Complement receptors 3 and 4 in kidney diseases

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ACR – albumin-to-creatinine ratio

ANCA-vasculitis – antineutrophilic cytoplasmic antibody-associated vasculitis

ANTN – accelerated nephrotoxic nephritis

BATF3 – basic leucine zipper transcription factor ATF-like 3

BMDC – bone marrow-derived dendritic cell

BSA – bovine serum albumin

BW – body weight

C3 – complement factor 3

C5aR – complement factor 5 receptor

CCL - chemokine (C-C motif) ligand

CCR7 – C-C chemokine receptor type 7

CD – a cluster of differentiation

CD40L – CD40 ligand

cDC – conventional dendritic cell type

cGN – crescentic glomerulonephritis

CFU – colony forming unit

Clec9A – C-type lectin domain containing 9A

CLIP-170 – cytoplasmic linker protein of 170 kDa

CR - complement receptor

CRISPR/Cas9 – clustered regularly interspaced short palindromic repeats and CRISPR-associated protein 9

CTLA-4 – cytotoxic T-lymphocyte-associated protein 4

CVF – cobra venom factor

CXCL – C-X-C motif ligand

DCs – dendritic cells

DCIR2 – dendritic cell inhibitory receptor 2

DN – diabetic nephropathy

DNA - deoxyribonucleic acid

EAE - experimental autoimmune encephalomyelitis

ELISA - enzyme-linked Immunosorbent Assay

F4/80 - EGF-like module-containing mucin-like hormone receptor-like 1

FBS – fetal bovine serum

FcγR – Fcγ receptor

fMLP – *N*-formylmethionine-leucyl-phenylalanine

FSGS – focal segmental glomerulosclerosis

GFP – green fluorescence protein

GFR – glomerular filtration rate

GM-CSF - granulocyte-macrophage colony-stimulating factor

gMFI – mean geometric fluorescent intensity

HC – healthy control

HTN – hypertension

ICAM-1 – intercellular adhesion molecule 1

iC3b – inactivated complement factor 3b

IreA – iron-responsive element A

ID2 – DNA-binding protein inhibitor 2

IFNβ – interferon β

IFNγ – interferon γ

IgA – immunoglobulin A

IgG – immunoglobulin G

IL – interleukin

i.p. – intraperitoneal injection

IRF4 – interferon regulatory factor 4

IRF8 – interferon regulatory factor 8

ITGAM – integrin alpha M

ITGAX – integrin alpha X

iNOS – inducible nitric oxide synthase

i.v. – intravenous injection

Klf4 – kruppel-like factor 4

L/D – life-dead staining of dead cells

LB medium – lysogeny broth medium

LN – lymph node

LPS – lipopolysaccharide

MAC – membrane attack complex

MBL – mannan-binding lectin

MCD – minimal change disease

mDia1 – mammalian diaphanous-related formin 1

mGN – membranous glomerulopathy
MHC – major histocompatibility complex
MIF – macrophage inhibitory factor
MMP-9 - matrix metallopeptidase 9
MPO -myeloperoxidase
MS – multiple sclerosis
mRNA – messenger ribonucleic acid
MyD88 – myeloid differentiation primary response 88
NADPH – nicotinamide adenine dinucleotide phosphate
NES - normalized enrichment score
NETs – neutrophil extracellular traps
NF-kB - nuclear factor kappa-light-chain-enhancer of activated B cells
NFIL3 – nuclear factor, interleukin 3 regulated
NGAL - neutrophil gelatinase-associated lipocalin
NK cells – natural killer cells
NLR – NOD-like receptor
NLRP3 – NLR family pyrin domain containing 3
NO – nitric oxide
NOD – nucleotide oligomerization domain
NOX – NADPH oxidizing complex
NTN – nephrotoxic nephritis
NTS – nephrotoxic sheep serum
OD – optical density
OVA – ovalbumin
pDC – plasmacytoid dendritic cell
PAMP – pathogen-associated molecular pattern
p.i. – post-infection
PBMC – peripheral blood mononuclear cell
PBS - phosphate-buffered saline
PMA - phorbol myristate acetate
PMNs – polymorphonuclear cells, neutrophils
PRR – pattern recognition receptor
PSP – phage shock protein
PTEC – renal proximal tubular epithelial cell

qRT-PCR – real-time quantitative reverse transcription polymerase chain reaction

RA – rheumatoid arthritis

Rab GTPase – Ras-associated binding GTPase

RCF – relative centrifugation force

RIG-I receptor – retinoic acid-inducible receptor 1

RNA - ribonucleic acid

ROS – reactive oxygen species

RORγT – retinoid-related orphan receptor γT

RPGN – rapid progressive glomerulonephritis

RT – room temperature

SLE – systemic lupus erythematosus

SYK – spleen tyrosine kinase

TCR – T cell receptor

Tfh - T follicular helper cell

TGFβ - transforming growth factor beta

T_h – T helper cell

TGFβ – transforming growth factor β

TLRs - toll-like receptors

TNFα – tumor necrosis factor betaT_{reg}

- regulatory T cell

V-ATPase – vacuolar ATPase

UPEC – uropathogenic *Escherichia coli*

UTI – urinary tract infection

WBCs – white blood cells

WT – wild type

ZEB2 – zinc finger E-box binding homeobox 2

Abstract

CR3 and CR4 are among the most abundant molecules on the cell surface of DCs, monocytes/macrophages, and PMNs. Both integrins recognize common ligands among which are iC3b, ICAM-1, and fibrinogen. iC3b is generated as a result of the activation of the complement cascade and can opsonize antigens. Among the diseases characterized by the activation of the complement system are glomerulonephritis and pyelonephritis. This project aimed to investigate the influence of CR3 and CR4 on the progression of these diseases using our newly generated *ItgamItgax*$^{-/-}$ mouse line.

During the project, we found that genes encoding CR3 and CR4 are upregulated in patients suffering from several types of glomerulonephritis. Also, mice lacking both CR3 and CR4 showed attenuated symptoms of the disease, as evidenced by lower ACR and concentration of NGAL in their urine compared to WT animals. Histological analysis of kidneys from *ItgamItgax*$^{-/-}$ mice showed a lower degree of crescent formation. Flow cytometric analysis revealed a decrease in the number of cDC2s and CD4^{+} T cells in the kidneys of *ItgamItgax*$^{-/-}$ mice compared to the WT group, which can indicate a selective decrease in inflammation. CD4^{+} T cells of nephritic *ItgamItgax*$^{-/-}$ mice had also a lower expression of genes engaged in IL-1-related signaling. cDC2s of *ItgamItgax*$^{-/-}$ mice captured less antigen during cGN compared to WT cells. Also, they were less efficient at inducing the proliferation of CD4^{+} T cells compared to WT DCs. In the case of pyelonephritis, *ItgamItgax*$^{-/-}$ animals had also attenuated symptoms of the disease as evidenced by the lower bacterial load in their kidneys compared to the WT group and decreased inflammation on day 7 p.i. *ItgamItgax*$^{-/-}$ BMDMs contained also less viable intracellular bacteria compared to WT cells, which can indicate that due to the absence of CR3 and CR4 bacteria are not efficiently phagocytose. This led us to the conclusion that UPEC can use macrophages as a niche to evade immune response in WT animals, which is not possible in the case of *ItgamItgax*$^{-/-}$ mice. This can explain the better outcome of knockout animals.

The findings presented above reveal the importance of CR3 and CR4 during the progression of crescentic glomerulonephritis as well as pyelonephritis and suggest that targeting these receptors may be a therapeutic strategy in the treatment of these diseases.

1. Introduction

1.1 Integrins

1.1.1 Structure and functions of integrins

Integrins belong to the group of transmembrane proteins and are present in most cell types, except for red blood cells. Extracellular domains of these proteins contain the ligand-binding sites. On the contrary, intracellular domains have regulatory functions. The ability of the integrins to bind to their ligands is regulated through conformational changes as well as by the clustering of these molecules. Integrins exist in three major conformational states depending on their activation, which could be mediated by chemokines or through TCR signaling. These states include inactive or bent-closed, intermediate or extended-closed, and active or extended-open confirmation [1].

Stimulation of the cells expressing integrins by particular chemokine or cytokine results in the recruitment of certain proteins among which are talin and kindlin to the intracellular part of the integrin. This process leads to the activation of the integrin through the separation of the cytoplasmic parts of its subunits. The active conformation is then stabilized thanks to the proteins mentioned above through binding of the integrin to the actin cytoskeleton [2].

The main functions of integrins include mediation of intracellular signaling pathways e.g., those important for migration of the cells, their differentiation, and proliferation [1]. Several studies also point out the importance of integrins in the regulation of angiogenesis [3] and cell death [4]. They take part in phagocytosis of complement-opsonized antigens too [5].

1.1.2 Role of complement receptors 3 and 4

Integrins that are mainly involved in the phagocytosis and infiltration of the immune cells are CD11b and CD11c which together with CD18 form complement receptor 3 (CR3) and 4 (CR4), respectively [6,7]. CR3 and CR4 are mainly expressed on the myeloid immune cells including dendritic cells (DCs) and monocytes/macrophages but can also be present on NK cells as well as activated T cells [5]. Both of the receptors have a similar structure as evidenced by 87% homology between CR3 and CR4 in their extracellular domains and

56% of homology between their intracellular domains. CR4 is the most abundant integrin on DCs and is widely used as a marker of this cell type [8]. Several studies also suggest that CR4 can be present in intracellular granules in neutrophils (polymorphonuclear granulocytes, PMNs) and can be released from the granules as a result of the activation of the granulocytes by *N*-formylmethionine-leucyl-phenylalanine (fMLP). This effect is the most prominent during sepsis. The same mechanism is true for CR3, although the receptor is expressed by neutrophils in a steady state too. An increase in the presence of CR3 and CR4 on the membrane of PMNs as a result of the inflammation helps the cells to uptake more pathogens opsonized by complement factors due to the activation of the complement system [9]. Another study dealing with the infection of neutrophils with *Candida albicans* (*C. albicans*) showed that the pathogen can be also taken up by PMNs through CR3, which recognizes β-glucan on the cell wall of the fungi [10].

Another prominent function of CR3 and CR4, as mentioned above, is their involvement in the adhesion and migration of the cells. Adhesion of leukocytes consists of several steps among which are rolling, arrest, crawling, and extravasation. The process is mediated via selectins on leukocytes and ICAM-1 on endothelial cells. First, CR3 and CR4 on immune cells are activated through selectins and chemokines, which results in the conformational changes of β_2-integrins leading to slow rolling of the cells. Subsequently, the conformation of CR3 and CR4 changes to the extended-open state resulting in the arrest of the immune cells. The next step of the migration of the cells is mainly dependent on CR3 and is characterized by the crawling of the leukocytes along the endothelial layer in search of the extravasation site [1].

However, several studies proved that migration of the immune cells is not always dependent on the same β_2-integrins and specific conditions can influence the strategy used by leukocytes to infiltrate organs. Kubo N *et al* showed utilizing *in vivo* model of atherosclerosis that infiltration of the aorta with macrophages during the disease was similar between mice reconstituted with bone marrow cells expressing CD11b and those reconstituted with $CD11b^{-/-}$ bone marrow cells, meaning that CR3 is not essential for the migration of leukocytes during the disease and other integrins can compensate for the lack of this β_2-integrin [11]. Another study showed that DCs deficient in all integrins exhibit the same ability to migrate into the draining lymph nodes (LNs) after injection into the footpad of the mouse. Instead, DCs migration was mainly influenced by the expansion of

the actin network and was dependent on CCR7 [12]. Altorki T *et al* utilizing the model of *Trichuris muris* (*T. muris*) colon infection in mice also showed that migration of the DCs to the mesenteric LNs in the case of Itgb2^{mut} mice lacking functional β_2-integrins is similar to those of WT animals. On the other hand, the ability of the DCs to infiltrate the site of infection was dependent on the integrins, which was evidenced by the increased number of these leukocytes in the colon of WT mice but not in Itgb2^{mut} animals post-infection (p.i.). The same observation was true for macrophages [13].

Adhesion of the immune cells, especially during inflammation, is also strongly associated with the ability of CR3 and CR4 to bind to the deposited inside the tissue fibrinogen. This results in the increased antimicrobial response of the leukocytes expressing CD11b and CD11c [14].

