using the Mann-Whitney U test, and the results are presented as means. (B) This figure complements Figure 7A by examining the expression of different effector molecules in various combinations using boolean gating. (C) Shown are cells expressing four, three, two, one, or no molecule(s) at a time. The mean frequencies from the respective datasets were statistically analysed using the Mann-Whitney U test and presented as means. The figure indicates significant differences with *p < .05, **p < .01, and represents non-significant differences as n.s. (Source: He, A., Sarwar, A., et al. AJT (2022). Graphical representation is modified. For detailed author contribution refer to the “Declaration of Own Contribution to the Publications” section in this thesis).

We conducted a polyfunctionality analysis using Boolean gating in FlowJo for each group and observed that NK cells exhibited a reduced polyfunctionality with age, while the opposite was true for $CD8^+$ and $CD4^+$ T cells. In particular, cells isolated from old kidneys displayed higher polyfunctionality, as shown in the pie chart in Figure 7B. We also present a graphical representation of polyfunctionality along with statistical analysis for each young and old group in Figure 7C.

4.6 Aged Graft is Differentially Repopulated by Recipient Cells

To gain a comprehensive understanding of age-related immune responses to grafts, we transplanted a graft from a wild-type mouse (allele form $CD45.2^+$) into a Pepboy mouse (allele form $CD45.1^+$) using an allogeneic kidney transplantation model. The grafts were harvested on days three and seven post-transplantation. As of day 3, $CD45.2^+$ donor cells can still be observed within the graft. However, by day 7, the graft had been completely repopulated by recipient cells ($CD45.1^+$) (91). Notably, on day 3, the recipient cells ($CD45.1^+$) that had infiltrated the graft began to exhibit markers associated with tissue retention and activation, such as integrin E (CD103) and CD69, indicating a phenotype characteristic of tissue residence (Figure 8).

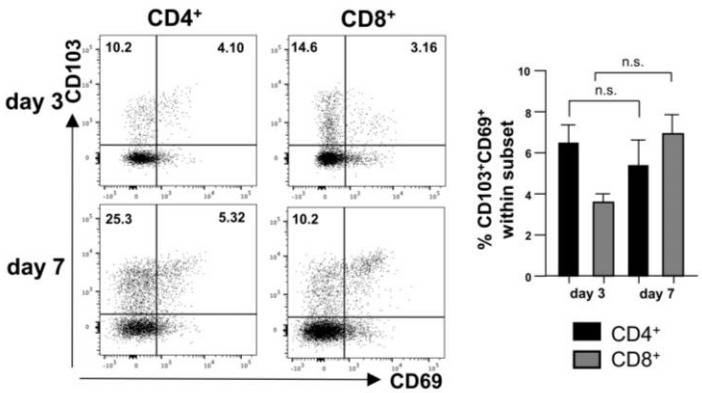

Figure 8: Repopulation of Recipient-Derived T Cells in the Allogeneic Graft at Day 7 Post-Transplantation. FACS dot plots demonstrating the increase of $CD69^+$ $CD103^+$ $CD4^+$ and $CD8^+$ tissue-resident memory (Trm) cells (left panel). The bar graph displays the frequencies of $CD4^+$ and $CD8^+$ Trm cells (right panel). Statistical analysis was conducted using the Mann-Whitney U test, and the results are presented as means. The figure indicates significant differences and denotes non-significant differences as n.s. (Source: He, A., Sarwar, A., et al. AJT (2022). For detailed author contribution refer to the “Declaration of Own Contribution to the Publications” section in this thesis).

4.7 Potent Proinflammatory and Cytotoxic Immune Response Elicited by Aged Graft

To characterise the immune response to young and aged grafts, we harvested the grafts on day 7 post-transplantation and performed *ex vivo* analyses of the cells infiltrating the graft.

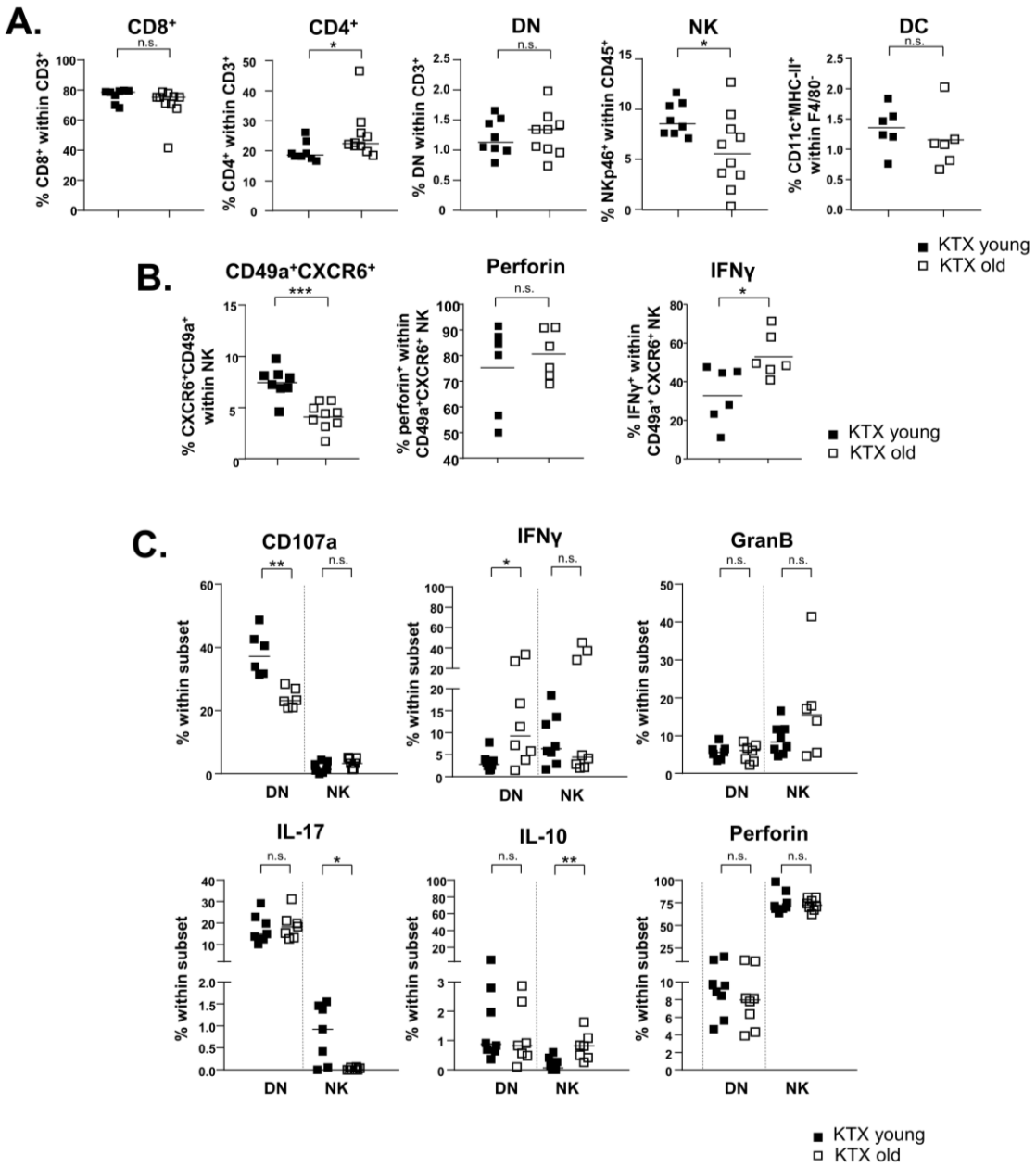

Figure 9: Induction of Intra-renal Inflammatory Profile by Aged Kidney Grafts. (A) Frequencies of $CD8^+$ and $CD4^+$ T cells, (B) NK cells isolated from young or aged C57BL/6-derived grafts transplanted into BALB/c recipients on day 7 post-transplantation (n = 8–10). Frequencies of $CD49a^+CXCR6^+$ NK cells isolated from either young or aged grafts and their production of perforin and IFNγ on day 7 post-kidney transplantation. (C) The effector profile (CD107a, IFNγ, granzyme B, IL-17, IL-10, and perforin) of $CD8^-$ $CD4^-$ T cells and NK cells isolated from young or aged renal allografts on day 7 post-transplantation (n = 5–9). Statistical analysis was performed using the Mann–Whitney U test or unpaired t-test, and the results are presented as means. Statistical analysis was conducted using the Mann–Whitney U test or unpaired t-test,

and the results are presented as means. The figure indicates significant differences with *p < .05, **p < .01, ***p < .001, and denotes non-significant differences as n.s. (Source: He, A., Sarwar, A., et al. AJT (2022). Graphical representation modified. For detailed author contribution refer to the “Declaration of Own Contribution to the Publications” section in this thesis).

We observed no significant differences in the frequencies of $CD8^+$, $CD8^-$ $CD4^-$ T, or DCs infiltration in young and old kidney graft (Figure 9A and B) and lower frequencies of $CD8^+$ $CD4^+$ T cells and NK cells were observed in the old graft (Figure 9B). However, the aged grafts accumulated more $CD4^+$ T (Figure 9A). Further analysis of the NK cell subset $CD49^+$ $CXCR6^+$ revealed differential repopulations in young and aged grafts after transplantation. The old graft exhibited lower frequencies of $CD49^+$ $CXCR6^+$ NK cells compared to the young graft. Of note, the frequencies of $CD49^+$ $CXCR6^+$ NK cells were lower in the old graft, these cells produced significantly higher levels of IFNγ upon activation, while perforin production remained unchanged (Figure 9C).

Moreover, when assessing *ex vivo* effector function, we found that cells isolated from aged grafts exhibited increased cytotoxic and pro-inflammatory capacities compared to those isolated from young grafts. $CD8^+$ T cells demonstrated significantly enhanced cytolytic activity, as evidenced by increased degranulation (CD107a), GranB, perforin, and IFNγ production. $CD4^+$ T cells exhibit elevated IFN-γ production (91). $CD8^-$ $CD4^-$ T cells show increased IFNγ production but decreased degranulation in aged grafts. Regarding NK cells, no significant differences were observed, except for decreased IL-17 production in the aged graft compared to the young graft. Interestingly, except for $CD8^-$ $CD4^-$ T cells, all the cells under study namely, $CD8^+$, $CD4^+$ and NK cells derived from the aged graft exhibited a significantly higher production of IL-10 (Figure 9C).

4.8 Senescence-Associated Markers Are Altered by ABT-263 Treatment in Aged Grafts

To investigate the impact of inflammaging on aged graft-related immunogenicity, aged grafts were pretreated with the senolytic drug ABT-263 and compared to untreated aged grafts. One kidney from each donor was analysed for a set of genes associated with senescence, and the other kidney was transplanted and harvested on day 7. The gene expression of three genes, $p16^{INK4a}$, KIM-1, and MCP-1, was analysed using qPCR, and all three showed increased expression with age compared to young and old donor kidneys. However, no significant difference was found in the expression of these three genes

between ABT-263 pretreated and untreated old donor kidneys (Figure 10B). β-galactosidase, a marker of senescence, was reduced in the ABT-263 pretreated old donor kidneys (Figure 10A).

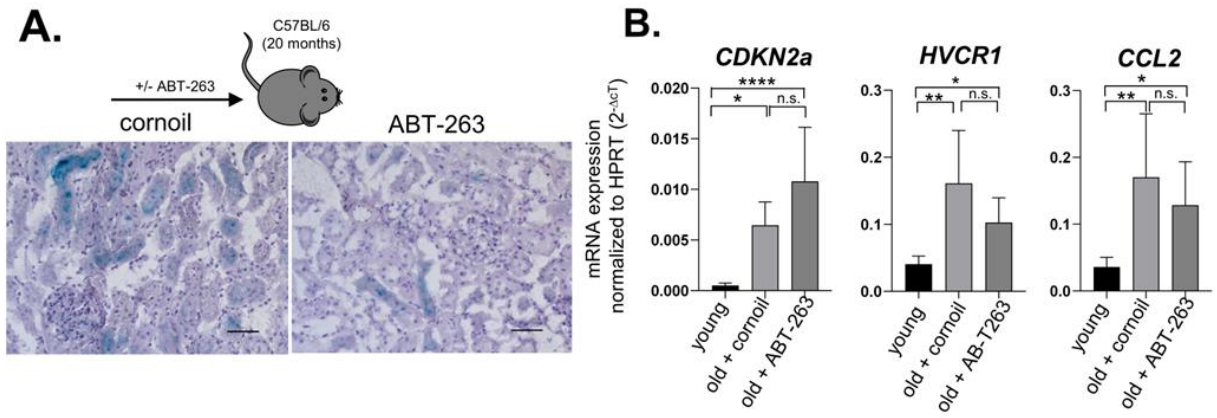

Figure 10: Modulation of Graft Immunogenicity by Treatment with the Senolytic Drug ABT-263. (A) Representative histological images of ß-galactosidase kidney staining comparing corn oil-treated and ABT-263-pretreated old C57BL/6 mice, magnification 200×, bar represents 50 µm. (B) Gene expression analysis of the candidate genes CDKN2a, HVCR1, and CCL2 in kidneys derived from naïve young (n = 6) or aged corn oil (n = 8) versus ABT-236-pretreated aged C57BL/6 mice (n = 11). Statistical analysis was conducted using the Mann–Whitney U test for two-group comparisons or Kruskal–Wallis test for multiple group comparisons, and the results are presented as means. The figure indicates significant differences with *p < .05, **p < .01, ***p < .001, ****p < .0001, and denotes non-significant differences as n.s. (Source: He, A., Sarwar, A., et al. AJT (2022). For detailed author contribution refer to the “Declaration of Own Contribution to the Publications” section in this thesis).

4.9 Modulation of Immune Response by ABT-263 Treatment

For further analysis, ABT-263 pretreated and untreated kidneys were transplanted, and the grafts were harvested on the day7 after kidney transplantation (KTx). Upon *ex vivo* analysis, we observed that frequencies of $CD8^+$ and $CD4^+$ cells remained unchanged in both ABT-263 pretreated and untreated grafts (91). However, there was higher infiltration of NK cells and $CD8^-$ $CD4^-$ T cells in the ABT-263 pretreated grafts. Interestingly, allograft repopulation varied for the tissue-resident subset of $CD49^+$ $CXCR6^+$ NK cells, which was significantly reduced in the pretreated group (Figure 11A). When the cells isolated from the ABT-263 pretreated and untreated groups were activated *ex vivo*, a significant immunomodulatory effect of ABT-263 treatment was observed, indicated by the reduced production of IFNγ and IL-10 in all T cell subsets (Figure 11B).

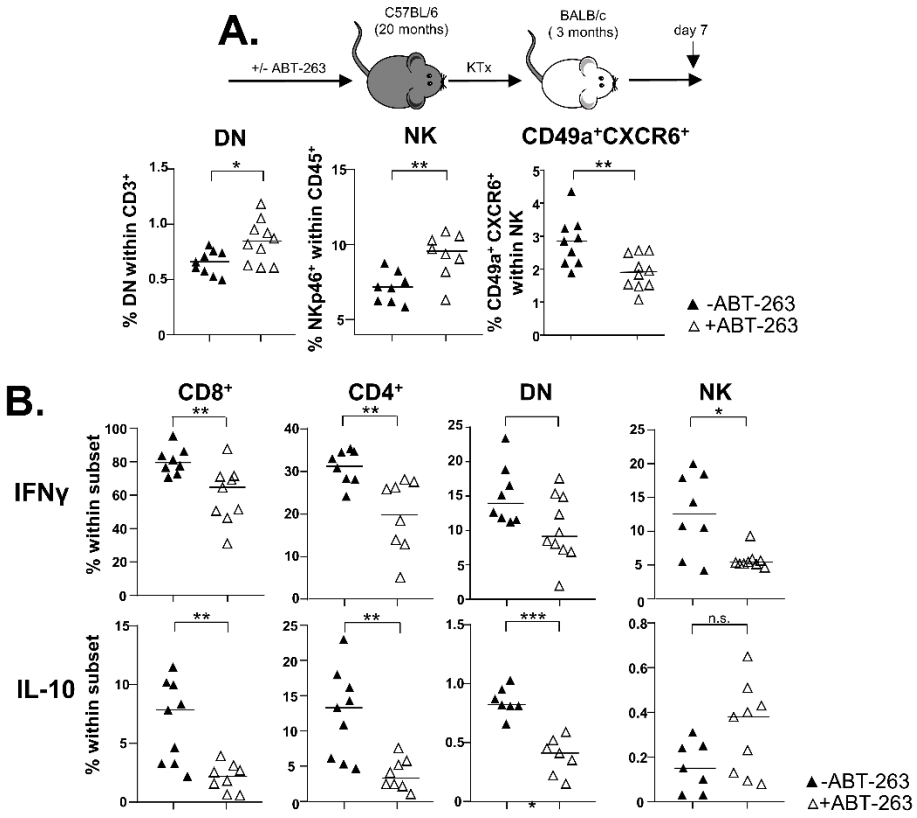

Figure 11: Improvement of Intra-graft Inflammation by ABT-263 Treatment in Senescent Kidneys. (A) Frequencies of $CD8^+$, $CD4^+$ (top panel), $CD8^-$ $CD4^-$ T, NK, and $CD49a^+$ $CXCR6^+$ NK cells (bottom panel), isolated from corn oil and ABT-263 pretreated aged C57BL/6-derived grafts on day 7 post-transplantation (8–10 animals/group). (B) Expression of IFNγ and IL-10 in CD8, CD4, $CD8^-$ $CD4^-$ T, and NK cells isolated from corn oil and ABT-263 pretreated grafts. Statistical analysis was conducted using the Mann–Whitney U test, unpaired t-test, or Kruskal–Wallis test, and the results are presented as means. The figure indicates significant differences with *p < .05, **p < .01, and denotes non-significant differences as n.s. (Source: He, A., Sarwar, A., et al. AJT (2022). For detailed author contribution refer to the "Declaration of Own Contribution to the Publications" section in this thesis).

4.10 ABT-263 Treatment Improves Long-term Graft Survival

To evaluate the long-term effects of ABT-263, grafts were harvested on day 28 after transplantation and *ex vivo* analysis was performed. We observed no significant difference in the T and NK cell population infiltrating the ABT-263 pretreated and untreated grafts. However, the group pretreated with ABT-263 demonstrated lower levels of creatinine and urea than the control group, indicating an improvement in graft function despite an unaltered histology. The figures are depicted in the original publication (91).

4.11 CyA Therapy Alters Frequencies of Leukocytes

In addition to investigating the effects of ABT-263 on aged allografts, we explored the influence of immunosuppressive treatments on immune cell populations in the context of kidney transplantation.

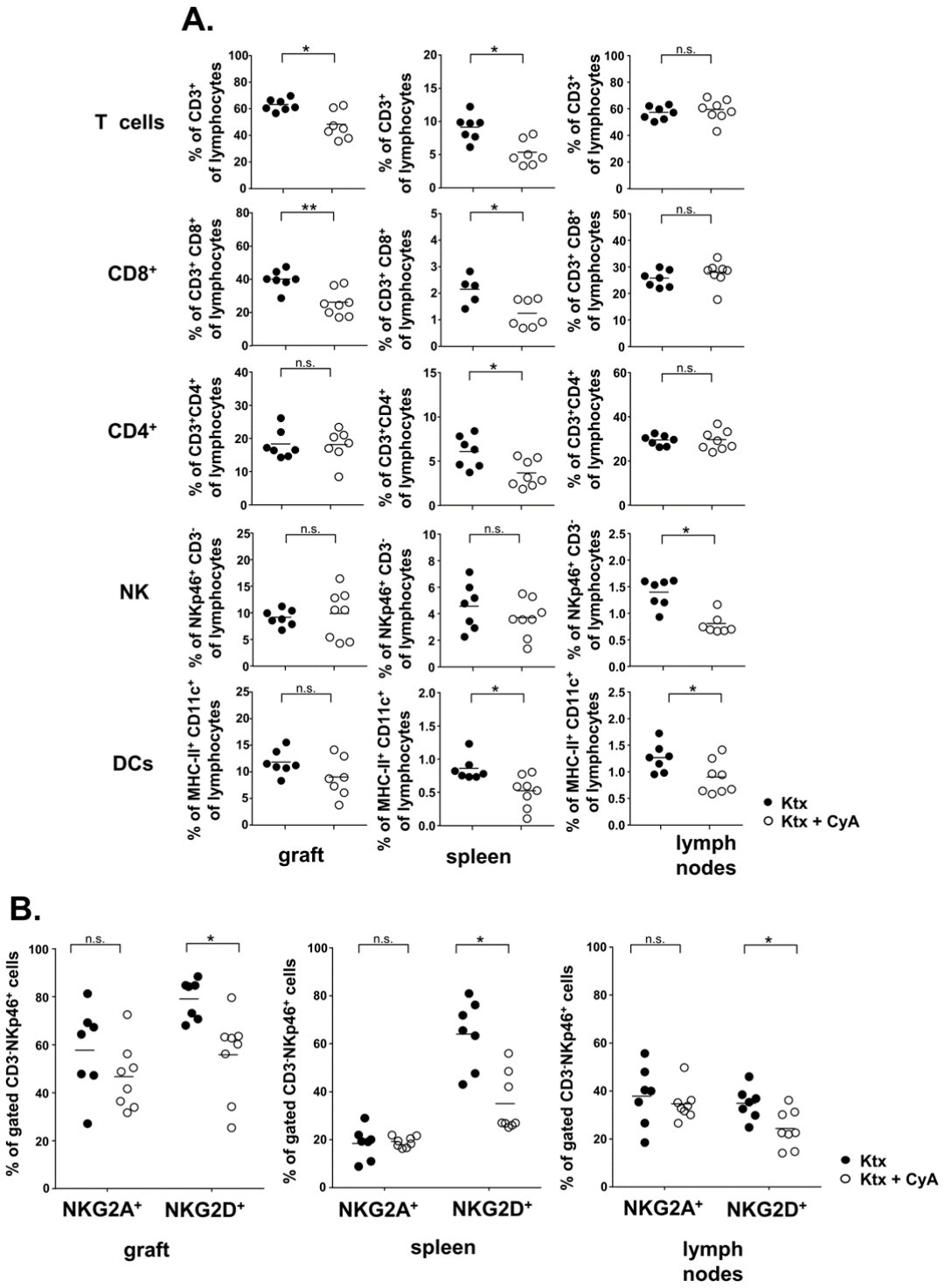

Figure 12: Modulation of Leukocyte Populations in the Graft by CyA Administration at Day 7. (A) After CyA administration, C57BL/6 recipient mice of BALB/c kidneys showed decreased frequencies of T cells in the allograft as well as in the spleen compared to untreated recipients on day 7. NK cell frequencies

were affected solely due to CyA treatment in the lymph nodes, whereas DC frequencies were significantly decreased in both the spleen and lymph nodes but not in the graft. (B) CyA treatment leads to a significant reduction in NKG2D$^+$ NK cells in all three investigated organs, whereas the NKG2A$^+$ NK cell subset remains unaffected. Data are presented as the mean of n = 7–8 animals/group. Statistical analysis was conducted using the Mann–Whitney U test, and significant differences are denoted as *$p < 0.05$ and **$p < 0.01$ (Source: Ashraf, M. I., Sarwar, A., et al. (2019). Natural killer cells promote kidney graft rejection independently of cyclosporine a therapy. Frontiers in immunology, 10, 2279). For detailed author contribution refer to the "Declaration of Own Contribution to the Publications" section in this thesis).

