Dissertation

Flow Cytometric B Cell Phenotyping before and after Kidney Transplantation – Potential Predictors of SARS-CoV-2 Vaccination Response within the Circulating B Cell Pool

submitted by

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice at the Medical University of Graz”.

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Disclosures

Parts of this thesis have been published as

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Abbreviations

ABMR - Antibody-mediated rejection

ACE2 – Angiotensin-converting enzyme 2

AID – Activation-induced cytidine deaminase

APC – Antigen presenting cells

ARDS – Acute respiratory distress syndrome

ATG – Antithymocyte globuline

AU – Arbitrary units

AZA – Azathioprine

BAFF - B cell activating factor

BAU - Binding antibody units

BC – B cells

B-CLL - B cell chronic lymphocytic leukemia

BCR – B cell receptor

BLyS - B lymphocyte stimulator

BX – Basiliximab

CD – Cluster of differentiation

CDC – Complement-dependent cytotoxicity

CKD G5 – Chronic kidney disease stage 5, formerly end-stage kidney disease

CLIA - Chemoluminesence Immunoassay

CMIA Chemiluminescent microparticle immunoassay

CMV – Cytomegalovirus

CNI – Calcineurin inhibitor

CSR – Class switch recombination

CTLA-4 - Cytotoxic T-lymphocyte-associated Protein

CXCR - C-X-C motif chemokine receptor

CyA – Cyclosporin A

DBD – Donation after brain death

DCD – Donation after circulatory death

DM – Diabetes mellitus

DN BCs – Double negative B cells

DSA – Donor-specific antibodies

E – Envelope protein

ECLIA - Electrochemical immunoassay

ESKD – End-stage kidney disease

EMA – European Medicines Agency

ESP – Eurotransplant Senior Program

EVE – Everolimus

FACS – Fluorescence activated cell sorting

FcRL4 - Fc receptor-like protein 4

FDC – Follicular dendritic cell

FMO – Fluorescence minus one

FXM – Flow cytometry crossmatch

GC – Germinal center

GFR – Glomerular filtration rate

HLA – Human leucocyte antigen

IFN – Interferone

Ig - Immunoglobulin

IGRA – Interferone gamme (γ) release assay

IL - Interleukine

IMPDH - Inosine-5′-monophosphate dehydrogenase

IQR – Interquartile range

IU – International units

KDIGO – Kidney Disease Improving Global Outcomes

KDPI - Kidney Donor Profile Index

KDRI - Kidney Donor Risk Index

KT – Kidney transplantation

KTR – Kidney transplant recipient

M – Membrane protein

mBC – Memory B cells

MFI – Mean fluorescence intensity

MHC – Major histocompatibility complex

MICA – MHC I chain-related antigen

MMF – Mycophenolate mofetil

MPA – Mycophenolic acid

Mpro – Main protease

N – Nucleocapsid protein

NA - Not applicable

NFAT - Nuclear factor of activated T-cells

NK cell – Natural killer cell

ORF – Open reading frame

PB – Plasmablasts

PBMCs – Peripheral blood mononuclear cells

PBS – Phosphate buffered saline

PC – Plasma cells

PRA – Panel reactive antibodies

PTDM – Posttransplant diabetes mellitus

RAG – Recombination activating gene

RdRP – RNA-dependent RNA polymerase

RNA – Ribonucleic acid

S – Spike protein

SAB – Single antigen beads

SEM – Standard error of the mean

SMH – Somatic hypermutation

TAC – Tacrolimus

TCs– T cells

TCMR – T cell mediated rejection

TCR – T cell receptor

TD – T cell-dependent

TdT - Terminal deoxynucleotidyl transferase

Tfh – T follicular helper cell

Th1 - T helper 1 cell

TI – T cell-independent

TLS – Tertiary lymphoid structures

TMPRSS2 - Transmembrane protease, serine 2

TMP/SMX – Trimethoprime/sulfamethoxazole

TrBC – Transitional B cells

Tregs – Regulatory CD4+ T cells

U - Units

UTI – Urinary tract infection

VDJ – Variability/diversity/joining region

WHO – Word Health Organization

XM – Crossmatch

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Zusammenfassung

Einleitung: Im Hinblick auf Morbidität, Mortalität und Lebensqualität stellt die Nierentransplantation die zu bevorzugende Form der Nierenersatztherapie dar. Um Abstoßungsreaktionen zu vermeiden ist eine chronische Immunsuppression notwendig, die mit einem erhöhten Infektionsrisiko einhergeht. Inhibition der T-Zellaktivierung ist das primäre Ziel gängiger immunsuppressiver Therapien. B-Zellen wurden historisch gesehen vernachlässigt oder reduziert auf ihre Rolle als Allo-Antikörper-Produzenten, obwohl sie humoralen Schutz und Antikörper-unabhängige Funktionen mit Bedeutung im Transplantationssetting bieten können. Die COVID-19 Pandemie stellt für Nierentransplantierte eine doppelte Bedrohung dar. Zum einen sind sie besonders anfällig für schwere Erkrankungsverläufe, zum anderen ist die Impfantwort auf SARS-CoV-2 Vakzine suboptimal. In der gegenwärtigen Studie haben wir die Veränderungen der B-Zellen und B-Zellsubpopulationen von vor Transplantation zu einem Jahr nach der Transplantation verglichen. Zudem haben wir nach potenziellen Prädiktoren innerhalb des peripheren B-Zellpools für ein späteres Ansprechen auf einen SARS-CoV-2 Impfstoff gesucht.

Methoden: Insgesamt wurden 105 Nierentransplantatempfänger*innen untersucht, und Blutproben wurden vor der letzten Hämodialysesitzung vor der Transplantation (T1) und ein Jahr nach der Transplantation (T2) entnommen. Nach Ausschluss aufgrund von unvollständiger Nachverfolgung, Tod oder Transplantatverlust konnten 71 Nierentransplantatempfänger*innen in unsere T1/T2-Kohorte eingeschlossen werden. Periphere mononukleäre Blutzellen wurden zu beiden Zeitpunkten isoliert und für die Durchflusszytometrie-Analyse von Leukozyten und B-Zellsubpopulationen unter Verwendung der Marker CD19, IgM, IgD, CD24, CD27, CD86, CD5 und CD38 untersucht. Für die Subkohorte der 40 Transplantierten, für die Antikörperdaten vorhanden waren, analysierten wir die Antikörperreaktion auf die mRNA-basierte SARS-CoV-2-Impfung (mRNA-1273 oder BNT162b2). Es wurden Spearman-Rangkorrelationen zwischen den B-Zellsubpopulationen bei T2 und den Antikörpertitern berechnet.

Ergebnisse: Von T1 nach T2 stiegen die Leukozytenzahlen aufgrund der Expansion von Monozyten und Granulozyten an, während die Lymphozyten stabil blieben. $CD19^+$ B-Zellen zeigten einen Trend zu vermehrtem Vorkommen bei T2. Naive B-Zellen, die größte B-

Zellsubpopulation, wurden in ähnlichen Frequenzen und absoluten Zahlen zu beiden Zeitpunkten gefunden. Plasmablasten und Transitionale B-Zellen (TrBCs) waren bei T2 deutlich reduziert, während doppelt negative B-Zellen in ihrer Häufigkeit zunahmen. Im Gedächtnis-B-Zell (mBC)-Pool waren IgM-only mBCs und class-switched mBCs bei T2 vermindert. IgD-only mBCs und unswitched mBCs blieben unverändert. Verringertes Vorkommen von aktivierten $CD86^{+}$ und tolerogenen $CD27^{-}$ $CD5^{+}$ BCs wurden bei T2 beobachtet.
Nur 50% der Nierentransplantatempfänger*innen (*n*= 20) in der Impf-Subkohorte entwickelten nach zwei Dosen der SARS-CoV-2-Impfung nachweisbare Antikörpertiter. Die Antikörperspiegel korrelierten mit den TrBC-Zahlen und -Häufigkeiten zu T2. Diejenigen, die eine Antikörper-Antwort zeigten, hatten eine erhöhte Anzahl von TrBCs im Vergleich zu denjenigen, bei denen keine nachweisbaren Anti-SARS-CoV-2 Antikörper vorhanden waren.

Schlussfolgerung: Die durchflusszytometrische Analyse von zirkulierenden B-Zellen zeigte deutliche Veränderungen vom Setting der hochgradig eingeschränkten Nierenfunktion zum stabilen Nierentransplantationsstatus. Insbesondere kleine Subsets wie PBs und TrBCs waren negativ betroffen. Indem wir zeigen konnten, dass TrBCs mit den Antikörperspiegeln nach SARS-CoV-2-Impfung korrelieren, liefern wir Hinweise darauf, dass quantitative und qualitative Veränderungen des B-Zellpools von klinischer Relevanz sein könnten. Unsere Erkenntnisse zu TrBCs als Prädiktoren für humorale Antwort, möglicherweise auch auf andere Impfstoffe, müssen in größeren und prospektiven Studien bestätigt werden.

Abstract

Introduction: Kidney transplantation (KT) is the preferred form of kidney replacement therapy considering morbidity, mortality and quality of life. To avoid allograft rejection, KT comes at the cost of chronic immunosuppression, which harbors an increased risk of infection. Current immunosuppressive treatments are primarily targeted towards T cells. B cells (BCs) have historically been disregarded or reduced to their, potentially detrimental, role as alloantibody producers, but BCs may also provide humoral immunity and exert antibody-independent functions with relevance in transplantation. The COVID-19 pandemic has posed a dual threat for kidney transplant recipients (KTRs), as they are particularly vulnerable to severe courses, while antibody response to vaccination has been suboptimal. In this study, we investigated the changes of BCs and BC subpopulations from before to one year after KT. Additionally, we screened for potential predictors within the peripheral BC pool for vaccination response.

Methods: A total of 105 KTRs were screened, and blood samples were taken prior to the hemodialysis session before transplantation (T1) and one year after transplantation (T2). Due to incomplete follow-up, death or graft loss 71 KTRs were included in our T1/T2 cohort. Peripheral blood mononuclear cells (PBMCs) were isolated at both timepoints and stained for flow cytometric analysis of leucocytes and BC subpopulations using the markers CD19, IgM, IgD, CD24, CD27, CD86, CD5 and CD38. For the vaccination subcohort, we analyzed antibody response to mRNA-based SARS-CoV-2 vaccination (mRNA-1273 or BNT162b2) in 40 out of 71 KTRs, for whom this data was available. Spearman rank-based correlation between BC subpopulations at T2 and antibody titers were calculated.

Results: From T1 to T2, leucocyte numbers increased by virtue of monocyte and granulocyte expansion while lymphocytes remained stable. $CD19^+$ BCs trended for increased abundance at T2. Naïve BCs, the largest BC subset, were found at similar frequencies and absolute numbers at both timepoints. Plasmablasts and transitional BCs (TrBCs) were profoundly reduced at T2, whereas double negative BCs increased in abundance. In the memory BC (mBC) pool, IgM-only mBCs and class-switched mBCs were reduced at T2. IgD-only mBCs and unswitched mBCs remained similar. Diminished frequencies and counts of activated $CD86^+$ and tolerogenic $CD27^-$ $CD5^+$ BCs were observed at T2.

Only 50% of KTRs (*n*= 20) developed detectable antibody titers after two doses of SARS-CoV-2 vaccination. Antibody levels correlated with TrBC counts and frequencies at T2. Responders displayed increased TrBC abundance compared to non-responders without any detectable antibodies.

Conclusion: Flow cytometric phenotyping of circulating BCs revealed profound compositional changes from the CKD G5 to the stable KT setting. Particularly small subsets like PBs and TrBCs were decreased after transplantation. By showing that TrBCs correlated with antibody levels after SARS-CoV-2 vaccination, we provide evidence that quantitative and qualitative changes of the BC pool may be of clinical relevance. Our findings of TrBCs as predictors of humoral response need to be confirmed in larger and prospective studies, potentially expanding to other vaccines.

1. Introduction

1.1. Kidney replacement therapy

The kidneys are paired organs located in the retroperitoneum, protected by a fibrous capsule. Anatomically, the outer cortex can be distinguished from the medulla and the central renal pelvis. Ultrastructurally, each kidney consists of approximately 1 million functional units called nephrons. A nephron consists of a glomerulus and its associated tubular apparatus. The glomeruli are located in the renal cortex and are composed of specialized cells to fulfill their function of plasma ultrafiltration (2).

Essentially, a glomerulus consists of an encapsulated capillary tuft surrounded by specialized cells called podocytes. The capillary endothelium, the basement membrane, and the processes of podocytes form a filtration barrier for higher-molecular-weight and negatively charged blood components. This barrier prevents the loss of cells or albumin, while low-molecular-weight components including metabolic waste products can pass through. In the subsequent tubular system, the filtered primary urine undergoes further processing. Depending on the body's needs, certain molecules such as glucose are reabsorbed and retained, while other substances are left behind or even actively secreted. Reabsorption of solutes also allows for the reabsorption of water from the filtrate. The tubular apparatus consists of histologically and functionally distinct portions, referred to as proximal, intermediate, or distal tubules, depending on their relative distance to the glomerulus. The distal tubule connects to collecting ducts via a connecting piece, which converges in the renal pelvis. The urine that enters the renal pelvis after fine-tuning throughout the tubular system is termed secondary urine. The kidneys filter around 180 liters of primary urine per day, underscoring the crucial role of extensive reabsorption in preventing excessive water loss. Around 1.5 liters of secondary urine (approximately 1% of primary urine) are formed daily (2).

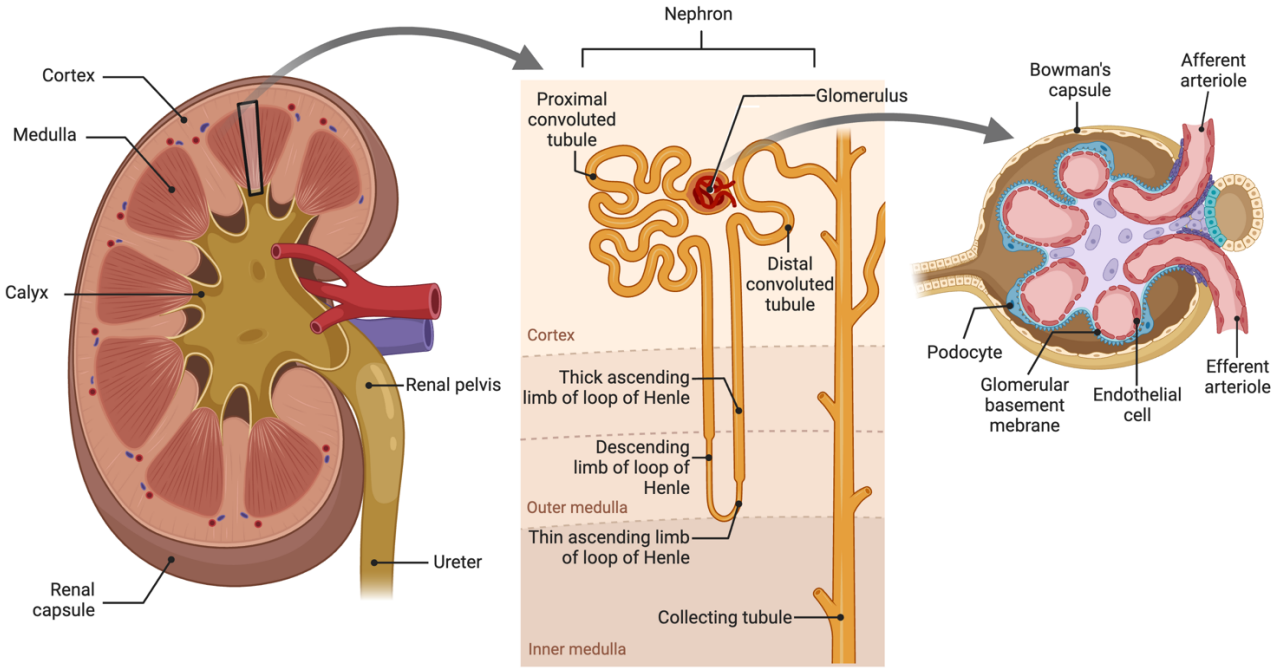

Figure 1. Macroscopical and microscopical anatomy of the human kidney. Each kidney consists of approximately one million functional units - nephrons. Each nephron is composed of a glomerulus and a tubular system. Capillaries of the glomerulus are fed by the afferent arteriole. Blood is filtered through the tripartite barrier, including the endothelium, basement membrane and podocyte. The resultant filtrate is termed primary urine, which undergoes compositional changes as it traverses the tubular system. Tubules converge into a collecting tubule which transports the modified filtrate, now termed secondary urine, into the renal pelvis. Filtered blood leaves the glomerulus through the efferent arteriole. Created with BioRender.com.

In addition to the well-known role of urine production, the kidneys fulfill other essential functions. By adjusting urine composition, they regulate the acid-base and electrolyte balance. Neurohumoral mechanisms, such as the renin-angiotensin-aldosterone system, modulate blood volume largely by influencing urine composition and water reabsorption. Bone metabolism and hematopoiesis are also critically influenced by Vitamin D3 and erythropoietin, which are activated and produced in the kidneys, respectively (2). In summary,

the kidney plays a vital role as a central homeostat, and its pivotal functions are gradually lost in the context of chronic kidney disease (CKD).

CKD is a worldwide health burden affecting over 800 million people around the globe (3). Major underlying diseases include diabetes mellitus (DM) and arterial hypertension. Other common causes of CKD are primary and secondary glomerulonephritis and polycystic kidney disease (3). Diagnosis of the underlying disease is ideally confirmed histologically, but often kidney biopsy is deemed not consequential and too unsafe. Frequently, diagnosis of CKD is made clinically (4). For example, in a patient with long-standing history of DM and albuminuria, reduced kidney function is considered a consequence of diabetic kidney disease and a kidney biopsy is often omitted. However, this approach has been questioned by some, as one may miss other conditions driving kidney function decline (5).

CKD is diagnosed and staged according to the Kidney Disease: Improving Global Outcomes (KDIGO) guidelines according to glomerular filtration rate (GFR) and albuminuria into G1-5 and A1-3 stages (4). Diagnosis of CKD G1 and G2 necessitates the existence of morphological kidney abnormalities and/or urine abnormalities. Higher G and A stages correlate with an increased cardiovascular risk and increased risk of CKD progression. CKD G5, formerly end-stage kidney disease (ESKD), is defined as a GFR < 15ml/min/1.73m^2(4). Once a patient reaches CKD G5, options for kidney replacement therapy ought to be discussed. There appears to be no optimal GFR cut-off for initiation of kidney replacement therapy (6), but it is frequently demanded by complications of reduced kidney function like volume overload or hyperkalemia. Furthermore, symptoms of uremia due to the accumulation of waste products (i.e., uremic toxins) like nausea, pruritus or fatigue are used as an indicator to start kidney replacement therapy (7). Options for kidney replacement therapy include hemodialysis (HD), peritoneal dialysis (PD) and kidney transplantation (KT).

Briefly, both HD and PD use the principles of diffusion for clearance of uremic toxins and other solutes. In HD, blood is pumped through an extracorporeal circuit into a dialyzer, which consists of a semipermeable membrane. This membrane separates patient blood from a specialized solution called dialysate. Low molecular uremic toxins and electrolytes diffuse along a concentration gradient. Additionally, excess fluid can be removed through pressure

gradients, where solutes are also dragged from the blood into the dialysate (8). Usually this is continuously done for 3-5 hours 3 times a week for chronic HD patients (7).

In PD, the peritoneum functions as a semipermeable membrane. The dialysate is filled through a catheter into the peritoneal cavity and removed again after waste products have diffused into the dialysate and an equilibrium has occurred. Filling and emptying of peritoneal dialysate are done several times a day either manually or automatically via a cycler at night (8).

Compared to hemodialysis (HD) or peritoneal dialysis (PD), kidney transplantation (KT) is the preferred form of kidney replacement therapy, as kidney transplant recipients (KTR) generally survive longer and at a better quality of life than HD or PD patients (9). A transplanted solid organ from a non-identical person is termed “allograft” or “graft”. KT is limited by the access to suitable organs and by patient comorbidities. Contraindications for KT include advanced cardiopulmonary disease limiting surgical fitness, active malignancy or infection, or psychiatric illness and active substance abuse (10). The paucity of suitable organs may be circumvented by living kidney donation, wherein a donor, most common a close relative, provides one kidney for the recipient. Kidney graft survival is superior after living kidney donation compared to cadaveric kidney donation (11, 12). For cadaveric kidney donation, donation after brain death (DBD) and donation after circulatory death (DCD) donors can be distinguished. DCD kidney require dialysis after transplantation more frequently (delayed graft function) compared to DBD, but reliable long-term outcome data on the superiority of either one are missing (13). The “quality” of the donor greatly affects transplant outcome. Historically, potential deceased donors were dichotomized into two categories: Standard criteria donor (SCD) and expanded criteria donor (ECD) (14). Anyone above the age of 60 or between the age of 50 - 59 with two out of three of the risk factors (creatinine > 1.5mg/dL, hypertension, or death from cerebrovascular death) was deemed ECD. This proved to be an oversimplified approach, and newer risk calculators like the Kidney Donor Risk Index (KDRI) and Kidney Donor Profile Index (KDPI) have been developed to aid clinicians in their evaluation of potential allografts (15, 16).

In the context of transplantation, it is crucial to assess the donor in correlation with the prospective recipient. These considerations range from size and weight compatibility to immunological tissue compatibility.

1.2. Immunological principals of transplantation

The human immune system has evolved to effectively eliminate foreign pathogens, while maintaining self-tolerance. Two primary branches – the innate and the adaptive immune system – work in concert to guard from pathogens or foreign substances. The innate immune system serves as a first defensive line and consists of granulocytes, monocytes/macrophages, mast cells and dendritic cells. Innate immune cells are activated by conserved pathogenic patterns. Apart from rapid neutralization through phagocytosis, innate immune cells are capable of cytokine and chemokine secretion to induce local and systemic inflammation. Innate immune cells offer immediate protection utilizing broad and unspecific recognition and defensive mechanisms. The adaptive immune system, on the contrary, provides targeted and pathogen-tailored protection, but necessitates a certain lead time. T cells (TCs) and B cells (BCs) are key components of the adaptive immune system, which also encompasses an immunological memory. Following exposure to a pathogen, pathogen-specific memory TCs and BCs are generated. These can eliminate the pathogen quickly and effectively upon re-encounter. The capacity to identify and respond to foreign antigens is essential for protection of the body against pathogens. However, the ability of the immune system to recognize non-self antigens also includes foreign blood group or differing human leukocyte antigen (HLA), which are of particular significance in transplantation (17).

For blood group, ABO is of most relevance in the transplantation field. The ABO blood group system, discovered by Karl Landsteiner in 1900, is determined by the presence of specific carbohydrates on the surface of erythrocytes. Natural antibodies against absent A or B antigens dictate blood and tissue compatibility between recipient and donor. For example, a subject with blood group A can receive erythrocytes from blood group A and O, which lacks any surface antigen. A person with blood group O has antibodies against antigens A and B. This person can only receive erythrocytes from the same blood group. In solid organ transplantation, organs are perfused, and donor erythrocytes are removed. However, vascular

endothelium and other cells of the graft also express ABO antigens (histo-blood group antigens), while they do not express Rhesus or other blood group antigens (18-20). Thus, ABO compatibility has been deemed a *conditio sine qua non* for a long time in the transplantation field to avoid hyperacute rejection (21). Desensitization protocols employing plasmaphereses, immunoadsorption, B cell depleting therapies and/or splenectomy to remove anti-A/B antibodies prior to transplantation have opened the door to ABO-incompatible KT (22). Naturally these protocols require a certain lead time, which often limits transplantation across ABO blood group barriers to living kidney donation. This has extended the pool of donors with excellent outcomes (23). Intriguingly, after an initial period of increased rejection risk, the ABO-incompatible graft is tolerated by the recipient's immune system (24). This status has been called "accommodation" and is, despite considerable effort, still incompletely understood (25, 26).