1.2 Complement system

1.2.1 Mechanisms of the activation of the complement system and its role in the innate immune response

Activation of the complement cascade belongs to one of the most crucial humoral mechanisms of the host defense against pathogens and it is important for the innate immune response. The complement system consists of around 50 different factors. There are three main pathways of complement activation: classical, lectin, and alternative pathways. The classical pathway is initiated due to the binding of C1q to the surface of the microorganism or antibody:antigen complexes. The activation of the lectin pathway is connected with the binding of the mannan-binding lectin (MBL) to the carbohydrates reached in mannose on the surface of the pathogens. Finally, the initiation of the alternative pathway happens as a result of the spontaneous breakdown of complement factor 3 (C3). Activation of this pathway of the complement system is also associated with the development of autoimmune diseases such as C3 glomerulopathy or rapidly progressive glomerulonephritis (RPGN). After subsequent steps of the reactions, activation of each type of the complement pathway leads to the formation of the C3 convertase. Then, C3 convertase cleaves C3, which leads to the generation of C3b and C3a. Afterward, C3b binds to C3 convertase leading to the formation of C5 convertase, which results in the production of the smaller anaphylatoxin C5a and larger fragment C5b. Assembly of C5 convertase can be prevented through cleavage of C3b to iC3b by factor

I which is present in the plasma. C5b is responsible for the initiation of the reactions which lead to the formation of a membrane attack complex (MAC) on the surface of the microorganism. Formation of MAC results in the creation of pores on the cell surface of the pathogen which leads to its lysis [15].

Another prominent function of the complement system in the innate immune response is the opsonization of antigens by complement factors such as C3b and iC3b, further uptake, and destruction of the pathogen by phagocytes (PMNs and macrophages). Among the receptors which can recognize particles coated with complement factors are CR3 and CR4. One of the studies demonstrated that in human monocytes CR3 and CR4, especially CR3, play an exceptional role in the mediation of phagocytosis of previously opsonized with human serum containing iC3b, *Staphylococcus aureus* (*S. aureus*) [16].

As mentioned above, activation of the complement cascade results in the generation of anaphylatoxins e.g., C3a and C5a in the plasma of the organism. C3a and C5a are recognized by C3aR and C5aR, respectively. These receptors belong to the group of G protein-coupled receptors (GPCRs) and they are expressed on myeloid immune cells but also on T cells, epithelial cells, and smooth muscle cells. Both complement factors can induce chemotaxis, and degranulation as well as they can promote the generation of reactive oxygen species (ROS) in myeloid cells such as PMNs, eosinophils, and monocytes. However, C5a much stronger inducer of these processes compared to C3a [17].

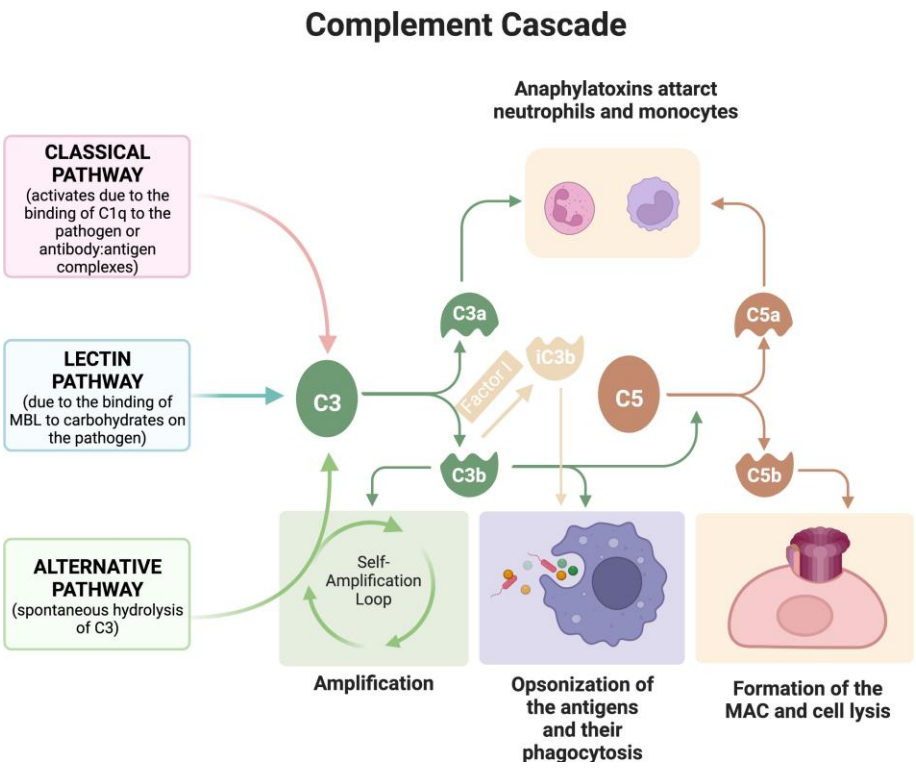


Figure 1. Complement cascade. Activation of the complement cascade occurs due to the presence of pathogens or spontaneously. There are three pathways of complement system activation: classical, lectin, and alternative pathways. All three pathways lead to the formation of C3 convertase. Activation of the complement cascade results in the phagocytosis of opsonized pathogens, formation of MAC, or attraction of other immune cells and their further activation. The figure was generated using BioRender. (Modified from Janeway CA Jr. *et al,* 2001)

1.2.2 Complement system in the adaptive immune response

Although activation of the complement system is mainly associated with innate immunity, it can regulate the activation and differentiation of adaptive immune cells too. Also, several studies showed that complement factors are not only available in the serum of the organism but can be synthesized intracellularly inside leukocytes [18].

Previous studies discovered that recognition of complement factors by the complement receptors on T cells such as C3aR and C5aR is important for the activation of these lymphocytes and induction of T_h immune response [19]. Interestingly, recognition of complement fragments by certain receptors on T cells relies on complement fragments that are generated inside this cell type. It is known that C3a and C3b can be produced by T cells in the process dependent on TCR activation, which results in the cleavage of C3

by cathepsin L [18]. Subsequent activation of C3aR and CD46 leads to the expression of IFNγ. Signaling pathways activated by C3aR and CD46 are engaged in IL-2R signaling as well as metabolic reprogramming of T cells due to mTORC1 induction [20]. Also, it was shown that T cells from mice with either C3aR or C5aR knockout are characterized by impaired production of IL-2 and IFNγ [19]. On the other hand, CD46 stimulation can also lead to IL-10 production. IL-10 is known for its anti-inflammatory properties [21]. Another interesting finding regarding the role of the complement system in $CD4^+$ T cells was its role in the formation of NLRP3 inflammasome inside this type of lymphocytes. Arbore G *et al* showed that NLRP3 inflammasome can form inside $CD4^+$ T cells leading to IL-1β production by them. This can further promote IFNγ expression by $CD4^+$ T lymphocytes. The whole process is dependent on the recognition of intracellular C5a by C5aR [22].

1.3 Characterization of the cells expressing CR3 and CR4 and their role in the immune response

1.3.1 Dendritic cells in the immune response

As previously mentioned, among the cells characterized by strong expression of integrins are DCs. The most common marker used to identify these cells is CD11c [8]. The main function of DCs is antigen presentation and shaping the adaptive immune response. DCs localize almost in all types of peripheral tissues, where they recognize and further capture pathogens via phagocytosis, micropinocytosis, or receptor-mediated phagocytosis. Then pathogens are processed into peptides, which are bound by a major histocompatibility complex (MHC) and displayed by it on the surface of the dendritic cells. Afterward, DCs migrate to the lymph nodes where they can present antigens to T cells [23].

To achieve effective activation of naïve T cells, except for antigen presentation two other types of signals are required [24]. One of them is a costimulatory signal. Among the costimulatory molecules expressed on DCs are CD80, CD86, and CD40. CD80 and CD86 recognize and bind to CD28 on T cells. This induces the production of IL-2. IL-2 is a cytokine responsible for the proliferation and survival of T cells. After activation, T cells start to upregulate CTLA-4, which is also recognized by CD80 and CD86. The affinity of CTLA-4 to the mentioned above costimulatory molecules is higher compared to the affinity to CD28. Binding of CTLA-4 to CD80 and CD86 on DCs results in the decreased T cell

response. The binding of CD40 to CD40L on T cells leads to the increased production of IL-12, which induces the proliferation of T cells. T cells with a low abundance of CD40L are characterized by increased expression of IL-10 that results in their decreased activation [25].

A third signal responsible for the activation of T cells by DCs is the presence of certain cytokines examples of which are IL-12, IL-23, IL-6, and TNFα [26]. IL-12 belongs to the family of IL-12 cytokines and constitutes 2 subunits: p35 and p40. The main function of this cytokine is the stimulation of the differentiation of T cells toward the T_h1 phenotype [27]. T_h1 cells are one of the main producers of IFNγ, which stimulates macrophages, induces antigen processing, and affects the infiltration of leukocytes [28]. Overproduction of IFNγ is associated with the progression of several autoimmune diseases such as multiple sclerosis (MS) [29], systemic lupus erythematosus (SLE) [30], and crescentic glomerulonephritis (cGN) [31]. On the contrary, decreased amount of this cytokine leads to susceptibility to influenza A [32] and urinary tract infections (UTIs) [33]. Another member of the IL-12 family of cytokines is IL-23. The cytokine also possesses a p40 subunit similar to IL-12 but instead of p35, it contains a p19 subunit. The abundance of IL-23 leads to the expansion of T_h17 cells that are known for their ability to initiate autoimmune reactions [27]. The most known cytokine produced by T_h17 cells is IL-17A. This cytokine was shown to be responsible for the development of rheumatoid arthritis (RA) [34]. IL-17A$^{-/-}$ mice are also known to be protected from the development of cGN on day 6 after the disease induction [35]. Except for IL-17A, T_h17 cells also express IL-6. IL-6 promotes further T_h17 response [36]. IL-6 is known for its engagement in the development and progression of such diseases as SLE [37] and RA [38] too. However, IL-6 deficiency is associated with the increased bacterial load in kidneys during pyelonephritis [39]. Another important cytokine for the activation and induction of proliferation of T cells is TNFα. TNFα was shown to be associated with autoimmune disorders as well as sepsis and tissue damage [36].

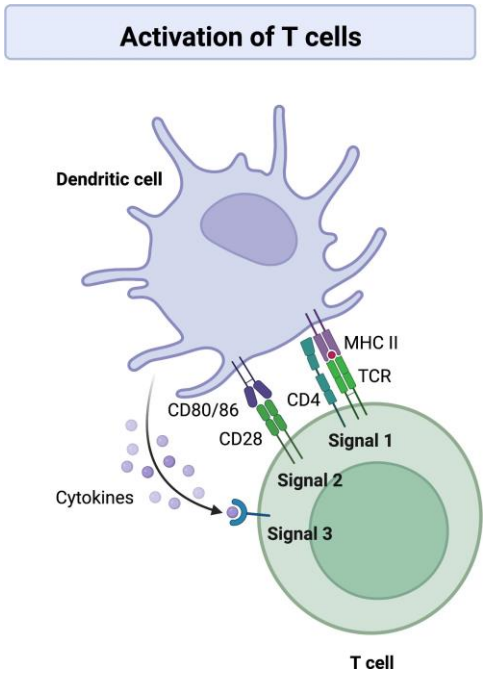


Figure 2. Activation of T cells by DCs. For the efficient activation of T cells, three signals are necessary: antigen presentation on MHC molecules, co-stimulation via CD80/86 on DCs and CD28 on T cells, and the presence of particular cytokines. The figure was generated using BioRender. (Modified from Janeway CA Jr. *et al,* 2001)

Interestingly, integrins were also shown to be important for the functionality of DCs and their ability to stimulate T cells. One of the studies demonstrated that mice with DCs lacking CD11c were not able to efficiently induce proliferation and further differentiation of T cells after contact with the antigen associated with CD47-deficient cells [40]. CD47 is a surface molecule, which gives phagocytes a signal preventing engulfment of the cells by phagocytic leukocytes [41].