The role of NK cells and the efficacy of CyA were examined. By analysing recipient responses and leukocyte dynamics, these studies provide valuable insights into enhancing graft outcomes through immunomodulation strategies. To analyse the effect of CyA treatment on the main leukocyte populations in the graft spleen and lymph nodes *in vivo*, C57BL/6 recipients engrafted with BALB/c kidneys were sacrificed on POD 7. T lymphocytes were significantly reduced in the grafts and spleens of CyA-treated recipients compared with those in untreated controls. Both organs exhibited decreased frequencies of CD8$^+$ T cells, whereas the spleen displayed substantial reductions in CD4$^+$ T cell frequencies. Furthermore, treatment with CyA resulted in significantly reduced frequencies of CD3-NKp46$^+$ NK cells in the lymph nodes and CD11c$^+$ MHC II$^+$ DCs in the spleen and lymph nodes (Figure 12A). Moreover, a comparison of NK cells expressing the cytotoxicity receptor NKG2D revealed considerably higher frequencies of NK cells expressing the inhibitory receptor NKG2A in all three organs studied in the CyA group (Figure 12B).

4.12 Effects of CyA on T and NK Cell Function

Spleen cells were isolated from CyA-treated or untreated recipients on POD 7 to investigate the impact of CyA on T and NK cell function *in vivo*. When comparing the two groups, CD4$^+$ and CD8$^+$ T cells from CyA-treated mice displayed a significant reduction in their ability to secrete IFN-γ, whereas NK cells maintained their capacity to produce IFN-γ. Importantly, the degranulation capacity of both NK and T cells was unaffected by CyA treatment (Figure 13A). Intra-graft mRNA expression analysis revealed significantly lower mRNA levels of the cytotoxic markers perforin and GranB in the CyA-treated kidneys ($p < 0.05$). Furthermore, grafts from the CyA group exhibited significantly reduced mRNA expression of the chemokine C-X-C motif chemokine 10 (CXCL10) and IFNγ, whereas no differences were observed for the inflammatory cytokines IL-1ß and TNF (Figure 13B).

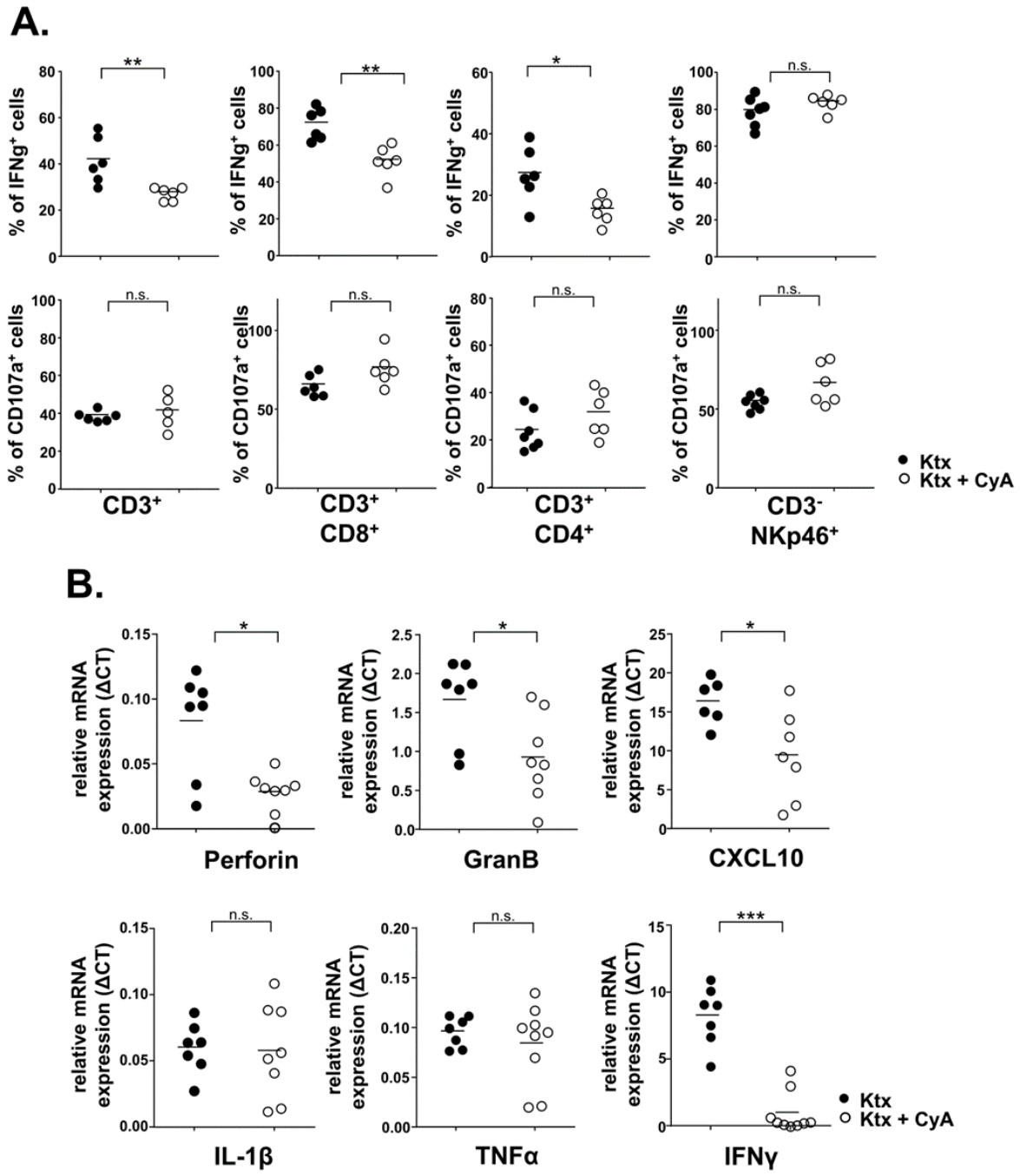

Figure 13: Impact of CyA Treatment on Splenocyte Stimulation. (A) Splenocytes derived from CyA-treated animals or untreated recipients were polyclonally stimulated with PMA and ionomycin. CD3+ CD4+ and CD3+ CD8+ T cells from CyA-treated animals showed a reduction in IFNγ production, while CD3− NKp46+ NK cells retained their capacity to produce IFNγ. No impact was observed on the degranulation capacity. (B) The mRNA profile of proinflammatory mediators, including perforin, granzyme B, CXCL10, and IFNγ, showed a significant reduction in CyA-treated recipients. Data are presented as the mean of n = 7–8 animals/group Statistical analysis was conducted using the Mann-Whitney U test, and significant differences are denoted as *p < 0.05 and **p < 0.01. (Source: Ashraf, M. I., Sarwar, A., et al. (2019). Natural killer cells promote kidney graft rejection independently of cyclosporine a therapy. Frontiers in immunology, 10, 2279. For detailed author contribution refer to the “Declaration of Own Contribution to the Publications” section in this thesis).

4.13 CyA and NK Depletion Improves the Graft Function

Although CyA therapy reduced the frequency of graft-infiltrating lymphocytes compared with that in untreated animals, the addition of NK cell depletion did not result in further reduction. All the groups exhibited diffuse C3d deposition, and a similar pattern was observed for $CD3^+$ T cells. The combined treatment of CyA and NK cell depletion did not enhance the improvement in the Banff Score observed in the CyA group compared to the untreated group. However, in terms of kidney function, measured by urea and creatinine levels, the CyA+NK group demonstrated significantly better outcomes than the untreated group (97). Although the combination of NK cell depletion with CyA treatment did not demonstrate substantial morphological advantages in our short-term survival groups, additional NK cell depletion led to a significant increase in overall survival until POD56 compared with the control group (Figure 14A). No significant differences were observed in the histological scoring among the surviving animals in the experimental groups (Figure 14B).

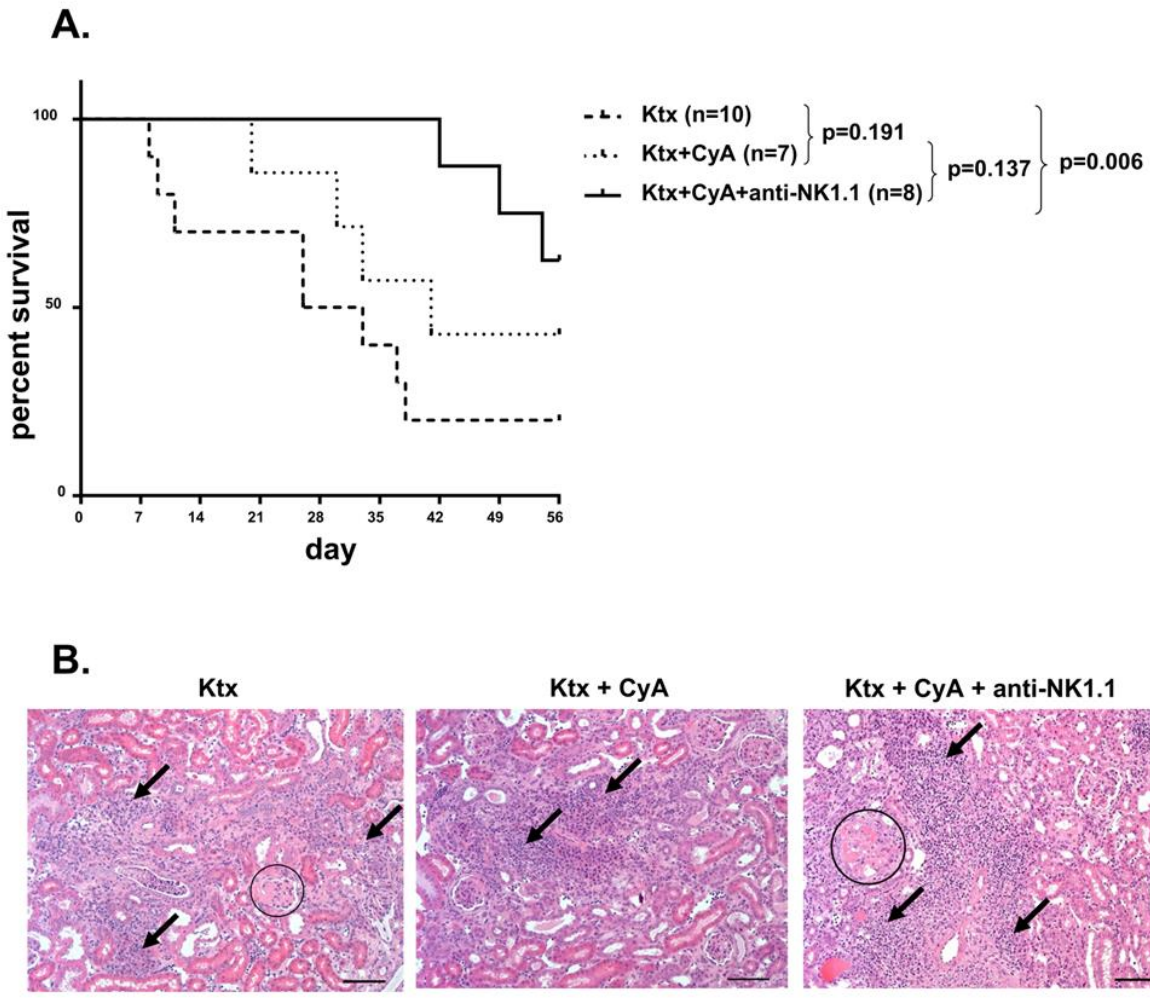

Figure 14: Dynamics of NK Cells and Graft Outcome under CyA Therapy. NK cell depletion in combination with CyA resulted in significantly improved long-term survival of kidney grafts until POD56 compared

to recipients left untreated. (Source: Ashraf, M. I., Sarwar, A., et al. (2019). Natural killer cells promote kidney graft rejection independently of cyclosporine a therapy. Frontiers in immunology, 10, 2279. For detailed author contribution refer to the “Declaration of Own Contribution to the Publications” section in this thesis).

In summary, Cyclosporine A (CyA) treatment effectively targeted T cells, leading to significant reductions in their frequencies. Notably, the impact on NK cells, particularly in terms of IFNγ production, was limited in the short term. However, in the long-term, a combined approach of CyA and NK cell depletion demonstrated enhanced allograft survival

.

5. Discussion

Donor age and poor outcomes after transplantation is not well elucidated at the cellular and molecular level (4, 95, 96). Given the age-related changes, understanding these mechanisms is crucial for improving graft outcomes. Inflammaging, which is characterised by age-related sterile inflammation, plays a critical role in tissue homeostasis, repair, and regeneration. Our study aimed to characterise inflammaging in aged kidneys and examine its contribution to donor-age-related immunogenicity with a particular focus on cellular senescence. We conducted phenotypic and functional characterisations of T and NK cells to determine their age-related impact on the composition and function of subsets isolated from the kidney and secondary lymphoid organs. Functional characterization of the cells revealed that NK and $CD8^-$ $CD4^-$ T cells are the primary sources of increased IL-17 production in lymphocytes isolated from aging kidneys (Figure 7A). This deviates from the conventional understanding that IL-17 production is solely attributed to $CD4^+$ T helper 17 (Th17) cells, where we found no significant differences between young and old $CD4^+$ cells (91). The production of IL-17 by $CD8^-$ $CD4^-$ T cells, which are implicated in age-related kidney damage, inflammation, and fibrosis (97-99), is influenced by age-related factors in an aging milieu. As aging progresses, there is an increase in Th17 cells driven by SASP factors, leading to an imbalanced Th17/Treg ratio in healthy aging (100). In our analysis, we observed a significant increase in the expression of NKG2D on NK and $CD8^+$ cells (Figure 5A). The signalling mechanisms of activating NK cell receptor NKG2D and IL-15 have been found to be linked (101). Furthermore, we investigated the influence of aging on the $CD49a^+$ $CXCR6^+$ double-positive NK cell subset, which is predominantly present in bulk NK cells of the aging kidney (Figure 5E). In humans, these cells have primarily been described in the liver, and there is growing consensus that $Eomes^{hi}$ $CD49a^+$ and $CXCR6^+$ NK cells represent trNK cells (102-106). This was further confirmed by parabiosis studies in mice examining trNK cells in the liver and kidneys revealed that $CD49a^+$ $DX5^-$ NK cells are confined to host tissues and do not circulate in the parabiont (107-109). CD49a CXCR6 double-positive NK cells in the liver and play a critical role in regulating hepatic inflammation, fibrosis, and regeneration, thereby contributing to liver physiology. In liver diseases, accumulation of $CD49a^+$ $CXCR6^+$ double-positive NK cells has been observed in the liver tissue of patients with chronic hepatitis B and C infections, as well as in patients with non-alcoholic fatty liver disease (NAFLD). These cells exhibit a pro-inflammatory phenotype and may contribute to the development of liver

fibrosis and cirrhosis (110). A study investigating patients with NAFLD demonstrated a significantly higher frequency of CD49a$^+$ CXCR6$^+$ double-positive NK cells in patients with advanced fibrosis than in those with early-stage fibrosis (111).

The differentiation of CD49a$^+$ CXCR6$^+$ double-positive natural killer (NK) cells is highly dependent on interleukin-15 (IL-15). This process of differentiation is driven by both IL-15 and IL-12 and mimics the pro-inflammatory cytokine milieu observed in liver disease and cancer, including the presence of IL-1α, IL-2, IL-6, IL-12, IL-15, IL-18, interferon alpha (IFNα), interferon gamma (IFNγ), and tumor necrosis factor alpha (TNFα/β) (112). This provides a plausible explanation for the enrichment of CD49a$^+$ CXCR6$^+$ NK cells in a microenvironment dominated by SASP cytokines, as it is conceivable that a similar phenomenon could occur in aging kidneys, leading to an increase in the abundance of CD49a$^+$ CXCR6$^+$ NK cells and explaining their accumulation with aging. IL-15 has also been shown to sustain other known immunosenescence hallmarks, such as the elevated expression of CD28$^-$ T cells in various inflammatory diseases, including inflammatory bowel disease (113-115).

Given the aforementioned findings, our subsequent study focused on investigating the immunogenicity of aged grafts compared to young grafts, and elucidating the underlying mechanisms involved. Our results revealed that aged grafts triggered robust pro-inflammatory and cytotoxic immune responses upon transplantation (Figure 9). These findings suggest that the heightened immune response observed in aged grafts is associated with increased immunogenicity compared to young grafts. To validate the contribution of SASP and immunosenescence to the inflammaging phenotype, we conducted additional experiments in which senolytics were used to eliminate senescent cells in donor mice prior to the transplantation of grafts into young recipients. Previous studies have shown that senolytics have beneficial effects in various experimental models, including diabetes, cardiac dysfunction, and AKI, (63, 116, 117), and heart transplantation (64) We used ABT-263, a senolytic agent that inhibits the pro-survival BCL-2 pathway, to pretreat aged kidneys before transplantation. The senolytic treatment effectively depleted senescent cells in the kidneys (Figure 11A). Following senolytic treatment, we observed a decrease in the production of pro-inflammatory cytokines, such as IFN-γ, and anti-inflammatory cytokines, such as IL-10, by the graft-infiltrating lymphocyte subsets (Figure 11B). Although the histology and overall renal function of the transplanted kidneys were not significantly influenced by ABT-263 pretreatment, we observed an improvement in graft

function on day 28 in the ABT-263 group. Overall, targeting senescent renal cells not only dampened the immune response but also improved the long-term function of the graft.

We conducted further research to identify the specific cell types targeted by senolysis within the complex cellular landscape of the kidney, which consists of approximately 20 distinct cell types and exhibits cellular heterogeneity with varying rates of senescence susceptibility (118).

Our research highlights the role of inflammaging and the alloimmune response in the context of an aged graft. SASP, which is characterised by a pronounced pro-inflammatory profile, has a pro-found impact on the immune system, promoting chronic inflammatory states, and potentially eliciting autoimmune responses in older individuals. Our findings show that the accumulation of pro-inflammatory T and natural killer cells in aging kidneys which are hallmarks of the aging process. Furthermore, we demonstrated that aged grafts elicit stronger immune responses than their young counterparts, indicating increased immunogenicity with aging. Interventions targeting senescent renal cells using senolytics are effective in attenuating the immune response and enhancing graft function. These findings provide valuable insights into potential strategies for improving allograft outcomes in aged donors and offer a deeper understanding of the interplay among cellular senescence, inflammation, and the immune system. Our research contributes to our understanding of age-related implications in organ transplantation and paves the way for advancements in therapeutic approaches aimed at enhancing patient outcomes.

The study aimed to investigate both the immunological changes associated with aging in donor organs and the significance of natural killer (NK) cells in modulating immune responses. The function of NK cells in transplantation and immunosuppression is not well understood, despite their important role in cell-mediated immunity. Understanding the intricate involvement of NK cells in the immune response will be crucial in developing targeted strategies for immunosuppression and optimizing kidney transplantation outcomes. In a prior study, we investigated the role of NK cells, specifically emphasizing their involvement in chronic rejection in Solid Organ Transplantation (SOT) (119). Conventional immunosuppressive regimens may not effectively control NK cell activity, leading to chronic rejection, but further comprehensive studies are required to support this hypothesis. Previous research on the influence of CyA on the number of Natural Killer (NK) cells in peripheral blood has primarily been conducted, but information on the presence and function of NK cells within transplanted organs is limited. For example, Neudoerfl et al. conducted a study on NK cells in the blood of kidney transplant recipients and found that

CyA treatment did not affect their percentage compared to healthy individuals. However, there is a lack of data on the specific role of NK cells in the context of organ transplantation, and further investigation is necessary to fully understand their contribution to the immune response in this context (83).

Our initial experiments involved administering CyA to recipients and observing a significant decline in the frequency of $CD3^+$ and $CD8^+$ T cells within the transplanted kidney and spleen (Figure 12A). Additionally, a notable decrease in $NKG2D^+NKp46^+$ NK cell frequencies was observed in all three organs (Figure 12B). Notably, previous studies have demonstrated that immunosuppressants such as mycophenolic acid and rapamycin can inhibit NKG2A and reduce NKG2D expression (82, 120). However, recent studies have shown that high concentrations of CyA in in vitro cultures lead to reduced IFN-γ production by NK cells. To investigate this further, we analyzed NK cells isolated from mice treated with clinically relevant doses of CyA. Interestingly, CyA treatment in vivo did not affect IFNγ production or the degranulation capacity of NK cells (Figure 13A), unlike recent in vitro studies of cyclosporine and tacrolimus (83, 121). Additionally, CyA-treated animals exhibited reduced intra-graft inflammation and IFNγ expression at the mRNA level (Figure 13B), which was associated with a decrease in graft-infiltrating $CD8^+$ T cells, rather than NK cells (Figure 13A). Our study investigated the effect of Cyclosporine A (CyA) treatment on kidney graft survival. While short-term results did not show significant differences between the CyA-treated and untreated groups, the combination of NK cell depletion with CyA significantly improved overall graft survival until postoperative day 56 (Figure 14A).

Our research presents novel insight about the potential benefits of reducing Natural Killer (NK) cells in combination with CyA for improving renal allograft function, which offers a promising approach for enhancing long-term kidney transplant outcomes. However, our study also has some limitations, such as not conducting a comprehensive analysis of NK cell phenotype and function at different time points following CyA treatment in kidney transplant recipients. Further investigations that consider earlier or later time points may lead to different results. Additionally, the timing and dosage of NK cell depletion were based on previous studies that focused on the risk of opportunistic infections associated with prolonged depletion. Therefore, it is necessary to explore alternative protocols for NK cell depletion with additional immunosuppressants to gain a better understanding of how modulating NK cell responses can improve allograft function after kidney transplantation.

In conclusion, our study demonstrates that NK cell depletion in combination with CyA treatment exhibits a synergistic effect on renal allograft function. However, further research is necessary to clarify the underlying mechanisms, including the roles of specific NK cell subsets and their interactions with other immune cells. Additionally, the impact of this therapeutic approach on long-term graft survival and production of de novo antibodies should be further investigated. By shedding light on the potential benefits of selective NK cell targeting, our study paves the way for future investigations aimed at optimizing immunosuppressive strategies and improving the outcomes of kidney transplantation.