The HLA genotype can be envisioned as "barcode", by which self and non-self are distinguished. This is also reflected by the broader, species-overlapping term for HLA, "major histocompatibility complex" (MHC) (27). The HLA gene locus can be separated into three distinct regions, class I, II and III, depending on the structural and functional differences in their gene products (28). While HLA class I and II encode for HLA class I and II cell surface proteins, respectively, HLA class III serves different functions like complement protein, cytokine and hormone generation (28).

HLA class I genes encompass HLA-A, HLA-B and HLA-C, giving riso to HLA class I protein expressed on all nucleated cells. HLA class II genes, including HLA-DR, HLA-DP and HLA-DQ, encode for HLA class II protein, which is selectively expressed by specialized cells of the immune system termed "antigen-presenting cells" (APC). BCs, monocytes/macrophages and dendritic cells can function as APC (27). HLA class I and II proteins constitute a platform for antigen presentation to TCs, and only when presented via HLA proteins can an antigen be recognized by TCs (27). HLA class I is necessary for the recognition of cytosolic proteins. Self and non-self cytosolic proteins are bound to HLA I and transferred to the outer surface of the cellular membrane. These antigens are scrutinized by TCs expressing cluster of differentiation (CD) 8 ($CD8^+$ TCs or precursors of cytotoxic TCs). In instances of cellular mutation or infection, aberrant antigens will be presented, triggering

$CD8^+$ TCs. The compromised cell will then be targeted for killing. Similarly, a missing MHC I complex on a cell's surface ("missing self") will lead to its destruction mediated by $CD8^+$ TCs or natural killer (NK) cells (17).
APC can take up antigens from their surroundings by various means and present those via HLA II to TCs expressing CD4 ($CD4^+$ TCs or T helper cells). Again, an activation of $CD4^+$ TCs should only occur, when exposed to a foreign or mutated antigen. Activated $CD4^+$ T helper cells orchestrate and coordinate the immune response by facilitating both cellular and humoral defense mechanism (17).

HLA genes stand out as exceptionally polymorphic within the human genome (29). Different variants for a specific HLA locus ("alleles") allow for a different spectrum of antigens to be presented, making certain HLA alleles an evolutionary advantage (30, 31). HLA alleles are inherited *en bloc* from father and mother in a mendelian fashion, which is called the HLA haplotype. The chance of a perfect HLA match between siblings is thus 25%, excluding recombination events and presuming that there are no similar HLA alleles between parents. HLA genes are expressed codominantly, meaning that all maternal and paternal HLA genes are transcribed and present as HLA molecules on a cell's surface. This allows for a broader antigen spectrum to be recognized by the immune system (17).

HLA typing has been historically done by serological testing. Antibodies of multiparous women, sensitized by the different HLA repertoire of their offspring, have been incubated with lymphocytes, complement factors and a vital dye (32). This approach allowed for a broad determination of HLA antigens. However, with the advent of DNA sequencing, a more granular detection of the HLA phenotype was made possible (33). Molecular HLA typing has led to the discovery of thousands of HLA alleles and the number of alleles is constantly growing. This sequence of developments explains the confusing nomenclature of HLA genes (34).

Even minor differences in the HLA genotype can lead to recognition by immune cells. The extent of HLA matching varies between centers, but HLA-A, HLA-B and HLA-DR constitute the routine examination. HLA-DR mismatches appear most relevant for kidney graft survival (35). Furthermore, with increasing mismatches (0 – 6), the risk of graft failure increases

linearly (36). However, the importance of HLA matching on graft survival has decreased over time, potentially due to improved immunosuppressive treatment strategies (37). Moreover, the effect of HLA matching has been deemed neglectable in the so called "Eurotransplant Senior Program" (ESP) (38). The ESP follows an "old-for-old" strategy, where kidneys from donors aged 65 years and older are allocated to recipients aged 65 years or older to combat organ shortage. Yet again, HLA-DR matching has been shown to improve outcomes in ESP transplantation (39).

Exposure to different HLA antigens generates anti-HLA antibodies. Pregnancies, blood transfusions and previous transplantations are classical immunization events (40, 41). Transplantation of an organ in a setting with preformed antibodies directed against the organ's HLA – donor specific antibodies (DSA) - results in hyperacute rejection (42). As preventive measure in preparation for transplantation, CKD patients are screened for the existence of anti-HLA antibodies. Serum of potential recipients has been historically tested regularly against panels of cells with known HLA phenotype representative of the population. "Panel-reactive antibodies" (PRAs) indicate the percentage of a reference population that an individual has antibodies against. Higher PRAs suggest an immunization against a larger proportion of the population and a reduced potential donor pool. The introduction of single antigen bead (SAB) detection of anti-HLA antibodies has greatly improved sensitivity and specificity. While prior PRA testing had to be done on cells expressing a multitude of HLA loci, SAB allows for the reliable determination of antibody specificity (43). This lead to the development of calculated PRA (cPRA) – the calculated proportion of kidney donors who express HLA antigens that are deemed unacceptable, i.e., the recipient has antibodies against (44). Furthermore, allocation omits donors with unacceptable antigens from the recipient (45).

Apart from an *a priori* limited donor pool, and despite the absence of DSA, retrospective data also indicate worse outcomes with higher pretransplant PRAs. Pereira et al. showed increased acute rejections in recipients with PRAs > 10% (46). Similarly, although only significant at cPRAs ≥ 98%, Lan et al. demonstrated heightened risk of death or graft loss for any cause in immunized patients compared to cPRAs 0% recipients (47). Together, these findings support the argument that cPRAs/PRAs may be an indicator of immunological risk.

Finally, crossmatching (XM) describes the definitive testing of histocompatibility between the donor lymphocytes and recipient serum. Pretransplant complement dependent cytotoxicity (CDC) XM was introduced by Patel and Terasaki in the late 1960s (42), and was further adapted to increase sensitivity, e.g. through the addition of antihuman immunoglobulin (48). In CDC XM total lymphocytes, TCs and BCs, are tested against recipient serum. TCs are equipped only with HLA class I antigens. BCs, which carry abundant HLA class I and class II antigens are less numerous. A positive total lymphocyte CDC XM can therefore not distinguish between anti-HLA class I or class II antibodies. To detect antibodies targeting HLA class II, CDC XM must be conducted independently on TCs and BCs. While a positive TC CDC XM poses substantial risk of acute rejection, making it a contraindication for transplantation (42), a positive BC CDC XM is more complex and it's prognostic significance still a matter of debate (49, 50). Additionally, a positive CDC XM may be attributed to Immunoglobulin M (IgM) alloantibodies, which pose no threat to the graft (51). This issue can be circumvented by incorporating dithiothreitol (DTT), which inactivates IgM (52, 53). Another limitation of CDC XM is its exclusive detection of complement-binding antibodies.

The emergence of flow cytometric XM (FXM) has improved sensitivity of alloantibody detection as FXM is able to detect antibodies independently of complement-binding capacity (54). In FXM, donor TCs and BCs are incubated with recipient serum and fluorescein-labeled antihuman IgG antibodies. Fluorescence intensity is then measured and typically reported as mean fluorescence intensity (MFI). However, interpretation of MFI are challenging and they do not necessarily correlate with antibody titers (55). Nonetheless, a positive FXM carries an increased risk of antibody mediated rejection and graft loss, even in the absence of a positive CDC XM (56).

Physical XM, encompassing CDC XM and/or FXM, is labor intensive, potentially delaying transplantation and increasing cold ischemia time. Consequently, virtual XM has been introduced as a time-efficient alternative for low-risk individuals. In contrast to physical XM, virtual XM relies on matching of recipient anti-HLA antibody data with donor HLA antigen data. In theory, a negative virtual XM indicates absence of DSA. However, there are specific limitations and assumptions that must be considered. First, donor HLA typing needs to be

performed at an adequate resolution. Second, DSA detection should be reliable, whereas due to current technical limitations relevant alloantibodies may be missed. Third, not all alloantibodies identified by SAB are biologically relevant (57, 58). Hence, in cases of uncertainty or a recent immunization event, a physical XM is recommended (58).

1.3. "The balance" in kidney transplantation

Recipient immune cells, particularly TCs, can recognize alloantigens through several pathways. First, direct allorecognition by recipient TCs depends on donor APCs expressing donor HLA. TCs capable of binding donor HLA are termed "cognate" TCs. When cognate TCs recognize and bind to donor HLA, it leads to a direct killing response (59). Second, indirect allorecognition alludes to the general concept of foreign antigen presentation. Here, recipient APCs present an alloantigen to TCs, that has been taken up from the graft and processed for presentation via host HLA (60, 61). TCs restricted to recipient HLA are called "non-cognate" (59). Non-cognate TCs coordinate various immune responses playing a role in both antibody formation and innate immune response. Of note, a third mode of recognition, the "semidirect" pathway, has been described. Here, recipient APCs acquire intact donor MHC complexes, which are then presented to TCs – a process also termed "cross-dressing" (62, 63).

It is generally believed that direct allorecognition is particularly important in the early stage after transplantation, when large numbers of APCs migrate to secondary lymphoid organs from the graft. While indirect allorecognition is also of relevance in the early phase, it is the primary mechanism responsible for chronic rejection and late-onset rejection. However, biological and time-dependent roles of these pathways remain debated (59).

Additionally, non-MHC antigens or "minor histocompatibility antigen" are an important factor in allorecognition beyond HLA. Non-MHC antigens include variable and immunogenic proteins like the male H-Y antigens, which consist of proteins encoded on the Y-chromosome non-existent on female recipients and MHC I-related chain A (MICA) antigens, which are

surface glycoproteins (64, 65). Mismatches in MICA and DSA against MICA have recently been associated with reduced graft survival and increased ABMR rates (66).

Despite efforts to minimize HLA mismatches, a complete elimination of incompatibilities remains challenging due to the polymorphic nature of HLA genes. Thus, suppression of the immune system is crucial to ensure graft survival. The remarkable increase in kidney graft survival in the last decades can be largely attributed to better immunosuppressive protocols, where an induction phase is followed by maintenance immunosuppression.
Induction treatment is administered in the immediate peritransplantat period together with high-dose corticosteroids (CS) to prevent early graft rejection. Two options for induction treatment are available: lymphocyte-depleting agents and interleukin (IL)-2 receptor blockers. Compared to no induction, administration of the lymphocyte-depleting antithymocyte globulin (ATG) has resulted in improved graft survival (67). The anti-CD52 antibody, Alemtuzumab, is an alternative effective lymphocyte-depleting option (68).
For IL-2 receptor blocker, after the withdrawal of daclizumab due to safety concerns (69), basiliximab (BX) is the only available option for induction treatment. Numerous studies have underlined the efficacy and relative safety of BX (70).
Brennan et al. compared ATG to Basiliximab (BX) as induction agent for high-risk KTRs. In this landmark trial, the ATG group experienced fewer rejections, while BX induction resulted in a lower number of infections and hematological complications. Overall, graft survival was comparable between both agents (71).
Alemtuzumab yielded similar results in high-risk patients as ATG. In immunological low-risk patients, Alemtuzumab induction resulted in fewer acute rejections at the cost of more severe infections and leukopenia than BX (68).
Accordingly, KDIGO guidelines recommend lymphocyte-depleting agents in immunological high risk situations, and BX for low risk recipients (72). Risk for rejection increases with the number of HLA mismatches (especially HLA-DR), existence of DSA, younger recipient age, older donor age, blood group incompatibility, delayed graft function (DGF) and longer cold ischemia time (72).

Maintenance immunosuppression ensures long-term graft survival and needs to balance toxicity with the suppression of the immune response to maintain graft function. In that

regard, combination therapies have been historically more successful and represent the current standard of care. The most common combination consists of a calcineurin inhibitor (CNI), an antiproliferative agent and corticosteroids. Potential CNI options include cyclsoporin A (CyA) and tacrolimus (TAC). Azathioprine (AZA), mycophenolic acid (MPA) and its prodrug mycophenolate mofetil (MMF) are routinely used antiproliferative agents in KT maintenance immunosuppression. Alternatively, though less frequently utilized, mammalian target of rapamycin (mTOR)-inhibitors or belatacept may be considered.
CS are a mainstay of induction therapy. Their suppressive effect on the immune system is exerted via a myriad of pathways (17). However, long-term steroid therapy comes with substantial associated toxicity. Out of concern for rejection and graft failure, many centers are reluctant to eliminate CS from maintenance therapy and it is continued at a dose of around 5mg/day (73, 74). In low immunological risk KTRs, early withdrawal of CS appears safe and reduces risk of post-transplant diabetes mellitus (PTDM) (75). The 5-year follow up of the HARMONY trial, confirmed the safety of a rapid steroid withdrawal, but failed to show a reduction in infections in those without CS (76).

TC activation is a complex multistep process. Effective suppression requires inhibition at multiple points of the activation cascade, which is the rationale for combination immunosuppression in transplantation.
Activation is initiated, when TCs engage an antigen through their TC receptor (TCR) when presented via MHC (Signal 1). Additional co-stimulatory ligand – receptor interactions, like CD28 – CD80/86, are essential for activation (Signal 2). Without co-stimulation, TCs may become anergic, i.e., unresponsive to the respective antigen. Similarly, cytotoxic T-lymphocyte-associated Protein 4 (CLTA-4) competes with CD28 for CD80/86 binding. When engaged, CTLA-4 dampens activation and proliferation signals.
Activation of the TCR in context of co-stimulation initiates various intracellular signaling pathways that support cell survival and stimulate proliferation including the calcineurin-nuclear factor of activated T-cells (NFAT) pathway. Consequently, transcription of IL-2 and the α subunit of the trimeric IL-2 receptor (IL-2R) are promoted. Association of the α subunit, also known as CD25, with the constitutively present β and γ subunit improves affinity for IL2. IL2-IL2R engagement provides signal 3 of TC activation and promotes mTOR signaling. mTOR further amplifies the activation status and provides proliferation signals (17).

TAC and CyA bind to FK binding protein and cyclophilin, respectively, which then attach to calcineurin. Consequently, calcineurin's phosphatase activity is hindered, preventing the dephosphorylation of NFAT and translocation of NFAT to the nucleus, where it operates as a pro-inflammatory transcription factor (77). Initially, CyA demonstrated significant potential in solid organ transplantation (78), but TAC has shown superior efficacy in preventing acute rejection (79, 80). Nevertheless, CNI can cause nephrotoxicity, arterial hypertension and hyperlipidemia, partly in a dose-dependent manner (77).

Therefore, several clinical trials have investigated the safety and outcomes of CNI sparing regimens. These include CNI dose reduction using MPA/MMF, CNI avoidance strategies using alternatives like mTOR-inhibitors or belatacept, a fusion protein consisting of CLTA-4 and IgG, or CNI withdrawal (81-83). These strategies appear to have a positive effect on graft function, but CNI sparing regimens overall showed higher rates of acute rejections (81, 83). However, many head-to-head comparisons were made with CyA, and findings may not be extendable to TAC treatment, which is the superior CNI in KT (84).

Belatacept is a potent immunosuppressant and alternative to CNI. It functions as a signal 2 inhibitor by preventing the engagement of co-stimulatory CD80/86 with CD28 on TCs (85). An increased risk of (viral) infections and increased rates of acute rejection warrant caution (86).
mTOR inhibitors, like sirolimus and everolimus, inhibit (TC) proliferation and have been investigated for CNI conversion, as part of non-CNI based regimen or as alternative antiproliferative agent. In a meta-analysis by Webster et al., graft function was improved with mTOR treatment, but at an increased risk of dyslipidemia and bone marrow suppression (87). Other potential benefits of mTOR-inhibitors include reduced risk of CMV infections and potentially lower rates of malignancy in KT (87, 88).

Lymphocytes are dependent on *de novo* purine synthesis for proliferation. This "weakness" is exploited by antiproliferative agents, also called antimetabolites. MPA or it's prodrug MMF inhibit inosine-5'-monophosphate dehydrogenase (IMPDH) and AZA interferes with glutamine phosphoribosyl pyrophosphate aminotransferase, respectively. Importantly, this

effect is not confined to TCs but extends to BCs as well (89). With regards to efficacy, MMF displayed superior rates of acute rejection and improved graft survival compared to AZA (90). Enteric coated MPA has been shown to be equivalent to MMF (91), making either one a first line drug in KT.

Consistently with the current body of evidence, routine maintenance immunosuppression consists of TAC, MMF/MPA and frequently low dose steroids. Significant improvements in early graft survival have not translated, however, into a substantial improvement of long-term graft survival (92, 93). Chronic graft failure is often multifactorial and key factors include antibody-mediated rejection (ABMR), TC mediated rejection (TCMR) and CNI toxicity (94). These findings highlight the need for optimized immunosuppressive strategies, which translate not only into a short-term but also a satisfactory long-term graft survival.

Current strategies need to balance the risk of rejection with the risk of infections (95), which are a major cause of death in the transplant population (96). Three distinct phases of post-transplant infections can be distinguished: an early phase within the first month, an intermediate period from month one to six and a late phase starting after month six (95, 97). Different phases yield distinct potential pathogens. Time-dependent vulnerability is further modified by donor and recipient factors including antimicrobial prophylaxis, degree of immunosuppression and hematological complications.
Early post-transplant infections occur during hospitalization and frequently involve wound infection, urinary tract infection (UTI), pneumonia and blood-stream infection. Importantly, nosocomial infections are often caused by resistant pathogens and pose a significant risk to the recipient (98).
The intermediate post-transplant period is characterized by infections caused by viral and opportunistic pathogens such as cytomegalovirus (CMV), polyomavirus and *Pneumocystis* (97). Beyond CMV syndrome and CMV end-organ disease, indirect effects of CMV include allograft rejection and vulnerability to additional infections (97). However, antimicrobial prophylaxis with valganciclovir or trimethoprim/sulfamethoxazole (TMP/SMX) can prevent herpes virus infections and *Pneumocystis* infections, respectively. Identification of recipients who benefit from prophylaxis may be challenging and side-effects of antimicrobial agents ought to be weighed in.

For CMV, serostatus of the recipient and the donor are the most important factors when considering valganciclovir prophylaxis (99, 100). For intermediate-risk recipients (recipient is seropositive, donor is either seropositive or seronegative) a watch and wait strategy may be equally effective with regards to graft and recipient survival (101). Lymphocyte-depleting induction is another major CMV risk factor (99, 100). Valganciclovir treatment is frequently complicated by leucopenia. The novel antiviral, letermovir, may be an alternative in high-risk CMV constellation (recipient seronegative and donor seropositive) at a substantially lower rate of hematological complications compared to valganciclovir (102). CMV prophylaxis is usually limited to the first three to six months post-transplant, and post-prophylaxis CMV may occur after cessation of prophylaxis.

TMP/SMX is the most effective option for prevention of *Pneumocystis* pneumonia. Again, the extent of immunosuppression impacts the *Pneumocystis* risk. Important risk factors include high dose corticosteroids and lymphocyte-depleting induction (103). Additionally, TMP/SMX may reduce the risk of UTIs (95, 103), but, like valganciclovir, poses the risk of cytopenia (104).

The spectrum of late post-transplant infections includes community-acquired pneumonia and UTIs, but also late viral infections like post-prophylaxis CMV. Generally, the risk of infections is reduced compared to earlier periods due to lower degree of immunosuppression (97).

Currently, there is no biomarker available that allows titration of immunosuppression in a manner to avoid overimmunosuppression and the risk of infection on the one hand, and underimmunosuppression at the risk of rejection on the other (105). Monitoring of the apathogenic torque teno virus may greatly aid in the management of immunosuppression in the future (106, 107).

1.4. The COVID-19 pandemic and kidney transplant recipients

The emergence and rapid dissemination of severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) in late 2019 lead to the coronavirus disease 2019 (COVID-19) pandemic. SARS-CoV-2 is a respiratory coronavirus that is capable to spread from human to human via respiratory droplets (108). The “corona” or “crown” appearance of the virus is

owed to the expression of the spike (S) protein as a homotrimer on the surface of viral particles. Two subunits S1 and S2 constitute the S protein, and S1 forms the receptor-binding domain (RBD), which is crucial for viral entry. The RBD binds to host angiotensin-converting enzyme 2 (ACE2), initiating endocytosis of the virus into the host cell. This interaction further depends on host transmembrane protease serine protease 2 (TMPRSS2) for priming of S protein (109). Upon entry, the viral envelope (E) protein is responsible for the release of viral ribonucleic acid (RNA) (110). The SARS-CoV-2 genome is organized in an open reading frame (ORF) 1a and 1b. These can be directly translated by the host machinery into polyproteins. After cleavage of polyproteins by viral proteases, several non-structural proteins assemble and form a replication and transcription complex. Non-structural proteins include RNA-dependent RNA polymerase (RdRP) and main protease (Mpro). They are essential for replication of new viral RNA and cleavage of polyproteins into individual functional proteins (110). In addition to S and E proteins, the membrane (M) and nucleocapsid (N) form the primary structural proteins. N proteins are essential for protection of the viral genome inside the virion (110). M proteins constitute the virion membrane. Structural proteins, non-structural proteins and viral RNA are assembled in the endoplasmic reticulum and Golgi apparatus of the host cell to form new virions, which are subsequently released via exocytosis (110).

Respiratory droplets enter the respiratory tract, where SARS-CoV-2 can bind to epithelial cells and migrate towards the lower respiratory tract (111). ACE-2 is expressed abundantly in lung epithelial cells and endothelium allowing for rapid spread of the virus (112). Apart from the direct cytopathic effect of SARS-CoV-2, the host immune response profoundly impacts on the severity of COVID-19 (108). A “healthy” immune response facilitates rapid viral clearance at minimal lung damage. A dysfunctional immune response is characterized by excessive release of cytokines, infiltration of immune cells, capillary leakage with pulmonary edema and substantial damage of the lungs and other organs (108). Clinical presentations may therefore range from asymptomatic disease to severe and life-threatening acute respiratory distress syndrome (ARDS). Severe presentations are accompanied by increased levels of circulating cytokines like IL-1β, IL-6, IL-10 and TNFα – the “cytokine storm” (113-115). The antiviral innate immune response poses a first line of defense. However, SARS-CoV-2 can specifically evade these strategies by reducing interferon (IFN) signaling thereby delaying

the immune response and allowing for viral replication and spread (114). Inhibiting the IFN antiviral response also delays priming of the adaptive immune system. SARS-CoV-2 specific TC and BC can be found approximately one week after onset of symptoms and a delayed lymphocyte infiltration may result in severe COVID-19 (116). Lymphocyte migration to the local site of inflammation may explain the frequently observed lymphopenia in COVID-19 patients (108, 117). SARS-CoV-2 induces a T helper 1 (Th1) polarized immune response, indicated by expression of IFNγ, tumor necrosis factor (TNF) and IL-2, which relies on cellular immunity (118). $CD8^+$ TC-mediated direct killing of infected cells and $CD4^+$ TC-mediated priming of $CD8^+$ TCs and BCs are paramount for an effective adaptive antiviral response. Exuberant inflammation and excessive tissue damage may ensue in individuals who have failed to control the early stages of viral replication and to prime adaptive immune response (116). The deleterious role of hyperinflammation in severe COVID-19 is further corroborated by the effectiveness of anti-inflammatory interventions like dexamethasone or anti-IL-6 antibodies (119, 120).

Initial reports of COVID-19 in KTRs reported a 20% risk of mortality in a European registry analysis (121), and a later meta-analysis of the first pandemic wave reported similar numbers (122). This high case fatality rate also resulted in an increased number of total KTR deaths: During the first year of the pandemic, 16% of deaths in KTRs were attributed to COVID-19 (123). Two putative factors may explain the high mortality of KTRs. First, KTRs are a population with a high prevalence of comorbidities like DM, arterial hypertension and cardiovascular disease. Age is another important risk factor for severe COVID-19 in KTRs (124). Second, immunosuppression could render KTRs particularly vulnerable. Contrarily, it is also conceivable that immunosuppression may protect from COVID-19-related cytokine storm and epidemiological and observational data are somewhat conflicting on the influence of immunosuppression.