1.3.2 Conventional dendritic cells type 1 (cDC1s) and 2 (cDC2s)

There are several types of dendritic cells present in the organism, including plasmacytoid dendritic cells (pDCs), cDC1s, cDC2s, and several other types [42]. In the dissertation, I will focus on cDC1s and cDC2s because they are the most relevant types of DCs for the research topic of the presented project.

cDC1s compose only a small part of leukocytes in human blood with around 0.05% of the peripheral blood mononuclear cells (PBMCs) [43]. Differentiation of DCs towards cDC1 phenotype depends on several transcription factors: BATF3, IRF8, NFIL3, and ID2. Among markers used to identify this type of DCs are CD103 in the case of cDC1s in nonlymphoid tissues and CD8 in the case of the cells residing in lymphoid organs. cDC1s are also characterized by high expression of MHC I. Their main function is cross-presentation and priming of $CD8^+$ T cells [44]. The process of cross-presentation plays a significant role in the defense against viral infections and cancer [45]. Also, cDC1s can present antigens generated during necrosis to T cells in the process dependent on the binding of the lectin Clec9A to the extracellular actin, which is usually present on the cell surface as a result of necrosis [43]. Several types of toll-like receptors (TLRs) such as TLR3, TLR9, and TLR10 are also abundant on cDC1s [44]. These receptors detect nucleic acids, which results in the production of type I interferons (IFNs) and IL-12 [46].

cDC2s are the most abundant subset of the DCs in blood. These cells are characterized by the expression of CD11b. Other common markers used to identify cDC2s are CD172a in the cells present in non-lymphoid tissues and DCIR2 in cDC2s in lymphoid organs. The expression of the following transcription factors is decisive in the differentiation of the DCs to cDC2s: IRF4, KLF4, and ZEB2. cDC2s induce the proliferation of naïve T cells and mediate differentiation of $CD4^+$ T cells towards T_h1, T_h2, T_h17, or T_{reg} phenotype depending on the secreted cytokines. cDC2s poses also a variety of TLRs (TLR2, TLR4-6, TLR8, and TLR9). Stimulation of TLRs on this cell type results in the production of cytokines such as IL-1, IL-6, IL-12, and IL-23 [44]. Also, CD11b expressed on cDC2s was shown to be engaged in TLR4 signaling after LPS stimulation. It was important for the induction of endocytosis as well as the MyD88 signaling pathway as evidenced by the fact that $CD11b^{-/-}$ bone marrow-derived dendritic cells (BMDCs) secreted lower amounts of IL-6 and TNFα. This can lead toward lower activation of T cells [47].

1.3.3 Immune response mediated by macrophages

Among other types of cells expressing CD11b and CD11c are macrophages [5]. They can originate from the yolk sac or blood monocytes. When entering the tissue and differentiating into macrophages, murine monocytes start to express F4/80. F4/80 is a major marker allowing the detection of macrophages in the tissues [48].

The major function of macrophages is the phagocytosis of antigens and apoptotic cells. On the surface of these leukocytes are present pattern recognition receptors (PRRs). They recognize pathogen-associated molecular patterns (PAMPs) such as LPS and peptidoglycan in the cell wall of bacteria. Examples of PRRs are TLRs. Other types of receptors responsible for the recognition and further elimination of microorganisms as well as apoptotic cells via phagocytosis are scavenger receptors. On the other hand, macrophages can recognize not only certain molecules expressed on the surface of the pathogen but also IgGs bound to the antigens presented on the pathogen via FcγRs [28].

Another example of the mechanism mediating the recognition of microorganisms is a coating of the pathogens with C3b and iC3b as a result of the activation of the complement system. As mentioned above, antigens coated with iC3b can be recognized by CR3 or CR4 and then phagocytosed via a type of phagocytosis called “sinking” phagocytosis. This process involves the utilization of actin and microtubule cytoskeletons without the formation of pseudopods. After the recognition of iC3b/C3b-coated antigens via CRs, follows the activation of RhoA GTPase. This results in the polymerization of F-actin. Also, Rho induces accumulation of mammalian diaphanous-related formin 1 (mDia1), which then binds to the microtubule-associated protein cytoplasmic linker protein of 170 kDa (CLIP-170). This leads to the polymerization of actin. After the successful phagocytosis of the antigen, follows the process of phagosome maturation, which consists of several steps. First, the early phagosome is acidified. This is needed for the creation of a suitable environment for the functioning of the enzymes and proteins needed for the destruction of the pathogen as well as the initiation of the antigen-presentation process. The molecules present in the acidified phagosomes are hydrolases, acidic proteases, Ras-associated binding GTPase (Rab GTPase), vacuolar ATPase (V-ATPase), and MHC II. Then, a phagolysosome is formed through its fusion with a lysosome. It is characterized by an acidic and oxidizing environment due to the presence of NADPH oxidizing complexes (NOXs), which generate ROS [49].

Except for the phagocytosis of the antigens, macrophages are also known for the production of a variety of cytokines after the stimulation of their PRRs by antigens. Such stimulation leads to the activation and induction of the NF-κB signaling pathway resulting in the expression of inflammatory cytokines such as TNFα and IL-6 [28].

1.3.4 Role of neutrophils during an immune response

Together with macrophages and DCs, neutrophils belong to professional phagocytes. They play a significant role in the innate immune response during infections and inflammatory diseases. PMNs are the largest population of leukocytes in the blood and they are recruited to the site of the infection in the first place [50].

Neutrophils eliminate pathogens through several mechanisms. Similar to macrophages they can phagocytose them. Among the most abundant receptors which mediate phagocytosis in PMNs are FcγRs and CR3. Activation of TLRs and NOD receptors on neutrophils can stimulate the engulfment of the pathogen. The process of phagocytosis is similar between neutrophils and macrophages, except for the fusion of the maturating phagosome with granules containing myeloperoxidase (MPO) and other antimicrobial proteins, which takes place in neutrophils [51]. Another mechanism of pathogens' elimination is the formation of neutrophil extracellular traps (NETs). NETs consist of chromatin as well as granule proteins including MPO and elastase. Neutrophils also synthesize chemokines (CCL2, CCL3, and CCL4) and cytokines (IL-6, TNFα, and IL-12) responsible for the attraction of other immune cells e.g., macrophages as well as stimulation of immune response [52].

Previous studies have identified several types of neutrophils. For example, in the case of infection with *S. aureus* scientists described two subsets of PMNs: PMN-1 and PMN-2. PMN-1s were shown to express IL-12 and several types of TLRs (TLR2, TLR4, TLR5, and TLR8). They were also $CD49d^{high}$ and $CD11b^{low}$. On the other hand, PMN-2 was characterized by a high expression of IL-10. On its surface were also present TLR7 and TLR9. The cell type was also characterized by low expression of CD49d and high presence of CD11b [53]. In the case of systemic inflammation, the third type of PMN is also present. It is characterized by hypersegmentation of the nucleus as well as by low expression of CD62 and high abundance of CD11b and CD11c on the cell membrane [54].

1.4 Role of CR3 and CR4 in kidney diseases

1.4.1 Immune response in crescentic glomerulonephritis

Among the inflammatory diseases that are known to be affected by the dysregulation of DCs' and T cells' immune response as well as by the activation of the complement system is crescentic glomerulonephritis, also known as rapidly progressive glomerulonephritis. Crescentic glomerulonephritis is a severe form of glomerulonephritis. Usually, the disease starts to develop as a result of the presence of another autoimmune disorder e.g., IgA nephropathy or ANCA-vasculitis, affecting kidneys but in rare cases, it can occur spontaneously. Patients suffering from RPGN have hematuria, low glomerular filtration rate (GFR), high creatinine levels in serum and urine, neutrophil gelatinase-associated lipocalin (NGAL) concentrations in urine, and proteinuria. The hallmark of the disease is the formation of crescents. They consist of infiltrating and proliferating immune cells, such as macrophages/monocytes, T cells as well as parietal and visceral epithelial cells [55].

To study cGN was developed a mouse model of the disease, which is called the nephrotoxic nephritis (NTN) model. It is induced by i.p. injections of nephrotoxic sheep serum (NTS) raised against glomerular antigens of murine kidneys. There are several stages of the development of NTN [56]. During the first few days after NTS injection, innate immune cells are recruited to the kidneys and renal damage occurs. Recruitment of PMNs is mediated by $\gamma\delta$T cells, which secrete IL-17A. Also, priming of the T cells, which are specific for antigens present in NTS, occurs in the lymphoid organs. Then, primed T cells infiltrate the kidneys. During the first 4-7 days dominates mainly the T_h17 immune response [57]. T_h17 cells are distinguished thanks to their high expression of a transcriptional factor RORγT. T_h17 immune response is induced due to the presence of IL-6, TGFβ , and IL-1 in the environment. Another important cytokine needed for the expansion of T_h17 cells is IL-23 [36]. Studies showed that IL-23 $p19^{-/-}$ mice had attenuated symptoms of NTN compared to WT mice [58]. During the later stages of NTN dominates T_h1 immune response induced by already matured DCs. T_h1 cells can secrete IFNγ, which activates macrophages to produce TNFα and nitric oxide (NO). Both molecules promote renal damage. Also, macrophages are strong modulators of fibrosis, which can cause kidney failure [57]. Mice with IFNγ knockout were characterized by lower glomerular damage and a

percentage of crescents after the NTN induction [31]. Two weeks later, autologous antibodies against antibodies present in NTS can be found in the blood of the nephritic mice, which leads to further kidney damage. *In vivo* studies also demonstrated that the adoptive transfer of $CD4^+$ and $CD8^+$ T cells can lead to kidney injury and the development of more severe symptoms of the disease [59].

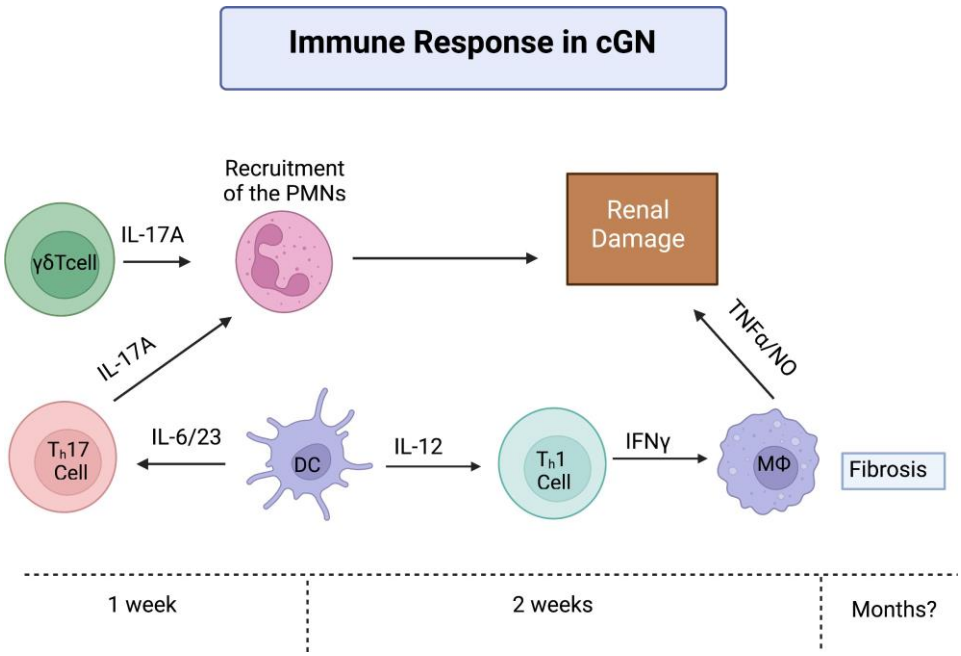


Figure 3. Immune response in cGN. During the first days after the disease induction PMNs are recruited to the kidneys due to the presence of IL-17A produced by γδT cells. Then T_h17 immune response is initiated by DCs leading to the secretion of IL-17A and the attraction of more PMNs. Two weeks later T_h1 immune response starts to dominate. IFNγ secreted by T_h1 cells activates macrophages to produce TNFα and NO leading to renal damage. Months after cGN induction occurs fibrosis. cGN, crescentic glomerulonephritis; DC, dendritic cell; MΦ, macrophage, PMNs, polymorphonuclear cells/neutrophils. The figure was created using BioRender. (Modified from Kurts *et al*, 2013)

1.4.2 Therapeutic strategies in cGN

Without treatment, crescentic glomerulonephritis can progress to terminal kidney failure [57]. Currently, several treatment options are available. Injections of cyclophosphamide are one of the methods to control immune response during RPGN [60]. Cyclophosphamide is a member of alkylating agents. The drug inhibits the proliferation of the cells and has cytotoxic properties, which results in a decreased count of PMNs, lymphocytes, and macrophages in the kidneys. In the case of the immune response, it

was shown to be especially effective in the regulation of T cell-mediated immune response. On the contrary, cyclophosphamide administration results in an increased risk of infections. This drug is often used in the combination with corticosteroids such as prednisone in the treatment of cGN [61].

Glucocorticoids are known immunosuppressors. Their anti-inflammatory effects are connected with their ability to upregulate the expression of anti-inflammatory cytokines e.g., IL-10, or inhibit the synthesis of inflammatory mediators such as TNFα and IL-6 through suppression of NF-κB [62]. Previous studies showed that glucocorticoid receptors are present in podocytes (type of glomerular cells), which means that administration of steroids could be beneficial in case of podocyte injury, which can happen as a result of kidney disease [63]. Although it seems that glucocorticoids should be highly effective in controlling glomerular disorders, they appeared to be not very beneficial in the case of the treatment of C3 glomerulopathy [62].