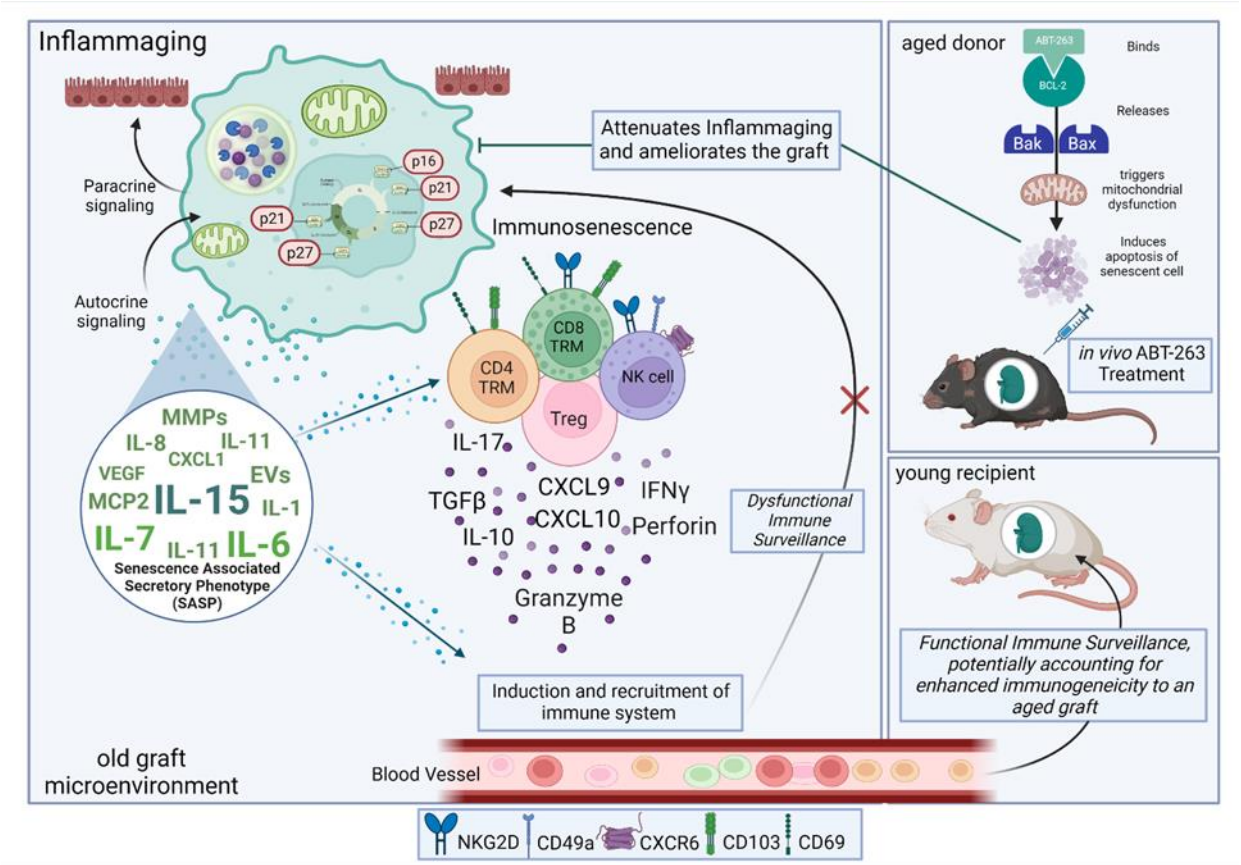

Figure 15: **Interplay Between Immunosenescence, SASP, and Organ Aging in Transplantation: A Schematic Diagram.** Key elements include the influence of SASP factors (IL-1, IL-6, IL-8, IL-7, IL-11, IL-15, GRO/, GM-CSF, MCP-1, MCP2, MMP-1, MMP-3, MMP-10, ICAM-1, PAI-1, VEGF, and IGFBPs) on immunosenescence pathways, involving T cells and NK cells. SASP, driving inflammaging and impairing immune surveillance, is also emphasized. Understanding SASP and immunosenescence is pivotal for successful transplantation, offering potential strategies to improve outcomes. SASP directly contributes to inflammaging and fuels immunosenescence, resulting in compromised clearance of senescent cells and the accumulation of SASP factors. This creates a proinflammatory microenvironment that perpetuates immunosenescence. Transplanting aged organs into young recipients triggers potent immune responses due to heightened immune surveillance, leading to increased immunogenicity. Understanding the intricate relationship between SASP, immunosenescence, and transplantation provides valuable insights into age-related immune dysregulation and informs strategies for successful transplantation. Attenuating SASP through senolysis presents a promising approach to decrease immunogenicity and facilitates organ regeneration, offering better long-term outcome (Created by Attia Sarwar in BioRender based on literature review cited in this thesis intended to create a graphical representation of the main concept of the thesis).

6. Conclusion

In this study, we investigated the role of senescent cells in the aged-donor graft-related immunogenicity. Using senolytics, such as Navitoclax, the targeted elimination of senescent cells resulted in the damping of the immune response and showed a better graft outcome. Depletion of senescent cells in animal models has shown impressive results over the past five years in a variety of tissues, which continues to inspire the conception and improvement of therapy (59, 64, 122-125). It is crucial to consider the unintended impact of senolytics on the immune system performance. As we grow older, immune system functionality declines, which has implications for immunosuppressive treatments in older individuals receiving transplantation. Research conducted in mice demonstrated that Navitoclax can revitalise haematopoietic stem cells (116). These findings suggest that such treatments may facilitate immune system rejuvenation in elderly patients, leading to the need for modified immunosuppressive regimens in transplant recipients. Although we treated the donors *in vivo* in the present study, senolytics may also be used for deceased kidney grafts. In addition to pretransplant therapy, senolytics can aid in the clearance of IRI-induced senescence, which otherwise hampers immunosuppression. In addition, the immunosuppressive regimen prevents the clearance of senescent cells, which could lead to aging of recipient organs. Senolytic therapy may help prevent nephrotoxicity and improve the recipient's overall health while receiving immunosuppression.

Moreover, our study reveals significant alterations in immune cell frequencies induced by Cyclosporine A (CyA) treatment in kidney transplant recipients. Notably, CyA impacts T cells, particularly $CD8^+$ T cells, and NK cells within grafts, spleens, and lymph nodes.

While short-term effects include reduced $NKG2D^+$ NK cell frequencies and altered T cell cytokine production, the combination of CyA and NK cell depletion does not show a substantial improvement in short-term kidney function. However, in the long term, this combination extends allograft survival, suggesting potential synergies between CyA and NK cell modulation.

Taken together, these findings lay the groundwork for future investigations aimed at refining therapeutic approaches for enhanced renal allograft outcomes in kidney transplantation.

7. Limitations / Future Prospects

The present study is not without its limitations.
The attempts to employ co-culture assays to understand the direct interactions between graft-infiltrating cells and target cells have been unsuccessful, highlighting a methodological challenge. The investigation failed to explore the direct interaction between graft-infiltrating cells derived from recipients and potential target cells in great detail.
The study primarily concentrates on the immunological aspects of aging in the kidney, potentially overlooking the systemic factors influencing inflamm-aging.
The reasons behind the lack of decrease in $p16^{INK4a}$ mRNA expression in kidneys pretreated with ABT-263, compared to controls, remain unclear.
The focus on renal senescence may not fully capture the broader implications of aging on the immune system, suggesting the need for a more comprehensive perspective.
Further investigation is required to elucidate the underlying mechanisms responsible for the effects of ABT-263 pretreatment on graft function observed in the study.
The potential avenues for further investigation, such as the effectiveness of alternative senolytics like Dasatinib and Quercetin, have not been evaluated.
The study did not assess the kinetic characteristics of NK cell phenotype and function after kidney transplantation, restricting the understanding of the temporal changes in NK cell modulation by CyA.
While acknowledging the limitations of a small sample size in specific experiments, the study underscores the need for caution in drawing general conclusions and emphasizes the necessity for larger follow-up studies.
Further confirmation is required to explore the relationship between NK cell depletion and the induction of $CD8^+CD122^+$ Tregs, as well as the implications of increased proliferation of $CD4^+$ Tregs, in larger follow-up studies.

The effects of alternative protocols for NK cell depletion in conjunction with immunosuppressants remain unexamined, presenting potential avenues for enhancing allograft function after kidney transplantation.

These shortcomings reveal opportunities for enhancement in future studies and provide directions for further investigation.

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Statutory Declaration

“I, Attia Sarwar, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic “Unravelling the Influence of Donor Age on Renal Allograft Survival” / “Entschlüsselung des Einflusses des Spenderalters auf das renale Allograftüberleben”, independently and without the support of third parties, and that I used no other sources and aids than those stated.
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Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; http://www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date Signature

Declaration of Own Contribution to the Publications

Attia Sarwar contributed to the publications listed below:

Publication 1: He A*, **Sarwar A***, Thole LML, Siegle J, Sattler A, Ashraf MI, Proß V, Stahl C, Dornieden T, Bergmann Y, Ritschl PV, Ebner S, Hublitz KW, Stamatiades EG, Bülow RD, Boor P, Kotsch K. Renal inflamm-aging provokes intra-graft inflammation following experimental kidney transplantation. Am J Transplant. 2022 Nov;22(11):2529-2547. IF=9.369 (2023)

*Shared First Authorship

Contribution:

- Experimental work
    - Mice Handling and Perfusion
    - Cell Isolation from murine organs (all groups)
    - Flow Cytometry Measurement:
        - Panel Establishment (PMT adjustment, Antibody Titration, Compensations)
        - Quality Control Check of the data
        - Data Acquisition (Figures contributed to the publication; Young vs. Aged Naïve Mice; Young vs Old KTx; ABT-263 Treated vs Ctrl KTx, specifically Figure 1C, D, E; Figure 2A-F; Figure 3A-C; Figure 5A-C; Figure 8A, 8D).
    - ABT-263 drug Preparation
    - Sample Preparation for Histology and β-galactosidase Analysis. Histology and statistical analysis were performed Peter Boor. β-galactosidase Analysis staining was performed by Yasmin Bergmann.
    - Gene Expression Analysis
- Data Analysis and Figure Creation

  Figure 1C, 1D, 1E: Phenotyping of Naïve ($CD4^+$, $CD8^+$, NK Cells and subsets, $CD8^-$ $CD4^-$ T cells, EM T cells, Regulatory T cells). Figure 2E, 2F; Figure 3A, 3B, 3C; Figure 4A, 4B; Figure 5A, 5B, 5C; Figure 8D; Figure 9A. tSNE plots used in the publication are generated by Linda Marie Laura Thole based on the Panel established, cell isolation acquisition, data analysis and quality control and compensation done by the author.

Publication 2: Ashraf MI, Sarwar A, Kühl AA, Hunger E, Sattler A, Aigner F, Regele H, Sauter M, Klingel K, Schneeberger S, Resch T, Kotsch K. Natural killer cells promote kidney graft rejection independently of cyclosporine a therapy. Front Immunol. 2019. 10, 2279.

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Contribution:

- Experimental work
    - Mice Handling and Perfusion
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- Data Acquisition
- Publication Revision Work

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Signature, date and stamp of first supervising university professor / lecturer

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Signature of doctoral candidate

Excerpt from Journal Summary List

Journal Data Filtered By: **Selected JCR Year: 2021** Selected Editions: SCIE,SSCI
Selected Categories: **"TRANSPLANTATION"** Selected Category Scheme: WoS
Gesamtanzahl: 25 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfaktor
1	JOURNAL OF HEART AND LUNG TRANSPLANTATION	15,150	13.569	0.02009
2	AMERICAN JOURNAL OF TRANSPLANTATION	31,958	9.369	0.04252
3	NEPHROLOGY DIALYSIS TRANSPLANTATION	31,696	7.186	0.02182
4	LIVER TRANSPLANTATION	12,263	6.112	0.01050
5	BIOLOGY OF BLOOD AND MARROW TRANSPLANTATION	16,477	5.609	0.02674
6	TRANSPLANTATION	29,058	5.385	0.02335
7	BONE MARROW TRANSPLANTATION	16,828	5.174	0.01411
8	STEM CELLS AND DEVELOPMENT	9,389	4.390	0.00491
9	CELL TRANSPLANTATION	8,435	4.139	0.00607
10	TRANSPLANT INTERNATIONAL	6,314	3.842	0.00699
11	ASAIO JOURNAL	5,107	3.826	0.00621
12	XENOTRANSPLANTATION	1,956	3.788	0.00229
13	CLINICAL TRANSPLANTATION	7,197	3.456	0.01064
14	Transplantation Reviews	1,251	3.066	0.00151

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Renal inflamm-aging provokes intra-graft inflammation following experimental kidney transplantation

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Abstract

Donor age is a major risk factor for allograft outcome in kidney transplantation. The underlying cellular mechanisms and the recipient's immune response within an aged allograft have yet not been analyzed. A comprehensive immunophenotyping of naïve and transplanted young versus aged kidneys revealed that naïve aged murine kidneys harbor significantly higher frequencies of effector/memory T cells, whereas regulatory T cells were reduced. Aged kidney-derived $CD8^+$ T cells produced more IFNγ than their young counterparts. Senescent renal $CD8^+$ T and NK cells upregulated the cytotoxicity receptor NKG2D and the enrichment of memory-like $CD49a^+CXCR6^+$ NK cells was documented in aged naïve kidneys. In the C57BL/6 to BALB/c kidney transplantation model, recipient-derived T cells infiltrating an aged graft produced significantly more IFNγ, granzyme B and perforin on day 7 post-transplantation, indicating an enhanced inflammatory, cytotoxic response towards the graft. Pre-treatment of aged kidney donors with the senolytic drug ABT-263 changed the recipient-derived effector molecule profile to significantly reduced levels of IFNγ and IL-10 compared to controls. Graft function after ABT-263 pre-treatment was significantly improved 28 days post kidney transplantation. In conclusion, renal senescence also occurs at the immunological level (inflamm-aging) and aged organs provoke an altered recipient-dominated immune response in the graft.

KEYWORDS
inflamm-aging, kidney transplantation, senescence, senolytic drug

Abbreviations: CKD, chronic kidney disease; DC, dendritic cell; DGF, delayed graft function; GBM, glomerular basement membrane; PTECs, proximal tubule epithelial cells; SASP, senescence-associated secretory phenotype; Trm, tissue-resident memory T cells.

An He and Attia Sarwar equally contributed as first authors.

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1 | INTRODUCTION

Worldwide, the number of individuals with an advanced age >65 years is steadily increasing. This results in a higher number of patients diagnosed with progressive chronic kidney disease (CKD) constituting a potential kidney transplant recipient group.[1,2] Chronological donor age is also a major risk factor for allograft dysfunction, as grafts from older donors are more susceptible to ischemic injury and prone to develop delayed graft function (DGF) post kidney transplantation.[3–5] Aged donor kidneys are characterized by structural changes, including glomerular basement membrane (GBM) permeability, changes in podocyte morphology and nephron loss, resulting in reduced glomerular filtration rate and altered homeostasis.[6–12]

Renal aging also entails chronic, sterile low-grade inflammation paralleled with the development of fibrosis occurring at different molecular and cellular levels including DNA damage, dysfunctional telomeres, and protein aggregation.[13] One major mechanism is mediated via *cyclin-dependent kinase inhibitor* p16INK4a resulting in growth arrest of viable cells and changes in the cell's secretory phenotype, called senescence-associated secretory phenotype (SASP).[14–16] In addition, the reduced regenerative capacity of stem cells and their progeny accelerates cellular senescence.[17]

Despite these findings, the exact mechanisms of age-dependent alterations in the kidney impacting long-term overall graft survival still remain unclear. Although it has been speculated that the SASP present in older allografts creates a pro-inflammatory milieu, literature on this topic is scarce. Some experimental data suggested that parenchymal changes seen in older allografts are associated with enhanced immunogenicity, therefore accelerating rejection, whereas other studies failed to detect such differences.[18,19] The process of progressive, multidimensional, physiological degeneration of the immune system resulting in a low-grade chronic inflammation has been described as inflamm-aging.[20,21] However, it has not been addressed as to how kidney-resident lymphocytes may undergo senescence. In this context we recently demonstrated that frequencies of tissue-resident memory CD8$^+$ T cells (T_{rm}) residing in human kidneys correlate with chronological age and that CD4$^+$ T_{rm} correlate with kidney function.[22] Here, using experimental mouse models, we show how age impacts immunity in the naïve kidney, but also assess how aged organs shape the recipients´ allo-response, with possible implications for therapeutic targeting.

2 | MATERIALS AND METHODS

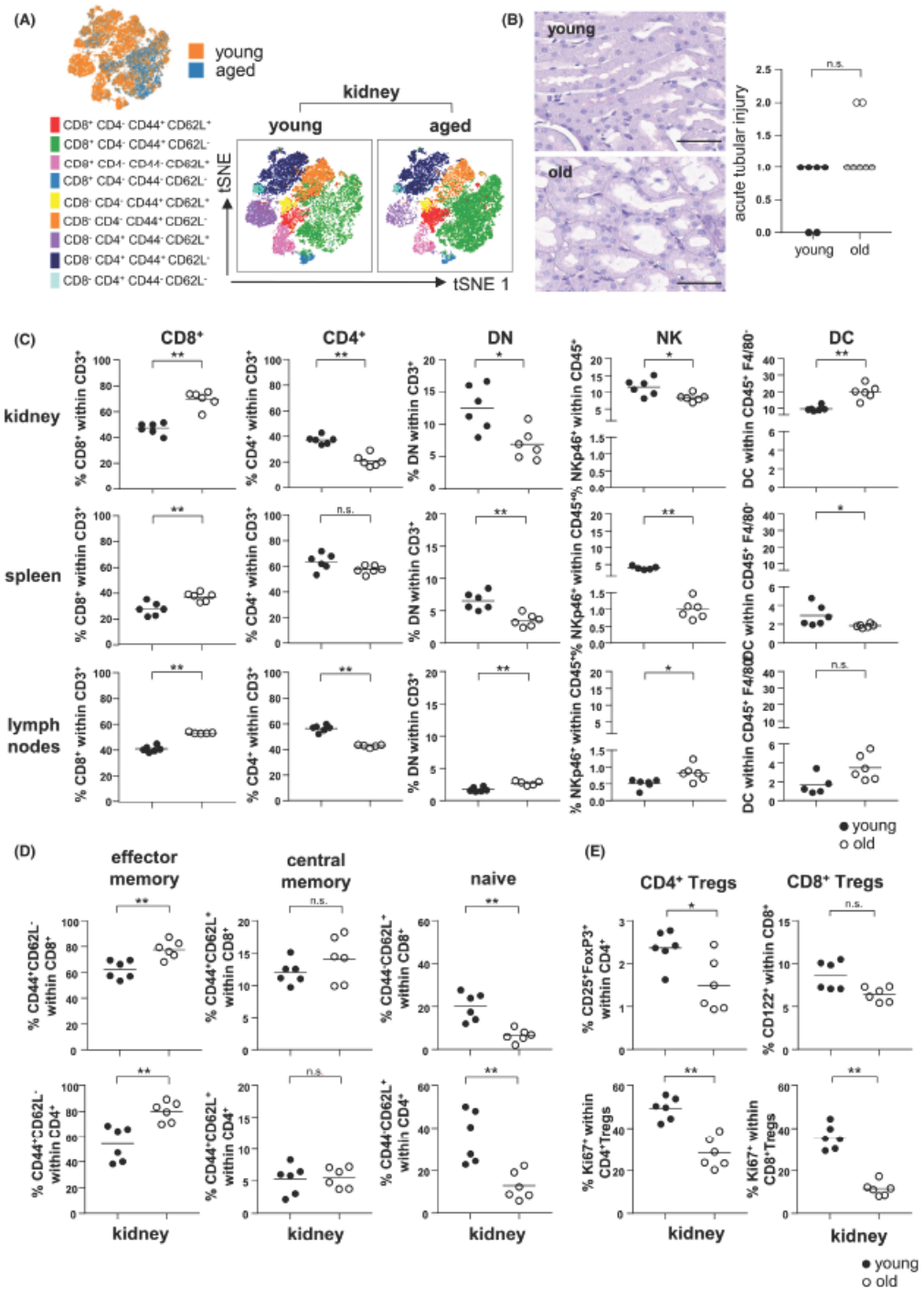
2.1 | Animals

Young BALB/c and C57BL/6 mice (8–12 weeks) and C57BL/6 mice (20 months) were purchased from Charles River Laboratories (Charles River). Male mice weighing 24–30 g were used. Animals were housed under standard conditions and received human care in compliance with the 'Principles of Laboratory Animal Care' prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86–23, revised 1985). All animal experiments were approved by the Landesamt für Gesundheit und Soziales Berlin, Germany.

2.2 | Kidney and heart transplantation

Renal transplantations were performed as previously described.[23] Briefly, after procurement of the left donor kidney, end-to-side anastomoses between the donor renal vessels and the recipient's abdominal aorta and inferior vena cava were performed following a knotless technique. Animals were either sacrificed on day 7 or on day 28 without receiving immunosuppression. For long-term

FIGURE 1 Aging affects the lymphocyte composition in various organs. (A) Visualization of t-distributed stochastic neighbor embedding (viSNE, left panel) considering the markers CD4, CD8, CD44, CD62L, CD103, CD69 and KLRG1 indicates the clustering of viable CD3$^+$ T cell subsets in C57BL/6 kidneys according to age; viSNE plots were generated on concatenated FCS files ($n = 5–6$) using an equal amount of 17.674 CD3$^+$events per file and Cytobank default parameters (1000 iterations, perplexity of 30, 0.5 Theta). Subsequent FlowSOM analysis of T cell subsets (right panel) performed on viSNE map using CD4, CD8, CD44, and CD62L for hierarchical clustering. (B) Representative images depicting kidney histology of young and old naïve (i.e., not transplanted) kidney showing slightly more acute tubular injury in kidneys of old mice. Bar represents 50 μm. (C) Frequencies of manually gated CD8$^+$, CD4$^+$, double negative (DN), NK cells from kidney and lymphoid organs (spleen, lymph nodes) derived from young (3 months) and aged (20 months) C57BL/6 mice (5–6 animals/group). (D, E) Frequencies of manually gated CD8$^+$, CD4$^+$ naïve, memory T cells and T_{regs} derived from young (3 months) and aged (20 months) C57BL/6 mice (5–6 animals/group). Statistically significant differences were tested with Mann–Whitney U test and presented as means, $^*p < .05$; $^{**}p < .01$; n.s. = not significant.