Findings that argue for a detrimental impact of immunosuppression include an observed trend for increased mortality in those with a shorter time since transplantation, during which immunosuppression is usually more aggressive (124, 125). After adjustment for other risk factors, organ transplantation was associated with a hazard ratio of 3.5 for COVID-19-related death in a large analysis of English health records (126). Similar to the general population, levels of IL-6 predicted disease severity and survival in KTRs providing further evidence

against the hypothesis that immunosuppression may counteract excessive cytokine release (127).
On the other hand, when compared to a propensity score matched non-transplant population, transplant recipients displayed a similar rate of death when admitted to the ICU (128). Furthermore, a large multi-center study concluded that age and other comorbidities, but not immunosuppression, were major drivers of mortality in transplant recipients (129).
The contrasting findings on the impact of immunosuppression may be reconciled by considering the temporal aspect: immunosuppression could initially hinder early viral clearance, yet potentially have a beneficial impact on hyperinflammation and cytokine storm in advanced COVID-19. However, as is common with other infections, immunosuppression is often reduced or paused during COVID-19 (124).

Few studies have investigated the immune response to SARS-CoV-2 in KTRs (130, 131). Candon et al. showed a robust TC and BC response to SARS-CoV-2 after tapering of the immunosuppression (131), while Hartzell and colleagues provided evidence of anti-SARS-CoV-2 antibodies despite continued immunosuppression (130). In agreement with findings from the general population, lymphocytopenia and reduced numbers of TCs and $CD8^+$ TCs were observed in KTRs with COVID-19 (108, 113, 130). More recently, blood transcriptomic analysis revealed a neutrophil activation signature coinciding with diminished adaptive immune response signaling in KTRs with acute COVID-19, independently of lymphocyte count and despite reduced immunosuppression in most cases (132). These findings mirrored blood transcriptomes of non-KTRs with COVID-19 (133). Gene signatures were also correlated with disease severity and were recovered with convalescence (132). Thus, SARS-CoV-2 *per se* induces an immunosuppressed state, which may be emphasized by immunosuppressive medication in transplantation.
Of note, the immune response to SARS-CoV-2 variants of concern or in vaccinated individuals may be different. In subsequent stages of the pandemic, when B.1.1.529 (Omicron variant) became dominant and threatened a broadly vaccinated population of KTRs, mortality rates were significantly lower (134). Presently, we investigated KTRs and SARS-CoV-2 vaccination response to the first two doses in early-mid 2021, which was prior to the emergence of Omicron or other variants of concern in Austria.

1.5. Vaccination in kidney transplant recipients

Since the emergence of SARS-CoV-2, several factors have profoundly altered the dynamics and impact of the pandemic. The rapid development and distribution of effective vaccines certainly marked a milestone in the global effort to combat COVID-19. Both, mRNA-1273 from Moderna and BNT162b2 from BioNTech and Pfizer, conferred a 94 to 95% protection from COVID-19 and showed minimal side-effects (135, 136). They also stand as the first successful mRNA-based vaccines in humans. Briefly, mRNA encoding for S protein is engulfed in lipid nanoparticles. Upon uptake by APCs, mRNA is translated into S protein, and can be presented via MHC class I or II. The MHC:antigen complex is recognized by TCs and BCs, which is followed by a robust cellular and humoral immune response (137). Additionally, a vector-based vaccine, ChAdOx1 nCoV-19 (AZD1222), provided 90% efficacy and presented a third option (138). Assessment of antigen-specific antibodies, like anti-S antibodies in case of SARS-CoV-2 vaccination, is an indirect, but readily available measure of vaccination success. Although antibody measurements indirectly assess TC response (Tfh-BC interaction), cellular immunity can also be quantified directly through IFNγ-release of $CD4^+$ and $CD8^+$ TC after stimulation with S protein. A major limitation of IFNγ-release assays (IGRA) is that they are time and labor-intensive (139). Similar limitations apply to measurements of S-specific BCs or TCs using flow cytometry. In the general population, there appears to be a more robust antibody response to mRNA-based vaccines, while ChAdOx1 nCoV-19 tends to evoke a stronger TC response (140).

KTRs have been excluded from initial SARS-CoV-2 vaccine trials, leading to a lack of data on the protective efficacy in this vulnerable subgroup. Consequently, early reports focused on measurement of anti-S antibodies as an indirect measure of protection. Benotmane et al. demonstrated that less than half of KTRs developed anti-S antibodies 28 days after two doses of mRNA-1273 (141). Similarly low numbers of responders among solid organ transplant recipients have been reported by Boyarsky et al. following mRNA-based vaccines (142). Cellular response in KTRs was consistently higher than antibody titers ranging from 30 – 79% after two doses (125). Contrary to the general population, there was no clear superiority of vector-based vaccination with regards to cellular immunity in KTRs (143, 144). A robust

cellular and humoral response was observed in KTRs vaccinated after a previous infection (145).
Nonetheless, KTRs evidently face a dual threat, as they are particularly vulnerable to COVID-19 and respond poorly to protective vaccination. Risk factors for non-response include reduced graft function, shorter time after transplantation and the immunosuppressive regimen. Particularly, MMF/MPA dose-dependently inhibit antibody generation in KTRs (125). Interestingly, seroresponse to mRNA-1273 is higher than to BNT162b2, potentially attributable to the higher dose of 100µg versus 30µg, respectively (144, 146).

Several strategies to optimize vaccination response have been investigated:
First, heterologous vaccination regimens potentially enhance response rates (143).
Second, repeated booster vaccinations have been shown to significantly improve cellular and humoral response (147, 148). Repeated doses increase the number of responders (149), and serological follow-up can aid in identifying KTRs who may benefit from additional booster vaccination (150).
Third, due to the detrimental impact of MMF/MPA, temporary withholding of antimetabolites has been investigated with conflicting results. While a peri-vaccination pause of five weeks has improved response rate, a two-week pause has not been beneficial (151, 152). Although, there were no safety issues in these studies, alloimmune activity remains an important concern, especially for high immunologic risk KTRs (151, 152).

Substantial uncertainties remain when interpreting indirect measurements of protection like antibody formation. First, seroresponse is defined differently between studies and depending on the employed testing platform. Second, there is no well-defined cut-off, which indicates protection from COVID-19.
While overall anti-SARS-CoV-2 antibodies are frequently reported, neutralizing antibodies are of particular interest, as these can prevent viral entry into host cells (153). Antibodies with neutralization capacity, usually by blocking the RBD of S protein and inhibiting the interaction with ACE2, are predictors of protection (154, 155).
Plaque reduction tests remain the gold standard for antibody-mediated virus neutralization, but they are time-consuming and difficult to scale as they are required to be handled in a biosafety level three facility. Therefore, surrogate methods for detection of neutralizing

antibodies like enzyme-linked immunosorbent assay (ELISA), which measure the competitive inhibition of the S protein/RBD – ACE2 interaction by antibodies, have been broadly used (156).

The World Health Organization (WHO) established an international standard to enable uniformity among various testing platforms and facilitate correlation with neutralizing activity. It advocates calibrating assays against this standard to generate results reported as international units (IU) or binding antibody units (BAU) (157). The objective is to create a close alignment between BAU measurements and actual neutralization levels, allowing for a correlation between the levels of anti-S antibodies detected and their functional neutralizing capacity.

Despite these efforts, the issue of a well-defined protective antibody threshold remains. To address this, Feng et al. introduced a correlate of protection after analyzing infection rates and antibody titers in ChAdOx1 nCoV-19-vaccinated and controls. Antibody titers of 264 BAU/ml or above correlated with an 80% protection from symptomatic SARS-CoV-2 infection (158). However, these results were derived using data from 2020 and early 2021 prior to the emergency of variants of concern with profound immune escape capabilities. Furthermore, cut-offs are derived from the general population and may not be transferable to the KT setting.

Early alarming reports of breakthrough-infections in fully vaccinated Israeli health care workers have challenged the concept of sterile immunity following vaccination (159). “Real world” data on the effectiveness of vaccination in the solid organ transplant population was first available in early 2022 (160). Data from United Kingdom showed that the risk of infection was not modified by vaccination status, but two-dose vaccination conferred a 20% risk reduction of COVID-19-related death. Interestingly, ChAdOx1 nCoV-19 but not BNT162b2 was associated with reduced mortality in transplant recipients (160). Compared to the general population, KTRs were at a substantially higher risk of breakthrough infections and hospitalization despite two-dose vaccination (161). Nonetheless, protection improves with the number of vaccine booster doses (162, 163), and the principle suggesting that elevated antibody levels equate to improved outcomes may still apply to KTRs after SARS-CoV-2 vaccination (150).

These studies have been conducted prior to the Omicron surge, limiting the applicability of these results to the pre-Omicron era. More recently, Hovd et al. could link anti-SARS-CoV-2 antibody titers with a reduced risk of severe COVID-19 and COVID-19-related death in KTRs during Omicron (164). Taken together, vaccination and repeated dose vaccination improve survival of KTRs with COVID-19. Non-responders to vaccination are at an increased risk, and efforts should be directed towards alternative means of protection (e.g., mask wearing in public places, social distancing during surges) and devising strategies to enhance vaccination response.

1.6. B cell biology and characterization of B cell subsets in peripheral blood

BCs constitute the humoral arm of adaptive immunity. Apart from their ability to produce antibodies, BCs serve a multitude of functions with relevance in transplantation (165).
The initial steps of BC maturation take place in the bone marrow. Pro-BCs constitute the first progenitor to express the BC transcription factor E2A (17, 166). At this stage, recombination of the Variable/Diversity/Joining (VDJ) region for the Ig heavy chain is mediated by Recombination-Activating Genes (RAG). Importantly, CD19, a standard marker for BC characterization and expressed throughout the entire BC lineage, is already present from this early stage (167). Additional variability into the BC receptor (BCR) repertoire is introduced by the enzyme terminal deoxynucleotidyl transferase (TdT), which adds random nucleotides during VDJ recombination (168). Successfully recombination results in the expression of the Ig heavy chain within the pre-BCR. Only precursor with functioning pre-BCR obtain survival and proliferation signals, which marks the transition to Pre-BCs. In Pre-BCs, successful recombination of the Ig light chain VJ region results in the association with the Ig heavy chain. Heavy and light chain form surface-bound IgM, a hallmark of immature BCs (17). BCRs that recognize locally expressed self-antigens are removed from the BC pool at this stage or become anergic (central tolerance) (169). Alternatively, autoreactive immature BCs can alter their BCR in a process called receptor-editing. Only when unresponsive to autoantigens, BCs will receive survival signals (170).

Immature BCs exit the bone marrow and undergo further maturation within secondary lymphoid organs. Transitional BCs (TrBCs), identifiable by their abundant expression of CD24 and CD38, represent a transitional subset between immature and mature BCs (17, 171). TrBCs can be further subsetted into three developmental stages (T1 → T3). As they relocate to the spleen, T1 TrBCs depend on B-cell-activating factor (BAFF, also known as B lymphocyte stimulator (BLyS)) provided by follicular dendritic cells (FDC) of the BC follicles. BAFF-signaling prompts the upregulation of IgD and CD21 (complement receptor 2) and downregulation of IgM, signifying the evolution to long-lived T2 TrBCs (172). At this point, T2 TrBCs can differentiate into two distinct BC fates: follicular BCs (also known as B2 BCs) and marginal-zone BCs (171, 173, 174). How exactly BC fate is determined remains elusive, but BCR specificity may influence their differentiation. Of note, the role and significance of the last TrBC stage, T3, remains elusive. T3 TrBCs are indistinguishable from naïve BCs using only surface markers (171).

Marginal zone BCs reside predominately in the marginal zone of the spleen, but can also be found in lymph nodes and Peyer's patches (175). They serve as a first line of defense against blood borne pathogens, as they can rapidly produce antibodies against bacterial polysaccharides independently of TC help (TC-independent, TI) (176). Marginal zone BCs appear to be generated independently of germinal center (GC) reactions, because they are found abundantly in CD40L deficient individuals, who are unable to form functioning GCs (177). In that manner, marginal zone BCs bridge the innate and adaptive immune system. Human marginal zone BCs also recirculate and account for 10 – 30% of all circulating BCs (176). They can be characterized by the expression of the memory marker CD27 and the markers CD21, CD1c, IgD and IgM. Thus, they have also been referred to as unswitched memory BCs (mBCs) (171, 176). However, within unswitched mBCs, certain subsets display genetic footprint of a GC reaction and the ability to respond to TC-dependent (TD) antigens hinting towards distinct marginal zone BC subsets capable of TI and TD antibody response, respectively (175, 178, 179).

Mature, antigen-naïve follicular BCs circulate through secondary lymphoid organs in search of their cognate antigen. They home to specific BC-enriched regions, the primary follicles. Opsonized antigens enter lymphatic tissue like the spleen or lymph nodes and bind to

macrophages or FDCs via complement receptors. Antigens are not phagocytosed but carried to the primary follicles for BC recognition. For peptide antigens, BCR engagement of the antigen leads to internalization of the BCR-antigen complex, which is subsequently fragmented. Peptide fragments are brought to the cell surface bound to MHC II for TC presentation. Simultaneously, the same antigen activates TCs within the TC zone of secondary lymphoid organs. Antigen-specific activated TCs and BCs migrate towards each other and meet at the border of the B and T zone by C-X-C motif chemokine receptors (CXCR5) guidance. MHC II-bound antigen interacts with the TCR. Furthermore, interaction of CD40-CD40L and co-stimulatory molecules like CD80/86-CD28, promotes mutual activation and proliferation (171).

The ensuing immune response can be divided into two phases:
First, the "extrafollicular response" leads to generation plasmablasts (PB) without the need of a GC reaction (180). PBs have shifted from producing membrane-bound BCRs to secreting antigen-specific antibodies (181). PBs are generated early after an infection but are relatively short-lived (182). They represent an important first line of humoral defense (183).

The second phase involves the generation of a GC. The GC comprises an outer dark zone, where GC BCs proliferate and undergo somatic hypermutation (SMH) (184). SMH refers to an active process in which the V region of the BCR gene is altered by the enzyme activation-induced cytidine deaminase (AID). AID is exclusively expressed in the GC (185). These random mutations of the complementary determining region (CDR) can greatly enhance antigen affinity (17). GC BCs with modified BCRs then enter the inner GC light zone, where they are again exposed to the antigen on FDCs (186). Based on the strength of their interaction with the antigen, BCs will receive signals for survival and differentiation by specialized TCs, Tfh, or face apoptosis (184). Another essential process within the GC is class-switch recombination (CSR), which alters antibody isotype by rearranging the DNA sequence of the BCR's constant region. During CSR certain gene segments are deleted, resulting in the permanent loss of IgM expression in BCs that have undergone isotype switching (185). Although CSR does not alter affinity, it impacts the functional properties of the antibody. Tfh cytokines influence the isotype of BCs. For instance, in mice, IL-4 promotes IgE isotype, while IFNγ favors IgG2a production (17). BC clones that received Tfh-

stimulation may re-enter the GC dark zone. Repeated cycling between dark and light zone further enhances antibody affinity (184). Alternatively, BC clones equipped with high-affinity BCRs can exit the GC and differentiate into mBCs or long-lived PCs (180).

While PBs produce mostly low affinity IgM antibodies and can undergo CSR and (to a small extent) SMH, PCs provide high affinity, heavily mutated class-switched antibodies in later stages of an infection. PCs originating from spleen and lymph nodes can migrate to the bone marrow and, to a lesser extent, other organs, where they can constantly produce antibodies for extended periods of time (187). Some PBs may also differentiate into long-lived PCs after clearance of infection (188).

The second pillar of humoral immunity is constituted by mBCs. When they re-encounter their specific antigen, mBCs can rapidly differentiate into PCs. Alternatively, they may form GCs with further refining of their BCRs to the antigen. This process results in the generation of mBCs and high-affinity PCs (180). Moreover, mBCs are particularly long-lived and can survive for decades (189), providing long-term protection after infection.
CD27 is a marker of mBCs, and around 40% of circulating BCs are $CD27^+$ (190). CD27 is upregulated during GC reaction and is also found on antibody-secreting cells like PBs and PCs (171). PBs and PCs can be distinguished from mBCs by their abundant expression of CD38, whereas mBCs are $CD38^-$ (171, 191).
The human mBC compartment is heterogenous and can be subsetted by the expression of surface IgM and IgD (or lack thereof) (171, 192). mBCs that have undergone isotype switch don't express IgM or IgD but IgG, IgE or IgA. These subtypes are called switched mBCs (171). IgM-only mBCs express IgM but not IgD. They are believed to arise early during GC reactions and to serve as substrate for switched mBCs (193-195). Contrarily, IgD-only mBCs are IgM^- and IgD^+ and their function in health and disease remains ominous (189). Due to heavily mutated IgD and CSR with the deletion of the IgM constant region, they are believed to be of GC origin (189, 196). IgD-only mBCs are found at low frequency (0.1 – 0.5% of BCs) in peripheral blood and have been implicated in the immune response to respiratory bacterial infections (197). Alternatively, they may constitute a "sink" for BCs with autoreactive BCR as a potential mechanism to control self-reactivity (198).

$CD19^+$ BC subsets in peripheral blood can be distinguished by CD27 and IgD expression, indicators of BC maturation. Naïve BCs express surface IgD but lack CD27. Conversely, antigen-experienced $CD27^+$ BCs comprise mBCs and antibody-secreting cells. BCs that express neither CD27 nor IgD are called double negative (DN) (171, 199). DN BCs are expanded in systemic lupus erythematosus (SLE) patients and in chronic infections like HIV and malaria (199-201). In chronic infections, DN BCs often express Fc receptor-like 4 (FcRL4), which can hinder BCR signaling, potentially leading to exhaustion (202), a trait shared with tissue-based mBCs (203). DN BCs were therefore regarded as atypical or tissue-based mBCs.

Recently, distinct DN BC subset have been identified. In SLE patients, surface expression of CXCR5, CD21 and CD11c, can distinguish DN1 ($CXCR5^+$ $CD21^+$ and $CD11c^-$) and DN2 ($CXCR5^-$ $CD21^-$ and $CD11c^+$). Through extensive transcriptomic, epigenomic and flow cytometric analysis, Jenks et al. provided evidence that DN2 cells are precursors of antibody-secreting cells derived from early activated BCs of extrafollicular origin. Moreover, DN2 BC abundance correlates with disease activity in SLE and with poor response to BC depleting therapy. DN1 BCs, on the contrary, have been described as early activated mBCs (204).

Apart from B2 BCs, encompassing MZ BCs and follicular BCs, a third distinct major BC population ought to be mentioned. B1 BCs are a developmentally and functionally unique BC subset that is well characterized in mice. In mice, B1 BCs produce natural, polyreactive IgM antibodies and reside mainly in the pleural and peritoneal cavity, where they can undergo self-renewal. CD5 expression aids in further subclassification of murine B1a and B1b BCs, which serve distinct functions (205). The putative marker profile of human B1 BCs is $CD20^+$ $CD27^+$ $CD43^+$ $CD70^-$ and this subset displays fundamental B1-like characteristics, i.e., tonic BCR signaling, spontaneous generation of polyreactive antibodies and efficient TC stimulation (206, 207). The existence of human B1 BCs has recently been corroborated in an extensive study on the developing human immune system (208).

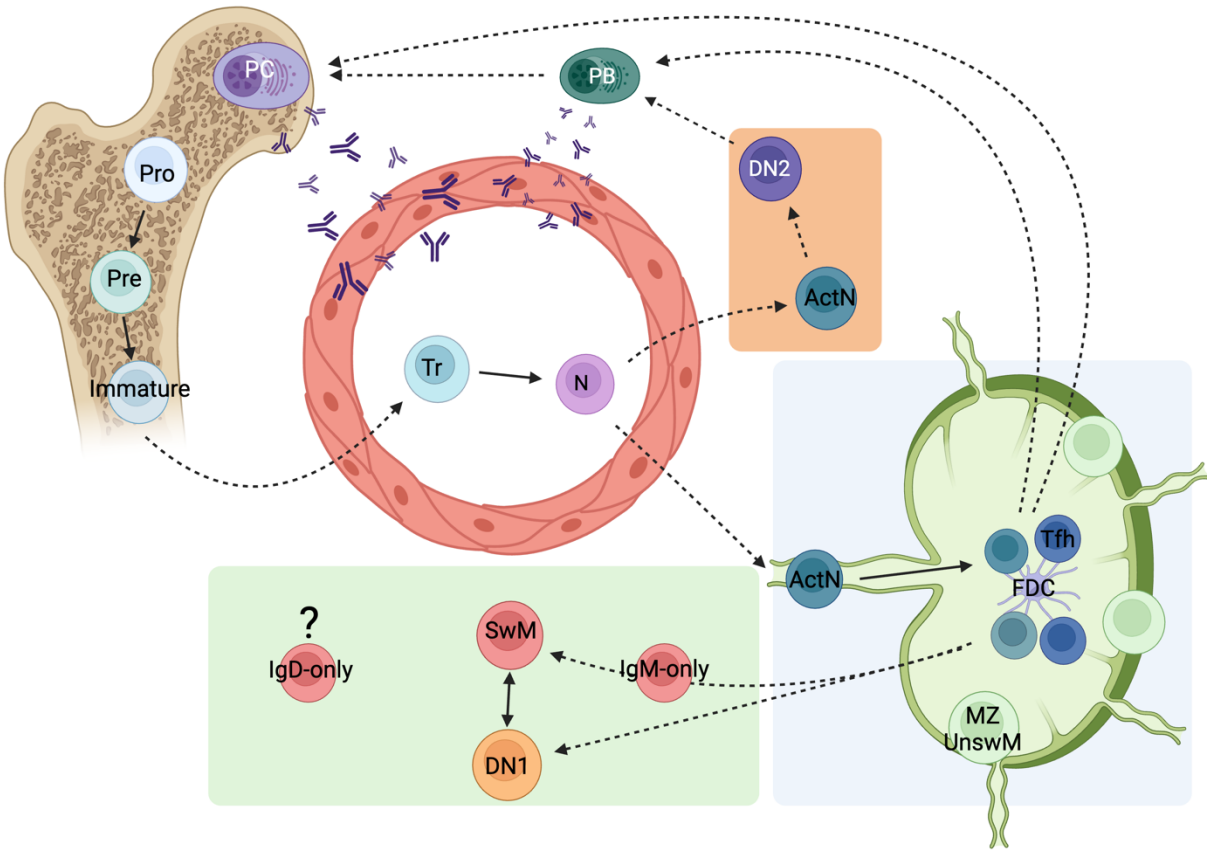

Figure 2. Schematic overview of BC subsets available from immunophenotyping of peripheral blood. BCs undergo stepwise maturation in the bone marrow from Pro-BC, Pre-BC to immature BCs, which exit the bone marrow niche and roam the periphery as TrBCs (Tr) for further maturation. Matured naïve BCs (N) become activated (ActN) upon antigen recognition. Together with T-follicular helper cells (Tfh), they can form germinal centers within secondary lymphoid organs. Follicular dendritic cells (FDC) play a central role in the follicular pathway (light blue box). Alternatively, extrafollicular activation pathways can give rise to PBs, likely via a DN2 intermediate (light orange box). PBs serve as first-line humoral defense by producing low-affinity antibodies, and some PBs can differentiate into long-lived PCs. Germinal center derived PCs are generated later during infection and can produce high-affinity antibodies for effective antigen neutralization. PCs migrate to the bone-marrow, where they provide long-term humoral defense. The memory compartment (light green box) consists of switched mBCs (SwM), generated during GC reaction, roaming the periphery in search of their antigen. While DN1 BCs are suggested to be early activated mBCs, IgM-only mBCs (IgM-only) serve as precursors of switched mBCs. The exact role of IgD-only mBCs (IgD-only) is unknown, whereas circulating unswitched mBCs (UnswM) are considered to be marginal-zone BCs (MZ). Created with BioRender.com.