Another drug that is used to treat cGN is rituximab. Rituximab is a monoclonal antibody that recognizes CD20 in B cells leading to their depletion. The drug was shown to be ineffective when given alone for the treatment of RPGN but in the combination with corticosteroids or cyclophosphamide, it showed promising results [64].

Medications targeting components of the complement cascade are a new class of drugs available for the treatment of glomerular diseases. An example of such a drug is Avacopan, which belongs to the class of small-molecular inhibitors and is an antagonist of C5aR. Avacopan acts by blocking C5a, which is a potent anaphylatoxin attracting PMNs to the sites of the inflammation. The antagonist of C5aR was shown to be effective for the treatment of ANCA-associated vasculitis in the murine model of the disorder [65]. In the randomized clinical trial, those patients with ANCA-associated vasculitis who received Avacopan had lower proteinuria compared to the untreated group. This means that the drug promotes attenuation of the disease [66]. Anti-C5 antibody Eculizumab is another example of a drug type that targets components of the complement system and it is used for the treatment of glomerular diseases. Although studies showed that usage of Eculizumab can help to reduce symptoms in patients with IgA nephropathy and C3 glomerulopathy, the efficacy of the treatment was not very high, and large international clinical trials are still missing. A more rational solution will be to target C3 or the receptors recognizing products of C3 convertase directly. However, currently, no humanized

monoclonal antibodies or small molecules that target these components of the complement cascade exist [65,67,68].

1.4.3 Immune response in urinary tract infections

Urinary tract infections (UTIs) belong to one of the most common infections worldwide. UTIs are more frequent in women. According to the statistics, 40-50% of women get infected with uropathogens during their life [69]. People with a light form of the infection may not develop any symptoms but in some cases, UTIs can lead to the development of pyelonephritis (infection of kidneys), or even urosepsis, which can be lethal [70].

Most often the disease is caused by uropathogenic *Escherichia coli* (UPEC) [71]. To invade the host, UPEC uses a wide range of virulence factors among which are flagella, fimbriae, siderophores, and several others. Fimbriae and flagella are important for the motility and adhesion of *E. coli* to uroepithelium, which is important for the efficient colonization of the host organism. Siderophores belong to the iron-acquisition system of bacteria. These molecules are responsible for iron uptake, which is important for the metabolism of UPEC. Uropathogenic *E. coli* is also known to create biofilms, which makes it more difficult for antibiotics to kill the microorganism since the biofilm prevents penetration of the drugs. It is also known that UPEC can survive intracellularly inside some cell types, evading elimination through immune cells [72,73].

To fight UTIs, the immune system has developed several strategies, including the release of antimicrobial peptides, chemokines, cytokines, and ROS after the activation of innate immune cells such as macrophages/monocytes and neutrophils through TLRs e.g., TLR4 [74].

Among the cells playing an important role in the progression of UTIs are macrophages. Previous studies demonstrated the existence of two types of these cells, which were beneficial during cystitis. $Ly6C^-$ macrophages, that reside in the kidneys, after encountering UPEC produce chemokines such as CXCL1, CCL2, and macrophage inhibitory factor (MIF) to attract PMNs and monocytes, which can further differentiate to inflammatory $Ly6C^+$ macrophages. Inflammatory macrophages are a source of TNFα, which can induce the expression of CXCL2 by tissue-resident macrophages. CXCL2, in

turn, stimulates the secretion of MMP9. Release of MMP9 facilitates migration of PMNs through uroepithelium towards the site of the infection [75]. On the contrary, another study showed that inflammatory macrophages cause disproportional to the severity of the infection inflammation that leads to renal damage [76]. Also, UPEC was demonstrated to be able to reside inside macrophages. Some strains of uropathogenic *E. coli* express phage shock protein (*Psp*) genes, which enable microorganisms to withstand the acidification of phagosomes, which allows them to survive and further replicate inside phagocytes [77].

One of the studies on pyelonephritis also demonstrated that UPEC influences NLRP3 inflammasome activation, which leads to the decreased release of IL-1β. This can result in increased survival of bacteria inside uroepithelium [78].

Opposite to macrophages, neutrophils were shown to be protective against bacterial pyelonephritis. Disbalance in the ratio between neutrophils and macrophages, and depletion of neutrophils led to the worsening of the infection due to increased inflammation [76]. Neutrophils can kill the bacteria through phagocytosis, and oxidative stress, caused by ROS, as well as by the formation of extracellular traps [50].

The complement system is also activated due to the presence of UPEC. Although it could be expected that activation of the complement cascade would be beneficial during UTIs, it was shown that $C3^{-/-}$ mice had attenuated symptoms of pyelonephritis. Also, kidney epithelial cells took up less *E. coli* when they were treated with C3-deficient serum. This result points to the detrimental role of the complement in this disease since UPEC can utilize epithelial cells as a niche for survival [79]. Another example of the benefits of complement inhibition is the fact that $C5aR^{-/-}$ mice had a lower presence of bacteria in kidneys compared to WT animals, which is explained by lower infiltration of kidneys with monocytes/macrophages [80].

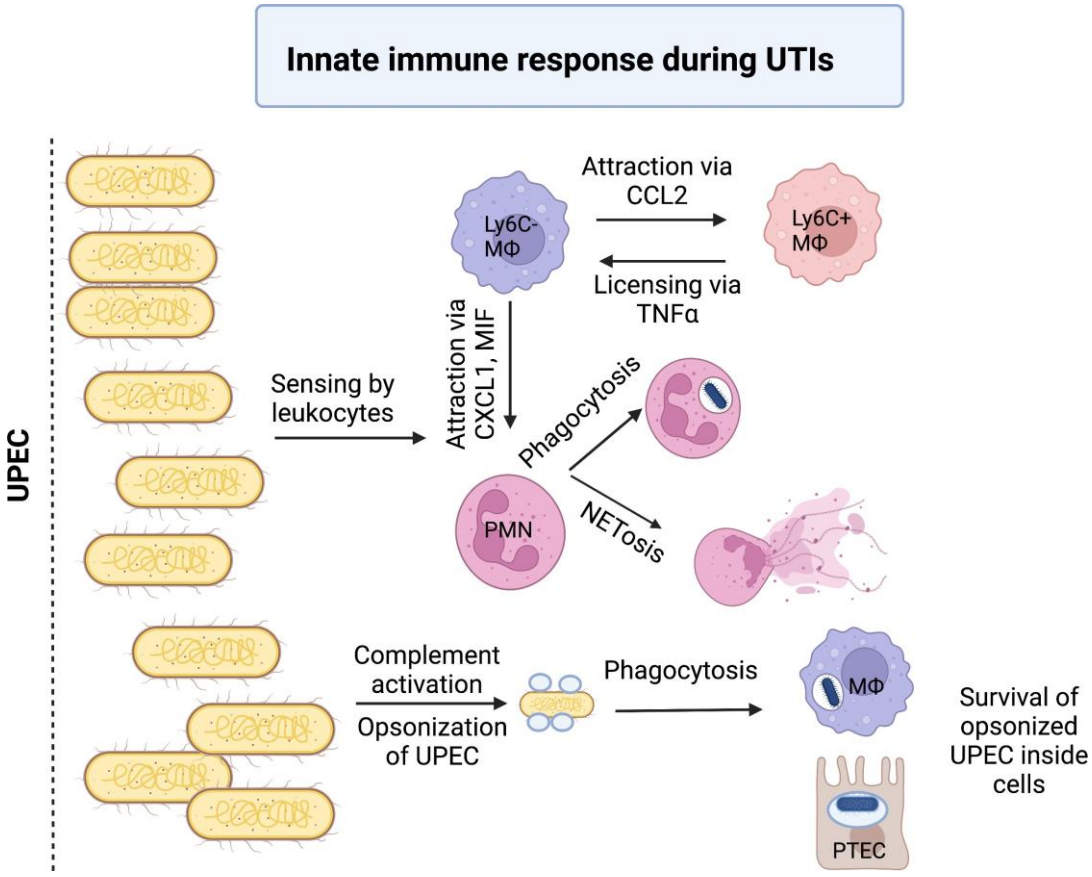


Figure 4. Innate immune response in pyelonephritis. Recognition of UPEC by Ly6C$^-$ macrophages leads to the increased CCL2 secretion and attraction of Ly6C$^+$ monocytes, which can further differentiate into macrophages. Production of CXCL1 and MIF by Ly6C$^-$ macrophages results in the infiltration of the site of the infection with PMNs. PMNs can then phagocytose UPEC and eliminate the bacteria, or produce NETs. Infection with UPEC can also lead to complement activation. Opsonization of the pathogen with the complement components helps macrophages and PTECs to phagocytose bacteria, where they can further survive and replicate. MΦ, macrophage, PMNs, polymorphonuclear cells/neutrophils; PTEC, renal proximal tubular epithelial cell; UPEC, uropathogenic *E. coli*. The figure was created using BioRender. (Modified from Schwab *et al*, 2017)

On the contrary, the adaptive immune response is not very well investigated in the context of UTIs. One of the studies showed that T cells transfer from mice with pyelonephritis to uninfected animals prevented them from developing severe disease after the infection [81]. Also, it is known that antibodies against uropathogenic *E. coli* can limit the binding of the bacteria to host cells [82].

1.4.4 Treatment of pyelonephritis

Pyelonephritis is usually treated with antibiotics such as fluoroquinolone and cephalosporine, and antipyretics e.g., ibuprofen. The main issue with antibiotics treatment of pyelonephritis is the increasing development of bacterial resistance to these drugs. Another problem is connected to the fact that UTIs are often recurrent [83]. To overcome this obstacle more attention is drawn toward the development of alternative treatment methods for the disease. Vaccines targeting siderophores were tested in a murine model of UTIs and several of them appeared to be effective. Among promising targets for vaccines are iron uptake transport aerobactin receptor (IutA) and siderophore receptor iron-responsive element A (IreA) [84].

Another treatment strategy for pyelonephritis could be targeting the complement system since previous studies showed the detrimental role of this part of the immune system during UTIs. However, currently, no clinical trials of inhibitors of the complement system or monoclonal antibodies against complement factors for the treatment of UTIs are conducted.

Aim

The following project aimed to evaluate the influence of the β_2-integrins CD11b and CD11c on the course of the following renal diseases: crescentic glomerulonephritis and pyelonephritis.

CD11b and CD11c are known to form together with CD18 complement receptors 3 and 4. CR3 and CR4 are characterized by high homology, which results in the ability of both receptors to recognize common ligands. Examples of their ligands include iC3b, ICAM-1, and fibrinogen. Widely used for studies of β_2-integrins, the $CD18^{-/-}$ mouse line has differences in the immune system compared to WT animals already in the steady state, which makes it not reliable. Due to the fact mentioned above, first, we needed to create a mouse line deficient in both integrins that has an immune system, which is not different from the one of WT animals and can be used e.g., for the evaluation of the importance of CR3 and CR4 in inflammatory diseases.

Since CR3 and CR4 recognize complement fragments that were shown to participate in the development of kidney autoimmune diseases as well as in kidney infections, the next aim of the project was to evaluate the progression and immune response in crescentic glomerulonephritis and pyelonephritis in *Itgam*$^{-/-}$*Itgax*$^{-/-}$ mouse line compared to WT animals. This study can also give preliminary hindsight into the possibility of the usage of CR3, CR4, and the ligands of the receptors mentioned before as targets for the treatment of complement-dependent kidney disorders.