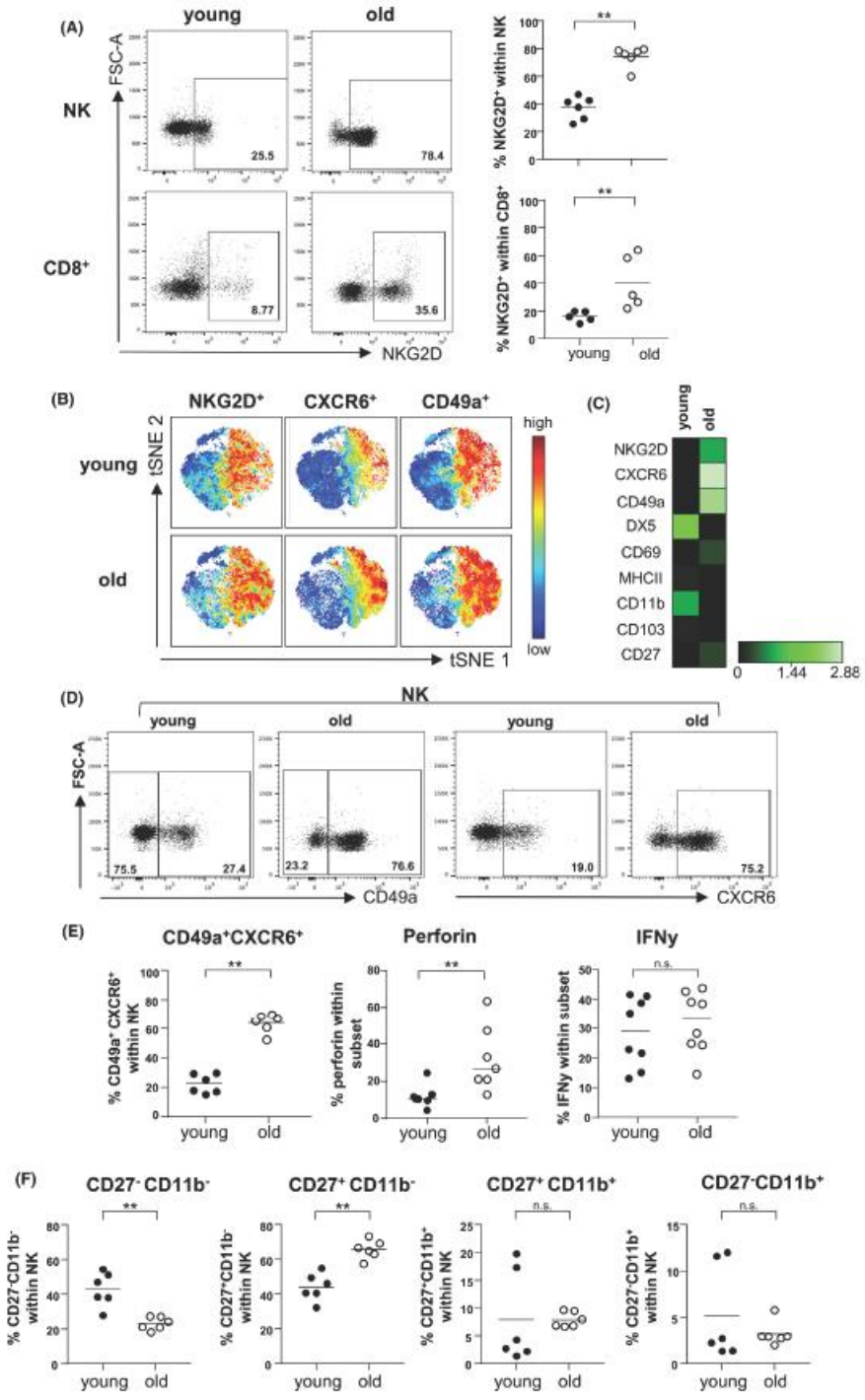


FIGURE 2 NKp46$^+$ NK cells upregulating NKG2D, CD49a and CXCR6 accumulate in the senescent murine kidney. (A) Representative FACS dot plots (left panel) and frequencies (right panel) of NKG2D$^+$ NK and CD8$^+$ T cells derived from young and aged C57BL/6 kidneys. (B) viSNE plots of viable NKp46$^+$ CD3$^-$ NK cells showing increased expression of NKG2D$^+$, CXCR6$^+$ and CD49a$^+$ in NK cells isolated from aged compared to young kidneys. Expression is shown by color coding in relative intensity. viSNE plots were generated on concatenated FCS files (n = 5–6) considering NKG2D, CXCR6, CD49a, DX5, CD69, MHC II, CD11b, CD103, CD27, an equal amount of 25 111 events were sampled per file. (C) Heatmap plot of flow cytometric analysis for NK cell populations illustrating log 2 transformed median of relative expression levels of measured markers. (D) Exemplary dot plots of CD49a and CXCR6 C57BL/6 expression on NK cells and (E) frequencies of CD49a$^+$CXCR6$^+$ kidney-derived senescent NK cells and their expression of perforin and IFNγ. (F) Frequencies of NK cell subtypes in young and aged C57BL/6 kidneys according to CD27 and CD11b expression, n = 5–8 animals/group. Statistically significant differences were tested with Mann–Whitney U test or unpaired t-test and presented as means, $^{**}p < .01$; n.s. = not significant.

survival experiments, the contralateral kidney was removed 1 day before animals were sacrificed, allowing determination of graft function after 24 h of observation. For sensitization of recipients, fully allogeneic BALB/c- (donor) derived hearts were transplanted into C57BL/6 recipients using a heterotopic cardiac transplantation model as described elsewhere.[24]

2.3 | In vivo treatment

ABT-263 (50 mg/kg/day Navitoclax, Hycultech GmbH) was administered in two cycles by oral gavage with a 1-week interval in-between. Control animals received corn oil. Animals were sacrificed either on day 7 (short-term survival) or on day 28 (long-term survival). In the long term group, the contralateral kidney was removed 1 day before animals were sacrificed in order to analyze serum creatinine and urea being indicative for graft function.

2.4 | Serum analysis of kidney function parameters

Serum samples were stored in aliquots at −20°C until serum creatinine and urea were measured using the CREP2 Creatinine Plus version 2 and Urea/BUN assays, respectively, on a Roche/Hitachi Cobas C 701/702 system (Roche Diagnostics).

2.5 | Isolation of cells

For isolating renal MNCs, tissues were mechanically dissociated and digested in 10 ml of RPMI medium supplemented with collagenases II and IV (Gibco/Invitrogen, Worthington) and DNase I (Roche Diagnostics) for 45 min at 37°C. Following digestion, recovered leukocytes were enriched using CD45 Microbeads over MACS LS columns (Miltenyi Biotec, Inc.). Leukocytes from spleen and lymph nodes were isolated by density gradient centrifugation. For analysis and culture of PTECs, kidneys were minced into 2 mm^3 pieces, processed through a 180 µm stainless steel sieve and collected in DMEM/F12 medium. Flow-through was applied to a 100 µm cell strainer from which renal tubular segments and glomeruli were recovered by reverse flushing with medium. After centrifugation, tubular segments were digested using collagenase II for 20 min at 37°C in a shaking waterbath. Thereafter, cells were either immediately analyzed by FACS or transferred to 6 well plates for outgrow cultures; 80% confluence of PTECs was reached after 5–6 days. Glomerular endothelial cells (gECs) were isolated as already published.[25] Briefly, glomeruli were digested using Trypsin–EDTA 0.25% (Life Technologies) for 23 min at 37°C in a shaking waterbath, with pellet resuspension every 5 min. gECs were harvested by filtering the suspension through a 40 µm strainer for immediate FACS analysis.

2.6 | Flow cytometry

Typically 1×10^6 cells were stained with antibodies listed in Table S1. Cells were measured using a FACS Fortessa X20 (BD Bioscience). FACS data analysis was conducted using FlowJo software 10.0 (Tree Star Inc.). A gating strategy for identification of the various lymphocyte subsets is illustrated in Figures S1 and S2. Polyfunctionality was assessed via Boolean gating. Generation of t-Distributed Stochastic Neighbor Embedding (t-SNE) plots, FlowSOM analysis and heatmaps was conducted using Cytobank (Beckman Coulter).

2.7 | In vitro assays

Functional analysis of T and NK was performed as previously described.[23] Cells were rested in 200 U/ml murine IL-2 (Miltenyi Biotec) over night, followed by stimulation with 50 ng phorbol 12-myristate 13-acetate (PMA) and 1 µg ionomycin (Sigma-Aldrich) for 4 h in the presence of 10 µg/ml brefeldin A and 2 µM monensin (Biolegend). CD107a expression was used as a correlate for degranulation. Following activation, cells were surface stained, fixed and permeabilized (Transcription Factor Staining Buffer Set; Thermofisher), followed by intracellular staining (representative raw data shown in Figure S3). To assess alloreactivity, DCs were isolated from young and old BALB/c kidneys by magnetic purification using the MojoSort panDC kit (Biolegend) in combination with MACS LD-type columns for depletion of unwanted cells (Miltenyi Biotec). Splenic CD3$^+$ T cells were equally enriched using the MojoSort T cell kit (Biolegend) from C57BL/6 mice sensitized with a BALB/c heart on day 7 post transplantation, followed by staining with the proliferation dye cell trace violet (CTV, 1 µM, Thermofisher). Allo-induced T cell proliferation

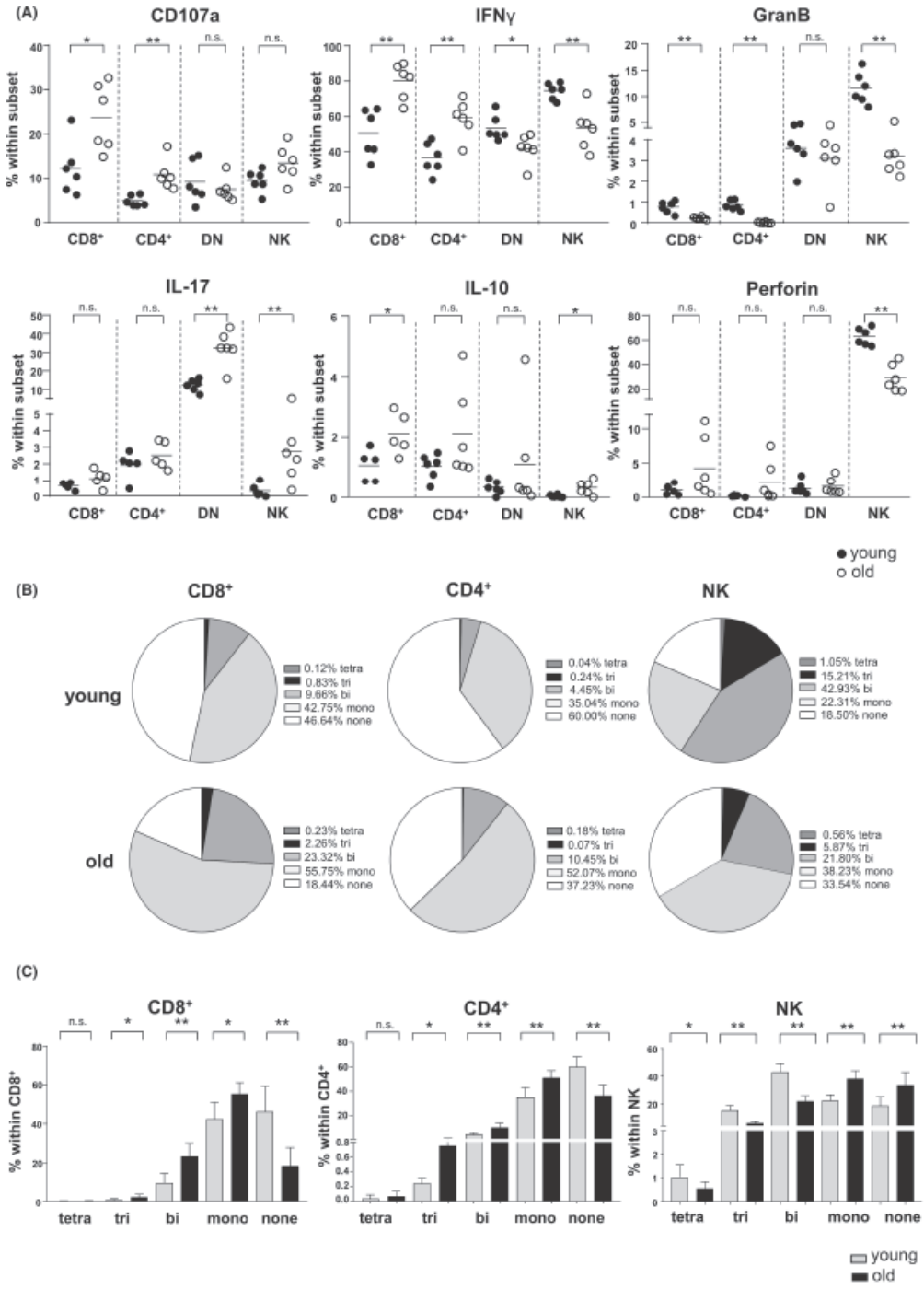


FIGURE 3 Kidney-resident lymphocytes derived from aged kidneys show an inflammatory effector profile. (A) Effector profile (CD107a, IFNγ, granzyme B, IL-17, IL-10, and perforin) of $CD8^+$, $CD4^+$, double negative (DN) T cells and NK cells isolated from young or aged C57BL/6 kidneys. (B) Analysis of $CD8^+$, $CD4^+$ T, and NK cell polyfunctionality was performed by boolean gating, including all of the above listed effector molecules. Shown are cells expressing four, three, two, one, or no molecule(s) at a time. Respective mean frequencies from $n = 5–6$ animals/group were used. (C) Statistical analysis of individual data sets as depicted in (B). Statistically significant differences were tested with Mann–Whitney U test and presented as means, $^*p < .05$; $^{**}p < .01$; n.s. = not significant.

was monitored by FACS based on loss of CTV after 4 days of co-culture of 10^4 DCs with 10^5 T cells.

2.8 | Real-Time RT-PCR

Real-Time PCR was performed as previously described.[26] Total RNA was extracted from snap-frozen samples using RNeasy Mini Kit (Qiagen). RT-PCR was performed for gene expression analysis on ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies) using Taqman® gene expression assays (Table S2). Gene expression was normalized ($2^{-\Delta Ct}$ formula) to hypoxanthine-guanine phosphoribosyltransferase (HPRT).

2.9 | Histology

Kidney tissues were formalin-fixed and paraffin-embedded; 1 µm thick sections were prepared, processed and stained with periodic acid schiff (PAS) and counterstained with hematoxilin as previously described.[27] Samples were assessed for necrosis, acute tubular damage, and glomerulitis. Semiquantitative scores in alignment to the Banff criteria were adapted and designed for each lesion. Following scores were used: acute tubular damage: 0 = non, 1 = mild, 2 = moderate, 3 = severe; necrosis: 0 = non, 1 = 1–10% of tissue, 2 = 11–20% of tissue, 3 = more than 20% of tissue; interstitial inflammation in non-fibrotic cortex: 0 = < 10%, 1 = 11–25%, 2 = 26–50%, 2 = more than 50%; glomerulitis: 0 = non, 1 = present in at least one glomerulus. Scoring was performed in a blinded fashion based on the whole slide of each sample. Staining of ß-galactosidase was performed according to the manufacturer's instructions (Abcam).

2.10 | Immunofluorescence microscopy

Immunofluorescence microscopy (IF) was done as previously published.[28] Briefly, transplanted kidneys were excised and a ¼ of each kidney was fixed overnight at 4°C with PLP fixative. Following extensive wash with PBS, kidneys were dehydrated in 30% sucrose in PBS overnight at 4°C, snap frozen in OCT and stored at −80°C. 16 µm kidney cryosections were mounted on Superfrost Plus microscope slides (Fisher) and dried overnight at room temperature. Sections were blocked and stained in a humidified box in the dark at room temperature overnight with purified or directly conjugated antibodies (Table S3). Next morning, following washes with PBS sections were stained with the respective secondary antibody for 1 h at room temperature, counterstained with Hoechst and mounted with Fluoromount-G (eBiosciences). Samples were scanned on a Zeiss LSM780 confocal microscope using a dry 20x 0.8 N.A. objective. Tiled, z-stacks were acquired at 1024x1024 pixels, with line averaging of 4 and pinhole size 1, and were stitched together using Zen Blue software (Zeiss). Fuji (version 1.53r) was used to process the acquired images and generate maximal intensity projection snapshots.

2.11 | Statistics

Statistical analysis was performed using GraphPad Prism 8 (GraphPad Software). Significant differences between groups were calculated applying either T- or Mann–Whitney U test (two groups) or Kruskal–Wallis test (multiple groups) after testing for normality distribution using the D'Agostino & Pearson test. Statistical significance was considered for the following p values: ns = $p > .05$, $^*p \leq .05$, $^{**}p \leq .01$, $^{***}p < .001$, $^{****}p \leq .0001$.

3 | RESULTS

3.1 | Solid and lymphoid organs are differentially affected by inflamm-aging

Applying a viSNE algorithm approach to assess FACS derived data for T cell diversity, we observed no clear differences between young and aged kidneys (Figure 1A, top). Next, we applied FlowSOM, a technique for unsupervised clustering of FACS data. Generated viSNE maps were overlayed with metaclusters identified in FlowSOM, allowing the identification of multiple T cell subpopulations based on their memory markers present in both young and aged kidneys (Figure 1A, bottom). Based on the annotated clusters, manual gating of FACS data for T cells revealed that frequencies of $CD8^+$ T cells were significantly increased in aged kidneys, spleen and lymph nodes whereas frequencies of $CD4^+$ T cells were decreased. Histology of young and old naïve kidneys did not reveal histopathological changes regarding necrosis, edema or interstitial inflammation (Figure S5A), however aged kidneys showed slightly more acute tubular injury (Figure 1B).

We further observed significantly reduced frequencies of double-negative (DN) T cells as well as $NKp46^+$ NK cells in aged kidneys and spleens compared to young organs, whereas both subsets were significantly increased in lymph nodes. Aged kidneys and lymph nodes were also characterized by significantly higher frequencies of $CD11c^+MHC\text{-}II^+$ dendritic cells (DCs) than their young counterparts (Figure 1C).

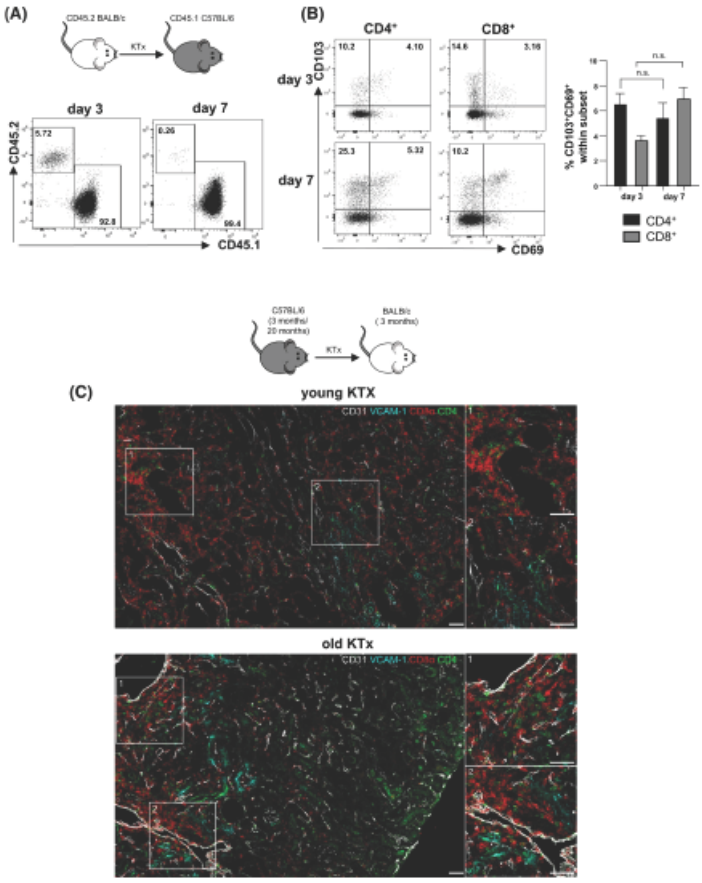


FIGURE 4 Recipient-derived T cells re-populate the allogeneic graft by day 7. (A) Representative FACS dot plots illustrating the intra-renal leukocyte distribution in a congenic BALB/c (donor, CD45.2$^+$) to C57BL/6 (recipient, CD45.1$^+$) combination on day 3 and day 7 post kidney transplantation. (B) Representative FACS dot plots showing the increase of CD69$^+$CD103$^+$ CD4$^+$ and CD8$^+$ T_{rm} cells (left panel). Bar graph represents frequencies of CD4$^+$ and CD8$^+$ T_{rm} cells (right panel, $n = 4$). Statistically significant differences were tested with Mann–Whitney U test and presented as means, n.s. = not significant. (C) Maximal intensity projection of confocal immunofluorescence snapshots from young or aged transplanted kidney cryosections (C57BL/6 into BALB/c, as depicted) stained for the indicated markers. Bars = 50 µm.

Both, FlowSOM and manual gating demonstrate that kidney, spleen and lymph nodes harbor significantly higher frequencies of effector memory CD4$^+$ and CD8$^+$ T cells (Figure 1A,D, Figure S5). In contrast, frequencies of natural CD4$^+$CD25$^+$FoxP3$^+$ regulatory T (T_{reg}) cells were significantly reduced in the aged kidney, being characterized by diminished portions of proliferating Ki67$^+$ cells; both features also applied to CD122$^+$ CD8$^+$ regulatory T cells (Figure 1E). As opposed to the kidney, CD8$^+$ T_{reg} frequencies were significantly higher in aged spleens and lymph nodes, but showing less proliferative capacity compared to their young counterparts (Figure S6). Thus, aging results in a change of the intra-renal lymphocyte compartment towards an effector memory phenotype paralleled with a loss of regulatory T cells.

3.2 | A distinct subset of NKp46$^+$ NK cells expressing CD49a and CXCR6 resides in senescent kidneys

We previously described increased mRNA expression of the activating cytotoxicity receptor NKG2D in human renal zero-hour biopsies as being indicative for biological donor age.[29] Here, we identified higher frequencies of NKG2D$^+$ NK and CD8$^+$ T cells in relation to age, confirming that NKG2D is upregulated on senescent renal lymphocytes (Figure 2A). This observation was also corroborated by viSNE analysis for NKp46$^+$ NK cells and equally applied to the chemokine receptor *C-X-C chemokine receptor type 6* (CXCR6) and the *alpha 1 subunit of α1β1 integrin* (CD49a) (Figure 2B). Heatmap analysis confirmed this induction, whereas other NK cell markers including DX5 and CD11b were downregulated (Figure 2C). Interestingly, CXCR6$^+$CD49a$^+$ NK cells have already been identified as tissue-resident NK cells in the human liver,[30] a feature that we confirm for murine kidneys and that quantitatively increases with age (Figure 2D,E). Aged CXCR6$^+$CD49a$^+$ NK cells contain significantly higher portions of perforin$^+$, but not IFNγ$^+$ cells (Figure 2E). Finally, despite lower overall frequencies of NK cells in aged kidneys (Figure 1B), we detected increased frequencies of differentiated CD27$^+$CD11b$^-$ NK cells (Figure 2F), being associated with greater effector function and responsiveness to chemokines.[31]

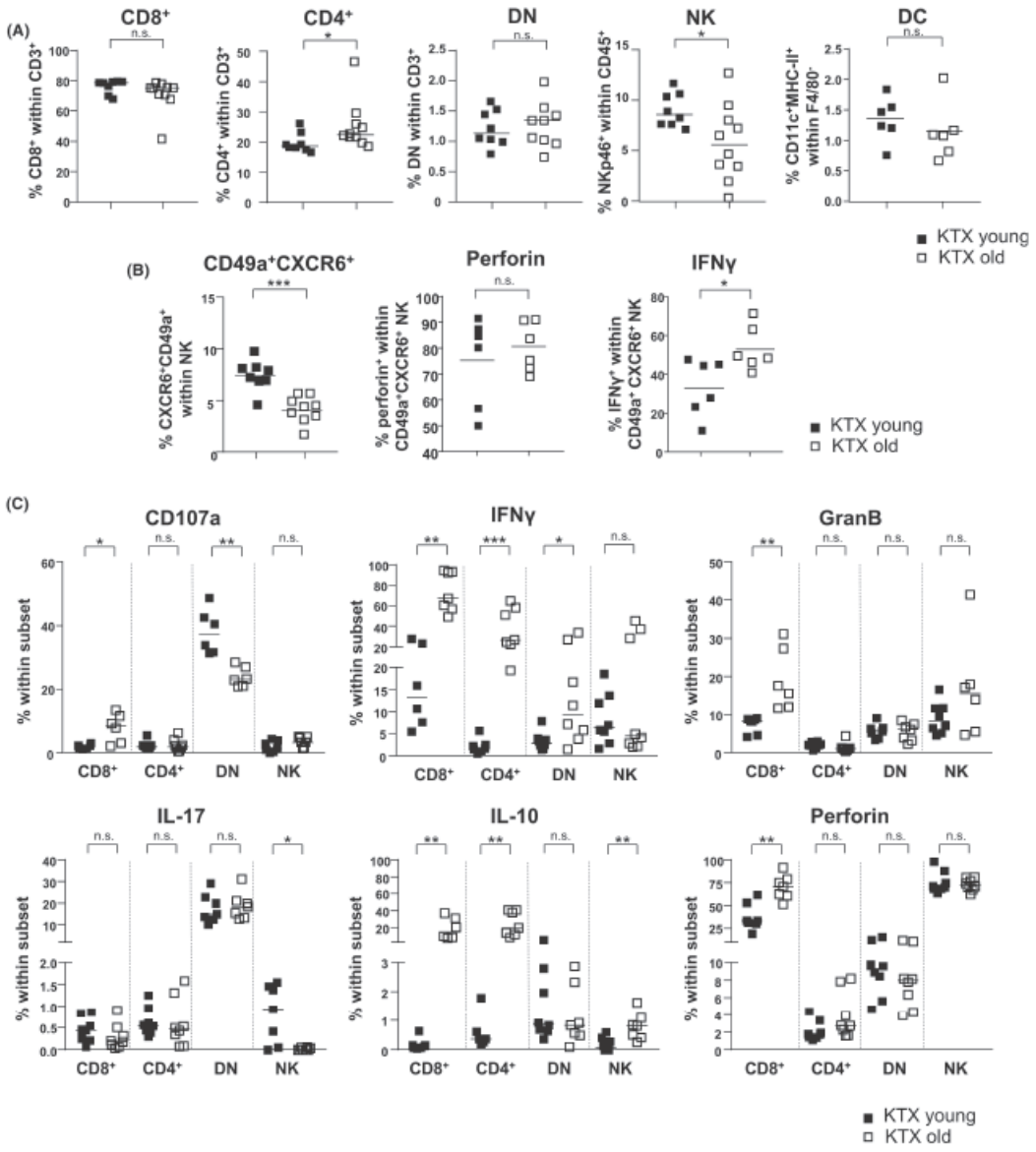


FIGURE 5 Aged kidney grafts provoke an intra-renal inflammatory profile. (A) Frequencies of $CD8^+$, $CD4^+$ T cells, DN, and NK cells isolated from either young or aged C57BL/6 derived grafts, transplanted into BALB/c recipients on day 7 post transplantation ($n = 8–10$). (B) Frequencies of $CD49a^+CXCR6^+$ NK cells isolated from either young or aged grafts and their production of perforin and IFNγ on day 7 post kidney transplantation. (C) Effector profile (CD107a, IFNγ, granzyme B, IL-17, IL-10, and perforin) of $CD8^+$, $CD4^+$ DN T cells, and NK cells isolated from young or aged renal allografts on day 7 post transplantation ($n = 5–9$). Statistically significant differences were tested with Mann–Whitney U test or unpaired *t*-test and presented as means, $^*p < .05$; $^{**}p < .01$; $^{***}p < .001$; n.s. = not significant.