1.7. B cells in kidney transplantation

BCs have been historically neglected in KT, which is reflected by the TC centered immunosuppressive protocols of today's practice. Although modern immunosuppressive drugs have significantly improved rates of acute rejection, long-term graft survival has remained suboptimal (209). Late transplant failure is commonly driven by ABMR (94). The occurrence of DSA after KT is associated with accelerated graft loss (210), but antibody-mediated damage can exist without identifiable DSA or complement activation (as indicated by histological C4d staining) (211, 212). Given the pivotal role of BCs as precursors of alloantibody-producing PCs, they are often perceived as adversaries in transplantation and considerable effort has been invested to inhibit BC and PC activity. Treatment options for late ABMR are frequently based on retrospective data and high-quality evidence is rare. The few prospective studies, involving treatment of bortezomib, eculizumab or intravenous immunoglobulins plus rituximab, have failed to convincingly improve eGFR trajectory in late ABMR (213-215).

BC depletion through rituximab induction in ABO compatible KTRs has resulted in a substantial increase of acute rejections prompting premature termination of the trial (216). These findings underscore the complexity of BC's role in KT. Certain BC subtypes can exert regulatory functions (Bregs) via secretion of IL-10, IL-35 and TGFβ (165). Bregs can be identified within several BC subsets including mBCs, naïve BCs and even PBs and PCs (217, 218). Importantly, Bregs are characterized by functionality, and at present, no specific surface marker can reliably pinpoint Bregs (171). However, TrBCs ($CD19^{+}$ $CD21^{++}$ $CD23^{++}$ $CD24^{++}$) and B10 BCs ($CD5^{+}$ $CD10^{+}$ $CD1d^{++}$) appear particularly enriched with Bregs (165). There is substantial evidence that enrichment of Bregs may be beneficial towards graft survival.

First, operationally tolerant KTRs, a rare subset of KTRs who have achieved graft tolerance without the need for immunosuppression, demonstrate a distinct BC signature. This "tolerance signature" is characterized by increased abundance of naïve and TrBCs (219). Additionally, BCs of tolerant recipients produce more IL-10, a cytokine known for its regulatory functions (220).

Second, Cherukuri et al. found that the relation of T1/T2 TrBCs is a strong predictor of graft survival. A relative increase in T1 TrBCs, which readily generate IL-10 compared to TNFα-producing T2 TrBCs was associated with improved graft function and offered more accurate prediction than DSA or eGFR (221). Subsequent investigations evaluated the IL10/TNFα ratio produced by T1 TrBC at 3 months post-transplant as a potential biomarker for rejection, demonstrating its reliability in predicting rejection within the first year (222). Moreover, KTRs with borderline rejection on graft biopsy, who are more vulnerable to acute rejection, showed a decreased IL10/TNFα ratio (223).

Third, in an innovative phase I study, Morath et al. used modified donor-derived immune cells prior to KT to induce donor unresponsiveness in recipients in an attempt to reduce the burden of unspecific immunosuppression. This tolerant-like phenotype was marked by a 68-fold higher frequency of TrBCs and increased IL-10 production (224). Subsequent *in vitro* experiments suggest a direct role of regulatory TrBCs in donor-specific tolerance, which appears long-lasting (225). Presently, a phase II trial is anticipated to prospectively investigate the effectiveness of modified immune cells in conjunction with a low-dose immunosuppressive protocol in living kidney donation (226).

Other BC functions with relevance in transplantation include antigen presentation and the formation of tertiary lymphoid structures (TLS) within the graft. Intriguingly, the presence of $CD20^+$ infiltrates has been associated with adverse outcomes in acute rejection (227). Furthermore, in a mouse model of cardiac transplantation, BCs could mediate rejection despite genetic knockdown of AID and their ability to produce alloantibodies (228). Although TLS are commonly found in kidney allografts, they do not necessarily indicate allograft injury. However, advanced TLS, featuring GC and/or FDCs, are notably abundant in KTRs with graft dysfunction (229). Experimental evidence suggests that TLS-associated BCs could secrete pro-fibrotic cytokines contributing to graft damage (230). Moreover, infiltrating BCs exhibit a distinct transcriptional profile, suggestive of their involvement in innate signaling pathways, breaking peripheral tolerance to organ-specific antigens driving allograft inflammation (231, 232).

1.8. Aims

BCs are a critical component of the immune system playing a pivotal role in alloimmunity and immune defense. Peripheral blood BCs are heterogenous and consist of functionally distinct subsets.

In this thesis, we aimed to:

1. Study the dynamics of BCs and previously not described BC subsets - from pre-transplantation in CKD G5 (ESKD) to one year post-transplantation.
2. Assess the peripheral BC pool for potential prognostic markers related to the antibody response to anti-SARS-CoV-2 vaccination.

2. Materials and Methods

2.1. Study Design

From 2016 to 2020, a total of 105 patients diagnosed with CKD G5 were prospectively recruited. They underwent KT at the Department of General, Visceral, and Transplant Surgery at the Medical University of Graz. All patients met the following inclusion criteria:

- Age ≥ 18 years
- Absence of immunosuppressive therapy prior to KT
- Reception of a cadaveric organ

After obtaining written informed consent in accordance with ethical guidelines outlined in the Declaration of Helsinki, peripheral blood samples were collected at two time points.

1.) Before the HD session prior to transplantation (**T1**)
2.) One-year post-transplantation (**T2**)

Prior to KT, each patient underwent a HD session to optimize their clinical condition. Only patients with complete follow-up and a functioning allograft at T2, defined as absence of dialysis dependency, were included in the study. The study protocol received formal approval from the Institutional Review Board of the Medical University of Graz, Austria, under the reference number 28-514ex15/16. The study was registered under the reference #DRKS00026238 in the German Register of Clinical Studies.

2.2. PBMC isolation, Flow Cytometry and Gating Strategy

Peripheral blood mononuclear cells (PBMCs) were isolated from freshly collected heparinized whole blood samples using BD vacutainer tubes containing lithium heparin (Becton Dickinson, Franklin Lakes, NJ, USA). Subsequently, whole blood was diluted at a 1:1 ratio with phosphate-buffered saline (PBS) and gently layered into a tube pre-filled with Lymphoprep density gradient media (Stemcell Technologies, Vancouver, Canada). A density gradient centrifugation step was performed, lasting 20 minutes at 800 × g at room

temperature. Next, the PBMC layer was carefully collected and washed with PBS. The viability and cell count were determined using an automated dual fluorescence cell counter, the LUNA-FL (Logos Biosystems in Anyang, South Korea), before proceeding to perform multi-parameter staining on 1×10^6 cells per fluorescence-activated cell sorting (FACS) panel. In addition, 0.5×10^6 cells were designated for unstained control purposes. Surface panel staining was carried out with BD Lyse/Fix buffer (Becton Dickinson), following the manufacturer's guidelines. All antibodies used in the study were sourced from Becton Dickinson, with specific details available in Table 1.

Antibody (Clone)	**Fluorochrome**	**Reference number**
CD19 (SJ25C1)	PE	345789
IgM (G20-127)	BB515	564622
IgD (IA6-2)	PerCP-Cy5.5	561315
CD24 (ML5)	BV711	563401
CD27 (L128)	BV786	563327
CD86 (FUN-1)	PE-CF594	562390
CD38 (HIT2)	APC-R700	564979
CD5 (L17F12)	PE-Cy7	348790

Table 1. Antibodies used for B cell phenotyping.

Furthermore, 50µl of fresh whole blood was subjected to staining with anti-CD45 APC-H7 antibodies (Becton Dickinson). For the determination of absolute numbers of leucocyte subpopulations, 123 count eBeads (Thermo Fisher Scientific, Waltham, MA, USA), were introduced. All samples were processed using a BD FACS Fortessa SORP instrument (Becton Dickinson) equipped with four lasers. The data were subsequently analyzed with the FlowJo software. For compensation purposes, UltraComp eBeads (Thermo Fisher Scientific) were employed, and Fluorescence Minus One (FMO) controls were employed to ensure accurate gating of BC subtypes. A detailed illustration of the gating strategy can be found in Table 2 and Figure 3.

Reported marker	As % of	Phenotype	Gating: initially gating cells, single cells and lymphocytes
$CD19^+$ B cells in Lymphocytes	Lymphocytes	$CD19^+$	(i) Display CD19, on $CD19^+$
IgD^+CD27^- Naive B cells in B cells	$CD19^+$ B cells	$CD19^+IgD^+CD27^-$	(i) Display CD19, on $CD19^+$, (ii) IgD vs. CD27, on IgD^+CD27^-
Transitional B cells in B cells	$CD19^+$ B cells	$CD19^+IgD^+CD27^-CD38^+CD24^+$	(i) Display CD19, on $CD19^+$, (ii) IgD vs. CD27, on IgD^+CD27^-, (iii) CD38 vs. CD24, on $CD38^+CD24^+$
IgD^-CD27^- double negative B cells in B cells	$CD19^+$ B cells	$CD19^+IgD^-CD27^-$	(i) Display CD19, on $CD19^+$, (ii) IgD vs. CD27, on IgD^-CD27^-
$CD38^+CD24^+$ Plasmablasts in B cells	$CD19^+$ B cells	$CD19^+IgD^{-/+}CD27^+CD38^+CD24^-$	(i) Display CD19, on $CD19^+$, (ii) IgD vs. CD27, combined gate of both $CD27^+$ populations, (iii) CD38 vs. CD24, on $CD38^+CD24^-$
$CD27^+CD38^{-/+}CD24^{-/+}$ Memory B cells in B cells	$CD19^+$ B cells	$CD19^+IgD^{-/+}CD27^+CD38^{-/+}CD24^{-/+}$	(i) Display CD19, on $CD19^+$, (ii) IgD vs. CD27, combined gate of both $CD27^+$ populations, (iii) CD38 vs. CD24, every event except Plasmablasts-Gate
IgD-only Memory B cells in Memory B cells	$CD27^+CD38^{-/+}CD24^{-/+}$ Memory B cells in B cells	$CD19^+IgD^{-/+}CD27^+CD38^{-/+}CD24^{-/+}IgM^-IgD^+$	(i) Display CD19, on $CD19^+$, (ii) IgD vs. CD27, combined gate of both $CD27^+$ populations, (iii) CD38 vs. CD24, every event except Plasmablasts-Gate, (iv) IgM vs. IgD, on IgM^-IgD^+
IgM-only Memory B cells in Memory B cells	$CD27^+CD38^{-/+}CD24^{-/+}$ Memory B cells in B cells	$CD19^+IgD^{-/+}CD27^+CD38^{-/+}CD24^{-/+}IgM^+IgD^-$	(i) Display CD19, on $CD19^+$, (ii) IgD vs. CD27, combined gate of both $CD27^+$ populations, (iii) CD38 vs. CD24, every event except Plasmablasts-Gate, (iv) IgM vs. IgD, on IgM^+IgD^-
switched Memory B cells in Memory B cells	$CD27^+CD38^{-/+}CD24^{-/+}$ Memory B cells in B cells	$CD19^+IgD^{-/+}CD27^+CD38^{-/+}CD24^{-/+}IgM^-IgD^-$	(i) Display CD19, on $CD19^+$, (ii) IgD vs. CD27, combined gate of both $CD27^+$ populations, (iii) CD38 vs. CD24, every event except Plasmablasts-Gate, (iv) IgM vs. IgD, on IgM^-IgD^-
unswitched Memory B cells in Memory B cells	$CD27^+CD38^{-/+}CD24^{-/+}$ Memory B cells in B cells	$CD19^+IgD^{-/+}CD27^+CD38^{-/+}CD24^{-/+}IgM^+IgD^+$	(i) Display CD19, on $CD19^+$, (ii) IgD vs. CD27, combined gate of both $CD27^+$ populations, (iii) CD38 vs. CD24, every event except Plasmablasts-Gate, (iv) IgM vs. IgD, on IgM^+IgD^+
$CD86^+$ B cells	$CD19^+$ B cells	$CD19^+CD86^+$	(i) Display CD19, on $CD19^+$, (ii) CD86 vs. SSC, on $CD86^+$
$CD27^-CD5^+$ B cells in B cells	$CD19^+$ B cells	$CD19^+CD27^-CD5^+$	(i) Display CD19, on $CD19^+$, (ii) CD5 vs. CD27, on $CD27^-CD5^+$

Table 2. Gating strategy for BC subsets in peripheral blood. mBC subset frequencies of total mBCs were converted manually to frequencies of mBC subsets of overall BCs for analyses.

Frequencies of BC subpopulation were reported as frequency of total $CD19^+$ lymphocytes. Absolute numbers were calculated from total BC counts (= $CD19^+$ lymphocytes) and relative frequencies of BC subpopulations.

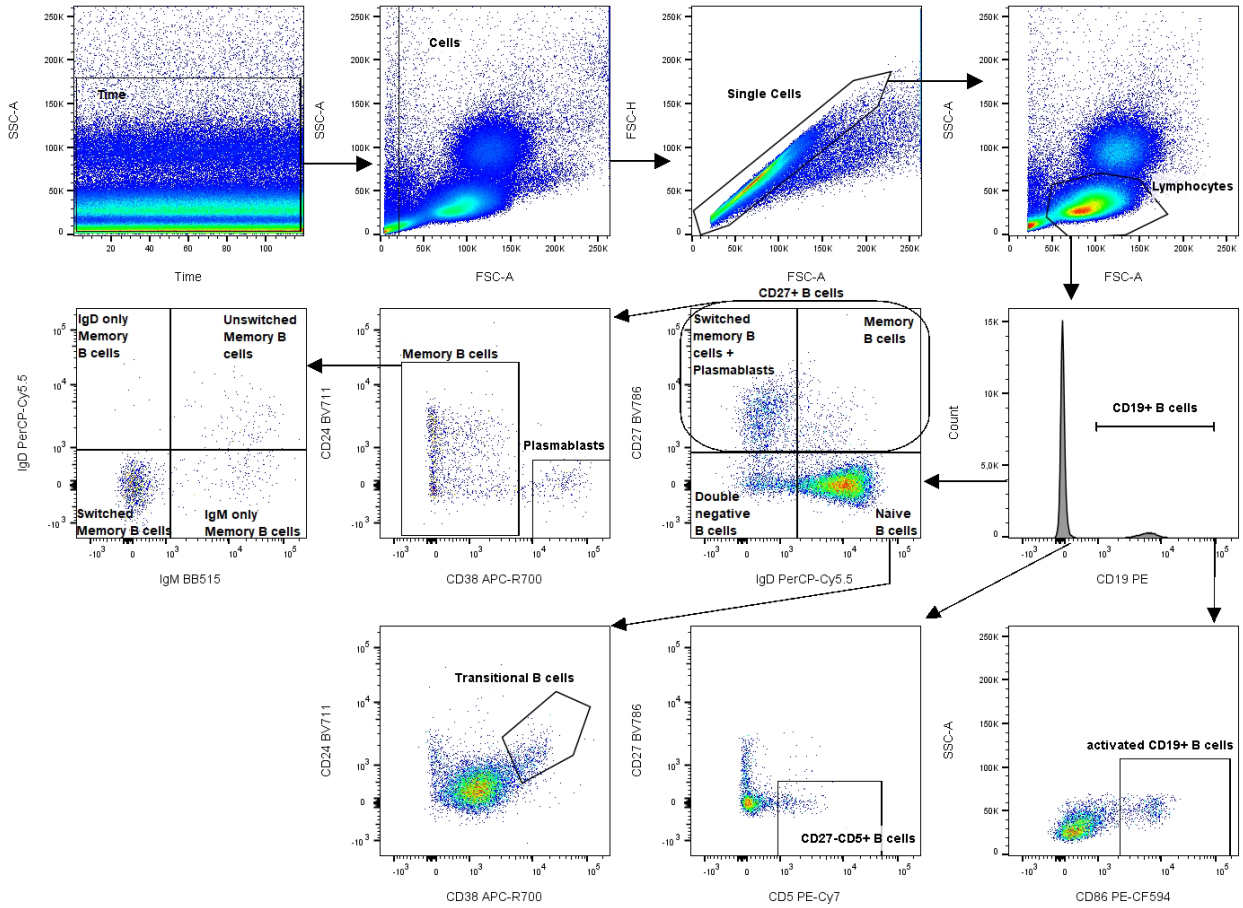

Figure 3. Gating strategy for BC subsets. Lymphocyte selection: Lymphocytes were initially isolated based on their morphological characteristics as indicated by the forward scatter (FSC-A) vs. side scatter (SSC-A) parameters. Data quality assessment: To ensure data integrity, the timeline was checked to identify measurement artifacts and to exclude doublets. BC identification: BCs were initially distinguished by their expression of CD19, and this was visualized using a histogram. Detection of activated BCs: Activated BCs were identified based on their $CD86^+$ expression. This characterization was achieved by examining the CD86 vs. SSC-A plot. $CD27^-CD5^+$ BCs: A specific subset of BCs displaying the $CD27^-CD5^+$

phenotype was discerned by evaluating their CD5 vs. CD27 expression. Categorization of naïve BCs, mBCs, and DN BCs: Further differentiation of BCs was carried out by assessing their IgD and CD27 expression. This allowed for the classification of BCs into subgroups, including naïve, memory, and DN BCs, using the IgD vs. CD27 plot. Classification of switched and IgM-Only mBCs, PBs: Within the $IgD^{-}CD27^{+}$ gate, BC subsets such as switched and IgM-only mBCs, along with PBs, were collectively identified. Refinement of mBC Subsets: To further refine the identification of mBCs and separate them from PBs, both $CD27^{+}$ gates were merged and utilized to distinguish these subsets. This was achieved through an analysis of CD38 vs. CD24 expression. Further subdivision of mBCs: The mBCs identified in the previous step were subsequently subdivided into IgD-only, IgM-only, switched, and unswitched mBCs, as determined by evaluating IgM vs. IgD expression. TrBCs: TrBCs with a $CD38^{+}CD24^{+}$ phenotype were isolated from the pool of naïve B cells using a CD38 vs. CD24 plot.

2.3. Vaccination Subcohort and Anti-SARS-CoV-2 Antibody Measurements

Our objective was to assess the correlation between BCs and their subpopulations with the humoral response to an mRNA-based SARS-CoV-2 vaccine. To achieve this, we selected a subset of KTRs with complete T1 and T2, who met additional criteria:

- Availability for follow-up until anti-SARS-CoV-2 antibody testing
- Having received two doses of either mRNA-1273 (Moderna) or BNT162b2 (Pfizer/BioNTech) vaccines
- Maintenance of a functioning graft at the time of vaccination. Return to dialysis was considered as graft loss.
- Completion of their last visit (T2) before vaccination

In instances where KTRs had experienced symptomatic or asymptomatic COVID-19, confirmed by either positive RT-PCR or positive serology for anti-N antibody at any point before, during, or after vaccination, those patients were excluded from the vaccination subcohort.

We defined the timepoint **T3** as the time of antibody testing. Our study's timeline including T1-T3 is visualized in Figure 4.

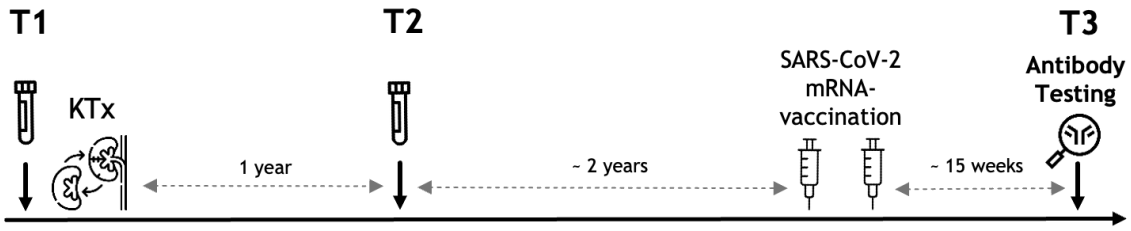

Figure 4. Blood samples were drawn prior to KT (T1) and one year after KT (T2). The time from T2 to SARS-CoV-2 mRNA-vaccination was variable. After another variable interval anti-SARS-CoV-2 antibody testing was performed (T3). Icons made by Freepik from www.flaticon.com.

Specifically, SARS-CoV-2 specific antibodies against the spike protein were quantified using various assays, including the LIAISON TrimericS IgG Assay (DiaSorin, Saluggia, Italy), Elecsys Anti-SARS-CoV-2S (Roche, Basel, Switzerland), Alinity I SARS-CoV-2 IgG II Quant (Abbott Laboratories, Chicago, IL, USA), or Atellica IM COV2G (Siemens Healthineers, Erlangen, Germany). Characteristics of different testing platforms are summarized in Table 3. To ensure comparability across the different test platforms, the respective units were converted to BAU/mL using conversion factors provided from the manufacturers. Individuals were categorized as "responders" if their antibody levels were detectable above the detection limit of the respective testing platform. Contrarily, individuals with no detectable antibodies after two vaccine doses were designated as "non-responders".

Platform	Provider	Assay Characteristics	Range	Limit of detection	BAU conversion
LIAISON TrimericS IgG Assay	DiaSorin	Quantitative CLIA detection of IgG against S1 and S2	1.85 – 800 AU/mL	0.72 AU/mL	2.4
Elecsys Anti-SARS-CoV-2S	Roche	Quantitative ECLIA detection of IgG against S-RBD	0.4 – 250 AU/mL	0.35 AU/mL	0.972
Alinity I SARS-CoV-2 IgG II Quant	Abbott	Quantitative CMIA detection of IgG against S1-RBD	21 – 40000 AU/mL	6.8 AU/mL	0,142
Atellica IM COV2G	Siemens Healthineers	Quantitative CLIA detection of IgG against S1-RBD	0.5 – 150 U/mL	0.05 U/mL	21.8

Table 3. Characteristics of anti-SARS-CoV-2 antibody testing platforms including BAU conversion factor are shown.
CLIA Chemoluminesence Immunoassay, AU Arbitrary units, BAU Binding antibody units, ECLIA Electrochemical Immunoassay, CMIA Chemiluminescent microparticle immunoassay, U Units, NA Not applicable.

2.4. Statistical analysis and Graphical Presentation

Statistical analyses were conducted using two software packages: Statistical Package for Social Sciences (SPSS v26, SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8.0.1 (GraphPad Software Inc., San Diego, CA, USA).
The normality of data distribution was assessed employing the Kolmogorov-Smirnov test. Results for normally distributed data are presented as the mean accompanied by the standard error of the mean (SEM), while non-normally distributed data is described using the median and interquartile range (IQR). Categorical data is presented as absolute values and relative frequencies (%).

To compare differences between two independent groups, t-tests, Mann-Whitney U-tests, $\chi 2$-tests, or Fisher's exact tests were applied depending on the data distribution and tested variables. When examining paired groups, a dependent t-test or Wilcoxon signed-rank test was utilized, depending on the distribution characteristics of the variables.
Spearman's rank-based correlation coefficient was employed to investigate associations between variables. Statistical significance was defined as p-values below 0.05. No formal adjustments for multiple testing were applied in the analysis. Statistical significance was signified by *p≤0.05, **p≤0.01, ***p≤0.001, ****p≤0.0001.
Graphical representations were generated using GraphPad Prism 8.0.1. Composition of figures was modified with Inkscape version 1.1 (Inkscape Project 2020). Illustrations were created with BioRender.com and icons were derived from flaticon.com.

3. Results

3.1. Clinical characteristics

Figure 5 illustrates the flow chart of our study and how we arrived at the final T1/T2 population. A total of 105 KTRs were screened, and blood samples were drawn at T1. In the first year of transplantation, one person died, nine KTRs experienced graft loss and 24 had incomplete follow-up until T2. The remaining 71 KTRs were included in the analysis of BC dynamics and constitute our T1/T2 cohort.