2. Materials and Methods

2.1 Materials

2.1.1 *In vivo* treatment		
Product	**Manufacturer**	**Comment**
Cobra venom Factor (CVF)	Quidel	-
Nephrotoxic sheep serum (NTS)	Sigma-Aldrich	-
Ovalbumin (OVA)	Sigma-Aldrich	-
Ovalbumin-AF647 (OVA-647)	Life Technologies	-
PolyI:C	Sigma-Aldrich	-

2.1.2 *In vitro* experiments and cell culture	
Product	**Manufacturer**
ACK lysing buffer	Lonza
β-mercaptoethanol	Lonza
$CD4^+$ T cells Isolation Kit	Miltenyi
CD45 MicroBeads	Miltenyi
CFSE	Sigma-Aldrich
Collagenase IV	Sigma-Aldrich
DNase	Sigma-Aldrich
Fetal Bovine Serum (FBS)	Sigma-Aldrich
Gentamicin	Sigma-Aldrich
LB-agar	Sigma-Aldrich
M-SCF	R&D Systems
Mouse Complement Serum	Antibodies Online
Naïve $CD4^+$ T cells Isolation Kit	Miltenyi
Neutrophil Isolation Kit	Miltenyi
Pan Dendritic Cell Isolation Kit	Miltenyi
Penicillin-Streptomycin	Sigma-Aldrich
RPMI 1640 Medium, GlutaMAX, HEPES	Thermofisher

2.1.3 Flow cytometry			
Product	**Manufacturer**	**Clone (if applicable)**	**Color (if applicable)**
B220	Biolegend	RA3-6B2	PE
Calibrate APC beads	BD		APC
C5aR	Biolegend	20/70	APC
CD4	Biolegend	GK1.5	PerCP-Cy5.5

CD8	Biolegend	53-6.7	APC
CD8	Biolegend	53-6.7	BV510
CD25	Biolegend	PC61	Pacific Blue
CD11b	Biolegend	M1/710	PECy7
CD11c	BD Biosciences	HL3	BUV737
CD19	Biolegend	6D5	APC-Cy7
CD44	Biolegend	IM7	FITC
CD45	Biolegend	30-F11	BUV395
CD80	Biolegend	16-10A1	FITC
CD86	Biolegend	GL1	AF700
CD103	Biolegend	2E7	PE
CD172a	Biolegend	P84	PE/Dazzle594
DCIR2	Biolegend	33D1	PE
F4/80	Biolegend	BM8	PerCP-Cy5.5
Fc block	Sigma-Aldrich	-	-
Fixable Viability Dye	eBioscience	-	eFluor 780
GolgiPlug	BD Biosciences	-	-
GolgiStop	BD Biosciences	-	-
IFNγ	Biolegend	XMG12	PE
IL-17A	Biolegend	TC11-18H10.1	APC
Ionomycin	Sigma Aldrich	-	-
Ly6c PerCPCy5.5	Biolegend	AL-21	FITC
Ly6g	Biolegend	1A8	BV510
MHCII	Biolegend	M5/114.15.2	AF700
NK1.1	Biolegend	PK136	APC-Cy7
Perm/Wash buffer	eBioscience	-	-
PMA	Sigma Aldrich	-	-
One Compensation Beads	eBioscience	-	-
TCRβ	Biolegend	H57-597	BV711
Thy1.2	Biolegend	30-H12	APC-Cy7
Vα_2	Biolegend	B20.1	Pacific Blue
Vβ_5	Biolegend	MR9-4	PE

2.1.4 Microscopy		
Product	**Manufacturer**	**Comment**
Acetic acid	Roth	-
B220 in PE	Biolegend	Clone RA3-6B2
CD3 in APC	Biolegend	Clone 17A2
Ethanol 99.9%	Roth	-

Hematoxylin	Roth	-
IHC Zinc Fixative	BD Biosciences	-
37% Hydrochloric acid	Roth	-
1% Periodic acid	Roth	-
Picric acid	Sigma-Aldrich	-
Roti Hystokit	Roth	-
Schiff's reagent	Roth	-
Sirius red	Sigma-Aldrich	-
Gelatin from cold water fish skin	Sigma-Aldrich	-
Normalized mouse sera	Invitrogen	-
PAP pen	Sigma-Aldrich	-
Tissue-Tek O.C.T. Compound	Sakura	-
Xylol	Roth	-

2.1.5 qRT-PCR		
Product	**Comment**	**Manufacturer**
cDNA Reverse Transcription Kit	-	Life Technologies
β-actin primers	5'-ACCCGCGAGCACAGCTTCTTTG-3' (sense) 5'-ACATGCCGGAGCCGTTGTCGAC-3' (antisense)	Invitrogen
CD11b primers	5'-ATGGACGCTGATGGCAATACC-3' (sense) 5'-TCCCCATTCACGTCTCCCA-3' (antisense)	Invitrogen
CD11c primers	5'-CTGGATAGCCTTTCTTCTGCTG-3' (sense) 5'-GCACACTGTGTCCGAACTCA-3' (antisense)	Invitrogen
Col1a1 primers	Cat-NR QT00162204	Qiagen
Col3a1 primers	5'-GGTGGTTTTCAGTTCAGCTATGG-3' (sense) 5'-CTGGAAAGAAGTCTGAGGAATG-3' (antisense)	Invitrogen
IL-1β primers	Cat-NR QT01048355	Qiagen
IL-6 primers	5'-GCCACTCCTTCTGTGACTCCAGC-3' (sense) 5'-AGACAAAGCCAGAGTCCTTCAGAGA-3' (antisense)	Invitrogen

IL-12 primers	5'-GGA AGC ACG GCA GCA GAA TAA-3' (sense) 5'-CTT GAG GGA GAA GTA GGA ATG-3' (antisense)	Invitrogen
IL-17A primers	5'-CTCCAGAAGGCCCTCAGACTAC-3' (sense) 5'-AGCTTTCCCTCCGCATTGACACAG-3' (antisense)	Invitrogen
IL-23 primers	Cat-NR qMmuCED0045759	BioRad
IFNγ primers	5'-AGTGGCATAGATGTGGAAGAAA-3' (sense) 5'-GACCTCAAACTTGGCAATACTC-3' (antisense)	Invitrogen
NLRP3 primers	Cat-NR QT00122458	Qiagen
NucleoSpin RNA Kit	-	Macherey-Nagel
SYBR Green	-	Life Technologies

2.1.6 Assessment of markers of renal damage and antibodies in kidney homogenates, serum, and urine	
Product	**Manufacturer**
Albumin ELISA	Bethyl
Anti-IgG antibody	Sigma-Aldrich
BSA (bovine serum albumin)	Sigma-Aldrich
Creatinine PAP FS kit	DiaSys
LEGENDplex	Biolegend
Mouse Lipocalin-2/NGAL Quantikine ELISA Kit	R&D Systems
OPD substrate	Sigma-Aldrich
Streptavidin-HRP	Sigma-Aldrich
Urea CT FS kit	DiaSys

2.2 Methods

2.2.1 Generation of *ItgamItgax*$^{-/-}$ mouse line

The *ItgamItgax*$^{-/-}$ mouse line was created in cooperation with prof. Marco Herold in the core facility MAGEC of the WEHI in Australia. The mouse line was generated using CRISPR/Cas9 technology. To delete sequences of *Itgam* and *Itgax* genes (located on chromosome 7 side by side), two sgRNAs with the following sequences were designed: TAACCCTGATGGTTCGGGCC and CTCCCAATAGTTTCGGTATC. For deletion was targeted 89, 984 bp of genomic sequence. First, Cas9 and both sgRNAs were injected into single embryos with a C57BL/6 background. Then, these embryos were transferred into female mice. After the birth, pups were checked for the presence of the deleted allele. For the detection of the deleted allele of F0 mice following primers were used: 5'-ACCAGCCTGATCCGAAACAC-3' (sense) and 5'-GTGCTGCTTGTGGGAACTTG-3' (antisense). PCR conditions were the following:

- 95°C, 2 min
  - 95°C, 30 sec
  - 60°C, 30 sec X35
  - 72°C, 30 sec
- 72°C, 5 min

To detect the WT sequence, the following primers were used: 5'-AGGGAGAGGTAGGAAGAGGC-3' (sense) and 5'-CTGCACCAGTGAGAGAGAGC-3' (antisense). PCR was performed under the conditions listed below:

- 95°C, 2 min
  - 95°C, 30 sec
  - 60°C, 30 sec X35
  - 72°C, 30 sec
- 72°C, 5 min

Material from the pups with a confirmed knockout of *Itgam* and *Itgax* was then used for next-generation sequencing to validate the deletion of the genes of interest. Mice in which deletions were confirmed were backcrossed with WT animals for 2 generations to

breed out potential off-target mutations. Afterward, offspring were intercrossed and further bred homozygously.

2.2.2 Data analysis of the gene array

Analysis of *Itgam* and *Itgax* expression in human kidney glomeruli was done using open databases from the European cDNA bank cohort, the Nephrotic Syndrome Study Network, and the Vasculitis Clinical Research Consortium [85]. Data were downloaded from NCBI (GSE104948) and analyzed by prof. dr. med. Sibylle von Vietinghoff.

2.2.3 *In vivo* experiments

Eight- to ten-week-old, age-matched male and female mice were used for the induction of nephrotoxic nephritis. In the case of the induction of pyelonephritis, exclusively female mice were used. Animals were either purchased from Janvier Labs or bred at the Haus for Experimental Therapy (HET) in the University Hospital in Bonn.

2.2.4 Nephrotoxic nephritis (NTN)

Mice were injected i.p. with 30 µl/ BW of NTS, which was previously diluted with PBS in the ratio 3:1. Injections were performed 2x on day 0 with the interval of 6 hours. On day 9 mice were placed into metabolic cages for 24 h to collect urine for further analysis. Ten days after NTN induction mice were sacrificed. During the time of the experiments, animals were monitored daily and their weight was measured on the daily basis.

2.2.5 Pyelonephritis

For the induction of pyelonephritis, the model established by Tittel A *et al* was used [86]. Uropathogenic *E. coli* (strain 536) was grown in LB medium for 5 hours at 37°C with shaking (180 rpm). After the incubation, bacteria were centrifuged (20°C, 10 min, 4000 rcf) and resuspended in 2 ml of PBS. The bacterial suspension was diluted 100x for the measurement of OD. To calculate the number of bacteria, it was assumed that OD=1 contains 10^9 *E. coli*. The concentration of bacteria was set to 10^{11} *E. coli*/ml. After the bacterial suspension was prepared, female mice were anesthetized using isoflurane and 100 µl of previously prepared suspension of *E. coli* was administered transurethrally. Three hours later mice were infected a second time. Animals were euthanized by carbon

dioxide after certain time points and the organs, plasma, and urine were collected for further analysis.

2.2.6 *In vivo* treatments with the compounds

To look into the differences between T cell-mediated immune response *in vivo*, WT and *ItgamItgax*$^{-/-}$ mice were injected with 5x10^4 naïve CD4$^+$ T cells isolated from the spleens of OT-II mice with Naïve CD4$^+$ T Cells Isolation Kit (Miltenyi) according to the manufacturer's protocol. The day after, a mixture of polyI:C together with OVA was prepared. For this purpose, polyI:C was first heated at 65°C for 10 min and then transferred on ice. Afterward, it was mixed with OVA in PBS. Each mouse was injected with 100 µg of polyI:C and 200 µg of OVA. One week later, the spleens of the studied mice were collected and stained with antibodies against CD45, TCRβ, and CD4 as well as with the antibodies that recognize Vα_2 and Vβ_5 (markers of OT-II cells). To determine the activity of CD4$^+$ T cells antibodies against CD44 and CD25 were used.

For the evaluation of the antigen uptake by dendritic cells, first, mice were injected with NTS as described in section 2.2.4. Ten days after the induction of NTN, animals received i.v. 500 µg/BW of OVA labeled with AF647. Thirty minutes later kidneys of the studied mice were collected for further flow cytometric analysis [87].

Cobra venom factor (Quidel) was injected into the mice i.p. in the concentration 0.8 µg/BW in 100 µl of PBS one day before induction of the NTN and later every other day till the day of final analysis [88].

2.2.7 Evaluation of colony forming units (CFUs)

On the day of the final analysis, kidneys were collected and then transferred into 1 ml sterile PBS. Afterward, collected organs were homogenized with the help of Ultra Turrax homogenizer (Sigma-Aldrich). Then, serial dilutions of the samples ranging from 1x to 10^{4}x were prepared and plated onto the LB agar plates. LB agar plates with diluted kidney homogenates were cultured for 24 h at 37°C. On the next day, CFUs were evaluated by manual counting.

2.2.8 Flow cytometric analysis

For the flow cytometric analysis, kidneys were collected and then transferred into a 24-well plate (Thermo Fisher Scientific) filled with RPMI 1640 Medium, GlutaMAX, HEPES (Thermo Fisher Scientific) containing 10% FBS (Sigma-Aldrich), DNase (Sigma-Aldrich) in the concentration 100 µg/ml, and collagenase (Sigma-Aldrich) in the concentration 1 mg/ml. Then, the organs were gently homogenized with a plunger and incubated at 37°C for 45 min. After the incubation, homogenates were resuspended by pipetting up and down and filtered into 15 ml falcons through 100 µm filters (BD Bioscience). To rinse filters, 1 ml of PBS was used. In the case of renal draining lymph nodes, cells were transferred into the 1.5 ml tubes. The samples were centrifuged at 300 rcf at 4°C for 5 min. After centrifugation was completed, cells from the kidneys were resuspended in 3 ml of PBS and 100 µl of the cell suspension was plated onto a round bottom 96-well plate (Thermo Fisher Scientific). Cells from renal draining lymph nodes were resuspended in 250 µl of PBS. The cell suspensions from collected organs were washed with FACS buffer (PBS, 1% BSA, and 0,1% NaN_3). In the case of the spleen, erythrocytes were lysed for 3 min at RT in 1 ml of ACK lysing buffer (Lonza). The lysis was stopped by adding 2 ml of PBS. Afterward, the cells were stained in 50 µl of FACS buffer that contained Fc blocker (Sigma-Aldrich) as well as appropriate antibodies at 4°C for 15 min in the darkness. After the incubation, the cells were washed with 150 µl of FACS buffer and resuspended in 150 µl of FACS buffer or FACS fixation buffer (PBS, 1% BSA, 1% formaldehyde, and 0,1% NaN_3), if the samples were going to be analyzed 24 h later. Samples were further measured by BD LSRFortessa (BD Biosciences) and analyzed using FlowJo software, version 10.4.2.