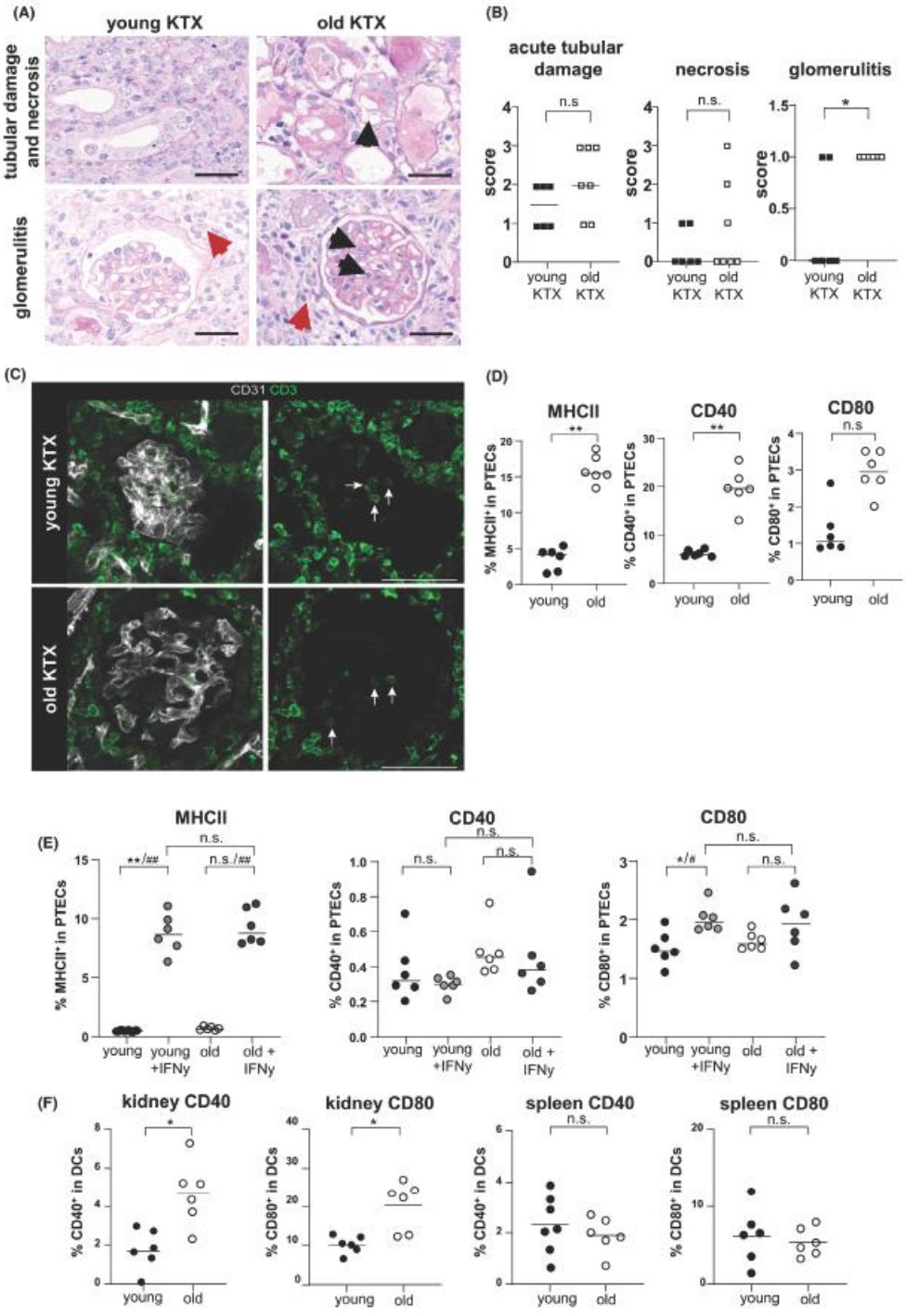


FIGURE 6 Aged PTECs and renal-derived DCs are characterized by induced expression of MHC class II and co-stimulatory molecules. (A) Representative histological images and (B) statistical analysis of tubular damage, necrosis (black arrow), glomerulitis (black arrows), and interstitial inflammation (red arrows) in young versus old C57BL/6 kidney grafts on day 7 post transplantation. 400× magnification, bar represents 20 μm. (C) Confocal immunofluorescence snapshots from young or aged transplanted kidney cryosections stained for CD31 (white) and CD3 (green), bars = 50 μm. (D) Surface expression of MHCII, CD40, and CD80 on freshly isolated PTECs from naïve C57BL/6 kidneys and (E) after 1 week of cell culture followed by IFNγ stimulation. (F) Ex vivo surface expression of CD40 and CD80 on $CD11c^+MHCII^+$ DCs isolated from naïve young or aged C57BL/6 kidneys and spleen. Data from $n = 6$–8 mice/group, respectively. Statistically significant differences were tested with Mann–Whitney U test with # indicating $p < .05$ and ## indicating $p < .01$ in a two-group comparison or, alternatively with Kruskal–Wallis in a multiple group comparison and presented as means and $^*p < .05$; $^{**}p < .01$; n.s. = not significant.

3.3 | Intra-renal lymphocytes display a distinct inflammatory effector function profile according to age

Both aged renal $CD8^+$ and $CD4^+$ populations contained significantly higher frequencies of degranulating $CD107a^+$ as well as $IFN\gamma^+$ cells, whereas portions of granzyme B producers were reduced (Figure 3A). Senescent NK cells less frequently expressed the cytotoxic mediators granzyme B and perforin, along with reduced proportions of $IFN\gamma^+$ cells, indicating an impairment of classical NK effector functions. Increased frequencies of $IL\text{-}17^+$ cells were confined to the senescent $CD4^-CD8^-$ DN T and NK cell compartment (Figure 3A). Aged kidney-derived $CD8^+$ and $CD4^+$ T cells further demonstrated an increase in polyfunctionality reflected by higher portions of cells secreting two or three effector molecules at a time, whereas senescent NK cells lost that ability (Figure 3B,C). In summary, our phenotypic analysis revealed a re-composition and altered effector profile of intra-graft lymphocytes in aged kidneys, which needs to be considered for their potential role as passenger leukocytes.

3.4 | The senescent kidney graft provokes an inflammatory immune response

In order to gain knowledge into the kinetics of graft infiltration, using congenic strains, we transplanted CD45.2 BALB/c kidneys into CD45.1 C57BL/6 recipients. Analysis of the intra-renal leukocyte composition revealed that whereas on day 3 post-transplantation donor-derived leukocytes were still detectable, the kidney was completely re-populated on day 7 by recipient-derived cells (Figure 4A). Thus, the majority of cells isolated from the graft in subsequent experiments are derived from the kidney recipient. Already on day 3, both recipient-derived, graft-infiltrating $CD45.1^+$ $CD4^+$ and $CD8^+$ T cells express the *integrin αE* (CD103) as well as the activation/retention marker CD69 being indicative for tissue-residency (T_{rm}). This expression increased for $CD8^+$ T cells until day 7, demonstrating that graft-infiltrating $CD4^+$ and $CD8^+$ T cells acquire a T_{rm} phenotype after entering the graft (Figure 4B). We hypothesized that aged grafts provoke the formation of higher intra-renal T_{rm} frequencies. However, whereas no differences between young and aged grafts were observed for $CD8^+$ T_{rm}, frequencies of $CD4^+$ T_{rm} were significantly decreased in aged kidney grafts on day 7 post-transplantation (Figure S7A).

Finally, we transplanted kidneys derived from either young (3 months) or aged (20 months) C57BL/6 mice into young (3 months) BALB/c recipients. Although we used in previous experiments BALB/c mice as donors,[22] this was not possible in this experimental set-up, as only aged C57BL/6 mice were available. On day 7 post-transplantation, $CD4^+$ and $CD8^+$ T cells were identified in young and aged organs. Both cell types were located in close proximity to $CD31^+$ (endothelial) cells and, although not exclusively, in close proximity to $VCAM\text{-}1^+$ tubuli in the cortex of kidney grafts (Figure 4C). Higher graft-infiltrating $CD4^+$ T cell frequencies were observed in aged organs, which was not observed for the spleen or lymph nodes (Figure 5A, Figure S7B). On the contrary, frequencies of total NK cells and $CD49a^+CXCR6^+NKp46^+$ NK cells were significantly decreased in aged kidneys compared with young grafts, but contained higher portions of $IFN\gamma^+$ cells (Figure 5B). Furthermore, we observed higher infiltration of effector memory $CD4^+$ T (T_{EM}) cells in aged kidneys (Figure S7C). Recipient-derived $CD8^+$ T cells infiltrating an aged kidney graft showed enhanced degranulation capacity reflected by CD107a, granzyme B, perforin and IFNγ expression with the latter feature also accounting for $CD4^+$ and DN T cells. Surprisingly, recipient-derived $CD4^+$ and $CD8^+$ T cell subsets isolated from a senescent kidney engrafted into a young recipient produced significantly more IL-10 than cells from a young graft (Figure 5C). Taken together, although frequencies of graft infiltrating cells are not dramatically different between young and aged kidneys, old grafts provoke a significantly higher inflammatory immune response mediated by graft-infiltrating recipient-derived T cells.

3.5 | Senescent proximal tubular epithelial cells upregulate MHC class II and co-stimulatory molecules

Aged allografts exhibited a higher incidence of glomerulitis, but not necrosis or tubular damage compared with young grafts (Figure 6A,B). Both young and aged grafts showed the presence of $CD3^+$ T cells in glomeruli (Figure 6C).

We further addressed whether proximal tubule epithelial cells (PTECs) might also contribute to age-related local alloinflammation. Freshly isolated PTECs from naive aged kidneys contained significantly higher portions of MHC class II^+, $CD40^+$ and $CD80^+$ cells than their young counterparts (Figure 6D). To gain sufficient cells for allo-stimulatory assays with T cells, we expanded PTECs from young versus old naïve mice for 5 days in vitro. As shown in

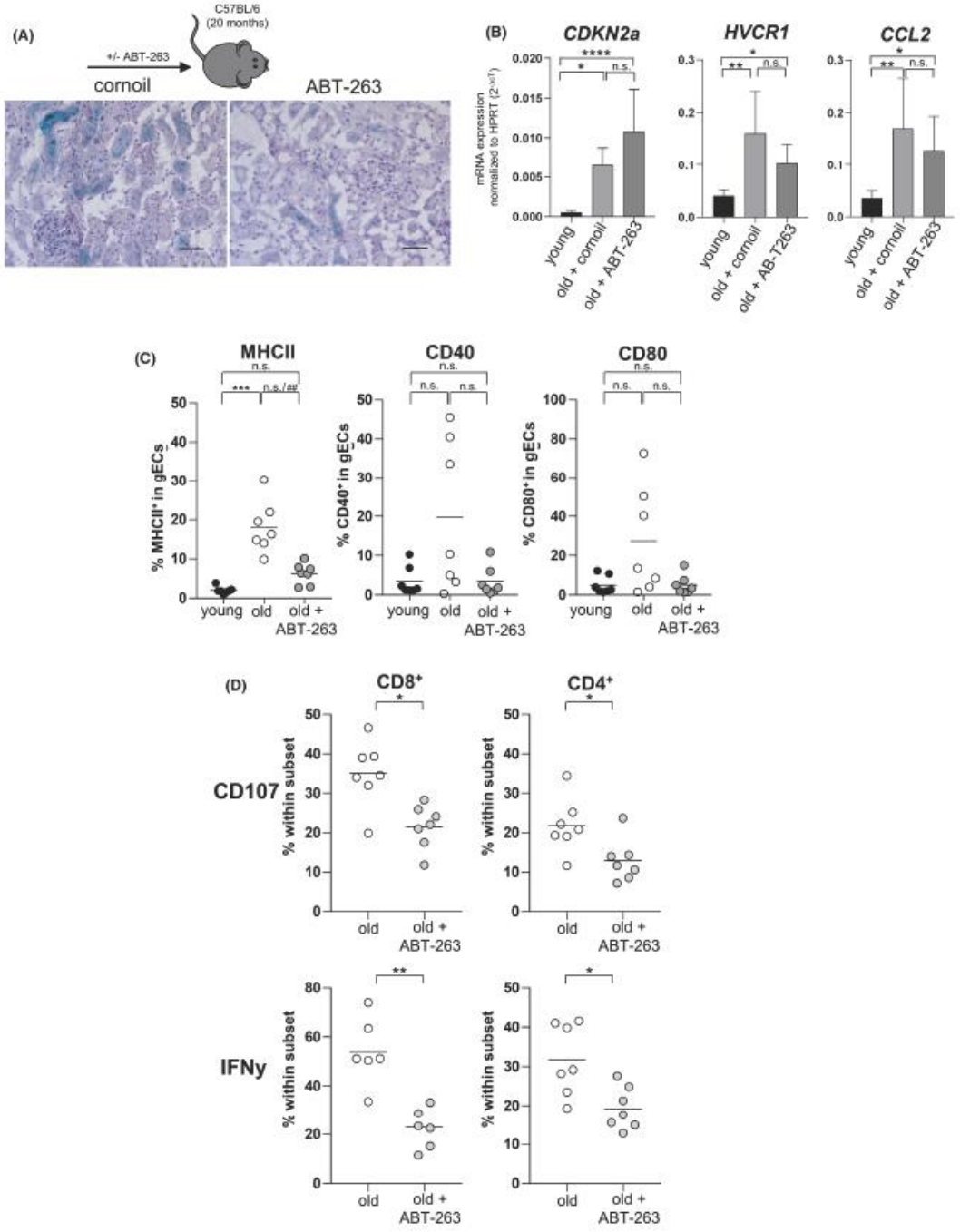


FIGURE 7 Treatment with the senolytic drug ABT-263 modulates graft immunogenicity. (A) Representative histological figures for ß-galactosidase kidney staining for cornoil versus ABT-263 treated old C57BL/6 mice, magnification 200x, bar represents 50 µm. (B) Gene expression analysis for the candidate genes *CDKN2a*, *HVCR1*, and *CCL2* analyzed in kidneys derived from naïve young ($n = 6$), or aged cornoil ($n = 8$) versus ABT-236 treated aged C57BL/6 mice ($n = 11$). (C) Ex vivo surface expression of MHCII, CD40, and CD80 on glomerular endothelial cells isolated from naïve young ($n = 6$), or aged cornoil ($n = 7$) versus ABT-236 treated aged C57BL/6 mice ($n = 6$). (D) Frequencies of $CD107a^+$ and $IFN\gamma^+$ $CD8^+$ and $CD4^+$ T cells isolated from aged cornoil ($n = 7$) versus ABT-236 treated aged C57BL/6 mice ($n = 7$). Statistically significant differences were tested with Mann–Whitney U where ## indicates $p < .01$ in a two-group comparison or, alternatively by Kruskal–Wallis-test in a multiple group comparison and presented as means. *$p < .05$; **$p < .01$; ***$p < .001$; ****$p < .0001$; n.s. = not significant.

Figure 6E, however, PTECs lose MHC class II and CD40 during culture as compared to ex vivo expression (Figure 6D). To re-induce costimulatory molecules, the expansion culture medium was supplemented with IFNγ, resulting in significant MHC class II upregulation; however, no differences between young and aged PTECs could be observed under these conditions (Figure 6E). CD80 expression was upregulated following IFNγ treatment solely on young kidney-derived PTECs but did not demonstrate higher expression levels than aged kidney-derived PTECs (Figure 6E). As an alternative cell-type for stimulation of allo-specific T cells, DCs were isolated. In line with data from PTECs, aged renal DCs showed significantly higher expression of CD40 and CD80 (Figure 6F), a feature not accounting for splenic DCs. However, the allo-stimulatory capacity of aged versus young kidney-derived DCs by adding allo-sensitized T cells was comparable (Figure S8).

3.6 | ABT-263 pre-treatment reduces inflammation in the aged graft

ABT-263 (Navitoclax) is a Bcl-2/w/xL inhibitor targeting the Bcl-2 pathway in senescent cells, resulting in cellular apoptosis.[32] Currently, ABT-263 is used in phase 1/2 clinical trials for both hematologic and solid organ malignancies, suggesting additional potential in other conditions requiring depletion of senescent cells, such as kidney transplantation.[33] We therefore treated naïve aged C57BL/6 mice with ABT-263 dosages. To examine potential effects of ABT-263 on renal senescent cells, we analyzed the contralateral kidney for classical markers indicative for renal senescence, tubular injury and inflammation.[18,34,35] Staining with ß-galactosidase, a classical senescence marker, demonstrated a reduction of senescent PTECs in ABT-263 pre-treated animals confirming recent data (Figure 7A).[34] Both aged mice treated with ABT-263 and untreated controls demonstrated a significantly higher mRNA expression of *CDKN2a* (p16INK4a), *HAVCR1* (kidney injury molecule 1, KIM 1) and *CCL2* (Monocyte Chemoattractant Protein-1, MCP-1) compared to untreated young kidneys (Figure 7B). Intriguingly, p16INK4a was induced, whereas KIM-1 and MCP-1 showed a reduced mRNA expression in ABT-263 kidneys. In order to evaluate the impact of ABT-236 treatment of renal endothelial cells, we isolated glomerular endothelial cells (gECs) from naïve young, old untreated and old ABT-263 treated mice. Comparable with PTECs, aged renal gECs demonstrated a significantly higher expression of MHCII compared with young gECs, which was partially observed for CD40 and CD80. This expression was downregulated in kidneys from ABT-263 pre-treated mice, although not statistically significant (Figure 7C). Interestingly, $CD4^+$ and $CD8^+$ T cells isolated from ABT-263 pre-treated untransplanted kidneys showed significantly less degranulation capacity and IFNγ production (Figure 7D).

On day 7 post kidney transplantation, higher frequencies of DN T cells and NK cells infiltrated ABT-263 pre-treated allografts. Despite the overall increase of NK cells, frequencies of $CD49a^+CXCR6^+$ $NKp46^+$ NK cells were significantly lower in grafts from ABT-263 treated mice (Figure 8A). Moreover, gECs isolated on day 7 post kidney transplantation from ABT-263 pre-treated mice showed still reduced levels of MHCII, CD40 and CD80 compared with aged untreated controls although not statistically significant (Figure 8B). Although no difference for graft function was detected on day 7 post transplantation (Figure 8C), recipient-derived, graft-infiltrating cells demonstrated a significant reduction of IFNγ and IL-10 for all T cell subsets (Figure 8D). We did not detect any histological changes between the ABT-263 treated and non-treated groups (Figure 8E). To prove a long-term effect of ABT-263 treatment, we repeated the aforementioned experiments, sacrificing the animals on day 28. Again, no dramatic changes for the various intra-renal lymphocyte populations analyzed were detected (Figure 9A), but creatinine and urea levels were significantly lower in the ABT-263 treated versus non- treated group, indicating an improvement of graft function after ABT-263 pre-treatment despite of unaltered histology (Figure 9A–C). Figure 10 summarizes the main findings of the study.