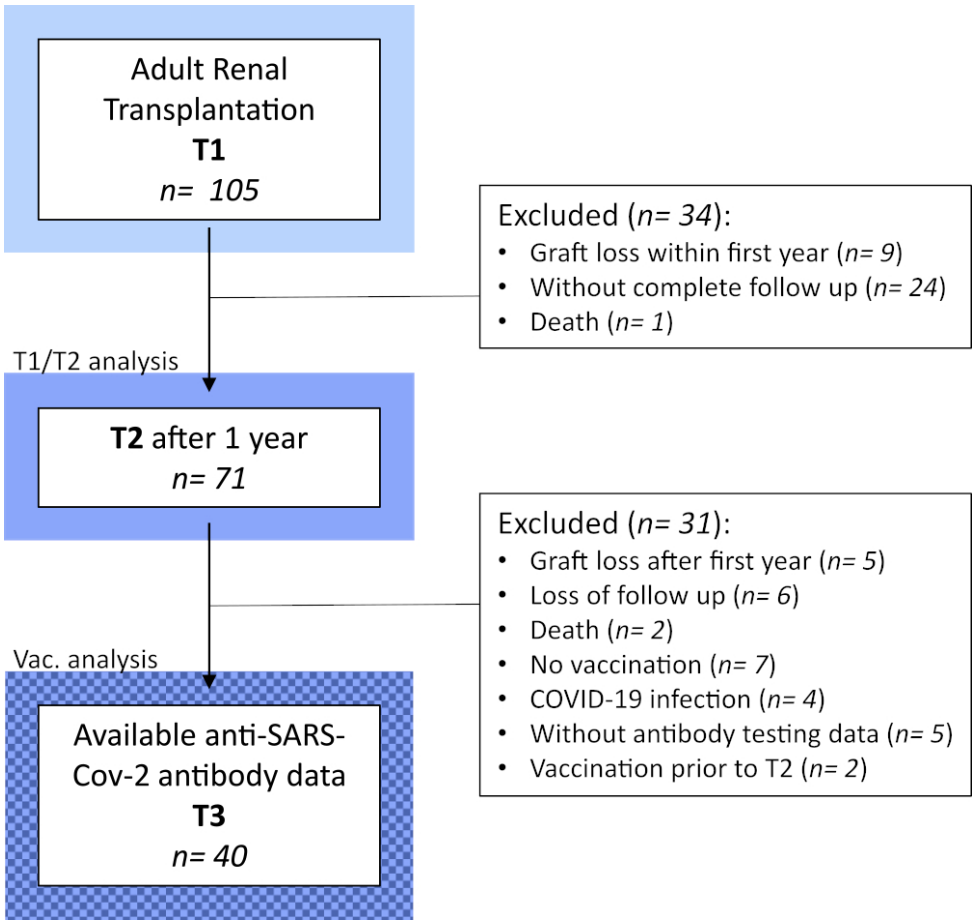

Figure 5. Flow chart of our study population.

Clinical and demographic information on the T1/T2 cohort are summarized in Table 4. The median age of the participants was 56 years, with approximately one-third (33.8%) being female. Among the KTRs, 22.5% had a pre-existing diagnosis of diabetes at the time of kidney transplantation. Median BMI was 26.4kg/m². The majority of participants were of Caucasian ethnicity (94.4%), and a small proportion (4.2%) received a pre-emptive transplant. For those who underwent dialysis prior to transplantation, hemodialysis was notably more common than peritoneal dialysis (80.9% vs. 19.1%). Median dialysis vintage prior to KT was 28 months.

The leading causes of CKD G5 in our cohort were glomerular disease (21.1%) and diabetic kidney disease (18.3%), followed by polycystic kidney disease (16.9%) and hypertensive kidney disease (7%). In 36.6% of cases, either an alternate cause of CKD was identified, or a specific diagnosis could not be determined.

Immunosuppressive treatment adhered to the recommendations outlined in the 2009 KDIGO guidelines (72). Following the guideline's criteria for high immunological risk, patients at a lower pre-transplant risk of rejection were administered BX (91.5%), while those at higher risk received recombinant ATG (9.9%) as induction therapy (72). Notably, only five individuals exhibited pre-transplant PRAs exceeding 0%. In those five KTRs, PRAs ranged from 12 to 81%. In one instance, an allergic reaction necessitated change of induction therapy from ATG to BX.

The majority of KTRs initiated immunosuppressive regimens comprising CS (100%), TAC (98.6%), and MPA or MMF (98.6%). There were two exceptions: one patient commenced with CyA (1.4%) in place of TAC, and another received AZA (1.4%) instead of MMF/MPA.

We observed 13 biopsy-proven rejection events in 12 individuals in the first year of KT. Of these, 12 were acute cellular rejections classified as BANFF1B (83.3%) and BANFF2A (16.7%) according to the 2015 Banff classification of renal allograft pathology (233). One ABMR was recorded. Treatment included high-dose CS and ATG with a duration ranging from seven to 14 days depending on the clinician's decision. Notably, rituximab was not administered to any of the KTRs in our study.

A detailed list of rejection episodes and treatment of rejection within the first year and beyond can be appreciated in Table 5. Importantly, no instances of rejection were observed within four months of T2 among the T1/T2 cohort.

Antimicrobial prophylaxis at T2 was uncommon. Only four individuals (5.6%) received valganciclovir and no KTR took trimethoprim/sulfamethoxazole (TMP/SMX) at T2.

Variable	**T1/T2 Cohort (n= 71)**
Age (years)	56 (48 - 64)
Female sex	24 (33.8)
Body-mass index (kg/m²)	26.4 (23.3 – 29.7)
Type 2 diabetes	16 (22.5)
Ethnicity	
Caucasian	67 (94.4)
Asian	1 (1.4)
Other	3 (4.2)
Dialysis prior KT	68 (95.8)
PD	13 (18.3)
HD	55 (77.5)
Dialysis vintage (months)	28 (20 - 43)
Renal disease	
Diabetic	13 (18.3)
Hypertensive	5 (7)
Glomerular	15 (21.1)
Polycystic kidney disease	12 (16.9)
Other/Unknown	26 (36.6)
Immunosuppression	
Induction (BX/ATG)	65/7 (91.5/9.9)
TAC	70 (98.6)
CyA	1 (1.4)
MMF/MPA	70 (98.6)
AZA	1 (1.4)
CS	71 (100)
PRAs > 0% prior KT	5 (7)
Anti-infectives at T2	
TMP/SMX	0 (0)
Valganciclovir	4 (5.6)
Rejection within 1st year of KT	13 (18.3)
Cellular/humoral Rejection	12/1 (92.3/7.7)
BANFF1B	2 (16.7)
BANFF2A	10 (83.3)

Table 4. Clinical and demographic data of T1/T2 Cohort. Continuous variables are shown as median (± IQR) and categorical variables as frequencies (%).

Subject-ID	Time from transplant	Type	Banff scores	DSA	Treatment
T1/T2 cohort					
N210	+ 218 days	Acute TCMR IIA	T3 i2 ti2 ptc2 v1 cv0 g0 cg0 mm0 ci1 ct1 ah0 C4d0	Borderline	ATG 10d, High dose-steroid
	+ 256 days	Acute TCMR IIA	T1 i0 ti0 v1 g0 cg0 ci1 ct1 cv0 cg0 mm0 ah0 ptc0 C4d0	Negative	ATG 8d, High-dose steroid
(>1 year post KT)	+ 1556 days	Chronic active TCMR + AMR	T3 i3 ti3 v3 cv3 g3 cg3 C4d2		TX-Nephrectomy
N232	+ 9 days	Acute TCMR IIA	T1 i1 ti1 ptc2 v1-2 cv0 g0 cg0 mm0 ci0 ct0 ah0 C4d0		ATG 10d, High-dose steroid
N237	+ 198 days	Acute TCMR IIA	T3 i2 ti2 ptc2 v1 cv0 g0 cg0 ci0 ct0 ah0 C4d0		ATG 10d, High-dose steroid
N245	+ 5 days	Acute TCMR IB	T3 i1 C4d0		High-dose steroid
N250	+ 8 days	Acute TCMR IB	T3 i2 ti2 ptc3 v0 cv0 g1 cg0 mm0 ci0 ct0 ah0 C4d0		High-dose steroid
N252	+ 8 days	Acute TCMR IIA	T3 i1 ti1 ptc1 v1 cv0 g0 cg0 ci1 ct1 aah2/3 ah0 C4d0		ATG 10d, High-dose steroid
N255	+ 114 days	Acute TCMR IIA	T1 ti1 i1 ptc2 v1 cv0 g1 cg0 ci0 ct0 ah0 C4d0	Negative	ATG 10d, High-dose steroid
(>1 year post KT)	+ 402 days	Transplant glomerulopathy	T0 i0 ptc0 b0 cv0 g1 cg3 mm0 ci1 ct1 ah1-2 c4d0	Negative	Steroid-dose increased
N259	+ 6 days	Acute TCMR IIA	T3 i1 ti1 ptc2 v1 cv0 g0 cg0 mm0 ci0 ct0 ah0 aah0 C4d0		ATG 10d, High-dose steroid
N261	+ 10 days	Acute TCMR IIA	T3 i1 ti1 ptc3 v1 cv0 g0 cg0 mm0 ci0 ct0 ah0 aah0 C4d0	Negative	ATG 10d, High-dose steroid
N281	+ 7 days	Acute TCMR IIA	T3 i2 ti1 ptc1 v1 cv0 g1 ci0 ct0 ah0 C4d0		ATG 7d, High-dose steroid
N287	+ 7 days	Acute ABMR	Ptc3 g1 v1 C4d3	Positive	ATG 14d, High-dose steroid, IA
N312	+ 17 days	Acute TCMR IIA	V1 t1 i0 ti0 C4d0		High-dose steroid

Table 5. Rejection episodes in subjects of the T1/T2 cohort within the first year and beyond (gray background). Banff scores were given according to the 2015 consensus (233). DSA are shown if available.

3.2. Leucocytes and major subpopulations at T1 and T2

Immune cell phenotyping in peripheral blood encompassed two critical time points: pre-KT (T1), reflecting the CKD G5 status, and one year post transplantation (T2).
We observed a substantial increase in total leucocyte counts among KTRs at T2 (4482 vs. 5658 x 10^3 cells/mL, p = 0.013, Figure 6A). This increase primarily stemmed from the expansion of monocytes and granulocytes (246 vs. 340 x 10^3 cells/mL, p = 0.003, Figure 6C, and 2960 vs 3818 x 10^3 cells/mL, Figure 6B, p = 0.002, respectively).
Of particular interest was the trend of decreasing BC numbers at T2, although this reduction did not reach statistical significance (67 vs. 55 x 10^3 cells/mL, p = 0.076, Figure 6E). In contrast, the total lymphocyte counts remained relatively stable between the two time points (948 vs. 1051 x 10^3 cells/mL, p = 0.984, Figure 6D).

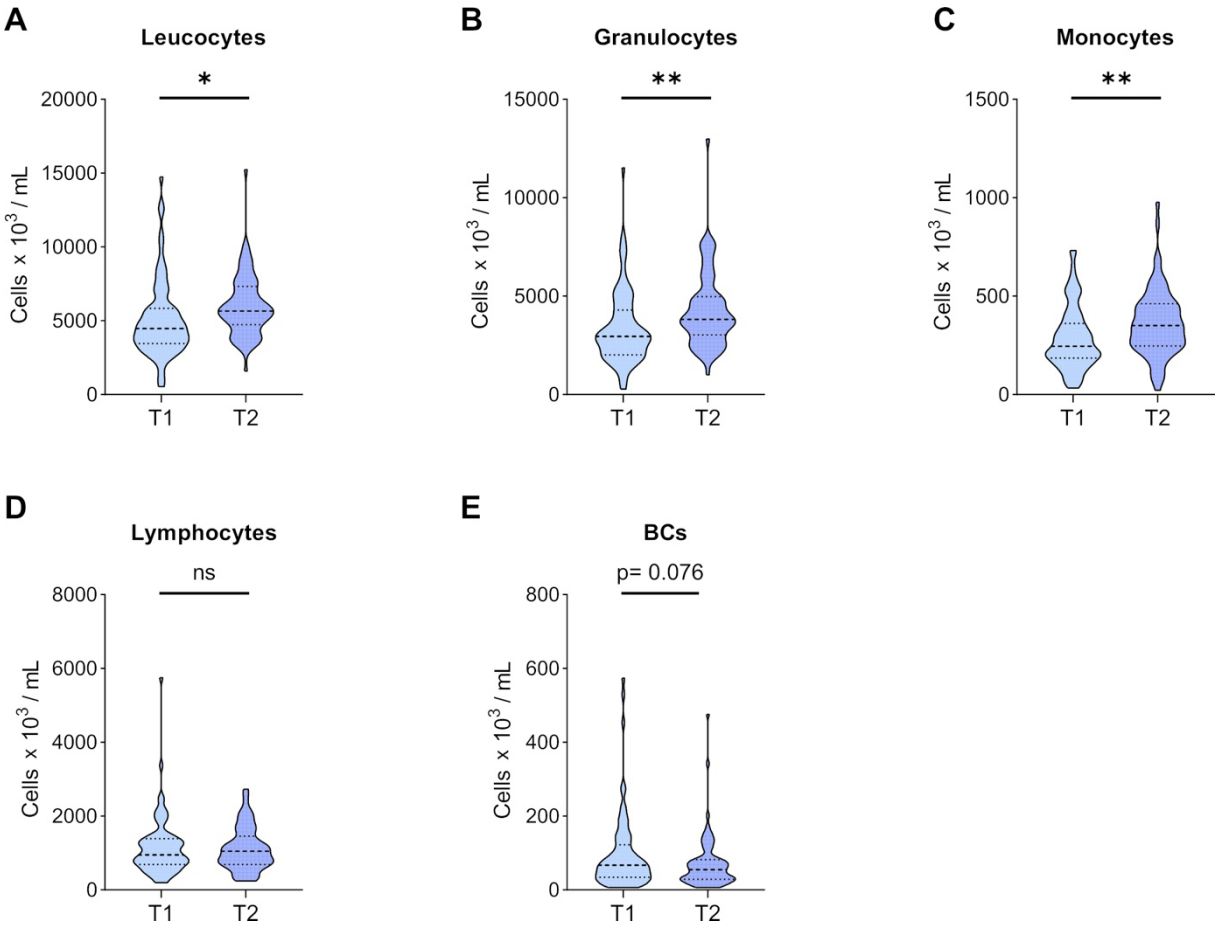

Figure 6. Dynamics of leucocytes and major subpopulation from T1 to T2. Counts are visualized using violin plots of (**A**) leucocytes, (**B**) granulocytes, (**C**) monocytes, (**D**) lymohocytes, and (**E**) BCs. Heavy dashed lines and light dashed lines indicate the median and IQR, respectively. Differences between the two timepoints were calculated using Wilcoxon signed-rank test.

3.3. B cell subpopulations at T1 and T2 as relative frequencies of $CD19^+$ B cells

BC subpopulations were tracked and shifts in relative BC frequencies are visually represented in Figure 7. Among peripheral BCs, naïve BCs made up the majority and were found at similar frequencies at both time points (66.9% vs. 68.2%, $p = 0.762$, Figure 7A). In contrast, TrBCs exhibited a highly significant reduction (1.92% vs. 0.37%, $p < 0.0001$, Figure 7B), while DN BCs showed a significant increase at T2 (10.7% vs. 14.4%, $p < 0.0001$, Figure 7C). The presence of PBs, although already at low frequencies during T1, experienced further

reduction after one year (0.73% vs. 0.13%, $p < 0.0001$, Figure 7D). In the mBC category, only IgM-only mBCs displayed a significant change over one year (3% vs. 1.82%, $p = 0.0005$, Figure 7F), while class-switched mBCs exhibited a non-significant trend towards lower frequencies at T2 (11.7 vs. 9.5%, $p = 0.063$, Figure 7G). Unswitched and IgD-only mBCs remained stable (2.39% vs. 2.31%, $p = 0.575$, Figure 7H and 0.47% vs. 0.51%, $p = 0.277$, Figure 7E, respectively).

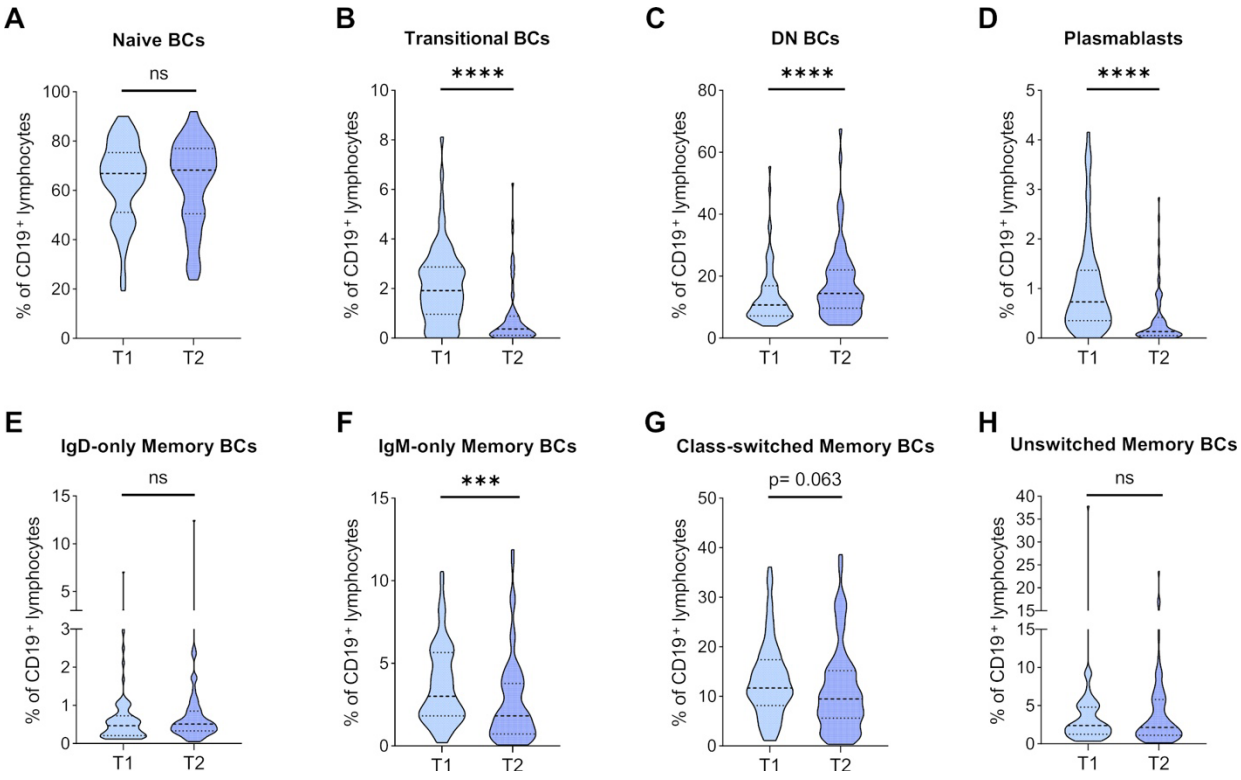

Figure 7. Relative frequencies of BC subpopulation before (T1) and one year after transplantation (T2) as violin plots. Frequencies (**A**) naïve BCs, (**B**) TrBcs, (**C**) DN BCs, (**D**) PBs, (**E**) IgD-only mBCs, (**F**) IgM-only mBCs, (**G**) class-switched mBCs, and (**H**) unswitched mBC are shown. The heavy dashed lines represent the median, while the light dashed lines indicate the IQR. Variances between the two timepoints were assessed using the Wilcoxon signed-rank test.

3.4. B cell subpopulations at T1 and T2 in absolute numbers

BC dynamics in terms of absolute numbers largely mirrored results derived from relative frequencies. In absolute values, naïve BCs displayed a trend towards reduction after the first year of KT (48.57 vs. 35.31 x 10^3 cells/mL, p = 0.077, Figure 8A). TrBC and PB counts were significantly lower at T2 (1.48 vs. 0.19 x 10^3 cells/mL, p < 0.0001, Figure 8B, and 0.30 vs. 0.08 x 10^3 cells/mL, p < 0.0001, Figure 8D, respectively). In contrast to the significant increase in DN BCs concerning relative frequencies, their absolute numbers remained similar at both time points (7.16 vs. 6.29 x 10^3 cells/mL, p = 0.722, Figure 8C). The quantification of mBC counts yielded results consistent with relative mBC frequencies. Specifically, post-transplantational IgM-only mBCs (2.27 vs. 0.9 x 10^3 cells/mL, p < 0.0001, Figure 8F) and class-switched mBCs (6.47 vs. 4.11 x 10^3 cells/mL, p = 0.004, Figure 8G) were significantly reduced, while IgD-only (0.25 vs. 0.26 x 10^3 cells/mL, p = 0.735, Figure 8E) and unswitched mBCs (1.84 vs. 1.26 x 10^3 cells/mL, p = 0.496, Figure 8H) exhibited relatively stable counts.

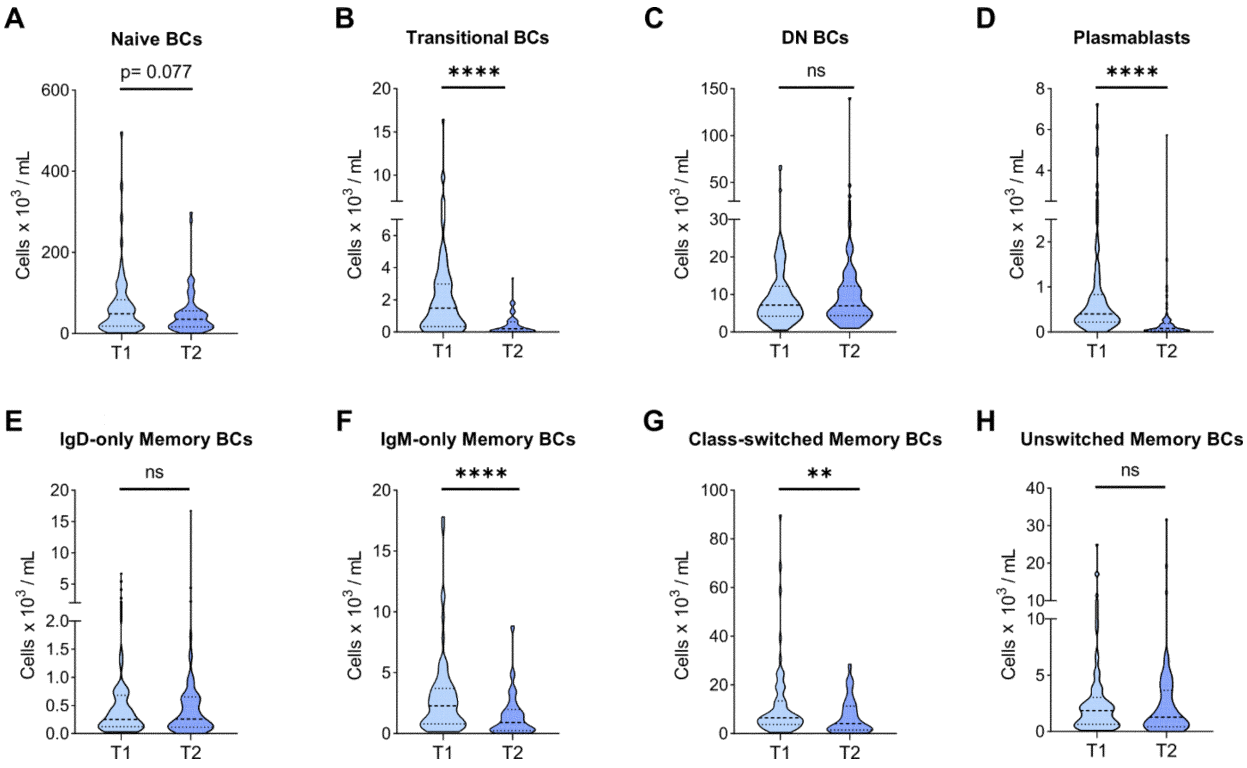

Figure 8. Absolute numbers of BC subpopulation before (T1) and one year after transplantation (T2). The absolute numbers were derived from total BC counts and the relative frequencies of BC subpopulations for 71 KTRs, both pre- (T1) and post-kidney transplantation (T2). Violin plots depict the distribution of (**A**) naïve BCs, (**B**) TrBCs, (**C**) DN

BCs, (**D**) PBs, (**E**) IgD-only mBCs, (**F**) IgM-only mBCs, (**G**) class-switched mBCs, and (**H**) unswitched mBCs per milliliter. The heavy dashed lines within the plots illustrate the median values, while the light dashed lines outline the IQR. Statistical analysis involved the Wilcoxon signed-rank test to determine variations between T1 and T2.