Cell types were identified based on the markers presented in the table below.

Cell type	Markers
B cells	$CD45^+CD172a^-B220^+$
$CD4^+$ T cells	$CD45^+CD172a^-TCR\beta^+CD8^-CD4^+$
$CD8^+$ T cells	$CD45^+CD172a^-TCR\beta^+CD4^-CD8^+$
cDC1s in kidney and renal draining LN	$CD45^+CD19^-Thy1.2^-NK1.1^-MHCII^{middle/high}F4/80^-CD172a^-CD103^+$
cDC1s in spleen	$CD45^+CD19^-Thy1.2^-NK1.1^-MHCII^{middle/high}F4/80^-DCIR2^-CD8^+$

cDC2s in kidney and renal draining LN	$CD45^+CD19^-Thy1.2^-NK1.1^-MHCII^{middle/high}F4/80^-CD103^-CD172a^+$
cDC2s in spleen	$CD45^+CD19^-Thy1.2^-NK1.1^-MHCII^{middle/high}F4/80^-CD8^-DCIR2^+$
Macrophages	$CD45^+CD19^-Thy1.2^-NK1.1^-MHCII^{middle/high}F4/80^+$
Monocytes	$CD45^+CD19^-Thy1.2^-NK1.1^-MHCII^{middle/high}F4/80^-Ly6C^+$
PMNs	$CD45^+CD19^-Thy1.2^-NK1.1^-MHCII^-Ly6C^{middle}Ly6G^+$

Table 1. Phenotyping of immune cells in different organs.

In case of measurements of cytokines expression via flow cytometry, cells were first incubated for 5 h at 37°C, 5% CO_2 in 200 µl of RPMI 1640 Medium, GlutaMAX, HEPES containing 10% FBS, PMA in the concentration of 5 [ng/ml], ionomycin in the concentration of 500 [ng/ml], GolgiPlug (BD Biosciences) in the concentration of 4.2 µl/600 µl of medium and GolgiStop (BD Biosciences) in the concentration of 6.1 µl/600 µl of the medium. After the incubation cells were washed and fixed in 150 µl of FACS fixation buffer and incubated for 24 h, at 4°C. On the next day, they were washed with FACS buffer and further permeabilized with Perm/Wash buffer (eBioscience) for 20 min at RT in the darkness. After 20 min cells were washed again and stained for 40 min at RT in the darkness in 50 µl of Perm/Wash buffer. When the incubation time was over additional 150 µl of Perm/Wash buffer was added to appropriate wells and cells were incubated for 5 min more. Subsequently, they were centrifuged (4°C, 300 rcf, 5 min), resuspended in 100 µl of FACS buffer, and measured on the same day using BD LSRFortessa.

2.2.9 Assessment of markers of renal damage and antibodies in kidney homogenates, serum, and urine

To collect urine mice were placed for 24 h into the metabolic cages. For the assessment of the albuminuria Mouse Albumin ELISA Kit (Bethyl) according to the manufacturer's recommendations was used. Creatinine in urine was measured as described in the manufacturer's protocol with the Creatinine PAP FS kit (DiaSys). To evaluate NGAL concentration in urine the following kit was used: Mouse Lipocalin-2/NGAL Quantikine ELISA Kit (R&D Systems). All procedures were performed as recommended by the manufacturer.

For the evaluation of anti-*E. coli* antibodies, cytokines' production as well as the concentration of creatinine in serum, and blood was collected from the hearts of studied mice. Then it was allowed to clot at RT for 2 h. Afterward, collected samples were centrifuged for 20 min at 4°C and 2000 rcf. Titers of anti-*E. coli* antibodies were measured by ELISA as described in section 2.2.14. Creatinine was measured with a Creatinine PAP FS kit (DiaSys) as recommended by the manufacturer. For the evaluation of the presence of certain cytokines in mouse serum LEGENDPlex (Biolegend) assay was performed according to the manufacturer's protocol. Samples were measured with BD FACSCanto II (BD Biosciences) and analyzed with LEGENDPlex Data Analysis Software (Biolegend).

2.2.10 Light microscopy

Kidneys for the microscopic analysis were put into 500 µl of IHC Zinc Fixative (BD Biosciences) and incubated overnight at RT. On the next day, they were transferred to 70% ethanol. Subsequently, organs were processed with STP 120 Spin Tissue Processor (ThermoScientific): using the following scheme 2x 70% ethanol for 1 h –> 80% ethanol for 1 h –>95% ethanol for 1 h –> 3x 99.9% ethanol for 1.5 h –> 3x xylene for 1.5 h –> 2x paraffin wax (60°C) for 2 h. Afterward, kidneys were embedded into paraffin blocks using modular paraffin embedding station (Myr EC 350). Kidney blocks were cut with Leica microtome HM355S. The thickness of the samples was 4 µm. Subsequently, samples were stained with PAS staining protocol to evaluate crescents' formation or with the Sirius red staining protocol for the assessment of fibrosis.

In the case of PAS staining samples were stained according to the following scheme: 3x xylol for 5 min –> 3x 99.9% ethanol for 5 min –> 2x 96% ethanol for 5 min –> 2x 70% ethanol for 5 min –> 3x deionized water for 5 min –> 1% periodic acid for 15 min –> washing under tap water for 2 min –> washing in deionized water –> Schiff's reagent for 40 min –> washing under tap water for 7 min –> washing in deionized water –> staining of the nuclei with hematoxylin for 45 sec –> washing under tap water for 2 min –> washing in deionized water –>samples were checked under the microscope to confirm staining of the nuclei –> 70% ethanol for 5 min –> 3x 96% ethanol for 5 min –> 3x 99.9% ethanol for 5 min –> 3x xylol for 5 min. After PAS staining was completed, coverslips were mounted with Roti Hystokit (Roth). Samples were evaluated for the crescents' formation in collaboration with prof. dr. med. Ulf Panzer from the Center for Internal Medicine at the University Hospital in Hamburg.

For the Sirius red staining the following scheme was used: 3x xylol for 5 min –> 3x 99.9% ethanol for 5 min –> 2x 96% ethanol for 5 min –> 2x 70% ethanol for 5 min –> 3x deionized water for 5 min –>samples were stained with Sirius red solution (0.1% Sirius red in 500 ml of picric acid) for 1 h –> washing 2x with acidified water (0.5% acetic acid in 1 L of deionized water) –> 3x dipping into 99.9% ethanol –> xylol for 10 min. After the staining was completed, coverslips were mounted with Roti Hystokit (Roth). Afterward, samples were evaluated under the microscope Olympus IX71. The area affected by fibrosis was assessed using ImageJ software (version 2.0.0-rc-69/1.52p).

2.2.11 Confocal microscopy

Collected spleens and lymph nodes were incubated overnight at 4˚C in PLP buffer (pH 7.4), which consisted of 1% PFA, 0.106 g of $NaIO_4$, 18.75 ml of L-Lysine solution, and 18.75 ml of 0.1 M P-buffer (81 ml of M Na_2HPO_4, 19 ml of M NaH_2PO_4 and 100 ml of H_2O). On the next day, the samples were washed twice with 0.1 M P-buffer. Afterward, the organs were transferred into 30% sucrose in a P-buffer and incubated overnight at 4˚C. After the incubation spleens and lymph nodes were put into cryomolds with Tissue-Tek O.C.T. Compound (Sakura). Then, they were frozen and stored at -80˚C till further preparation. Cryosections with a thickness of 7 µm were cut with a cryostat (Leica, CM1520) and transferred onto the glass slides. The samples were stored at -20˚C. On the day of the staining samples were dried for 10 min and then circled with a PAP pen (Sigma-Aldrich). Afterward, they were rehydrated using 0.1 M TRIS-HCl buffer (pH 7.4) for 10 min at RT. After the incubation, blocking buffer (1% BSA, 1% gelatin from cold water fish skin (Sigma-Aldrich), 1% normalized mouse sera (Invitrogen), and 0.3% Triton-X100 (Sigma-Aldrich) in 0.1 M TRIS-HCl buffer) was added onto the sections for 1 h, at RT. Then, a master mix containing 1:100 anti-B220 antibody in PE (Biolegend) and 1:100 anti-CD3 antibody in APC (Biolegend) in blocking buffer was prepared and subsequently transferred onto the slides. Samples were incubated overnight in a humid chamber at 4˚C. On the next day, they were washed 3x for 5 min with 0.1 M TRIS-HCl buffer. Afterward, 30 µl of Fluoromount (Sigma-Aldrich) was added to the slides and then they were covered with cover glasses. The samples dried overnight at RT and were stored at 4˚C until further analysis by LSM 780 microscope (Zeiss).

2.2.12 Real-time PCR analysis

To extract RNA from kidney samples, a Nucleo Spin RNA kit (Macherey-Nagel) was used. RNA extraction was performed according to the manufacturer's protocol. The concentration of RNA was measured by Nanodrop (Thermo Fischer Scientific). The next step was to generate cDNA. For this purpose, High-Capacity cDNA Reverse Transcription Kit (Life Technologies) was utilized according to the manufacturer's protocol. For the reverse transcription from RNA to cDNA, the following scheme was used: 25°C for 10 min –> 37°C for 120 min –> 85°C for 5 min. Afterward, the cDNA was diluted five times. For the qPCR, the following mix was prepared: 5 µl of SYBR Green (Life Technologies), 1 µl of each 10x diluted primer, 3 µl of DEPEC water, and 1 µl of cDNA. Relative expression of the genes of interest was measured by QuantStudio 6 Flex Real-Time-PCR-Systeme (Thermo Fischer Scientific) using the following scheme: 50°C for 2 min –> 95°C for 10 min –> 45x 60°C for 1 min –> 4°C limitless. As a reference gene was chosen gene encoding β-actin.

2.2.13 3'mRNA sequencing of $CD4^+$ T cells

First, $CD45^+$ cells were isolated from the kidneys of nephritic WT and *ItgamItgax*$^{-/-}$ mice using CD45 MicroBeads (Miltenyi) by magnetic sorting. Next, cells were stained with anti-CD45, anti-CD172a, anti-TCRβ, and anti-CD4 antibodies. After staining, they were sorted using FACSAria III (BD Biosciences). $CD4^+$ T cells were defined as $CD45^+CD172a^-$ $TCR\beta^+CD4^+$. After the sorting was completed, RNA was isolated using RNeasy Micro Kit (Qiagen) according to the recommendations of the manufacturer. The concentration of RNA was measured by Nanodrop (Thermo Fischer Scientific). Libraries were prepared using QuantSeq FWD 3´-mRNA-Seq Kit (Lexogen). The 3'mRNA sequencing was performed in the UKB NGS core facility, Bonn on the NovaSeq 6000. The length of the read was 1x100bp.10 M raw reads were generated per sample. To align FASTQ files StarAligner was used. Analysis of 3'mRNA sequencing data was performed using Bioconductor. Differentially expressed genes were determined using DESeq2. Data were analyzed by dr. Jian Li.

2.2.14 ELISA for anti-*E. coli* antibodies

First, a high-binding 96-well plate (Sarstedt) was coated with the suspension

containing 5x10^8 UV-inactivated *E. coli* strain 536 in a volume of 200 µl/well. The plate was then incubated for 75 min at 37°C. In the meantime, samples were diluted in the blocking buffer (PBS + 1% BSA). After the incubation, the plate was washed three times with the washing buffer (PBS + 0.05% Tween20). Samples were put into the plate in the volume of 100 µl/well and incubated for 1 h at 37°C. Afterward, the plate was washed three times with the washing buffer. Then, 100 µl of anti-IgG antibody diluted 10^4x in the blocking buffer was put into the plate, and incubated for 2 h at 37°C. After the incubation plate was washed again three times with the washing buffer and 100 µl/well of streptavidin-HRP, which was previously diluted 5000x in the blocking buffer was added. The plate was incubated for an additional 30 min at RT and then washed three times with washing buffer. After this step was completed 100 µl of OPD substrate was put into each well. Two minutes later the reaction was stopped by adding a stopping solution (50% H_2SO_4 in deionized water). Absorbance was measured at the wavelength 450 nm with the reference wavelength 540 nm using Safire^2, Tecan (Thermo Fisher Scientific).