4 | DISCUSSION

Strategies to address the current organ shortage request a critical re-examination of donor eligibility, especially including the elderly.[36,37] Immune activation within a renal allograft may be mediated via the vasculature and/or passenger immune cells, yet no experimental studies have determined the impact of aging on any of these compartments. As molecular mechanisms of senescence have already been documented,[38–40] we aimed to understand the immunological component first, by assessing the intra-renal cellular composition according to kidney senescence. A shift of the CD4:CD8 ratio has already been described in association with aging and obesity in humans,[41] however, our analysis confirms higher frequencies of $CD8^+$ T cells, paralleled by a reduction of

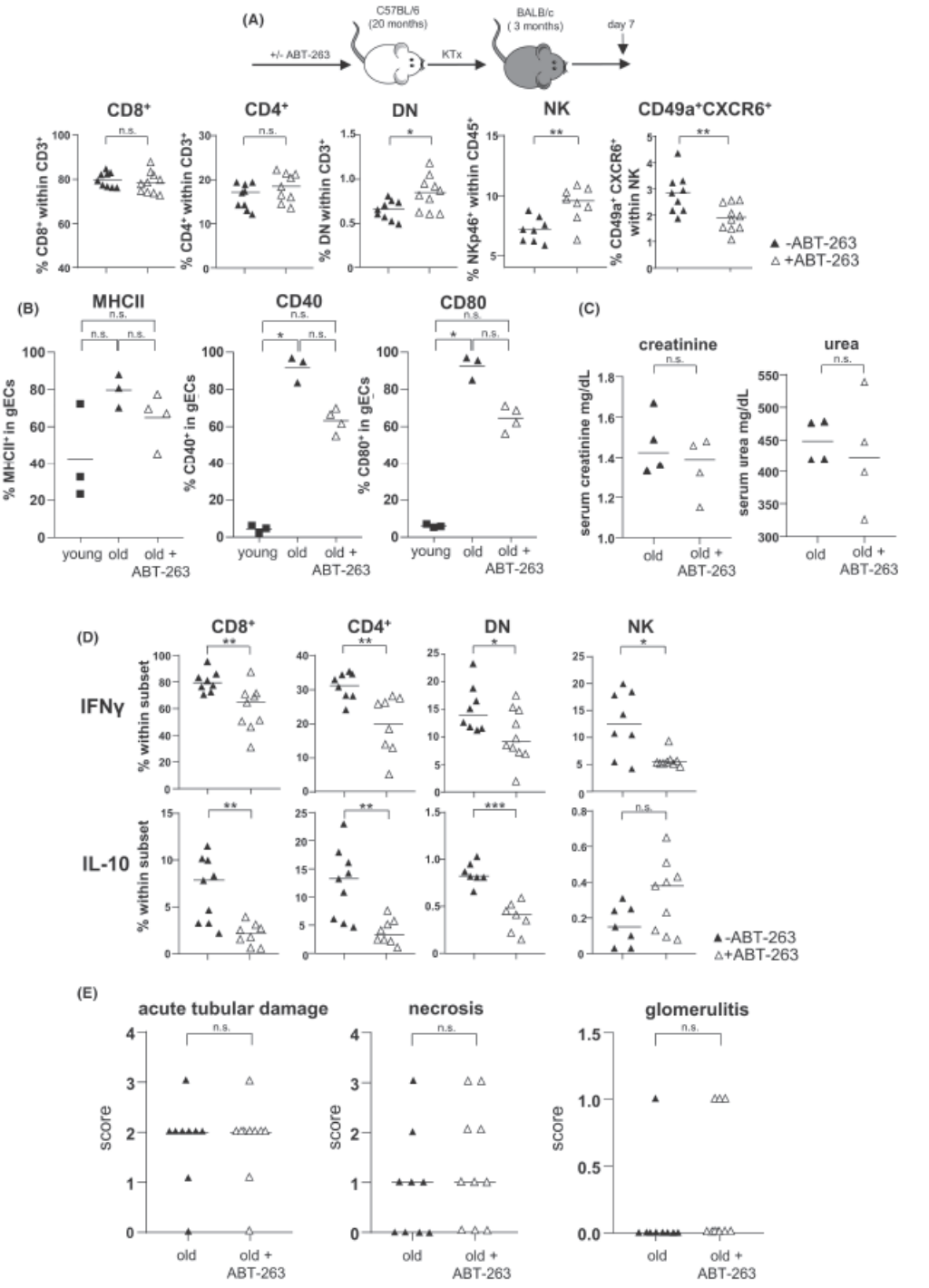


FIGURE 8 ABT-263 treatment ameliorates intra-graft inflammation of senescent kidneys (A) Frequencies of $CD8^+$, $CD4^+$, DN, NK, and $CD49a^+CXCR6^+$ NK cells isolated from either cornoil or ABT-263 pre-treated aged C57BL/6 derived grafts on day 7 post transplantation (8–10 animals/group). (B) Ex vivo surface expression of MHCII, CD40, and CD80 on glomerular endothelial cells isolated from young ($n = 3$) or aged cornoil ($n = 3$) versus ABT-236 treated aged C57BL/6 grafts ($n = 4$) transplanted into BALB/c recipient mice on day 7 post transplantation. (C) Functional parameters (serum creatinine, urea) from aged cornoil ($n = 3$) versus ABT-236 treated aged C57BL/6 grafts ($n = 4$) on day 7 post kidney transplantation. (D) IFNγ and IL-10 expression of $CD8^+$, $CD4^+$, DN T, NK cells isolated from either cornoil or ABT-263 pre-treated grafts. (E) Histopathological scoring for acute tubular damage, necrosis and glomerulitis between cornoil and ABT-263 pre-treated senescent grafts derived from day 7 post kidney transplantation. Statistically significant differences were tested with Mann–Whitney *U*-test, unpaired *t*-test or Kruskal–Wallis test and presented as means. $^*p < .05$; $^{**}p < .01$; n.s. = not significant.

$CD4^+$ T cells, in the aged kidney. According to studies performed in human peripheral blood and murine studies, we detected a significantly higher portion of $CD4^+$ and $CD8^+$ T_{EM}, whereas naïve T cells were decreased.[42,43] Our findings further demonstrate that renal NK cells were significantly reduced in naïve, older kidneys displaying reduced cytotoxicity and impaired polyfunctionality. Although an age-dependent decline of peripheral blood NK cells in humans has been described,[44,45] alternative studies document a significant increase in the percentage and/or absolute number of $CD3^-$ $CD56^+$ NK cells according to age.[46,47] We could also identify the presence of $CD49a^+CXCR6^+$ NK cells in the aged kidney. Both CD49a and CXCR6 have been originally described as markers of murine and human liver NK cells, the latter being critical for their long-term homeostasis.[48,49] These liver $CXCR6^+$ NK cells can mediate intense skin inflammation, suggesting that $CXCR6^+$ NK cells possess a memory potential.[48] As NK cells are emerging as powerful drivers of immune-mediated kidney allograft rejection,[50–53] future studies will be necessary to determine which defined NK cell subpopulation might be responsible for these effects. In summary, our findings document that the aging renal compartment is characterized by a defined composition of lymphocytes, which is not necessarily reflected in spleen or lymph nodes. Thus, inflammaging is an organ-specific characteristic, which needs to be considered in future studies addressing senescence.

The interaction between senescent cells and the intra-renal immune milieu post-transplantation is complex. Potential targets of recipient-derived graft-infiltrating cells including PTECs or gECs were characterized by significantly higher expression of activating and co-stimulatory markers indicating a premature aging phenotype. Although it can be assumed that senescence can also be triggered actively when cells are exposed to excessive inflammatory stimuli, such as IFNγ, aged versus young PTECs did not demonstrate a significant difference in their expression of MHC class II or CD80 in vitro. Similarly, we did not detect a difference in the stimulatory capacity of young versus aged renal-derived DCs towards allo-sensitized T cells. Thus, these data suggest that a subtle chronic inflammatory status shapes and maintains a senescent intra-renal micromilieu in vivo resulting in a mature phenotype of PTECs and gECs, which is difficult to mimic in vitro, in contrast to recently published data within a heart transplantation setting.[54] With the exception of infiltrating $CD4^+$ and $CD8^+$ T cells acquiring a T_{rm} phenotype,[55] we did not detect dramatic differences in lymphocyte frequencies infiltrating a young versus an aged organ. However, recipient-derived T cells isolated from aged donor grafts were characterized by an inflammatory phenotype. Based on the observation that a higher activation status was still observed for old gECs on day 7 post kidney transplantion, we postulate that recipient-derived T cells will become activated by the higher stimulatory capacity of the local endothelium/epithelium present in aged grafts.

Murine studies have shown that depletion of senescent cells delays age-associated disease.[56] We therefore pre-treated aged kidneys by the direct inhibition of the pro-survival Bcl-2 pathway applying ABT-263 to donor animals. This resulted in a depletion of senescent PTECs and gECs as well as in a reduction of activated graft-resident $CD4^+$ and $CD8^+$ T cells. Thus, it can be assumed that in naïve aged kidneys, activated PTECs and gECs maintain a pro-inflammatory phenotype of resident T cells. Interestingly, a reduction of allostimulatory gECs as a consequence of ABT-263 donor-pretreatment was still detected on day 7 post kidney transplantation. Targeting of renal senescent cells resulted in reduced IFNγ and IL-10 production by graft-infiltrating lymphocyte subsets, although graft histology and renal function were not influenced. Similar observations were made for day 28, however, an improvement of graft function could be observed for the ABT-263 pretreamtent group.

The application of senolytics has already been demonstrated to ameliorate numerous conditions in experimental models, including diabetes, cardiac dysfunction or acute kidney injury[32,34,57] as well as experimental heart transplantation.[58] Here, we demonstrate that targeting of renal senescence might also be a valuable approach to improve donor organ function, bearing the potential to reduce maintenance immunosuppression.

We are aware that our study has several limitations. First, it remains unclear why *p16INK4a* mRNA expression is not lower in ABT-263 pre-treated kidneys compared to controls, although this has been shown e.g. in lungs of aged ABT-263 treated mice.[32] As p16INK4A is not found in all senescent cells and can also be expressed in some non-senescent cells, the induced expression remains to be clarified.[59,60] Although the effectiveness of alternative senolytics, such as Dasatinib and Quercetin has been proven in experimental models as well as in clinical studies,[54,61–63] it needs to be evaluated whether their application might result in comparable—or even better results. Second, we failed with potential co-culture assays to understand the direct interaction between recipient-derived graft-infiltrating cells and potential target cells explaining their enhanced effector functions. Finally, it remains to be determined how very subtle changes in tissue integrity could be uncovered by advanced histological techniques.

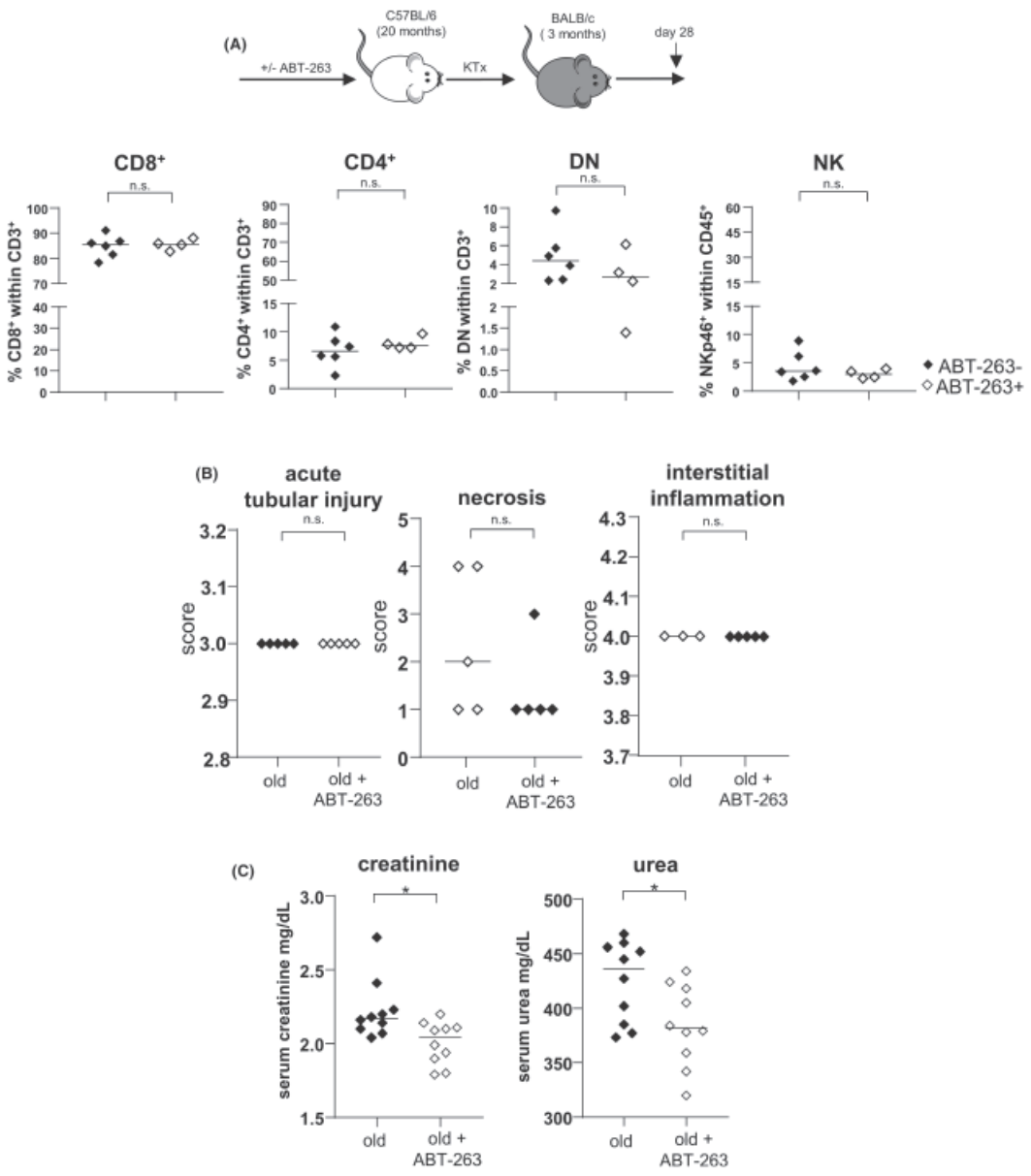


FIGURE 9 ABT-263 pre-treatment of senescent kidneys improves graft function in the long term but does not change graft histology. (A) Frequencies of $CD8^+$, $CD4^+$, DN T, and NK cells isolated from either cornoil or ABT-263 pre-treated aged C57BL/6 derived grafts on day 28 post transplantation ($n = 4–6$ animals/group). (B) Histopathological scoring for acute tubular damage, necrosis, and glomerulitis between cornoil and ABT-263 pre-treated senescent grafts derived from day 28 post kidney transplantation (3–5 animals/group). (C) Functional parameters (serum creatinine, urea) on day 28 post kidney transplantation ($n = 10–11$ animals/group). Statistically significant differences were tested with Mann–Whitney U test or unpaired *t*-test and presented as means. $^*p < .05$; n.s. = not significant.

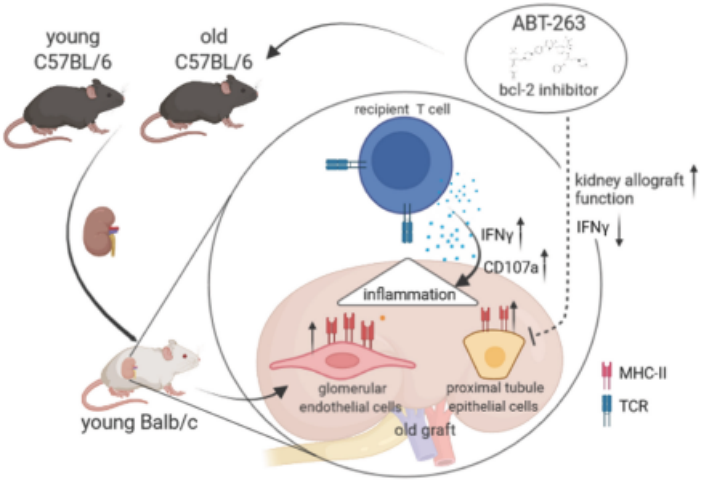


FIGURE 10 Graphical summary of the main manuscript findings.

Our current understanding of the influence of age on the size of the memory compartment entirely relies on studies of peripheral blood and lacks information on resident memory cells. It is therefore mandatory to better understand the molecular and cellular pathways that are responsible for linking the aging immune system with the kidney. On that background, pre-conditioning of senescent organs, as demonstrated here, bears a high potential for clinical translation due to its regulation of the inflammatory cellular response and improvement of organ function.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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Natural Killer Cells Promote Kidney Graft Rejection Independently of Cyclosporine A Therapy

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Natural Killer (NK) cells have recently been recognized as key players in antibody-mediated chronic allograft failure, thus requiring a comprehensive understanding whether NK cells can escape conventional immunosuppressive regimens. Influence of cyclosporine A (CyA) on NK cell function was studied in a mouse model of allogeneic kidney transplantation (KTX, BALB/c to C57BL/6). Recipients were treated daily with CyA (10 mg/kg) for seven or 14 days for long term survival (day 56). Administration of CyA in recipients resulted in significantly reduced frequencies of intragraft and splenic $CD8^+$ T cells, whereas the latter illustrated reduced IFNγ production. In contrast, intragraft and splenic NK cell frequencies remained unaffected in CyA recipients and IFNγ production and degranulation of NK cells were not reduced as compared with controls. Depletion of NK cells in combination with CyA resulted in an improvement in kidney function until day 7 and prolonged graft survival until day 56 as compared to untreated controls. Surviving animals demonstrated higher intragraft frequencies of proliferating $CD4^+FoxP3^+Ki67^+$ regulatory T (T_{REG}) cells as well as higher frequencies of $CD8^+CD122^+$ T_{REG}. We here demonstrate that NK cell depletion combined with CyA synergistically improves graft function and prolongs graft survival, suggesting that NK cell targeting constitutes a novel approach for improving KTX outcomes.

Keywords: kidney transplantation, Natural Killer (NK) cells, cyclosporine A, graft rejection, immunosuppression

INTRODUCTION

Following kidney transplantation (KTX), commonly used immunosuppressive regimens are prevalently based on initial calcineurin inhibitors (CNIs), either cyclosporine A (CyA), or tacrolimus (Tac) (1). Following this approach, the incidence of acute T cell-mediated rejection has been effectively reduced, resulting in 1-year graft survival rates between 88 and 95% for renal allografts (2). However, CNIs primarily target T cells, whereas their efficacy in suppressing other immune subsets—such as Natural Killer (NK) cells—might be limited (3, 4).

While it is well known that NK cells are the primary effector cells of rejection after MHC-mismatched bone marrow (BM) transplantation (5, 6), until recently the role of NK cells in solid organ transplantation (SOT) has been widely underrated (7). However, increasing evidence now substantiates the notion that NK cells are involved in several facets of graft rejection, also after SOT, although their exact function remains controversial, suggesting a dichotomous role in transplantation (8). Whereas, inactivation of CD28-mediated co-stimulation of T cells failed to achieve acceptance of allogeneic vascularized cardiac grafts, additional inhibition of NK cells resulted in long-term allograft survival (9).

A potential contribution of NK cells to graft rejection was suggested by the observation that $NK1.1^+$ cells provide help for alloantigen-specific T cells (10). Especially with respect to kidney injury, NK cells can recognize and kill tubular epithelial cells based on NKG2D ligand expression being upregulated in response to ischemia-reperfusion injury (IRI) (11). Despite the fact that these observations underline a pro-inflammatory role of NK cells, contrarily also pro-tolerogenic properties of NK cells in the context of SOT have been observed by killing donor antigen-presenting cells (12). More importantly, evidence of NK cells and their role in antibody-mediated rejection of kidney grafts is accumulating, as it was recently demonstrated that antibody-mediated rejection (ABMR) associates with an intrarenal expression signature enriched with NK cell pathways (13, 14).

However, data addressing the effect of CyA on NK cells are mostly drawn from peripheral blood NK cells as well as from *in vitro* studies and remain conflicting (15–19). Therefore, this study aims to delineate the effects of CyA on NK cells for the first time in a murine model of KTX in order to define the influence of NK cells on renal allograft outcome *in vivo*.

METHODS

Animals

BALB/c and C57BL/6 mice were purchased from Charles River Laboratories (Charles River, Sulzfeld, Germany). Male mice weighing between 24 and 30 g were used for all experiments. Animals were housed under standard conditions and received humane care in compliance with the "Principles of Laboratory Animal Care" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86–23, revised 1985). All animal experiments were approved by the Landesamt für Gesundheit und Soziales Berlin (G0089/16) or by the Austrian Federal Ministry of Science and Research (BMWF-66.011/0163-II/3b/2012).

Kidney Transplantation

Allogeneic renal transplantations were performed as previously described (20). Briefly, the left donor kidney was flushed *in situ* with histidine-tryptophane-ketoglutarate solution (Custodiol®, Dr. Franz Köhler Chemie GmbH, Bensheim, Germany) and procured. End-to-side anastomoses between the donor renal vessels and the recipient's abdominal aorta and inferior vena cava were performed following a knotless technique (21). For urinary tract reconstruction the ureter was directly anastomosed into the bladder. The duration of cold and warm ischemia of allografts was maintained at 30 min. each. The contralateral native kidney was removed 24 h before sacrificing the animal on post operative day (POD) 7. For long-term surviving animals the contralateral kidney was removed on POD7 and the surviving animals were sacrificed on POD56. Animals with surgical complications were excluded from the study.

In vivo Treatment

Beginning on the day of KTX (POD0), CyA was administered to C57BL/6 recipients until POD7 using daily subcutaneous injections at dosages of 10 mg/kg body weight. However, to prevent acute rejection in the long survival groups, recipients were treated daily with CyA for 14 days, as previously described (22). Depletion of NK cells *in vivo* was performed by intraperitoneal injection of an anti-mouse NK1.1 monoclonal antibody (200 μg; PK136, BioXCell, Lebanon, NH, USA) on POD−2 and POD+2.

In vitro Assays

Functional analysis of NK and T cells was performed as recently described by using isolated splenic mononuclear cells (MNC) (23). Cells were stimulated in the presence of 200 U/ml mIL-2 either with 50 ng phorbol 12-myristate 13-acetate (PMA), 1 μg ionomycin calcium salt (Sigma-Aldrich, St. Louis, MO, USA), alternatively with murine YAC-1 cells as target cells (with an effector:target ratio 2:1), 5 μg/ml brefeldin A (Sigma Aldrich), and 2 μM monesin (Biolegend) for 4 h at 37°C and 5% CO_2. After stimulation, cells were stained with antibodies listed in **Supplemental Table 1**. Degranulation capacity was assessed by CD107a lysosome-associated membrane protein-1 (LAMP-1) expression. Cell activation was assessed by first fixing and permeabilizing cells using Transcription Factor Staining Buffer Set (eBioscience) and then by staining intracellularly for IFNγ.

Flow Cytometry

MNCs from spleen and lymph nodes were isolated by Ficoll-Histopaque (Sigma Aldrich) density gradient centrifugation. To obtain single cell suspension from kidneys the tissue was digested with collagenase IV (Gibco/Invitrogen, Darmstadt, Germany) plus DNase (Ambion/Applied Biosystems, Darmstadt, Germany) in 10 ml of supplemented RPMI for 45 min at 37°C. Released leukocytes were first separated by passing through a cell strainer (100 μm) and leukocytes were enriched using CD45 MicroBeads (Miltenyi Biotec, Inc., Auburn, CA, USA). For flow cytometry, 1×10^6 cells were incubated for 20 min at 4°C for surface stainings and 30 min at room temperature for intracellular stainings with respective antibodies as listed in **Supplemental Table 1**. Cells were analyzed on a FACSFortessaX20 (BD Bioscience, Heidelberg, Germany), collecting a total of 100.000 events in a live gate, and data were analyzed using FlowJo software 10.0 (Tree Star Inc., Ashland, OR, USA). An exemplary gating strategy is provided in **Supplemental Figure 1**.

Measurement of Antibody Concentrations

Antibody concentrations were assessed in recipient serum by a flow cytometry bead-based analysis applying a mouse immunoglobulin isotyping panel (LEGENDPlex Multi-Analyte Flow Assay Kit, Biolegend) according to the manufacturer's instructions and measured on a FACSFortessaX20 (BD Bioscience).

Histology and Immunohistology

For immunohistochemistry, 5 µm paraffin tissue sections were deparaffinized and incubated for 1 h at 25°C with monoclonal rabbit anti-CD3 antibody (clone SP7, Thermo Scientific, Waltham, MA, USA) and with polyclonal goat anti-mouse C3d antibody (R&D Systems, Minneapolis, MN, USA). Controls using normal sera were run to exclude non-specific staining. Slides were processed using the Promark rabbit-on-rodent HRP Polymer Kit and the Promark goat-on-rodent HRP Polymer Kit (Biocare Medical, Concord CA, USA) followed by HistoGreen (Linaris, Wertheim, Germany) as substrate and counterstained with hematoxylin (24). In order to evaluate histomorphology, 1–2 µm kidney sections were cut, dewaxed, and histochemically stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and the Elastica van Gieson staining kit (Merck) for connective tissue. Histological lesions were scored according to the definition of standard BANFF Classification (25).