3.5. Activated $CD86^+$ BCs and Tolerogenic $CD27^-CD5^+$ BCs

Next, we examined if the expression of CD86, an activation marker on BCs, is influenced by post-transplant immunosuppressive treatment, as compared to the T1 stage that reflects CKD G5. We found a substantial decrease in the frequency of $CD86^+$ B cells at T2 compared to T1 (3.45% vs. 2.26%, $p = 0.011$, Figure 9A). This decline was also reflected by a reduction in the absolute numbers of $CD86^+$ B cells (2.00 vs. 1.01 x 10^3 cells/mL, $p < 0.0001$, Figure 9B).

In recent years, $CD5^+$ B cells have garnered attention in the context of transplantation and organ tolerance. Our findings demonstrated a substantial decrease in both numbers (0.36 vs. 0.16 x 10^3 cells/mL, $p < 0.0001$, Figure 9D) and frequencies (0.48% vs. 0.29%, $p < 0.0001$, Figure 9C) of $CD27^-CD5^+$ BCs after KT.

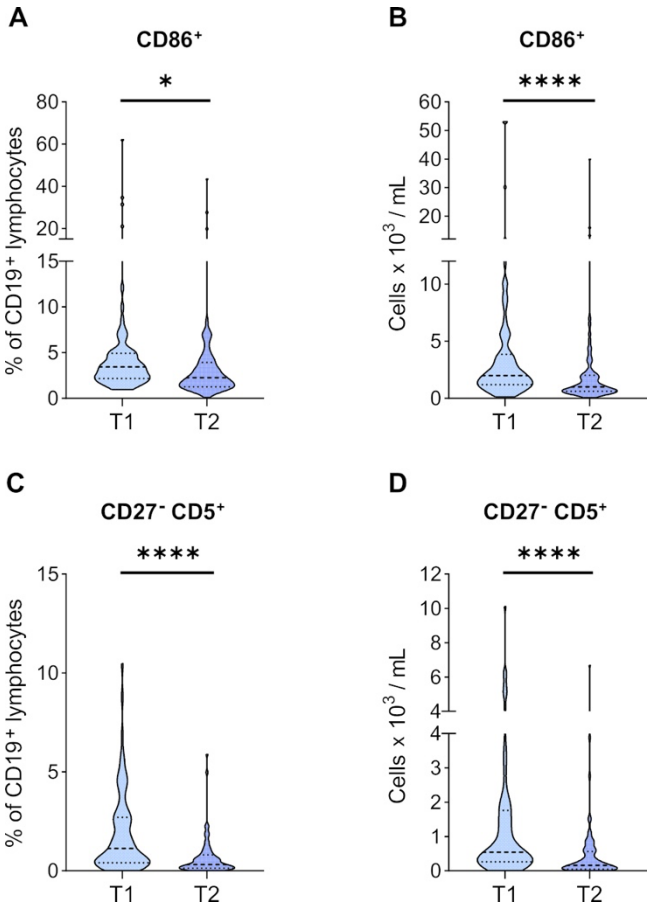

Figure 9. Relative frequencies and absolute numbers of activated $CD86^+$ BCs (**A** and **B**) and $CD27^-CD5^+$ BCs with an ostensibly tolerogenic phenotype (**C** and **D**). Heavy dashed lines indicate the medians, while light dashed lines represent the IQR in violin plots. The differences between T1 and T2 were evaluated using the Wilcoxon-signed rank test.

3.6. Impact of antimetabolites on B cells at T2

IMPDH inhibitors like MMF and MPA are integral components of long-term immunosuppression in KTRs. Consequently, MMF/MPA was highly prevalent within our cohort (91.5% at T2). However, the dosages administered varied among patients based on considerations of individual tolerability and the presence of infectious complications. Median daily MPA dosage was 1080mg/day with an IQR of 810mg/day.

IMPDH is universally expressed across all lymphocytes, thus its inhibition has an impact on both TCs and BCs (89). Consequently, our investigation sought to discern whether

MMF/MPA dosage has an influence on the absolute and relative counts of B cells one year after KT.

Performing rank-based correlations, we found several correlations between MMF/MPA dosage and BC subpopulations. Specifically, the frequencies of naive BCs exhibited a positive correlation, while the frequencies of DN BCs and PBs exhibited negative correlations with MMF/MPA dosage (Figure 10A). In absolute terms, only PB counts displayed a negative correlation with the MMF/MPA dose (Figure 10B).

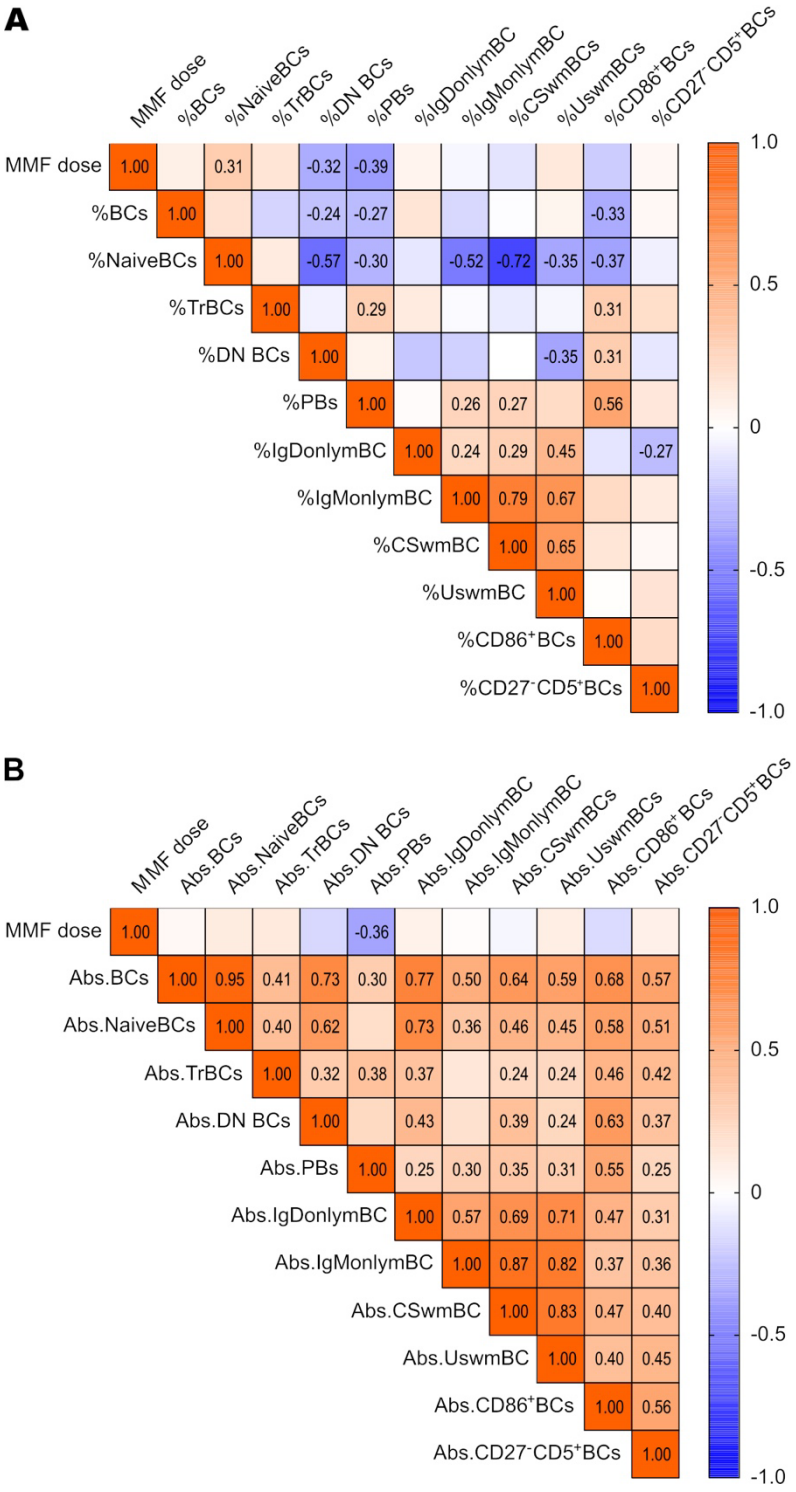

Figure 10. Spearman correlation matrix of MMF/MPA dose with BCs and BC subsets as relative frequencies (**A**) and absolute numbers (**B**). Significant correlations are indicated by numbers.

3.7. Vaccination subcohort

Next, we were interested if the changes in BCs and BC subsets after transplantation could be used as potential biomarkers for vaccination response in KTRs. Therefore, we conducted a retrospective analysis of anti-SARS-CoV-2 antibody testing data within our study population in KTRs who had received two doses of an mRNA-based vaccine (mRNA-1273 or BNT162b2), administered four weeks apart. Out of 71 KTRs in the T1/T2 cohort, we had to exclude five due to graft loss, two due to death, six were lost to follow-up, seven had not been vaccinated, four experienced COVID-19, five did not have antibody testing data and two were vaccinated before T2 (Figure 5). Finally, we arrived at a subcohort of 40 individuals with a functioning graft at T3, two mRNA-based vaccinations and anti-SARS-CoV-2 testing data. Demographic and clinical characteristics at T1 of the vaccination subcohort were overlapping with the overall T1/T2 cohort (Table 6). A notable exception is the comparatively lower rate of rejections within the first year compared to the T1/T2 cohort (5% vs. 18.3%) underlining the detrimental impact of rejection episodes for long-term graft survival.

Variable	**Vaccination Subcohort (n= 40)**
Age (years)	59.5 (49.5 - 64)
Female sex	13 (32.5)
Body-mass index (kg/m²)	27 (25.2 - 31)
Type 2 diabetes	12 (30)
Ethnicity	
Caucasian	37 (92.5)
Asian	1 (2.5)
Other	2 (5)
Dialysis prior KT	38 (95)
PD	9 (23.7)
HD	29 (76.3)
Dialysis vintage (months)	28 (20 - 42.8)
Kidney disease	
Diabetic	10 (25)
Hypertensive	3 (7.5)
Glomerular	7 (17.5)
Polycystic kidney disease	7 (17.5)
Other/Unknown	13 (32.5)
Immunosuppression	
Induction (BX/ATG)	36/5 (90/12.5)
TAC	40 (100)
MMF/MPA	40 (100)
CS	40 (100)
Rejection within 1st year of KT	2 (5)
Cellular/humoral Rejection	2/0 (100/0)
BANFF2A	2 (100)

Table 6. Characteristics of Vaccination Subcohort at T1. Data are shown as median ± IQR and categorical variables as frequency (%).

3.8. Leucocyte and B cell subpopulations at T1 and T2 in vaccination subcohort

To explore whether leucocytes and BC subsets followed similar kinetics as in the T1/T2 cohort, we examined the dynamics of these cell types separately for the vaccination subcohort.

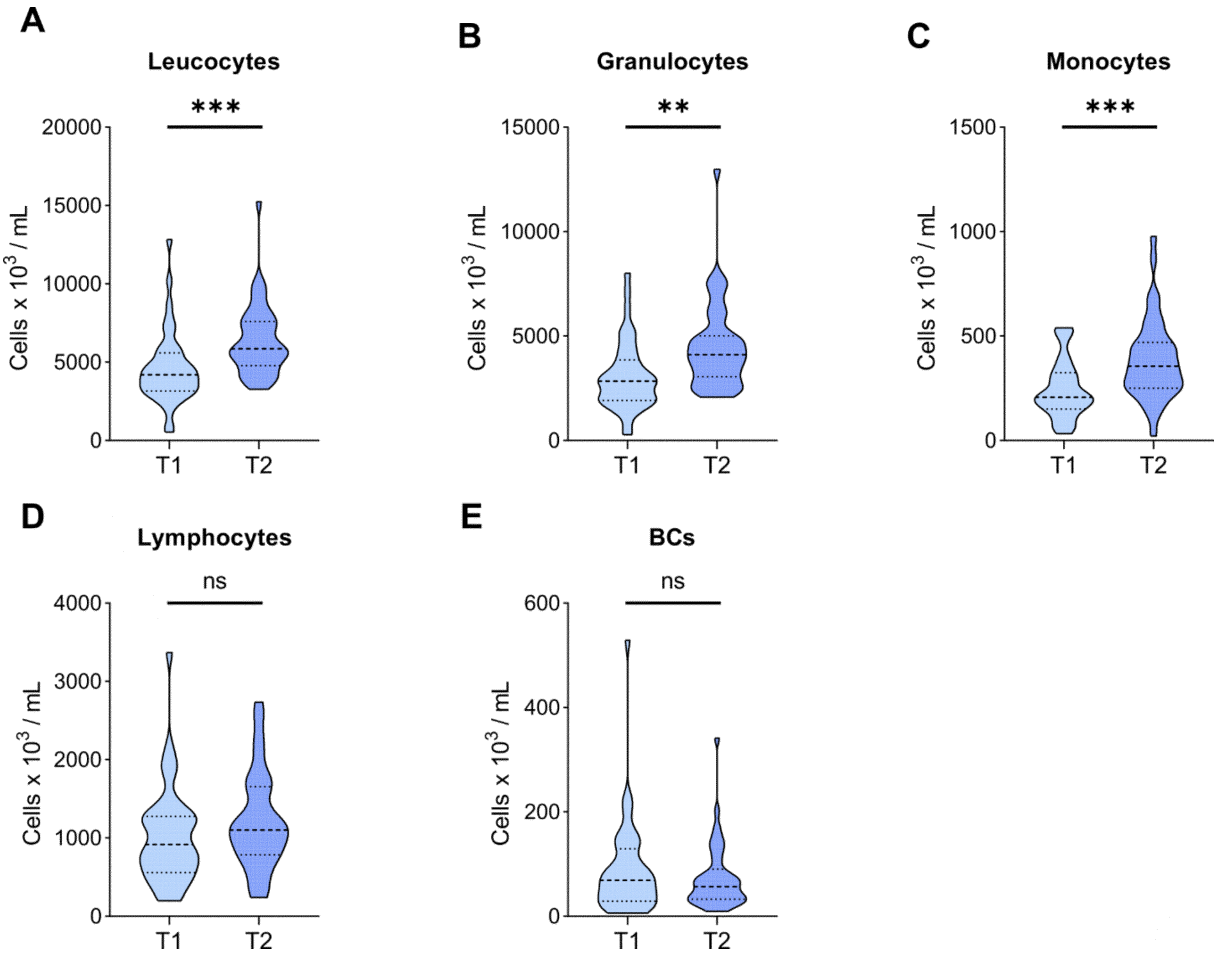

Figure 11. The changes in leucocytes and their major subpopulations between T1 and T2 were analyzed in the vaccination subcohort. Violin plots display the counts of (**A**) leucocytes, (**B**) granulocytes, (**C**) monocytes, (**D**) lymphocytes, and (**E**) BCs. The median and IQR are marked by heavy dashed lines and light dashed lines, respectively. The two timepoints were compared using the Wilcoxon signed-rank test.

Similar to the T1/T2 cohort, individuals with available vaccination data displayed an increase in leucocytes (4185 vs. 5862 x 10^3 cells/mL, p= 0.001, Figure 11A) granulocytes (2843 vs. 4105 x 10^3 cells/mL, p= 0.011, Figure 11B) and monocytes (206 vs. 356 x 10^3 cells/mL, p= 0.0001, Figure 11C) from T1 to T2. In this subcohort, lymphocytes also remained stable (915 vs. 1103 x 10^3 cells/mL, p= 0.136, Figure 11D) and the trend for BC reduction observed in the T1/T2 cohort was not appreciable (65 vs. 57 x 10^3 cells/mL, p = 0.685, Figure 11C).

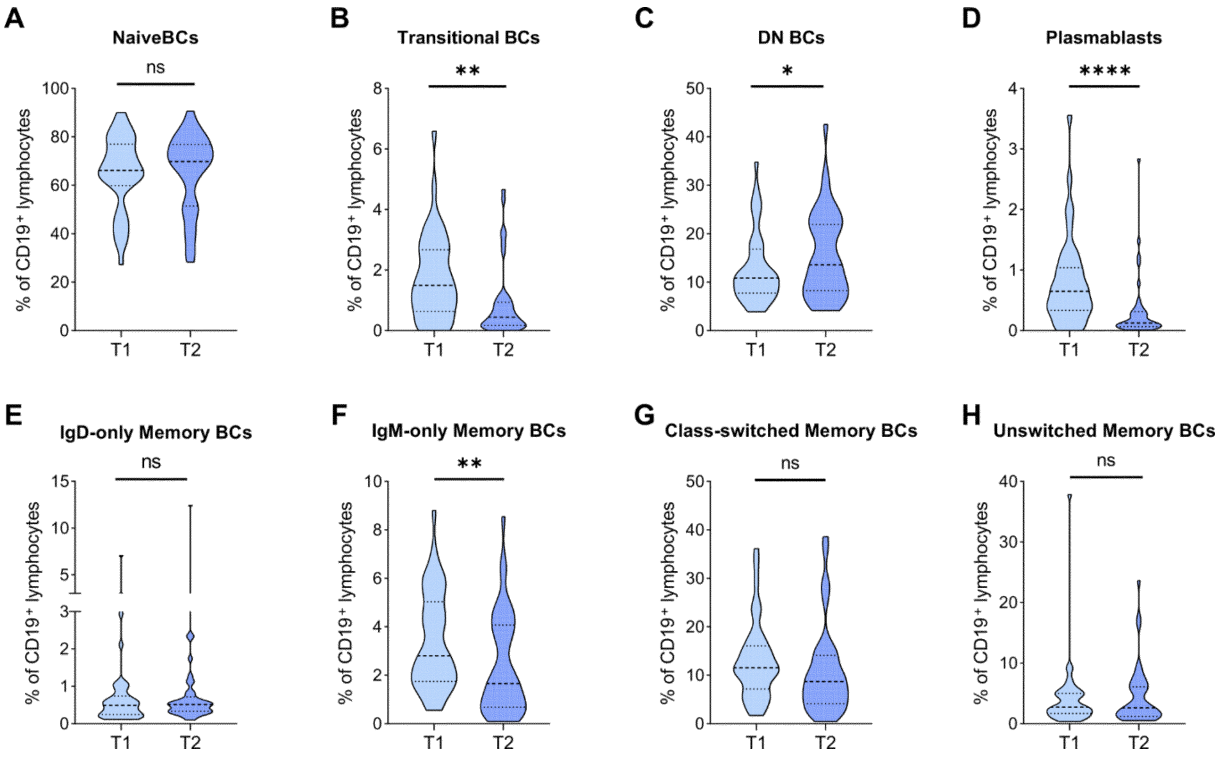

Figure 12. Visual representations of the relative frequencies of various B cell subpopulations before (T1) and one year after transplantation (T2) are depicted using violin plots in the vaccination subcohort. Distributions of (**A**) naïve BCs, (**B**) TrBcs, (**C**) DN BCs, (**D**) PBs, (**E**) IgD-only mBCs, (**F**) IgM-only mBCs, (**G**) class-switched mBCs, and (**H**) unswitched mBC are showcased. Median values are denoted by heavy dashed lines, while the IQR is indicated by light dashed lines. Wilcoxon signed-rank test was used to assess for potential differences between T1 and T2.

Relative BC frequencies show comparable dynamics in the vaccination subcohort. Briefly, naïve BCs remained at similar frequencies (66.2% vs. 69.8%, p = 0.819, Figure 12A), whereas TrBCs and PBs were diminished at T2 (1.5% vs. 0.45%, p= 0.002, Figure 12B and 0.65% vs. 0.13%, p <0.0001, Figure 12D, respectively). DN BCs increase in frequency at T2 (10.9% vs. 13.6%, p= 0.029, Figure 12C). IgM-only mBCs remained significantly altered in this subcohort (2.8% vs. 1.65%, p = 0.006, Figure 12F). Class-switched mBCs, unswitched and IgD-only mBCs remained stable (11.5% vs. 8.67%, p = 0.142, Figure 12G; 2.71% vs. 2.62%, p = 0.656, Figure 12H and 0.49% vs. 0.51%, p = 0.493, Figure 12E, respectively).

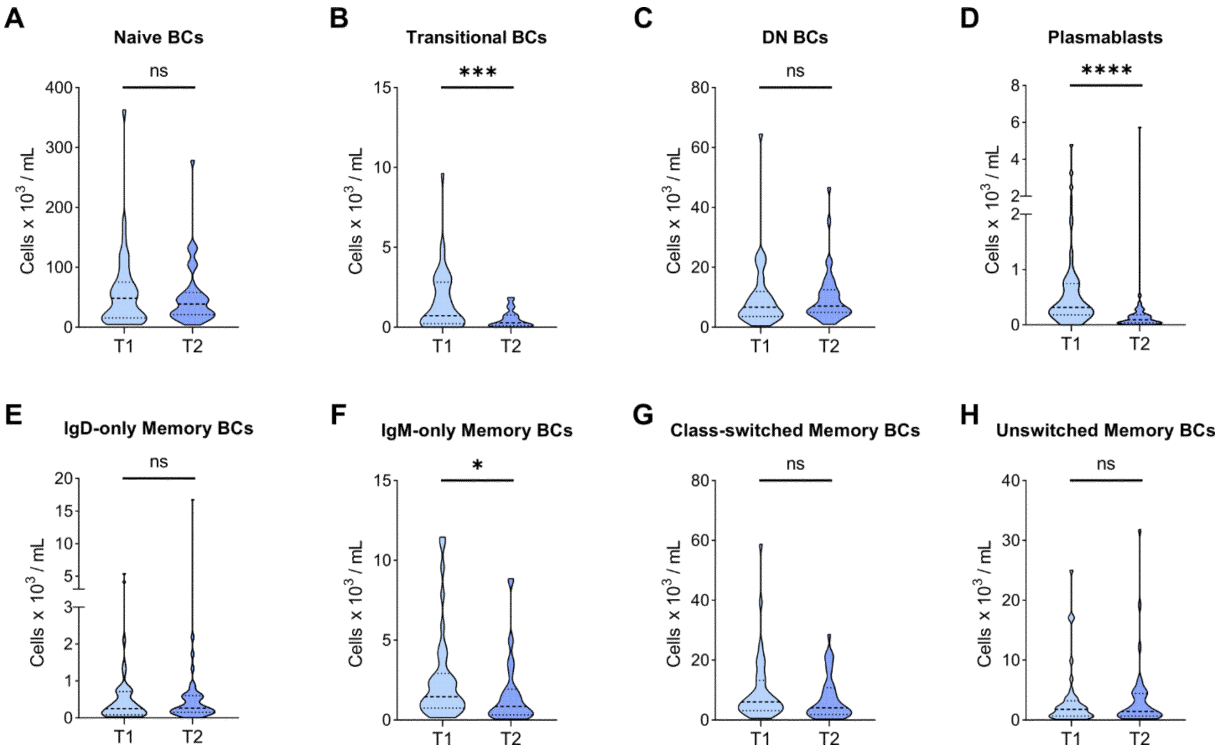

Figure 13. BC subpopulations from T1 to T2 are displayed in absolute counts in the vaccination subcohort. Calculated from from total BC counts and relative frequencies of BC subpopulations the violin plots demonstrate the distribution of (**A**) naïve BCs, (**B**) TrBCs, (**C**) DN BCs, (**D**) PBs, (**E**) IgD-only mBCs, (**F**) IgM-only mBCs, (**G**) class-switched mBCs, and (**H**) unswitched mBCs per milliliter. Heavy and light dashed lines represent median values and IQR, respectively. The statistical comparison between T1 and T2 was conducted using Wilcoxon signed-rank test.