2.2.15 Evaluation of OT-II cells proliferation in co-culture with dendritic cells

Dendritic cells were isolated from the spleen of WT and *ItgamItgax*$^{-/-}$ mice with Pan Dendritic Cell Isolation Kit (Miltenyi) according to the manufacturer's protocol. Then, cells were resuspended in RPMI 1640 Medium, GlutaMAX, and HEPES containing 10% mouse complement serum (Antibodies Online) or 10% heat-inactivated mouse complement serum and counted. Dendritic cells were then seeded into a U-bottom 96-well plate (Thermo Fischer Scientific) in the density 5x10^4/200 µl/well. Afterward, OVA was added into the appropriate wells in the final concentration of 500 µg/ml. Subsequently, cells were incubated for 2.5 h at 37°C, 5% CO_2.

In the meantime, $CD4^+$ T cells were isolated from the spleen of the OT-II mouse with $CD4^+$ T cells Isolation Kit (Miltenyi) according to the manufacturer's protocol. After the isolation, cells were washed with 15 ml of PBS and then resuspended in 10 ml of PBS containing 10 µl of CFSE (Sigma-Aldrich). $CD4^+$ T cells were incubated for 10 min at 37°C in the water bath in the darkness. After the incubation, the reaction was stopped with 40 µl of ice-cold FBS. Cells were centrifuged for 5 min at 300 rcf, at 4°C. Afterward, they were resuspended in 1 ml RPMI 1640 Medium, GlutaMAX, HEPES containing 10% FBS, and 1% β-mercaptoethanol and counted.

After 2.5 h, the plate with the dendritic cells was centrifuged for 5 min at 300 rcf, at 4°C, and then washed with PBS. Later, 10^5 $CD4^+$ T cells were added to the appropriate wells. Dendritic cells and $CD4^+$ T cells were co-cultured for 3 days at 37°C, 5% CO_2. After this time point, cells were stained with antibodies. Samples were then measured using BD LSRFortessa and analyzed with FlowJo software, version 10.4.2.

2.2.16 Evaluation of the ability of neutrophils to phagocytose and kill *E. coli*

Neutrophils were isolated from the bone marrow of WT and *ItgamItgax*$^{-/-}$ mice with a Neutrophil Isolation Kit (Miltenyi) according to the manufacturer's protocol. For the evaluation of the phagocytosis capacity of both groups of neutrophils, 10^5 neutrophils were cultured in RPMI 1640 Medium, GlutaMAX, HEPES containing 20% Mouse Complement Serum (Antibodies Online) for 3 h with $2.5x10^6$ heat-killed *E. coli* expressing GFP and then measured using LSRFortessa. Phagocytosis efficacy was evaluated based on the value of geometric mean fluorescent intensity (gMFI) of GFP in neutrophils. The percentage of neutrophils containing *E. coli* expressing GFP was also assessed.

To evaluate the number of viable intracellular bacteria, the gentamycin protection assay was performed. In this case, 10^5 neutrophils were infected with $2x10^5$ *E. coli* and then cultured for 2 h at 37°C, 5% CO_2. After the incubation, the cells were washed with PBS, and a medium containing 50 µg/ml gentamicin (Sigma-Aldrich) was added to kill extracellular bacteria. Neutrophils were further incubated for 1 h longer at 37°C, 5% CO_2. Then, cells were washed with PBS and lysed using sterile deionized water. Subsequently, serial dilutions of the cell lysates were prepared and then they were plated onto the LB-agar plates. Plates with the lysates were further cultured at 37°C for 24 h. On the next day, CFUs were manually counted.

2.2.17 Evaluation of the ability of bone marrow-derived macrophages (BMDMs) to kill *E. coli*

First, cells from the bone marrow (BM) of WT and *ItgamItgax*$^{-/-}$ mice were isolated. For this purpose, BMs from both groups of mice were centrifuged at 10 000 rcf for 45 sec at 4°C, and then resuspended in 1 ml of PBS and filtered through a 40 µm sterile cell strainer (BD Biosciences). The cells were centrifuged again at 300 rcf for 5 min at 4°C and resuspended in 1 ml of ACK lysing buffer (Lonza) to lyse erythrocytes. After 3 min, the

reaction was stopped by adding 2 ml of PBS. Isolated cells were centrifuged again under the same conditions and resuspended in 1 ml of PBS. Subsequently, they were filtered through a 70 µm sterile cell strainer (BD Biosciences) and centrifuged, as described before. Afterward, the cells were resuspended in RPMI 1640 Medium, GlutaMAX, and HEPES containing 10% FBS and 1% Penicillin-Streptomycin. The cells were counted and 2 mln of cells/well were seeded into a 24-well plate (Thermo Fischer Scientific). To each well 2 ng/ml of M-CSF (R&D Systems) was added to differentiate monocytes into macrophages. The cells were ready for the experiments one week later.

On the day of the experiment, cells were washed with PBS, and the medium was changed to RPMI 1640 Medium, GlutaMAX, HEPES containing 20% Mouse Complement Serum (Antibodies Online). Then *E. coli* was added to the appropriate wells in the MOI 1:10. BMDMs were then cultured for 2 h at 37°C, 5% CO_2. After the incubation, the cells were washed with PBS, and a medium containing 50 µg/ml gentamicin (Sigma-Aldrich) was added to kill extracellular bacteria. The cells were incubated for 3 h longer at 37°C, 5% CO_2. Then, they were washed with PBS and lysed with sterile deionized water. Subsequently, serial dilutions of the lysates were prepared and then plated onto the LB-agar plates. Plates with cell lysates were further cultured at 37°C for 24 h. On the next day, CFUs were counted manually.

2.2.18 Statistical analysis

Provided results are presented as mean ± SEM. First, the results were checked for the presence of outliers with the Grubbs test. If present, outliers were excluded from further analysis. Before calculations of the statistical significance, the Shapiro-Wilk normality test was performed to check whether the results were distributed normally. In the case of normal distribution, statistical significance was calculated with Student's t-test. When the results were not distributed normally, the Mann-Whitney test was used. For the comparison of three or more groups, statistical significance was evaluated with a one-way ANOVA test. All the calculations were performed in GraphPad Prism, version 9. Statistical significance was marked as: * - $P<0.05$; **- $P<0.01$; ***- $P<0.001$; **** - $P<0.0001$.

3. Results

3.1 Generation of *ItgamItgax*$^{-/-}$ mice and characterization of their immune system

As discussed before, CD11b and CD11c together with CD18 form CR3 and CR4. Both of the receptors have several common ligands including iC3b, fibrinogen, and ICAM-1 [5]. This means that it would be beneficial to study their role in the progression of inflammatory disorders together. However, until this time point, there was no mouse line without CD11b and CD11c available. To overcome this problem, many researchers used CD18$^{-/-}$ mice, although this solution has several disadvantages. First, CD18 forms heterodimers not only with CD11b and CD11c but also with CD11a and CD11d. Second, mice with CD18 knockout are characterized by an elevated number of neutrophils, smaller peripheral lymph nodes, and splenomegaly already in a steady state, which can affect experiments and influence their results due to the initial differences in the immune response [89]. Thus, we decided to generate a new mouse line, which lacks genes encoding CD11b and CD11c.

3.1.1 B and T cell zones are present in lymphoid organs of *ItgamItgax*$^{-/-}$ mice. *ItgamItgax*$^{-/-}$ mice are not characterized by splenomegaly

Since other mouse lines which lack β_2-integrins e.g., CD18$^{-/-}$ mice are characterized by defective lymph nodes and splenomegaly [89], first, we looked at whether it is also true for *ItgamItgax*$^{-/-}$ animals. For this purpose, we isolated inguinal LNs from this mouse line and stained them with anti-CD3 and anti-B220 antibodies to visualize B and T cells zone in this organ. We also performed the same procedure with the spleen.

Microscopic analysis revealed the presence of well-defined B and T cell zones in inguinal LNs and spleen of *ItgamItgax*$^{-/-}$ mice. Representative pictures of both organs are presented below (Fig. 5A and B). When we measured the weights of the spleen, we did not identify significant differences between knockout and WT animals (Fig. 5C). This means that *ItgamItgax*$^{-/-}$ animals do not have splenomegaly, which is characteristic of CD18$^{-/-}$ mice.

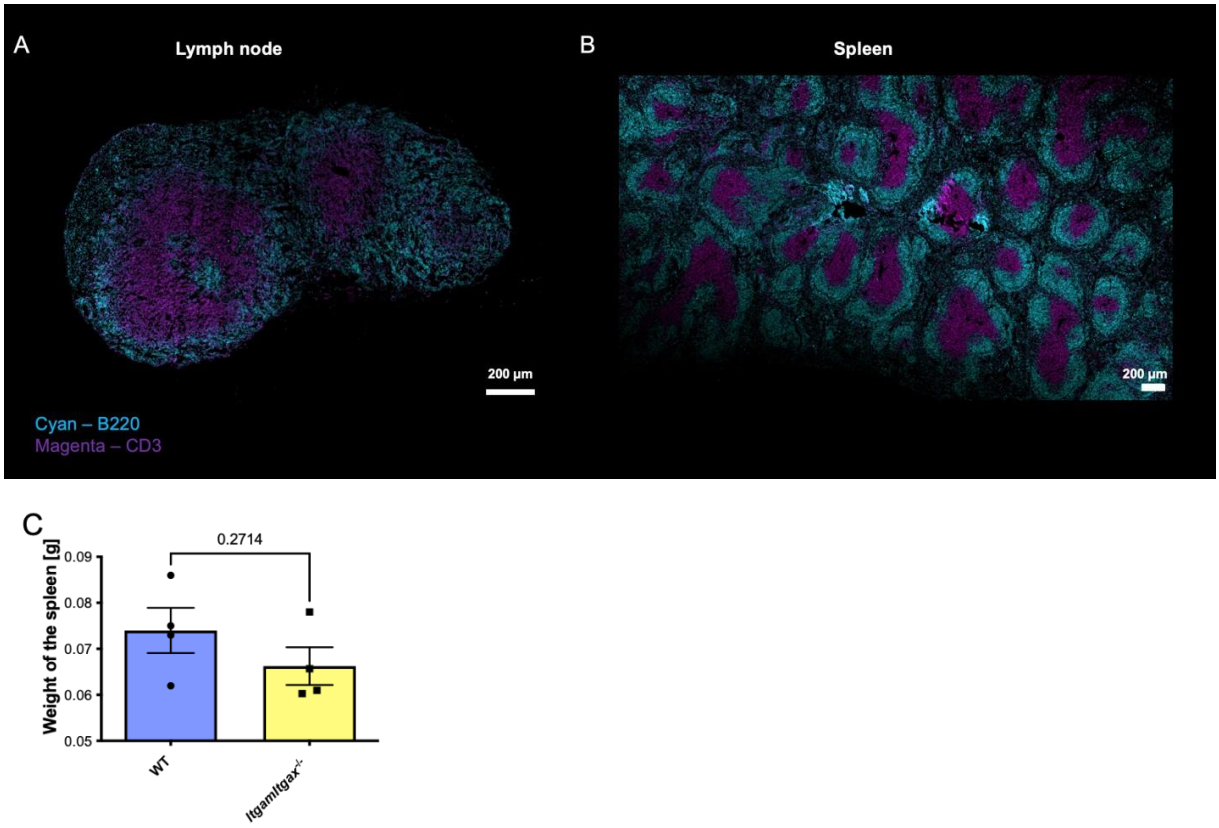


Figure 5. **B and T cell zones are present in LN and spleen of *ItgamItgax*$^{-/-}$ mice**. **Splenomegaly is not present in *ItgamItgax*$^{-/-}$ animals.** (A) Representative picture of B and T cells zone of the lymph node of *ItgamItgax*$^{-/-}$ mice, (B) representative picture of B and T cells zone of the spleen of *ItgamItgax*$^{-/-}$ mice, (C) weights of the spleen of WT and *ItgamItgax*$^{-/-}$ mice. Data are presented as mean ± SEM and analyzed using an unpaired t-test.