Serum Analysis of Kidney Function Parameters

Serum samples were stored in aliquots at −20°C until serum creatinine and urea were measured using the CREP2 Creatinine Plus version 2 and Urea/BUN assays, respectively, on a Roche/Hitachi Cobas C 701/702 system (Roche Diagnostics, Mannheim, Germany).

Real-Time RT-PCR

Real-time RT-PCR was performed as recently described (24). In brief, total RNA from snap-frozen biopsies was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Integrity of RNA was checked using a NanoDrop™ 2000c spectrophotometer. For cDNA synthesis, 2 µg of total RNA was reverse transcribed in a 40 µl reaction volume using oligo(dT) primer and the RevertAidTM H Minus M-MuLV Reverse Transcriptase (Fermentas GmbH, St. Leon-Rot, Germany). Samples were tested for genomic DNA contamination and if tested positive excluded from the study. Real-time reverse transcription polymerase chain reaction (RT-PCR) for gene expression analysis was performed with the ABI PRISM 7500 Sequence Detection System (Life Technologies, Carlsbad, CA, USA). Primers were directly purchased as Taqman® gene expression assays (Life Technologies, Carlsbad, CA, USA) (**Supplemental Table 2**). Specific gene expression was normalized to the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) using the formula $2-\Delta Ct$. The mean Ct values for the genes of interest and the housekeeping gene were calculated from double determinations. Samples were considered negative if the Ct values exceeded 40 cycles.

Statistics

Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). Kaplan-Meier plots were used to analyze graft survival, and the log-rank test was applied to assess the statistical significance of differences between survival curves. Statistical significance between two groups was calculated using the Mann-Whitney *U*-test. For multiple comparisons, Kruskal-Wallis test with Dunn's *post-hoc* was used. Statistical significance was considered for the following *p*-values: ns = $p > 0.05$, $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

RESULTS

CyA Treatment Modulates Leukocyte Frequencies in Graft, Spleen, and Lymph Nodes

In order to determine whether treatment with CyA results in a modification of the major lymphocyte frequencies *in vivo*, we sacrificed C57BL/6 recipients of BALB/c kidneys on postoperative day (POD) 7. Compared to untreated controls, T cells were significantly reduced in grafts as well as in the spleens of CyA-treated recipients. Especially frequencies of $CD8^+$ T cells were reduced in both organs, whereas a significant reduction in $CD4^+$ T cells was detected solely for spleens. Contrarily, CyA treatment resulted in significantly reduced frequencies of $CD3^-NKp46^+$ NK cells in lymph nodes and $CD11c^+MHCII^+$ dendritic cells (DCs) in both spleen and lymph nodes (**Figure 1**). Focusing on T cell subsets, we detected reduced frequencies of $CD8^+$ $CD44^+CD62L^-$ effector memory T cells (T_{EM}) in all investigated organs derived from CyA-treated animals. A reduction in $CD4^+CD44^+CD62L^-$ T_{EM} cells was detected solely in lymph nodes (**Supplemental Figure 2**).

In vivo Treatment With CyA Reduces $NKG2D^+$ NK Cell Frequencies

Although we could not observe changes in the graft for bulk NK cell frequencies (**Figure 1**), we detected slightly reduced frequencies of $CD27^+CD11b^-$ immature NK cells in the kidney transplant as well as significantly reduced levels in the spleen derived from CyA animals, whereas no changes were observed for mature $CD27^+CD11b^+$ NK cells (**Figure 2A**). Recently, we reported on the functional importance of the activating Natural killer group 2 member D (NKG2D) C-type lectin receptor for allograft survival (23). In contrast to NK cells bearing the inhibitory NKG2A, frequencies of NK cells expressing the cytotoxicity receptor NKG2D were significantly decreased in all three investigated organs derived from the CyA group (**Figure 2B**). This observation was further underlined by the significantly decreased surface expression levels of NKG2D, as reflected by their mean fluorescence intensity, on NK cells (**Figures 2C,D**). Contrarily, $CD3^+$ $NKG2D^+$ T cell frequencies and surface expression levels of NKG2D on T cells were not affected by CyA treatment (**Supplemental Figure 3**).

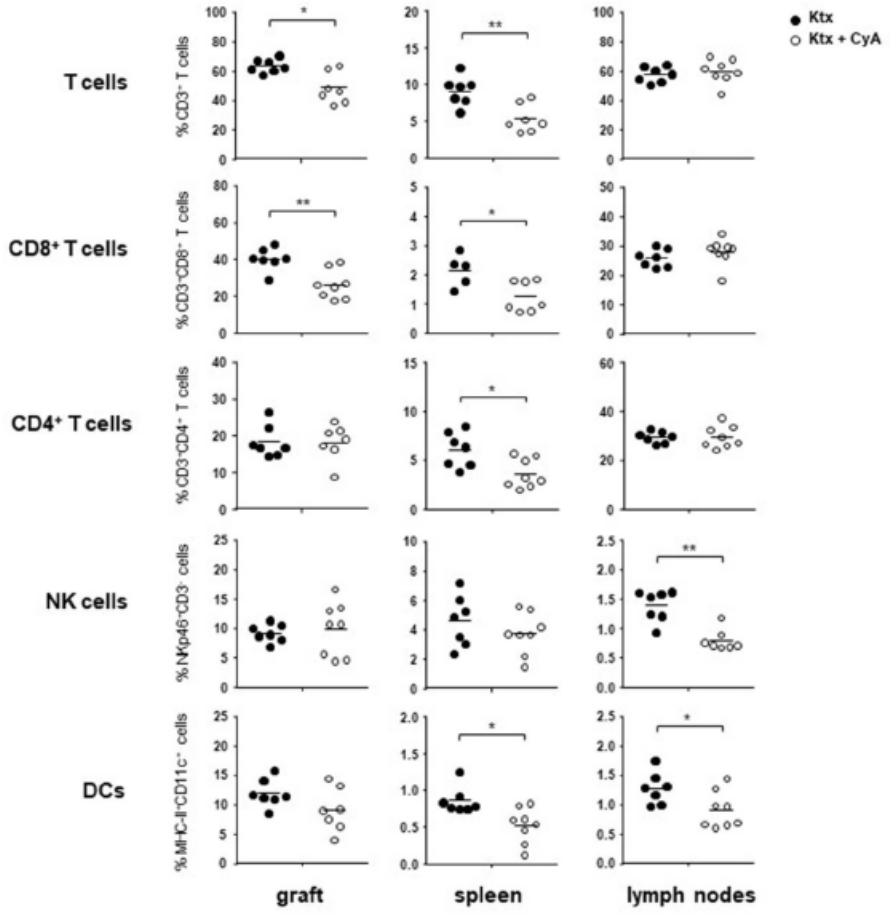

FIGURE 1 | Administration of CyA leads to modulation of intragraft lymphocyte frequencies. After CyA administration, C57BL/6 recipient mice of BALB/c kidneys showed on POD7 significantly decreased frequencies of T cells in the allograft as well as in the spleen as compared with untreated recipients. NK cell frequencies were solely affected as a consequence of CyA treatment in the lymph nodes, whereas DC frequencies were significantly decreased in both spleen and lymph nodes, but not in the graft. Data are presented as mean of $n = 7$–8 animals/group. Cells were pre-gated on live CD45$^+$ events. Statistically significant differences between naïve and CyA-treated animals were tested with the Mann-Whitney *U*-test; $^*p < 0.05$, $^{**}p < 0.01$.

CyA Decreases IFNγ Production of T Cells but Not of NK Cells

In order to examine whether CyA affects T and NK cell function *in vivo*, we isolated spleen cells from CyA or untreated recipients on POD7. Whereas, both CD4$^+$ T cells and CD8$^+$ T cells isolated from CyA-treated animals illustrated a significant reduction in their potential to secrete IFNγ as compared to that of untreated animals, IFNγ production by NK cells was not affected. No influence of CyA treatment was observed on the degranulation capacity of NK and T cells (**Figure 3A**). Using the murine cell line YAC-1 as target cells, a significant reduction of IFNγ was observed for CD8$^+$ T cells, however no differences for degranulation and IFNγ production of NK cells derived from CyA treated animals compared with naïve recipients (**Supplemental Figure 4**).

Analysis of intragraft mRNA expression revealed that CyA kidneys showed significantly reduced mRNA levels of the cytotoxic markers perforin and granzyme B ($p < 0.05$). Moreover, mRNA expression of the chemokine *C-X-C motif chemokine 10* (CXCL10) and of IFNγ was also significantly reduced in grafts

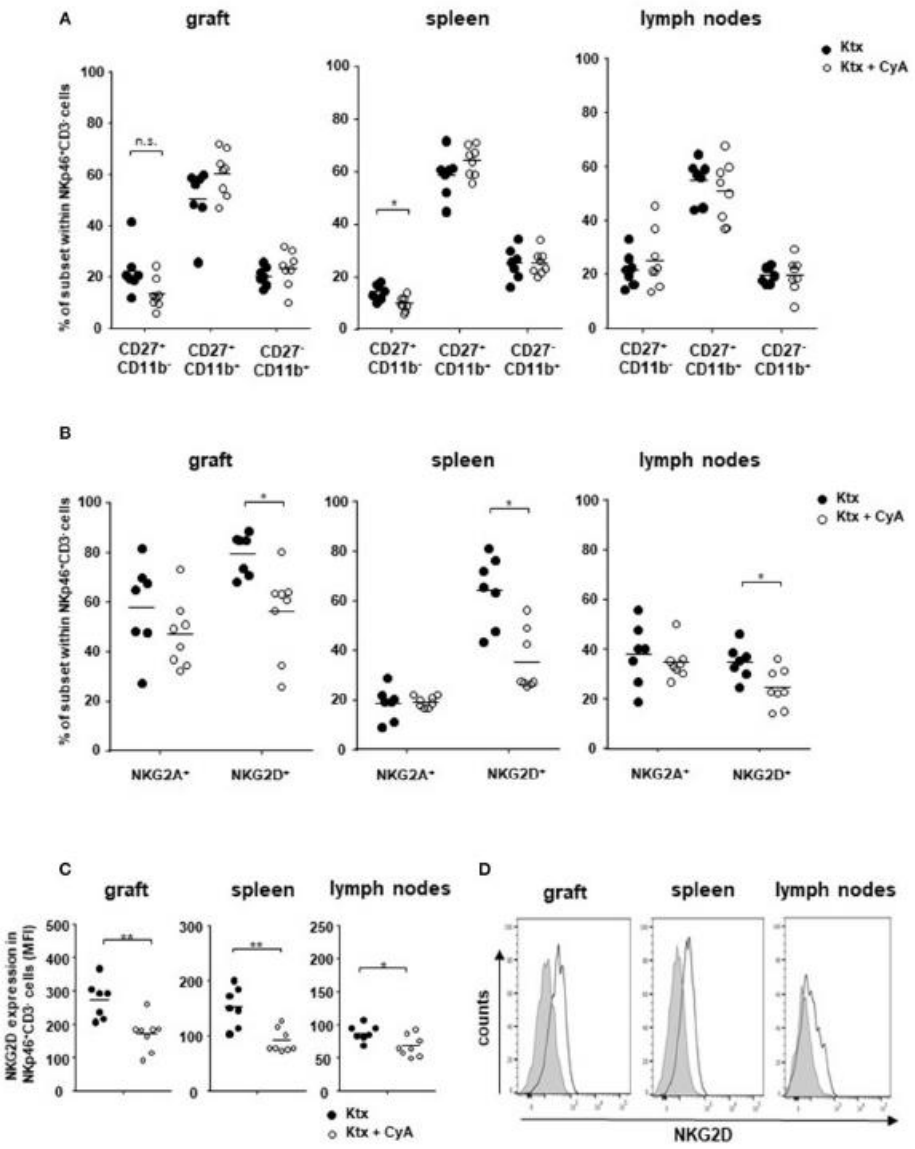

FIGURE 2 | Impact of CyA on NK cell subsets. **(A)** CD3−NKp46+ NK cell subsets were analyzed according to their CD11b and CD27 expression. An influence of CyA treatment was detected for immature CD11b−CD27+ cells in the spleen. **(B)** CyA leads to a significant reduction in NKG2D+ NK cells in all three investigated organs, whereas the NKG2A+ NK cell subset remains unaffected. **(C,D)** NKG2D is significantly down-regulated on NK cells as a consequence of CyA, as reflected by mean fluorescence intensity (MFI). Data are presented as mean of $n = 7–8$ animals/group. Cells were pre-gated on live CD45+ events. Statistically significant differences between naïve and CyA-treated animals were tested with the Mann-Whitney *U*-test; $^{*}p < 0.05$. Black line corresponds to NKp46+ NK cells derived from untreated animals, gray area to NK cells derived from CyA-treated animals. $^{**}p < 0.01$.

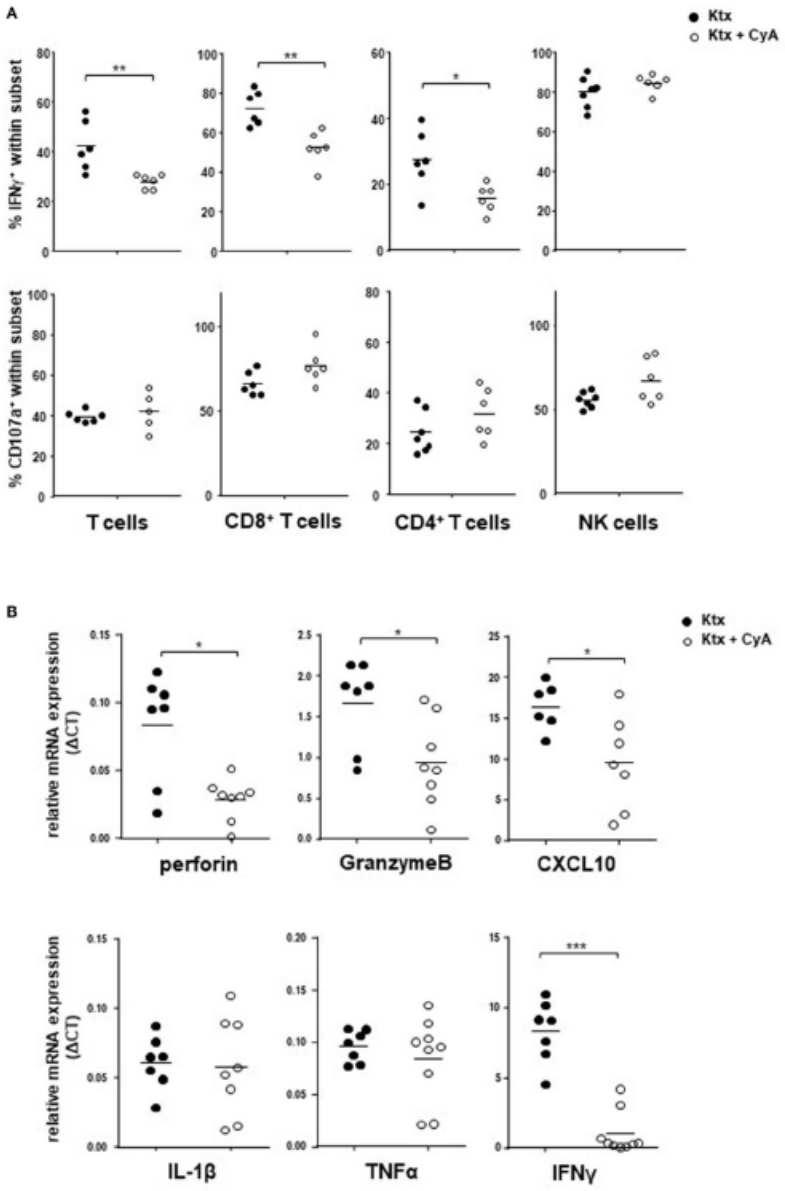

FIGURE 3 | IFNγ production of NK cells is unaffected in CyA-treated recipients. **(A)** Splenocytes derived from CyA animals or recipients left untreated were polyclonally stimulated with PMA and ionomycin. Whereas both $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells derived from CyA animals illustrate a clear reduction in IFNγ, $CD3^-NKp46^+$ NK cells retain their capacity to produce IFNγ. No impact was observed for the degranulation capacity. **(B)** Assessment of the intragraft mRNA profile revealed a significant reduction in inflammatory mediators including perforin, granzyme B, CXCL10, and IFNγ in CyA-treated recipients. Data are presented as mean of $n = 7$–8 animals/group. Cells were pre-gated on live $CD45^+$ events. Statistically significant differences between naïve and CyA-treated animals were tested with the Mann-Whitney *U*-test; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

derived from the CyA group (**Figure 3B**), whereas no differences were observed for the inflammatory cytokines IL-1ß and TNFα.

CyA in Combination With NK Cell Depletion Does Not Improve Kidney Function in the Short Term

Our results illustrated so far suggest that NK cells in the recipient are insufficiently targeted by CyA treatment. In order to evaluate whether the additional NK cell neutralization results in improved graft survival, we depleted NK cells in CyA-treated C57BL/6 recipients of BALB/c kidneys on day POD-2 as well as on POD+2. The two-fold application of NK1.1 antibody resulted in efficient depletion of NK cells in the graft, spleen, and lymph nodes (**Figure 4A**, **Supplemental Figure 5**). Compared with graft recipients receiving CyA only, analysis on POD7 revealed a significant increase in $CD4^+$ T cells in the allograft, whereas $CD8^+$ T cells were increased in lymph nodes (**Figure 4A**). More interestingly, overall frequencies of $CD3^+NKG2D^+$ T cells were significantly reduced in the allograft as well as in the spleen (**Figure 4B**), whereas NKG2D expression levels were not affected on T cells (**Figure 4C**).

Although CyA treatment of the recipient resulted in decreased frequencies of graft-infiltrating lymphocytes as compared with untreated animals, this was not further improved by NK cell depletion. A similar picture was observed for $CD3^+$ T cells, whereas a diffuse C3d deposition was detected for all groups (**Figure 5A**). However, the CyA group demonstrated an improved Banff Score compared to the untreated group, an observation which was not improved by combined CyA+NK treatment. In contrast, kidney function based on urea levels and creatinine were significantly improved in the CyA+NK group compared to the untreated group (**Figure 5B**). As NK cells are currently discussed as important contributors to humoral rejection post-kidney transplantation (13, 14), we therefore assessed the antibody response in the short-term surviving animals. CyA treatment of the recipient resulted in a clear decrease in IgG3 antibody concentrations, whereas the additional depletion of NK cells resulted in a significant decrease in the majority of antibody subclasses as compared with graft recipients left untreated ($p < 0.05$, $p < 0.01$, respectively) (**Figure 6**).

CyA in Combination With NK Cell Depletion Improves Allograft Survival in the Long Term

Although our findings in the short-term survival groups did not indicate a superior morphological benefit of NK cell depletion in combination with CyA treatment, the additional depletion of NK cells resulted in a significant overall survival until POD56 as compared with the control group (**Figure 7A**). However, histological scoring revealed no significant differences among the surviving animals in the experimental groups (**Figure 7B**, **Table 1**).

In order to explore mechanisms involved in the improved graft survival in the CyA+NK group, we analyzed animals that survived until POD56 for their regulatory T cell (T_{REG}) composition. Intriguingly, in all investigated organs, frequencies of $CD4^+CD25^+FoxP3^+$ T_{REG} were decreased as compared with the CyA group, but showed increased *ex vivo* proliferation as displayed by their Ki67 expression (**Figure 8A**). No differences in T_{REG} frequencies were detected by immunohistology (**Figure 8B**). Contrarily, as compared with the CyA group, intragraft $CD8^+CD122^+$ were clearly increased when NK cells were initially depleted in the early post-transplantation period, but displayed a reduced proliferation profile, although not significant (**Figure 8C**).

DISCUSSION

We recently summarized the various facets in which NK cells are involved in SOT and particularly emphasized their relevance for chronic rejection, which may result from NK cells escaping from conventional immunosuppressive regimens (26). However, controversies in the literature make comprehensive studies necessary to substantiate this hypothesis. Conclusions concerning the influence of CyA on NK cell numbers *in vivo* have been specifically drawn from the analysis of peripheral blood cells derived from patients, whereas data on NK cells residing in other compartments including solid organ transplants remain elusive. For instance, Neudoerfl et al. analyzed peripheral blood NK cells from kidney recipients, illustrating that CyA treatment did not affect the percentage of NK cells in the lymphocyte population among KTX patients by comparison to healthy individuals (27).