Absolute numbers of BC subsets in vaccination subcohort correlated well with relative frequencies and the overall T1/T2 cohort. As an exception, naïve BCs did not show any tendency in the vaccination subcohort (48.63 vs. 38.61 x 10^3 cells/mL, p = 0.952, Figure 13A), but TrBC and PB counts were similarly lowered at T2 (0.72 vs. 0.27 x 10^3 cells/mL, p = 0.0002, Figure 13B, and 0.32 vs. 0.09 x 10^3 cells/mL, p < 0.0001, Figure 13D, respectively). No change was observed in DN BC numbers (6.72 vs. 7.0 x 10^3 cells/mL, p = 0.519, Figure 13C). IgM-only mBCs (1.46 vs. 0.84 x 10^3 cells/mL, p = 0.020, Figure 13F) were the only mBC subset with reduced numbers at T2. Contrary to observations in the T1/T2 cohort, class-switched mBCs (6.01 vs. 4.02 x 10^3 cells/mL, p = 0.166, Figure 13G) were not significantly

reduced in the vaccination subcohort. IgD-only mBCs (0.25 vs. 0.26 x 10^3 cells/mL, p = 0.237, Figure 13E) and unswitched mBCs (1.78 vs. 1.40 x 10^3 cells/mL, p = 0.656, Figure 13H) showed similar abundance at both timepoints and in T1/T2 cohort and vaccination subcohort.

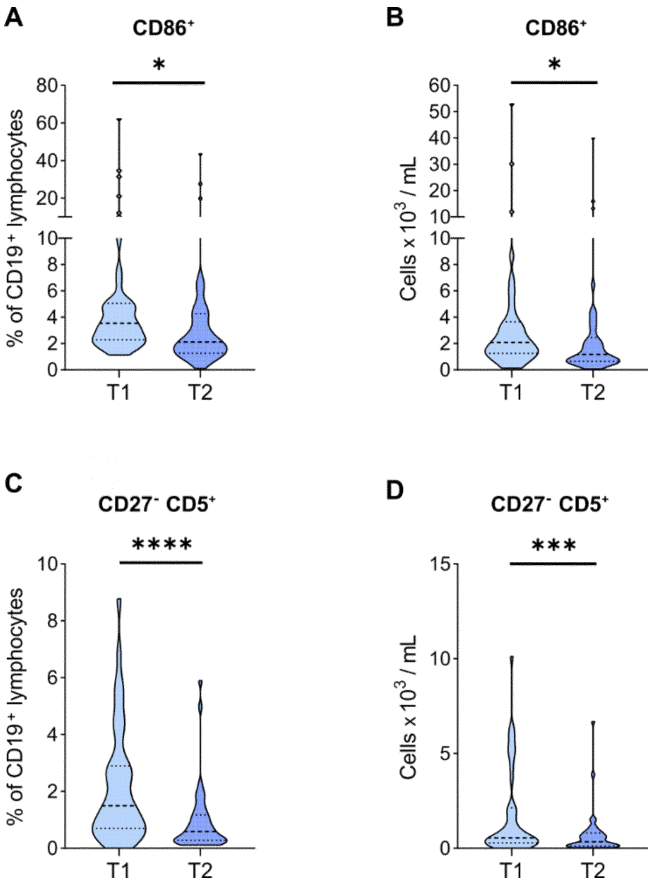

Figure 14. Activated $CD86^+$ BCs (**A** in absolute numbers and **B** in frequencies) and tolerogenic $CD27^-CD5^+$ BCs (**C** in absolute numbers and **D** in frequencies) are shown at T1 and T2. Medians and IQR are depicted as heavy and ligh dashed lines, respectively. Potential differences between timepoints were assessed using the Wilcoxon-signed rank test.

Dynamics of activated $CD86^+$ BCs and $CD27^-CD5^+$ BCs with regards to relative frequencies and absolute numbers mirrored findings from the T1/T2 cohort. $CD86^+$ BCs were significantly lower at T1 compared to T2 (3.55% vs. 2.12%, p = 0.027, Figure 14A and 2.21 vs. 1.16 x 10^3 cells/mL, p = 0.019, Figure 14B, respectively).

Similarly, $CD27^{-}CD5^{+}$ BCs were reduced at T2 (0.36 vs. 0.22 x 10^3 cells/mL, p = 0.0003, Figure 14D and 0.7% vs. 0.36%, p < 0.0001, Figure 14C, respectively).

Overall, these findings show that the vaccination subcohort displayed comparable numbers and frequencies of leucocytes, BCs and BC subsets as the T1/T2 cohort and are representative of our whole study population.

3.9. Anti-SARS-CoV-2 antibody testing and responder versus non-responder

Data on the vaccination subcohort at T3 is summarized in Table 4. mRNA-1273 was given to 67.5% and 32.5% received BNT162b2. Of note, only homologous vaccination strategies were followed, and doses were administered four weeks apart. We could not control for the interval between T2 and first vaccination as it was dependent on the timing of KT. The median interval between T2 and the first vaccination was 106.5 weeks. Furthermore, the interval between second vaccination and T3 was variable, and the median was 15 weeks. The extended timeline is visualized in Figure 4.
Antibody testing was performed on different platforms and results were converted to BAU/mL as recommended by the WHO (234). The majority was tested with Roche Elecsys (60%) and DiaSorin Trimeric S (30%). Two patients were tested with Siemens Healthineers and two with Abbott Alinity (5% each). We observed very low antibody titers and decided to dichotomize patients into responders and non-responders depending on the detectability of anti-SARS-CoV-2 antibodies. Half of the vaccination subcohort could be classified as responders with a median of 21.75 BAU/mL, while the other 50% did not exhibit any detectable antibodies.

Next, we were interested if there are any differences between responders and non-responders concerning clinical characteristics, immunosuppressive medication or kidney function at T3 (Table 7). Interestingly, we found that age, female sex, BMI and the proportion of diabetics were comparable between both groups, although these are generally considered to influence vaccination response. Responders did not differ from non-responders with regards to kidney function as assessed by creatinine and creatinine-based eGFR and proteinuria assessed by urinary albumin to creatinine ratio (U-ACR) and protein to creatinine ratio (U-PCR). Furthermore, immunosuppressive regimen was similar. Considering the impact of MMF/MPA on circulating BCs, we categorized MMF/MPA dosage into two variables: Below or above 1g/d. Still, we found no apparent influence of MMF/MPA dosing on vaccination response.
Notably, the mRNA-1273 vaccine was more frequently administered to responders (80% vs. 55%), while non-responders received proportionally more BNT162b2 (45% vs. 20% in responders).

There was no discernable difference in the timing of T2 and T3 relative to vaccination between responders and non-responders. Although there was a trend indicating a longer duration of dialysis vintage prior to T1 among responders (31.5 vs. 26 months), both preemptively transplanted individuals were part of the non-responder group.

Variable	**Vaccination Subcohort (n= 40)**	**Responder (n= 20)**	**Non-Responder (n= 20)**	**p-Value**
Age (years)	64 (54 – 68)[a]	65 (57 – 67)[a]	63 (52 – 68)[a]	0.588
Female Sex	13 (32.5)	6 (30)	7 (35)	1.000
Body-mass index (kg/m^2)	27.8 (24.8 – 29.6)	27.9 (26.2 – 29.7)[b]	27.6 (24.5 – 30.9)[b]	0.860
Type 2 diabetes	12 (30)	5 (25)	7 (35)	0.731
Dialysis prior KT	38 (95)	20 (100)	18 (90)	0.487
PD	9 (23.7)	6 (30)	3 (16.7)	0.451
HD	29 (76.3)	14 (70)	15 (83.3)	1.000
Dialysis vintage (months)	28 (20 – 42.8)	31.5 (21 – 52.75)	26 (15.25 – 40.5)	0.102
Kidney disease				
Diabetic	10 (25)	4 (20)	6 (30)	0.716
Hypertensive	3 (7.5)	1 (5)	2 (10)	1.000
Glomerular	7 (17.5)	5 (25)	2 (10)	0.407
Polycystic kidney disease	7 (17.5)	5 (25)	2 (10)	0.407
Other/Unknown	13 (32.5)	5 (25)	8 (40)	0.501
Rejection	3 (7.5)	0	3 (15)	0.487
eGFR (mL/min/1,73m^2)	47.5 (36.6 – 58.7)	49.3 (39.5 – 63.9)	46,8 (36.3 – 54.9)	0.301
Creatinine (mg/dL)	1.44 (1.18 – 1.76)	1.42 (1.11 – 1.78)	1.47 (1.2 – 1.73)	0.602
U-ACR (mg/g)	26.5 (7.3 – 45.5)[c]	28 (14 – 98)[c]	20 (4 – 43)[c]	0.583
U-PCR (mg/g)	107 (77 – 137)[d]	120.5 (78.5 – 161)[d]	93 (77 – 130)[d]	0.298
Immunosuppression				
TAC	39 (97.5)	20 (100)	19 (95)	1.000
EVE	3 (7.5)	2 (10)	1 (5)	1.000
MMF/MPA	36 (90)	17 (85)	19 (95)	0.605
< 1g/d	17 (47.2)	8 (47.1)	9 (47.4)	1.000
> 1g/d	19 (52.8)	9 (52.9)	10 (52.6)	1.000
CS	40 (100)	20 (100)	20 (100)	NA
Vaccination				
mRNA-1273/ BNT162b2	27/13 (67.5/32.5)	16/4 (80/20)	11/9 (55/45)	0.176
Interval 2nd dose – T3 (weeks)	15 (9 - 22)[e]	17 (10.3 - 22)[e]	12 (7.5 – 20.8)[e]	0.336
Interval T2 – 1st dose (weeks)	106.5 (85.5 - 151.5)[f]	94 (81 - 148)[f]	132 (85.5 – 159.5)[f]	0.303

Antibody testing				
Anti-SARS-CoV-2 antibody level (BAU/mL)	0.59 (0 - 21.78)	21.75 (5.93 - 83.22)	0	<0.001
LIAISON TrimericS IgG Assay	12 (30)	6 (30)	6 (30)	1.000
Elecsys Anti-SARS-CoV-2S	24 (60)	12 (60)	12 (60)	1.000
Alinity I SARS-CoV-2 IgG Quant	2 (5)	1 (5)	1 (5)	1.000
Atellica IM COV2G	2 (5)	1 (5)	1 (5)	1.000

Table 7. Overview of the vaccination subcohort and comparison between responders and non-responders to SARS-CoV-2 vaccination. Clinical and laboratory data at T3 are summarized. Data are reported as median ± IQR and as frequency (%), respectively. NA, not applicable.
Missing values: [a] 39/19/20 values; [b] 31/14/17 values; [c] 38/19/19 values; [d] 37/18/19 values; [e] 39/19/20 values; [f] 38/20/18 values.

3.10. Correlations between antibody titer and B cell subsets at T2

Finally, we aimed to correlate anti-SARS-CoV-2 antibody levels with absolute numbers and relative frequencies of BCs at T2. TrBCs (in % of $CD19^+$ lymphocytes) showed a modest positive correlation with antibody levels of (rs = 0.4, Figure 15A). Likewise, absolute TrBC counts were correlated positively with antibody titers (rs = 0.42, Figure 15C). No other association between antibody levels and BC subsets were observable.

Next, we compared TrBCs at T2 between responders and non-responders. At T2, individuals who successfully mounted a humoral response to vaccination exhibited significantly higher absolute TrBC counts (0.61 vs. 0.15 x 10^3 cells/ml, p= 0.011, Figure 15D) and a notably increased relative TrBC frequency (0.81% vs. 0.23%, p = 0.012, Figure 15B) compared to non-responders.

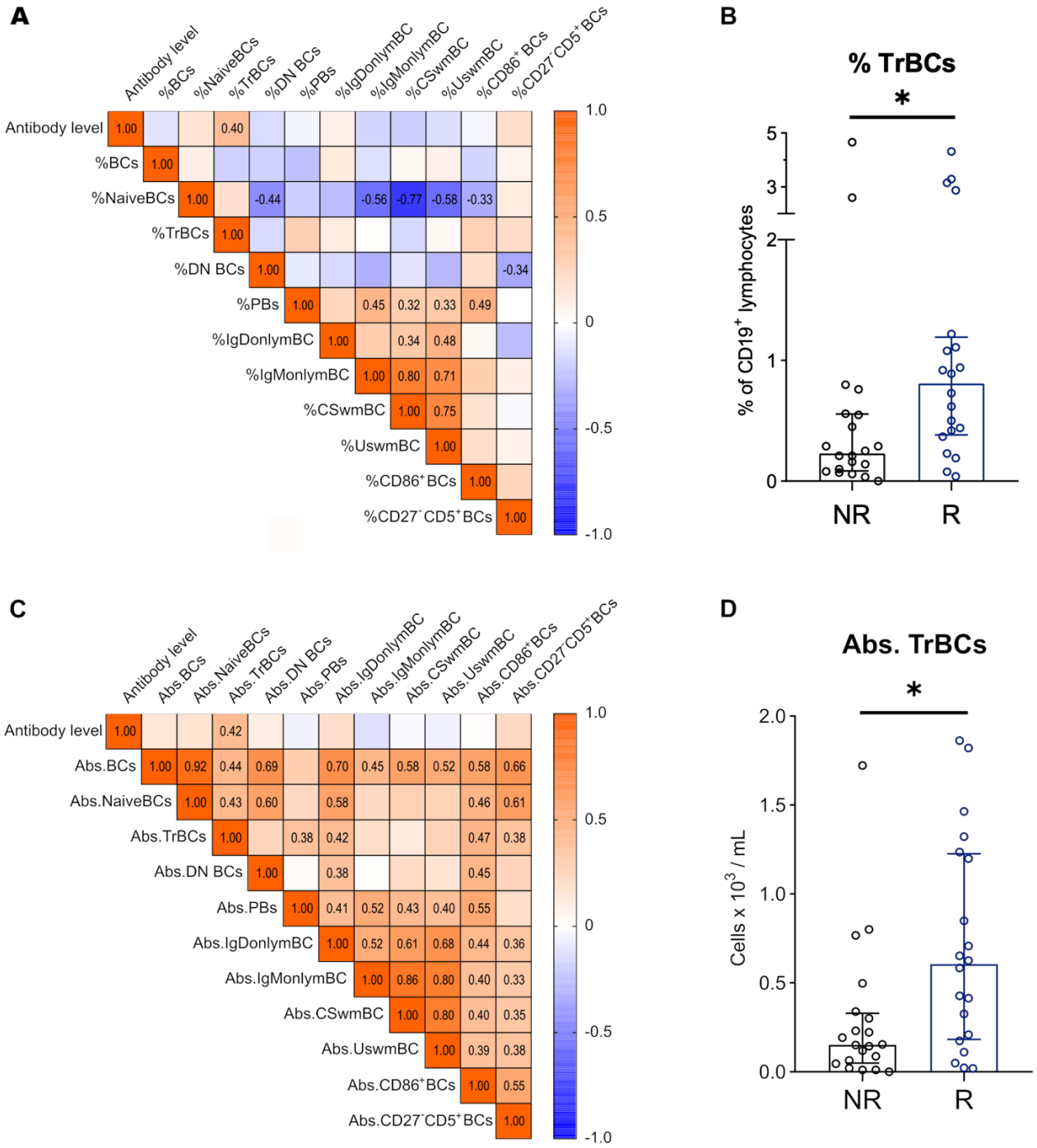

Figure 15. Correlation matrix of anti-SARS-CoV2 antibody levels and BC subpopulations at T2 (**A** and **C**). Significant correlations are indicated by numbers. Comparison of relative TrBC frequency (**B**) and absolute TrBC numbers (**D**) between non-responders (NR) and responders (R). Differences between NR and R were assessed using Mann-Whitney U tests.

4. Discussion

Historically, the focus of transplant immunology has primarily centered around TCs, recognizing their pivotal role in graft rejection. Yet, the significance of BCs in KT has garnered increasing attention in recent years. The role of BCs has been often reduced to their ability to produce antibodies and mediate humoral anti-allograft immunity, but recent observations have led to more nuanced perspective on the influence of BCs on both allograft acceptance and rejection. Simultaneously, BCs are critically implicated in antimicrobial defense and response to vaccination while infections remain a major contributor to recipient morbidity and mortality (235-237). Our findings provide insight into the dynamics of the blood BC compartment in KTRs and its implications for immune responses to SARS-CoV-2 vaccination. Furthermore, different immunological steady states are reflected by the two timepoints. In the same individual, T1 represents ESKD, while T2 marks KT after one year.

Studying the peripheral blood is attractive because it is easy to access and minimally invasive. Previous studies have also investigated kinetics of peripheral BCs in KTRs, and results are summarized in table 8.

Longitudinal studies on B cell dynamics in kidney transplant recipients			
First Author, Publication date	Study Design	Antibodies	Findings
Ishida et al., 2006 (238)	10 ABO incompatible KTRs, 5 with stable function vs. 5 with graft dysfunction function and rejection Flow cyt.: Before, + 1 wk., +1 mo.	CD19, CD5	"B1" ($CD19^+CD5^+$): ↑ in both groups at +1mo. "B2" ($CD19^+CD5^-$): ↑ only in rejection group
Van de Berg et al., 2012 (239)	65 KTRs (rejection, chronic allograft dysfunction, stable) Flow cyt.: +6mo., +2yrs, +5yrs compared to 15 HC	CD27, IgD, CD25, CD19, CD20	- KTRs: ↓ BCs after KT compared to HC - More differentiated BC pool (Naïve ($CD27^-IgD^+$) ↓, $CD27^+$ ↑) - No difference between stable and rejection
Legris et al. 2013 (240)	307 KTRs: ATG induction, - MMF/TAC (n= 151) - AZA/CyA (n= 156) Flow cyt.: Before, +15days, +12mo 96 HCs	CD19	BCs: initially ↑ then ↓ (at +12mo) MMF/TAC: ↑ BCs at +15days than AZA/CyA, then ⇔ BCs ↑ in HCs than KTRs (at all timepoints)
Chung et al., 2014 (241)	21 (stable) KTRs Flow cyt. + *ex vivo* stimulation (TAC and MMF): before and +1 mo.	CD19, CD38, CD24	mBCs ($CD24^+CD38^-$): ⇔ Mature BCs ($CD24^+CD38^{int}$): ⇔ Immature BCs ($CD24^+CD38^+$): ↓ TAC: suppressed proliferation of immature BCs *ex vivo*
Kamburova et al, 2014 (242)	RCT, 26 KTRs: (CNI+MMF+CS), + Rituximab (n= 14) - Rituximab (n= 12) Flow cyt.: Before, +3 mo., +6 mo., +12 mo., +24 mo	CD3, CD19, CD24 Bm classification (IgD, CD38)	- Rituximab: BCs ⇔ Bm1: ↑ Bm2: ↓ (+12 mo., +24mo.) Bm3+4: initally ↓ then ⇔ + Rituximab: All BCs ↓↓ until +12mo., TrBCs first to repopulate.
Longshan et al., 2014 (243)	16 KTRs (BX induction) Flow cyt.: Before, +1day, +3days, +7days, +14days; +1mo, +3mo, +6mo	CD19, CD5, CD27 Bm-classification (IgD, CD38)	BCs: initially ↑ then ↓ (at +6mo) $CD5^+$ BCs: ↓ mBCs ($CD27^+$): ↑ Bm1: ⇔ Bm2: ↓ Bm3: ↓ Bm4: ↓ Bm5: initially ↑ then ⇔
Heidt et al., 2015 (244)	21 KTRs with acute rejection vs. 22 stable KTRs Flow cyt.: before, at discharge, at time of rejection (or corresponding time point for control)	CD3, CD19, CD24, IgD, CD5, CD27, CD1d, CD38	% BCs: initially ↓ then ⇔ TrBCs ($CD24^{++}CD38^{++}$): ↓ (permanently) B10 ($CD19^+CD27^+CD24^{hi}$): ↑ at discharge then ⇔ Similar kinetics between groups
Shabir et al., 2015 (245)	Prospective, 73 KTRs: Flow cyt.: Before, +14days, +3mo., 6mo., +9mo., +12mo.	CD19, CD24, CD38, CD27	TrBCs ($CD24^{++}CD38^{++}$): ↓ TrBCs correlate with protection from rejection
Svachova et al., 2016 (246)	Prospective, 98 KTRs Protocol biopsy: +3 mo. Flow cyt.: Before, +1 week, + 3 mo., +1 yr	CD45, CD19, IgD, CD27, CD38, CD24, IgM, CD21	$CD19^+$BC: inititally ↓, then ⇔ Naïve BC ($CD19^+IgD^+IgM^+CD27^-$): ⇔ mBC ($CD27^+$): initially ↑ then ⇔ PB ($IgM^-CD38^{++}CD27^{++}$): initially ↓↓ then ⇔ TrBC ($IgM^+CD38^{++}CD24^{++}$): initially ↓↓ then ↓ TrBC levels at month 3 predict rejection

Schlößer et al., 2017 (247)	Prospective, 37 KTRs - ABO-incompatible KTRs (n= 10) - ABO-compatible KTRs (n= 27) Flow cyt.: Before, +3wks, +100days + 1 yr	CD19, CD20, CD86, CD27, IgD, CD20, CD38	ABO-incompatible (+ Rituximab): At 1yr compared to ABO-compatible: BCs: ↓ % Subsets: ⇔ ABO-compatible (no Rituximab): BCs: ⇔ Activated BCs ($CD86^{+}$): ↓ then ⇔ at 1yr mBCs ($CD27^{+}IgD^{-}$): ⇔ PBs ($CD27^{+}CD38^{++}CD20^{-}$): ↓↓ then ↓
Alfaro et al., 2021 (248)	41 KTRs (5 Rejection vs. 36 Non-Acute Rejection) Flow cyt.: Before, + 3 mo. and + 6 mo.	CD19, CD27, CD24, CD38, IgD, IgM, CD45, CD3	Total BCs: ↓ Naïve BC ($CD27^{-}IgD^{+}$): ↓ mBC ($CD27^{+}CD38^{+}$): ⇔/↑ TrBC ($CD27^{-}IgM^{+}CD38^{++}CD24^{++}$): ↓↓ PBs ($CD27^{++}CD38^{++}IgD^{-}IgM^{-}$): ⇔ Distinct BC clusters: - TrBCs high and class-switched mBC low → graft function ↑ - PB high → rejection risk ↓
Zielinski et al., 2021 (249)	52 (low immunized) KTRs, Follow Up: every 3 mo. up to 2 yrs (Flow cyt.) and up to 8 yrs (Blood samples)	CD5, CD19, CD27	Higher $CD5^{+}$BC correlate with alloantibody production; but not with kidney function Top 10% CD5 levels: associated with acute Rejection
Thukral et al., 2021 (250)	52 AB0 incompatible KTRs, -26 low dose Rituximab (100mg) - 26 high dose Rituximab (200mg) Flow cyt.: Before, +1mo., +3mo., +6mo., +12mo.	CD20	CD20 counts: ↓↓ and slow repopulation, similar in both groups Low dose Rituximab: Infection risk ↓, rejection risk ⇔

Table 8. Overview of studies investigating BC dynamics in KTRs including a brief summary of BC-relevant findings.

Mo: month(s), yr(s): year(s), HC: healthy control; Flow cyt.: Flow cytometry; RCT: randomized controlled trial

Bm classification system of BCs:

Bm1: $IgD^{+}CD38^{-}$ (Naïve, unswitched mBC, IgD-only mBC); Bm2: $IgD^{+}CD38^{+/++}$ (TrBC., naïve BC, Pre-GC BC); Bm3 and Bm4: $IgD^{-}CD38^{++}$ (GC BC, PB); BM5: $IgD^{-}CD38^{-}$ (IgM-only mBC, switched mBC, DN BC)

Generally, comparability between flow cytometric studies is often complicated by different protocols, gating strategies and varying degree of subset resolution. For example, some authors may characterize circulating BCs based on the Bm-classification system, while others used the CD27 vs. IgD gating approach. We decided against using the Bm-classification system as it was not designed for characterization of circulating BCs (251). Furthermore, the

Bm-classification is not able to discern certain subsets and significant overlap between different BC subpopulations complicates analysis and interpretation (171).
Regarding dynamics of overall $CD19^+$ BCs, a transient increase of $CD19^+$ BCs followed by a reduction to pretransplant levels is frequently reported (240, 243, 246). Importantly, pretransplant levels are already lower than in healthy controls (240), which underlines the abnormal immunological state in ESKD (252).
Prior research has demonstrated a shift in the composition of circulating BCs in KTRs towards a more "differentiated" profile (239). Experimental data also indicate that immature BCs may be more vulnerable to the effects of immunosuppressive medications compared to mature phenotypes (241). Consistent with these findings, our study revealed stable numbers of naïve BCs and a notable decrease in TrBCs. In contrast to some prior research showing stable or increasing mBCs (239, 246), we observed a reduction in IgM-only and switched mBCs, while IgD-only and unswitched mBCs remained constant.
These discrepancies could potentially be attributed to variations in how mBC subsets were characterized using IgM and IgD. Most studies describe switched (IgM^- IgD^-) and unswitched (IgM^+ IgD^+) mBCs. Here we also report minor subsets, namely IgM-only (IgM^+ IgD^-) and IgD-only (IgM^- IgD^+) mBCs, in KTRs. While IgM-only mBCs are widely considered to be pre-switched mBCs and serve as a substrate for switched mBCs (194), IgD-only mBCs remain an enigmatic subset that is consistently found in peripheral blood (189). Given some evidence suggesting potential autoreactivity, IgD-only mBCs represent an intriguing subset to investigate in the context of transplantation (253). Taken together, although the size of the peripheral BC pool remains similar, the composition is profoundly altered.

DN BCs exhibited an increase in frequency in our hands from T1 to T2, while their absolute numbers remained similar. DN BCs can be further characterized into functionally distinct subsets, DN1 and DN2, based on CXCR5 and CD21 expression, as proposed by Sanz et al. (204). DN2 are regarded as precursors of antibody-secreting cells through an extrafollicular pathway, while DN1 are considered to constitute early activated mBCs of follicular origin (171). DN BCs have been recognized as significant contributors in autoimmunity and infectious diseases (171, 254). DN2 BCs are expanded during SLE flares (204) and have been found in abundance in nephritic kidneys (255). Recently, extrafollicular response and DN2 BCs have been associated with antibody response and morbidity in COVID-19 (256). It

is hypothesized that the extrafollicular pathway activation in severe COVID-19 reflects failed early viral control with reduced GC formation and salvage through extrafollicular antibody formation (256)

Notably, others have distinguished four DN subsets (DN1 – DN4) depending on the markers CD21 and CD11c. The proposed DN3 subset ($CD21^{-}$ $CD11c^{-}$), which is implicated in extrafollicular response and rapid antibody generation, was related to COVID-19 severity. However, intensive activation of the extrafollicular pathway could result in autoreactivity as it has been shown for SLE (257, 258).

Unfortunately, our panel lacked these markers, preventing us from distinguishing between different DN subsets. However, it would be intriguing to monitor DN and DN subsets throughout the course of renal transplantation, especially in the context of rejection and infection.

Effector B cells, including PBs and PCs, play a pivotal role in generating antigen-specific antibodies. Distinctive markers of PCs include CD138 and the lack of the proliferation marker Ki-67, which overlaps with surface HLA-DR (259, 260). PCs primarily reside in the bone marrow, making them less accessible for study in humans. Conversely, PBs, while present in peripheral blood in limited numbers in the absence of stimulation, can be investigated more conveniently (181). As PBs constitute the bulk of antibody-secreting cells characterized by the $CD27^{+}$ $CD24^{-}$ $CD38^{++}$ marker profile, we termed this population accordingly. However, we recognize that this may have included PCs, which we could not differentiate due to the lack of CD138 or HLA-DR in our panel. Importantly, bone marrow-resident long-lived PCs downregulate CD19 (261), and circulating $CD19^{-}$ antibody-secreting cells have also been found in peripheral blood shortly after antigenic stimulation (262). $CD19^{-}$ antibody-secreting cells have been put forth as transitional stage between PBs and PCs (262), but would be missed on conventional gating of $CD19^{+}$ BCs as in our study. Of note, PCs also downregulate CD20, which makes them inaccessible to conventional BC-depleting agents, like the chimeric anti-CD20 antibody, rituximab (263). Yet, alloreactive GC-derived PCs are critically implicated in graft loss, as demonstrated by the heightened risk of allograft loss in patients with IgG3 anti-HLA antibodies compared to those who only display IgM alloantibodies (264). These findings may partly explain the disappointing results of BC-targeted therapy in transplantation and in the context of late ABMR (265, 266). On the other hand, PC-targeted

therapy has also failed to improve eGFR trajectory in late ABMR (213), potentially due to a robust compensatory GC response (267). Thus, novel treatment approaches for ABMR may potentially need to target both, BCs and PCs.

In contrast to previous research, our data reveals a further reduction in PBs one year after transplantation, whereas some studies suggested a repopulation of PBs to pre-transplant levels during the same time frame (246). Rincon-Arevalo et al. highlighted the role of PB induction as a key event for antibody response to SARS-CoV-2 vaccination in a study on KTRs and CKD G5 patients compared to healthy controls. After the second BNT162b2 booster vaccination, both KTRs and CKD G5 patients display an increase of RBD^+ BCs within the naïve and pre-switched (IgM-only) mBC compartment, whereas healthy controls, who were able to mount an early and robust antibody response, show predominant expansion of RBD^+ PBs and switched mBCs. Additionally, RBD^+ PBs, both in absolute terms and relative to total BCs, correlated with humoral response. While CKD G5 patients showed a delayed response, almost all KTRs remained non-responders (268).
In our study, pre-vaccination PB levels did not exhibit a correlation with subsequent antibody production. Taken together, these findings suggest that a deficiency in generating new effector BCs, exemplified by PBs, may contribute to the suboptimal response to SARS-CoV-2 vaccination. Importantly, our data suggests that the size of the pre-existing PB pool does not serve as a predictive marker for serological response.

Activated BCs may be characterized by the expression of CD86 among other markers (171). In our study, CD86 was downregulated at T2, while Schlößer et al. showed that one-year post-transplant CD86 expression was comparable to pre-transplantation levels (247). Counterintuitively, tolerant KTRs are enriched with $CD86^+$ BCs compared to non-tolerant KTRs (219, 269), although co-stimulatory ligands, CD80/CD86, are critically involved in TC activation. A possible interpretation could be that BC expression of CD80 and CD86 is also necessary for the induction and maintenance of peripheral Treg populations (270, 271). CD80/CD86 are neither pro- or anti-inflammatory, but they facilitate the TC-BC interaction, and, in the context of Bregs, may polarize TCs towards a Treg phenotype (272).

Considerable interest in transplantation has revolved around $CD5^+$ BCs, as they are enriched in tolerant KTRs compared to KTRs who remain on stable immunosuppressive regimens (269). Additionally, a decrease in $CD5^+$ BCs has been linked to cases of rejection in ABO-incompatible KT (238).

CD5 is a marker of murine B1a cells, which are primarily found in peritoneal and pleural cavities. The existence and characterization of human B1 cells remains somewhat controversial (207). Yet, similar to murine B1 cells, 75% of the proposed human B1 cells express CD5 (206). However, several other BC subpopulations include $CD5^+$ cells in humans. Particularly immature subsets like TrBCs and "pre-naïve" BCs, which act as proposed link between TrBCs with naïve BCs are enriched with $CD5^+$ cells (273-275). In adults, frequency of $CD5^+$ BCs has been reported to range from 5 – 30% of BCs (276, 277). Due to its expression across different subsets, it has been suggested, that in humans CD5 upregulation may signify a fluctuating BC activation signal rather than indicating a distinct subset (275). CD5 is also expressed abundantly by BC malignancies, like B cell chronic lymphocytic leukemia (B-CLL) (278). Additionally, $CD5^+$ BCs are increased in peripheral blood in a variety of autoimmune disorders including SLE or type 1 diabetes mellitus (275, 279). Functionally, CD5 acts as a negative regulator of BCR signaling (280), leading to the plausible hypothesis that higher CD5 expression could potentially mitigate alloreactive responses in the context of transplantation (280). Moreover, CD5 plays a pivotal role in promoting the production of IL-10 (281-283). On the other hands, $CD5^+$ BCs produce poly- and autoreactive antibodies including single-stranded DNA antibodies (276). To reconcile this paradox, regulation of BCR signaling depending on alternative transcripts of CD5 have been proposed (284). While conventional full-length CD5 promotes BC anergy, truncated CD5 may augment BCR signaling (285). However, regulation of these alternative transcripts remains engimatic.

$CD27^+$ memory BCs have been shown to downregulate CD5 (286). Thus, our focus centered on $CD5^+$ B cells within the $CD27^-$ BC population, whereas other investigations have taken a broader approach by examining CD5 expression across BCs more inclusively (238, 249). These discrepancies may explain why we found a significant reduction in $CD27^-CD5^+$ B cells at T2, while others have reported total $CD5^+$ BCs at similar levels before and after the first year of KT, despite displaying some fluctuations in the early post-transplantation period

(249). Congruently with finding from the ESKD population, we found low levels of $CD27^-$ $CD5^+$ B cells at T1 (252).

Lederer et al. elegantly showed through serial analysis of draining lymph node and blood samples in healthy controls and KTRs, that the antibody response to SARS-CoV-2 vaccination is directly dependent on GC formation. Crippled GC BC response, diminished mBC formation and failed seroresponse were found in KTRs underlining the detrimental impact of immunosuppression in this context (287).
Our study population received a very homogenous immunosuppressive regimen consisting of BX induction (91.5%) and standard triple maintenance immunosuppression TAC, MMF/MPA and low dose CS. Thus, we were not able to investigate the impact of different immunosuppressive drugs on the BC pool size or composition. Previous studies have investigated the effect of different induction and maintenance agents:
Unsurprisingly, rituximab induction in ABO-incompatible KTRs leads to a long-lasting depletion of $CD19^+$ BCs (242, 247, 250). TrBCs are the first subtype to repopulate peripheral blood after rituximab treatment (242). ATG is commonly employed in KTRs with high immunological risk. ATG mechanism of action is not restricted to TC depletion, but includes apoptosis induction of BCs by binding to surface markers like CD19 and CD20, modulation of dendritic cell function and induction of Tregs (288). The overall effect of ATG on BC counts is minimal, but the mBC subset may be reduced as consequence of lymphocyte-depleting induction (289).

BX is currently the only available anti-IL-2R antagonist available for KTRs, as daclizumab has been deemed unsafe by the European medicines agency (EMA) (69). BX specifically targets CD25, the alpha chain of the IL-2 receptor, thereby inhibiting signal 3 of TC activation. Although primarily targeted at CD25 on activated TCs, around 30% of peripheral BCs also express CD25 (290). Moreover, IL-2 is critically involved in PC differentiation (291). When comparing the effect ATG to BX longitudinally, Svachova et al. found not difference except for increased mBCs on the 7^{th} day after KT in the ATG group. One year after transplantation, neither size nor composition of the BC pool was distinguishable (246). Contrarily, Cherukuri et al. took a snapshot of the peripheral BC pool approximately two years after KT and compared two steroid-sparing regimens, BX vs. Alemtuzumab. They

reported lower numbers of total BCs, and subsets including naïve BCs, TrBCs and subsets with a regulatory phenotype. However, an alternative explanation of this lasting effect might be the employment of TAC and MMF in the BX arm, whereas the Alemtuzumab arm received only TAC maintenance therapy (292).

For maintenance therapy, the suppressive effect of MMF/MPA on BCs is widely recognized. Antimetabolites like MMF/MPA and AZA target *de novo* purine synthesis, which is necessary for lymphocyte proliferation (293). AZA and MMF appear to have comparable long-term effects on peripheral BCs (240). Antimetabolite treatment has been recognized as a risk factor for non-response to vaccination (125). A temporary withhold of antimetabolite during SARS-CoV-2 vaccination has therefore been proposed to increase response rates (151). While TAC exerts indirect effects on BCs via inhibiting TC activation and proliferation, antimetabolites and mTOR inhibitors may directly inhibit BC response (294). In that manner, MPA has been been shown to inhibit early activation of BCs by arresting them in the G0/G1 phase and to prevent naïve and mBC expansion and PC differentiation. However, in terminally differentiated PCs, IMPDH is downregulated, and they become unsusceptible to MPA (295). We observed a negative correlation of MMF/MPA dose with PB abundance, which hints towards a dose-dependent inhibition of PB proliferation by MMF/MPA. This PB-specific effect of MMF has also been reported in SLE patients, where MMF treatment coincided with lower levels of PBs compared to AZA or cyclophosphamide (296, 297). We did not observe an influence of MMF/MPA dose on vaccination response, but, as has been shown recently, IMPDH activity may be an even better predictor of seroconversion. Furthermore MMF/MPA dose correlates only weakly with IMPDH activity, underlining the non-linear dose-response relationship of immunosuppression (298).
mTOR-inhibitors like EVE are known for preserved humoral response and superior antiviral activity (299), but safety issues make it a second line drug in KTRs (300, 301). In our vaccination subcohort three patients received EVE, as it is only used in our center when antimetabolites are not tolerated or in infectious or tumor complications, thus not allowing for comparison with regards to BC subsets or vaccination outcome.

Consistently with the existing literature, our study observed a reduction in TrBCs after transplantation (246, 248). TrBC reduction has been linked to graft rejection (221, 245), while

tolerant KTRs have been found to have a higher frequency of TrBCs (220). Due to the enrichment of Bregs within the TrBC subpopulation, they may be critically implicated in graft tolerance (302).
Intriguingly, we found that TrBCs in stable KTRs one-year post-transplantation were significantly correlated with antibody levels following SARS-CoV-2 vaccination. Moreover, individuals who responded positively to vaccination, with detectable antibody titers, had higher absolute TrBC numbers and a greater TrBC frequency in their blood compared to non-responders. At the time of publication, this was the first investigation into the potential of stable KTRs' BCs as biomarkers for subsequent SARS-CoV-2 vaccination success.
Interestingly, in the CKD G5 setting (at T1), TrBCs were considerably higher in our patients, allowing us to speculate that improved vaccination response in dialysis patients may be linked to superior TrBC abundance compared to KTRs (146, 303, 304).
In a similar context, Tsang et al. developed a predictive model for the serological response to influenza vaccination in healthy individuals, and TrBCs emerged as one of the most predictive blood cell populations (305). However, evidence regarding pre-vaccination TrBCs in immunocompromised individuals is limited, predominantly observational, and sometimes contradictory. For instance, patients with acute myeloid leukemia, exhibiting higher TrBC frequencies than healthy subjects, demonstrated a poorer response to H1N1 vaccination (306). Similarly, in HIV-infected children, TrBCs correlated negatively with H1N1 vaccination response (307). In contrast, patients treated with rituximab, despite having abundant TrBCs, responded equally well to H1N1 vaccination as controls with lower TrBC counts (308).

In KTRs specifically, Egli et al. reported HLA-DR and CD86 expression as predictive markers of seroconversion following H1N1 vaccination (309). Total BCs were correlated moderately with titers after tetanus vaccination in one study (299), whereas another found no predictor BC subset in a heterogenous study population (310). Lower numbers of pre-vaccination PBs coincided with reduced seroconversion rate and antibody titers in KTRs compared to healthy controls after administration of the trivalent influenza vaccine (311). In immunocompromised children, including two KTRs, numbers of circulating $CD27^+$ BCs predicted response to pneumococcal conjugate vaccine (312). Thus, a systematic review and meta-analysis from 2021 on biomarkers of future vaccination response, excluding studies on

SARS-CoV-2, concluded that there was no strong predictor within BCs while acknowledging the scarcity and heterogeneity of the available data (313).

For COVID-19, Schulz et al. reported that naïve BCs ($CD27^-$ IgD^+) were predictive of humoral response to mRNA vaccination in a heterogenous cohort of immunocompromised individuals in multivariable analysis. However, this study did not include KTRs and immunosuppression was highly variable including a third of patients treated with BC-depleting agents (314). Low BC counts have been reported as an indicator of non-response in systemic rheumatic diseases with rituximab or belimumab, a BAFF inhibitor (315). In patients treated with rituximab, presence of repopulating BCs may be a better predictor of humoral response than time from last infusion (316). However, these findings may not be transferable to the KT setting, as rituximab is rarely used, apart from induction in ABO-incompatible KT. Nonetheless, $CD19^+$ BCs predicted and correlated with antibody response to BNT162b2 vaccination in KTRs and HD patients (317). Although they did not directly assess the association to vaccination response, Rincon-Arevalo et al. found that pre-vaccine numbers of total BCs were reduced in KTRs and dialysis patient compared to healthy controls. Additionally, whereas dialysis patients could mount a delayed response, almost all KTRs were unable to. Intriguingly, the peripheral BC pool prior to vaccination was composed differently, with reduced frequencies of naïve BCs and increased frequencies of switched mBCs in KTRs compared to dialysis patients (268).

Finally, the predictive value of TrBCs for SARS-CoV-2 vaccination response has been reaffirmed in a study involving KTRs who received three doses of BNT162b2. Corresponding to our results, Stai et al. demonstrated that individuals who exhibited a positive vaccination response had elevated levels of TrBCs before vaccination (318).

Several limitations of our study need to be addressed.
First, our study did not possess adequate power or design to measure antibody titers after SARS-CoV-2 vaccination prospectively in KTRs. KTRs have already been shown repeatedly to respond insufficiently to vaccination (141, 146, 304). Similarly, we found an alarmingly low rate of responders in our cohort, and a trend for improved seroconversion rates after mRNA-1273 compared to BNT162b2 vaccination (146). Presumably owing to our small

cohort, we could not find an impact of other proposed risk factor for vaccination failure like age or time from transplantation (146, 319). Because of the homogenous immunosuppressive regimen, we could not evaluate for distinct drug effects on circulating BCs or vaccination response.

Second, we did not collect blood samples at any time point after T2 including the time of vaccination. Previous reports suggested very little fluctuations in circulating BC composition from the first year of KT onward (239, 242), thus we assumed a stable BC pool after T2. Inclusion criteria for the vaccination subcohort demanded a functioning graft at vaccination and T3, but immunosuppression (medication and/or dosing) may have changed between T2 and T3, potentially influencing circulating immune cells.

Flow cytometric characterization of cell types is inherently constrained by that a limited number of surface (or intracellular) markers may not be informative on the function of a specific cell. Thus, transcriptional analysis or *ex vivo* determination of cytokine release or response to antigenic stimulation would have been of great value. Specifically, functional characterization of TrBCs may have allowed us to determine if there is a shift in the prevalent subtype (i.e., T1 TrBCs or T2 TrBC) between the two timepoints. It is conceivable that responders to vaccination show a preferential expansion of T2 TrBCs with a pro-inflammatory phenotype which allows them to respond more efficiently to antigenic stimulation. Although a differentiation between T1 and T2 TrBCs may also be feasible by the intensity of CD24 and CD38 expression with T1 TrBCs being $CD24^{+++}$ $CD38^{+++}$ and T2 TrBCs being $CD24^{++}$ $CD38^{++}$, we decided against it as the cut-off remains arbitrary and the distinction ought to be confirmed by functional assessment (221).

Different assays, testing platforms and laboratories were used for the analysis of anti-SARS-CoV-2 antibodies, which were then converted to International Standard units according to WHO recommendation (234). Although results from different platforms have shown a good correlation, they may not provide interchangeable results (320, 321). Of note, testing platforms were equally distributed between responders and non-responders. The so-called "correlates of protection" may not be applicable to our study cohort, as (i) they have not been validated in immunosuppressed patients, (ii) relied on neutralizing antibody tests or (iii) were

derived from a vector-vaccine based cohort (154, 158, 322). Therefore, and due to the low antibody concentration in our cohort, we defined KTRs with detectable anti-SARS-CoV-2 antibodies (i.e., above the detection limit of the respective test) as responders. Our rationale was that even low antibodies would indicate a minimal response to vaccination and may offer at least some protection compared to a complete non-response. Notably, the threshold of 264 BAU/mL, which proposedly confers 80% protection from symptomatic infection (158), was only achieved by three KTRs in our cohort.

Another limitation is the variable interval between T2 and vaccination and between vaccination and antibody testing. Antibody generation in the general population following the second dose of mRNA-based SARS-CoV-2 vaccination underlies certain kinetics with a peak concentration found after around two weeks. Thereafter antibody levels gradually decline, unless boostered by another vaccine dose or natural infection (323, 324). Longitudinal analysis of anti-SARS-CoV-2 antibody kinetics in KTRs are scarce, but existing data does not indicate a more rapid decline in antibody titers and that they persist for at least six months after two doses (325). Similarly, to findings from dialysis patients (268, 304), KTRs may have a delayed antibody response to vaccination (325). Taken together, these findings highlight the importance of the timing of antibody testing following vaccination. Presently, we did not observe timing differences between responders and non-responders.

Apart from vaccination, natural COVID-19 infection is another potential source of anti-SARS-CoV-2 antibodies (326, 327). Therefore, we have only included those who did not have a history of symptomatic or asymptomatic COVID-19 infection, as tested by PCR or anti-nucleocapsid-antibodies. KTRs are in regular follow-up care and are instructed to report any incidents at their regular outpatient visits, so that even oligosymptomatic infections should have been recorded. However, as anti-nucleocapsid-antibodies were only available for 22 subjects (all of them negative), we cannot rule out prior asymptomatic and untested COVID-19 in the other individuals. Moreover, we did not test for cellular immunity in our cohort, while it has been consistently demonstrated that assessment of only humoral response may underestimate responders (143, 303).

In conclusion, we have reported significant quantitative and compositional alterations in the circulating BC pool after KT. Adding to the existing literature, we have included

subpopulations like DN BCs and mBC subsets, which have become recently recognized players in autoimmunity and inflammation. We have provided a potential link between pre-vaccination TrBCs in stable KTRs and response to SARS-CoV-2 vaccination, as antibody levels correlate with TrBC abundance and responders display higher TrBC counts and frequencies than non-responders.

We acknowledge that the COVID-19 pandemic has fundamentally changed since its emergence, due to the appearance of virus variants with altered transmissibility and virulence, widespread and repeated vaccinations (including variant-adapted vaccines) and availability of effective medication. These factors have contributed to the lower morbidity and mortality of KTRs with COVID-19, who, nonetheless, remain a risk population. Furthermore, poor response to antigenic stimulation is not restricted to SARS-CoV-2 but has been reported with influenza and hepatitis B vaccines in KTRs as well (328). This underlines the need for biomarkers to identify non-responders either for rapid and repeated booster vaccinations or to emphasize on alternative protective measures. Considering our study's results, we advocate for further evaluation of BCs and BC subsets, particularly TrBCs, as biomarkers of clinical events like vaccination response after KT.

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