In our first set of experiments, recipient treatment with CyA significantly reduced intragraft bulk $CD3^+$ and $CD8^+$ but not $CD4^+$ T cell frequencies, whereas no reduction in bulk NK cells was observed for the kidney graft and spleen (**Figure 1**). Nevertheless, it appeared that distinct NK cell subsets were influenced by CyA, as we detected lower frequencies of intragraft and intrasplenic immature $CD27^+CD11b^-$ NK cells than in untreated controls (**Figure 2A**). More interestingly, the significant decrease in $NKG2D^+NKp46^+$ NK cell frequencies was obvious in all three investigated organs (**Figures 2B–D**). The inhibition of NKG2A and a reduction in NKG2D expression have been demonstrated solely for the immunosuppressants mycophenolic acid and rapamycin (18, 28), but it was recently demonstrated that IFNγ production is reduced for NK cells cultured *in vitro* in the presence of high concentrations of CyA (27). We therefore analyzed whether this also applies for NK cells isolated from mice treated with clinically relevant doses of CyA. Importantly, CyA treatment *in vivo* clearly affected IFNγ production of T cells, but not of NK cells. Moreover, the degranulation capacity of both T and NK cells remained unaffected (**Figure 3A**). Our data therefore stand in contrast to recent *in vitro* studies reporting that treatment of NK cells with cyclosporine and tacrolimus (FK506) resulted in inhibition of both degranulation and IFNγ production (27, 29). Intragraft inflammation of CyA-treated animals was significantly reduced at the mRNA level, which was also observed for IFNγ (**Figure 3B**). We assume that the decreased IFNγ expression is associated mainly with reduced graft-infiltrating $CD8^+$ T cells and their reduced capacity of IFNγ production in the CyA group, as

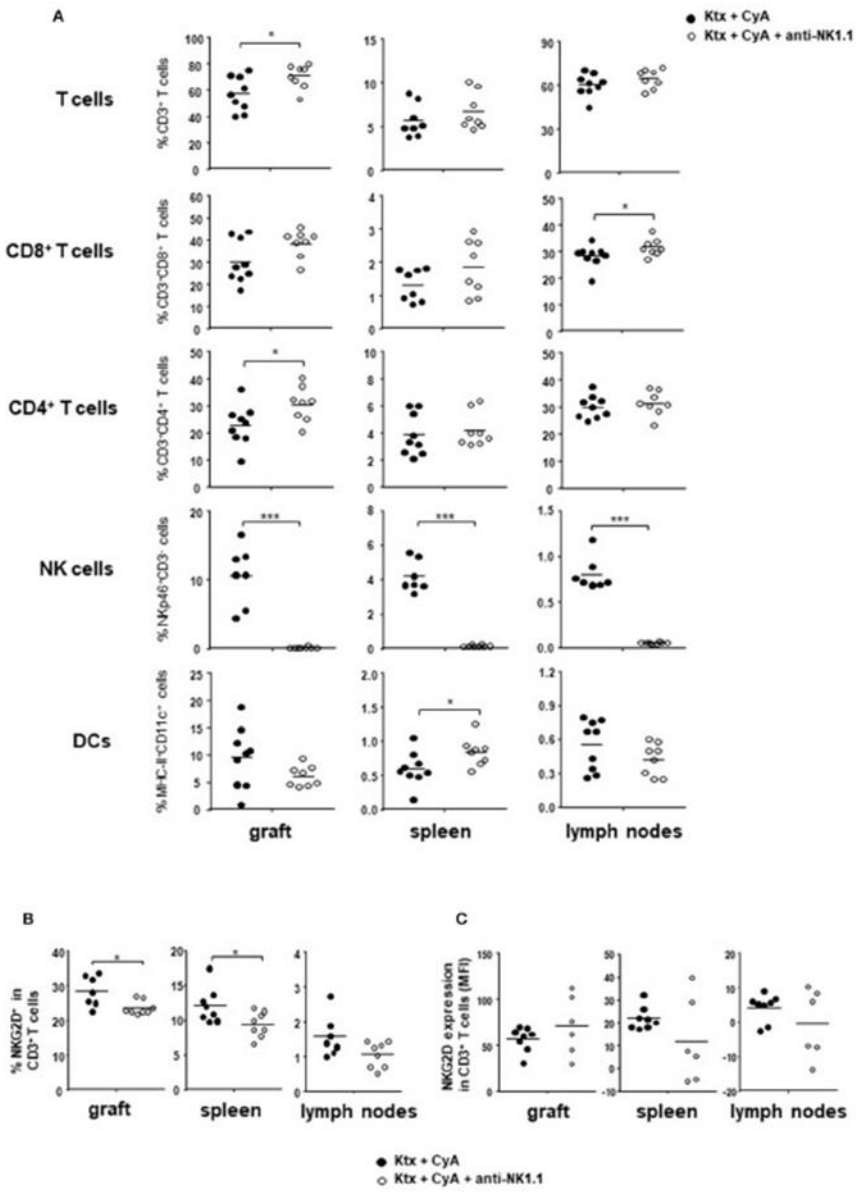

FIGURE 4 | Short-course depletion of NK cells in combination with CyA increases intragraft CD4$^+$ T cells. **(A)** NK depletion at POD-2 and POD+2 results in increased intragraft T cell frequencies at POD7, whereas NK cells were efficiently depleted in the kidney, spleen and lymph nodes. **(B)** Frequencies of NKG2D$^+$CD3$^+$ T cells are significantly reduced in graft and spleen following NK cell depletion, whereas **(C)** NKG2D is not downregulated on T cells as reflected by mean fluorescence intensity (MFI). Data are presented as mean of $n = 7$–8 animals/group. Cells were pre-gated on live CD45$^+$ events. Statistically significant differences between naive and CyA-treated animals were tested with the Mann-Whitney *U*-test; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

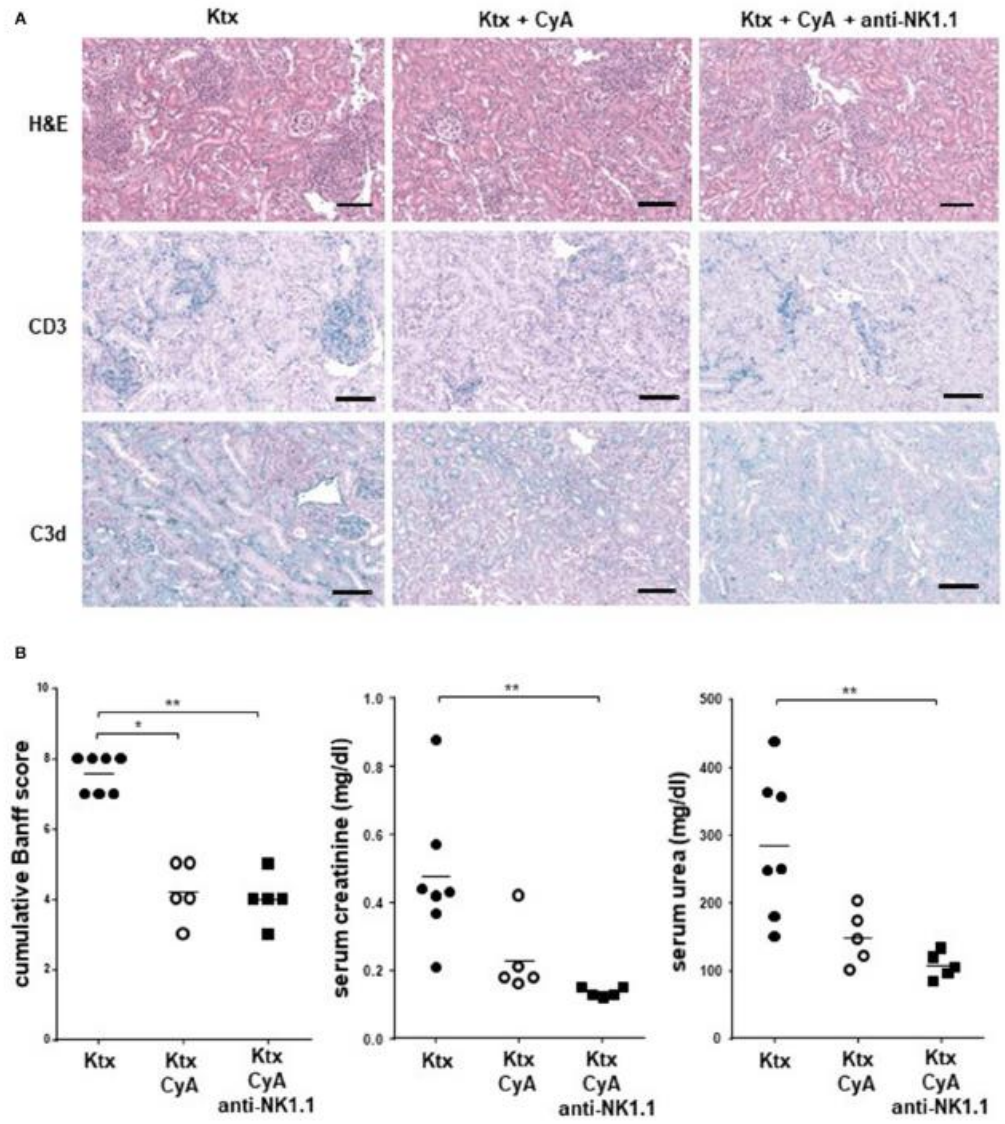

FIGURE 5 | CyA in combination with NK cell depletion results in improved kidney function. **(A)** Sections of kidney grafts on POD were stained with hematoxylin and eosin (H&E), CD3 and C3d deposition. Histological analysis of renal allografts illustrates a massive cell lymphocyte infiltration in untreated animals. The photographs are representative of 7–8 animals in each group, magnification × 250 (scale bar = 100 μm) **(B)** Compared with the untreated group, a reduced BANFF Score, serum creatinine (mg/dl) and serum urea (mg/dl) were detected in the CyA as well as in the CyA+NK cell group. Data are presented as mean of $n = 5$–7 animals/group. Statistically significant differences between experimental groups were tested applying the Kruskal-Wallis test with Dunn's *post-hoc* test; $^{*}p < 0.05$, $^{**}p < 0.01$.

neither NK cell frequencies nor their capacity to produce IFNγ were affected by CyA (**Figures 1**, **3A**). Interestingly, it has been demonstrated that IFNγ neutralization itself does not alter the kinetics of renal allograft rejection (30). However, our data are in line with *in vivo* data demonstrating that NK cell cytotoxicity under CyA treatment is preserved after the transplantation of

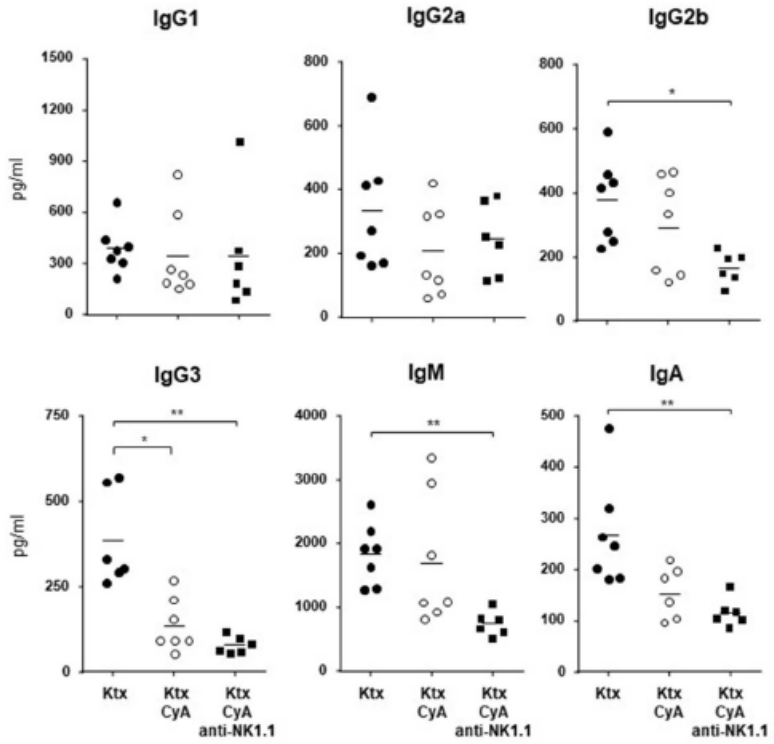

FIGURE 6 | Antibody concentrations of kidney graft recipients. NK cell depletion in combination with CyA results in significantly lower frequencies of IgG2b, IgG3, IgM, and IgA as compared with naïve kidney recipients. No difference was detected for the CyA vs. the CyA+NK cell group. Data are presented as mean of $n = 6–7$ animals/group. Statistically significant differences between experimental groups were tested applying the Kruskal-Wallis test with Dunn's *post-hoc* test; $^{*}p < 0.05$, $^{**}p < 0.01$.

multipotent adult germline stem cells (maGSCs) into the heart of RAG2$^{-/-}$ and C57BL/6 mice (31).

Having observed that CyA does not impair NK cell frequencies or function, we hypothesized in a second set of experiments that an additional selective targeting of these cells might beneficially affect kidney graft function. Whereas, CyA treatment alone resulted in an improvement of tissue damage compared with the untreated group, this was not further ameliorated by additional NK cell depletion. In contrast, the CyA+NK group demonstrated a significant improvement in graft function (urea, creatinine) as compared to untreated controls (**Figure 5**). However, additional NK cell depletion reversed the effect of decreased frequencies of antigen-presenting cells in the spleen, but not in the graft or lymph nodes (**Figure 4**). We can only speculate whether donor-derived APC killing by recipient-derived NK cells accounts for this observation as we could not distinguish between donor and recipient cells in our model (12, 32). Moreover, this would not explain the lack of differences for APCs in graft and lymph nodes between CyA and CyA+NK cell-depleted recipients (**Figure 4**).

However, by conducting long-term survival experiments until POD56, graft survival was significantly improved in recipients treated with CyA+NK cell depletion as compared with untreated recipients, whereas CyA treatment alone was not sufficient to significantly prolong allograft survival (**Figure 7A**). Despite the presence of activated intragraft CD4^{+} T_{REG} and higher frequencies of CD8^{+} T_{REG} in the CyA+NK group, we were not able to detect a significant improvement in graft histology (**Table 1**). With respect to CD4^{+} T_{REG}, the presence of FoxP3^{+} cells has already been comprehensively demonstrated in the murine KTX model (33, 34), but their proliferative capacity was never addressed. Speculating on the fact that T_{REG} proliferate in response to antigenic stimuli *in vivo* (35) and that these cells illustrate a greater suppressive capacity *in vitro* (36), we hypothesize that the prolonged long-term survival in the

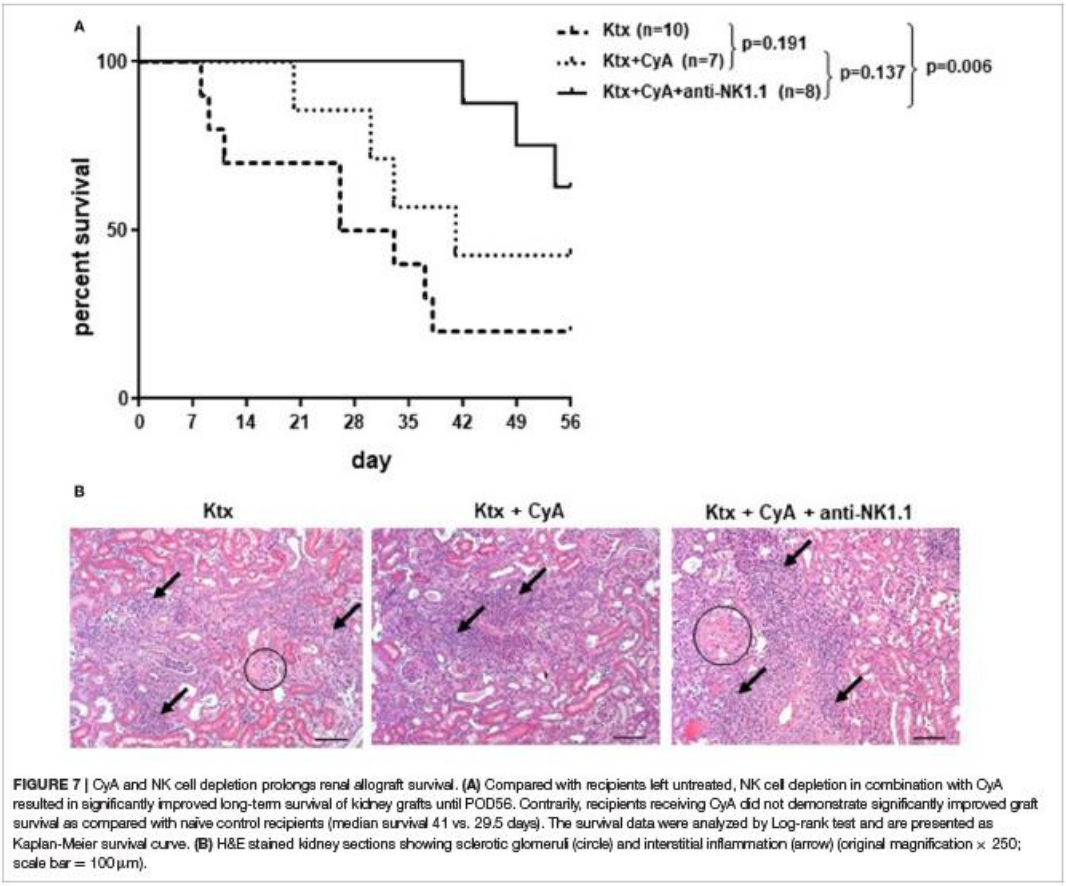

FIGURE 7 | CyA and NK cell depletion prolongs renal allograft survival. **(A)** Compared with recipients left untreated, NK cell depletion in combination with CyA resulted in significantly improved long-term survival of kidney grafts until POD56. Contrarily, recipients receiving CyA did not demonstrate significantly improved graft survival as compared with naive control recipients (median survival 41 vs. 29.5 days). The survival data were analyzed by Log-rank test and are presented as Kaplan-Meier survival curve. **(B)** H&E stained kidney sections showing sclerotic glomeruli (circle) and interstitial inflammation (arrow) (original magnification × 250; scale bar = 100 µm).

TABLE 1 | Histopathological evaluation of renal allografts in the mice surviving until POD56 using standard Banff Classification (median).

Histological lesions	KTX ($n = 2$)	KTX + CyA ($n = 3$)	KTX + CyA+anti-NK1.1 ($n = 5$)
Interstitial inflammation	3	3	2
Tubulitis	3	3	3
Glomerulitis	1.5	1	2
Arteritis	0.5	0	0
Capillaritis	2	3	2
Fibrosis	0	0	0
Tubular atrophy	2	3	2
Glomerulopathy	0.5	1	1
Mesangial matrix increase	0	0	0
Intimal fibrous thickening	0	0	0
Total	12.5	14	12

Banff Score (0–3; 0 – Nil, 1 – below 25%, 2 –25–50%, 3 – above 50%).

CyA+NK group does not rely solely on the presence of $CD4^+$ T_{REG}, but instead on the frequency of antigen-specific and proliferating $CD4^+$ T_{REG}. Although this result is not statistically significant, the induction of $CD4^+FoxP3^+Ki67^+$ T cells in the graft, spleen and lymph nodes was obvious in the CyA+NK group as compared with the two other groups. Intriguingly, NK cell depletion resulted in a clear induction of intragraft $CD8^+CD122^+$ Treg, a subset, which has been shown to be more potent in suppression of allograft rejection than their $CD4^+CD25^+$ counterparts (37). As numbers of surviving animals was low, both the observation of increased frequencies of proliferating $CD4^+$ T_{REG} as well as the induction of $CD8^+$ T_{REG} and its relation to NK cell depletion need to be confirmed in independent follow-up studies.

Currently, NK cell transcripts have been associated with antibody-mediated rejection of renal allografts (13, 14). Interestingly, a decrease in the antibody response in the short-term course was not detected for CyA treatment only, but

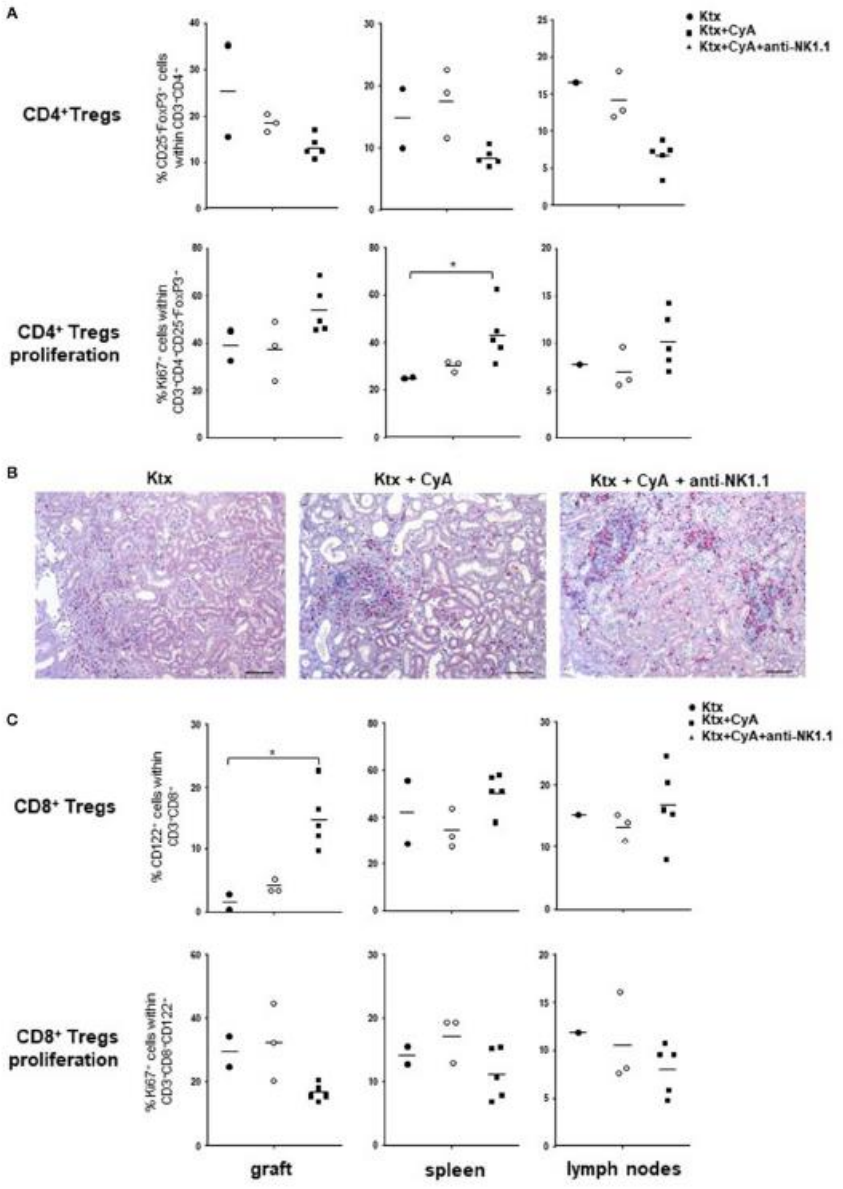

FIGURE 6 | CyA in combination with NK cell depletion increases T_{REG}. **(A)** Compared with recipients treated with CyA only, lower frequencies of $CD4^+$ T_{REG} were detected in grafts, spleen and lymph nodes when NK cells were depleted in the early operative period. $CD4^+$ T_{REG} were highly activated as reflected by Ki67 expression. **(B)** $CD4^+$ (red) $Foxp3^+$ (brown) T_{REG} cells were diffusely distributed in the interstitial infiltrate within the graft. Original magnification × 250 (scale bar = 100 μm). **(C)** In contrast to $CD4^+$ T_{REG}, intragraft $CD8^+CD122^+$ were increased, but demonstrate decreased proliferation. Data are presented as scatter dot plot with mean of surviving animals ($n = 2$–5)/group. Cells were pre-gated on live $CD45^+$ events. Statistically significant differences between experimental groups were tested applying the Kruskal-Wallis test with Dunn's *post-hoc* test; $^*p < 0.05$.

associated mainly with CyA+NK treatment (**Figure 6**), thus suggesting a positive influence of NK cell depletion on *de novo* antibody generation. We have to admit that the number of animals analyzed in this experiment is small, but the obtained data suggest that the influence of CyA on antibody production needs to be carefully revisited, as CNI agents should suppress the humoral immune response by interfering with T-helper cell signaling (38). Nevertheless, the induction of *de novo* antibodies despite a CyA regimen is still a frequent observation in clinical kidney transplantation (39).

We show for the first time that NK cell depletion combined with CyA synergistically improves renal allograft function, suggesting that selective NK cell targeting might constitute a novel approach to ameliorating outcomes after KTX in the long term. Nonetheless, we are aware of the fact that our study has limitations. First, we did not perform a kinetic analysis of NK cell phenotype and function in CyA-treated recipients post-kidney transplantation. Therefore, we cannot rule out that an earlier or later time point would have brought alternative results. Second, the time point and dosage of NK cell depletion were chosen on the basis of previous studies, where it was observed that application of neutralizing NK1.1 antibody administered daily for seven days resulted in signs of opportunistic infections (data not shown). We therefore decided to apply only a double dose of NK cell-depleting antibody as this was sufficient to efficiently deplete NK cells (**Supplemental Figure 4**). However, data from infection models suggest that the time point at which NK cells are depleted greatly influences the anti-viral immune response. For instance, delayed NK cell depletion improves the control of persistent LCMV infection more efficiently than does NK cell depletion at day 4 or 5 *post infectionem* (40). Consequently, alternative protocols of NK cell depletion combined with additional immunosuppressants need to be studied in order to gain more insights into the question whether modulation of NK cell response will improve allograft function post-kidney transplantation.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

ETHICS STATEMENT

The animal study was reviewed and approved by Landesamt für Gesundheit und Soziales, Berlin, Germany.

AUTHOR CONTRIBUTIONS

MA, TR, KKo, and SS were responsible for experiment design and acquisition and analysis and interpretation of data. ASat, AK, EH, ASar, HR, MS, and KKl assisted with experiments and data analyses. FA provided experimental resources. KKo and TR were responsible for writing of the manuscript. All authors reviewed the manuscript before submission.

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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.2019.02279/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Curriculum Vitae

My curriculum vitae is not included in the electronic version of the dissertation for data protection reasons.

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