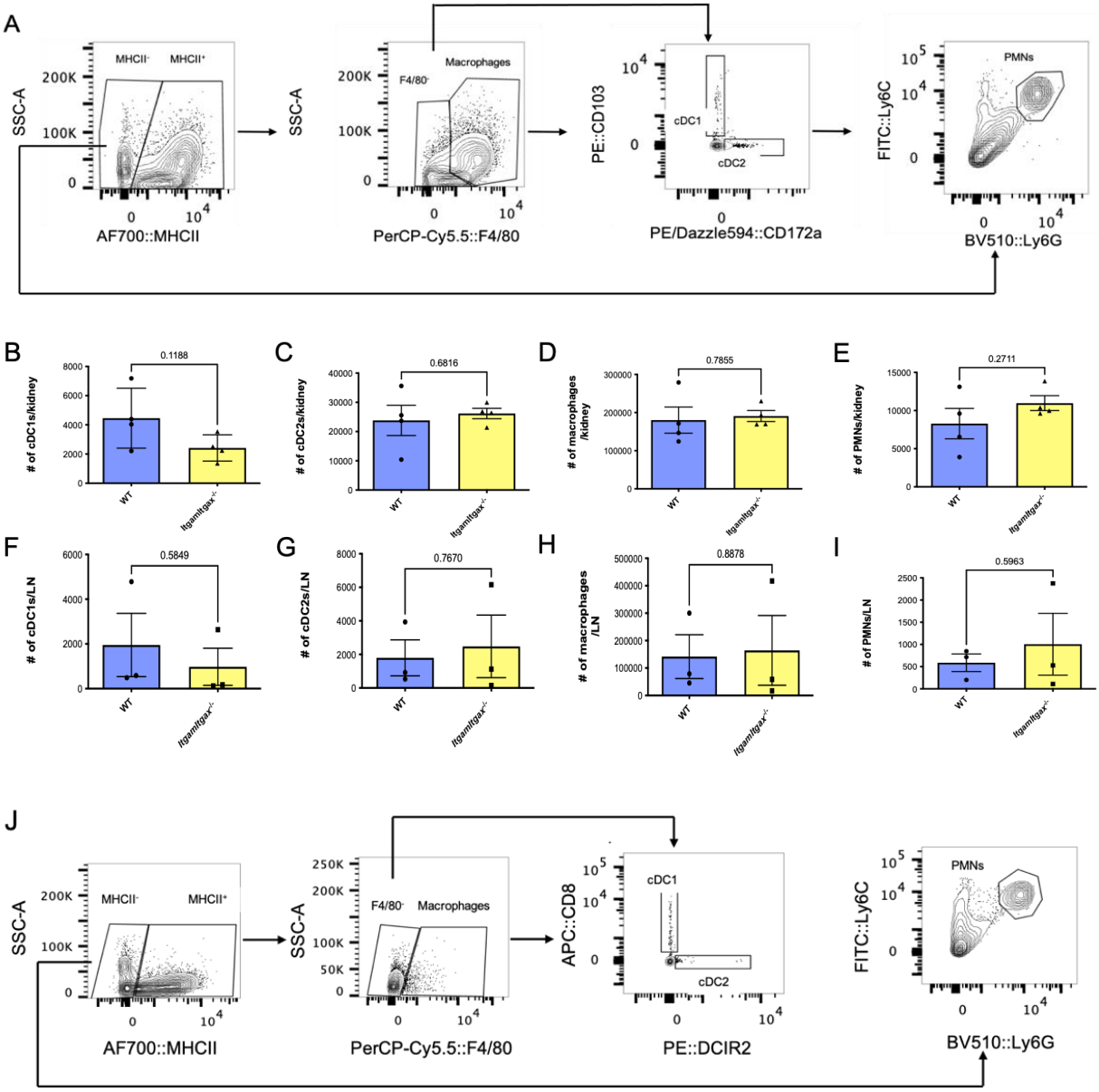
3.1.2 Immune cell composition of *ItgamItgax*$^{-/-}$ mice is similar to those in WT animals

To investigate whether knockout of CD11b and CD11c influences the composition of the immune cells in different organs, we used our newly generated *ItgamItgax*$^{-/-}$ mouse line and performed FACS analysis of the populations of immune cells in several organs.

Since *ItgamItgax*$^{-/-}$ mice lack CD11b and CD11c, it is not possible to use these molecules as markers of DCs in the studied mouse line. To overcome this obstacle, we used CD103 as a marker for cDC1s in kidneys and LNs (Fig. 6A) and CD8 – in the case of the spleen (Fig. 6J). CD172a and DCIR2 were used as markers of cDC2s in kidneys, LNs (Fig. 6A), and spleen (Fig. 6). The usage of these markers to identify cDC1s and CD2s was initially proposed by Fabio F. Rosa *et al* [90].

Flow cytometric analysis revealed that the *ItgamItgax*$^{-/-}$ mouse line exhibits a similar number of myeloid and lymphoid immune cells as WT mice in kidneys, renal-draining lymph nodes, and spleen (Fig. 6A-X).

The obtained results indicate that *ItgamItgax*$^{-/-}$ mice can be a suitable line to study the influence of CD11b and CD11c on the immune response in the kidneys, LNs, and spleen.



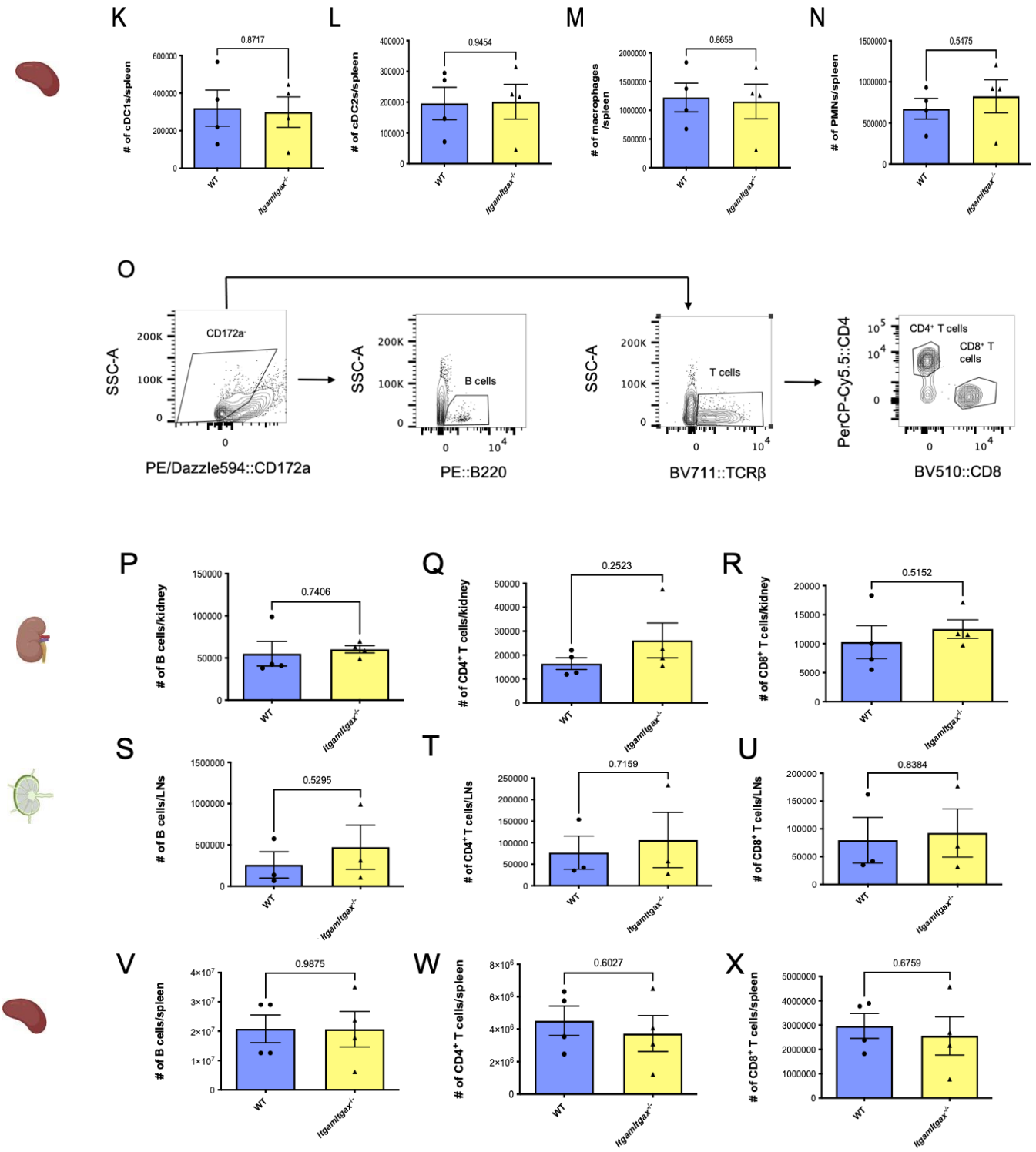


Figure 6. Characterization of the immune system in *ItgamItgax*$^{-/-}$ mice. (A) Gating strategy for myeloid immune cells in the kidney and lymph nodes, (B-I) numbers of myeloid immune cells in the kidney and kidney-draining lymph node measured via flow cytometry, (J) gating strategy for myeloid immune cells in spleen, (K-N) numbers of myeloid immune cells in spleen, (O) gating strategy for

lymphoid immune cells (P-X) numbers of lymphoid immune cells in the kidney, kidney-draining lymph node, and spleen. Data are presented as mean ± SEM and analyzed using an unpaired t-test. LN, lymph node; DCs, dendritic cells; PMNs, polymorphonuclear leukocytes or neutrophils. *P<0.05.

3.1.3 Expression of cytokines in *ItgamItgax*$^{-/-}$ mice in steady-state

Since CD11b can regulate T_{reg}/T_h17 balance and inhibit the production of IL-6 in animal models of inflammatory diseases such as rheumatoid arthritis [91], we decided to look at whether *Itgam* and *Itgax* knockout can lead to higher production of IL-6 and IL-17A as well as IFNγ, TNFα , and IL-10. For this purpose, we took plasma from healthy mice and performed a LEGENDplex assay.

Results obtained during the assay revealed no differences in the concentration of studied cytokines in the plasma between healthy WT and *ItgamItgax*$^{-/-}$ mice. This means that the lack of CD11b and CD11c does not influence immune reactions in steady-state (Fig. 7A-E), which makes the *ItgamItgax*$^{-/-}$ mouse line suitable for studies of the role of CR3 and CR4 in the immune response.

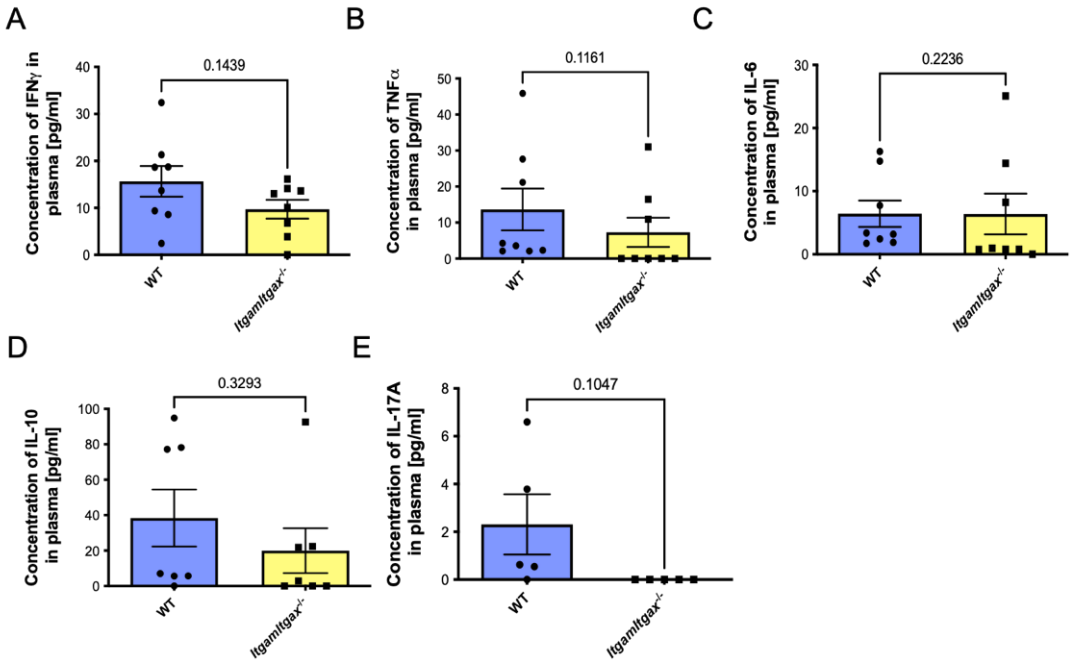


Figure 7. Production of cytokines in *ItgamItgax*$^{-/-}$ mice compared to WT animals in steady-state. (A-E) Concentrations of cytokines in plasma measured by LEGENDplex assay. Data are presented as mean ± SEM and analyzed using Mann-Whitney or an unpaired t-test dependent on the distribution of the data.

3.1.4 Characterization of the induction of adaptive immune response in *ItgamItgax*$^{-/-}$ mouse line

To check whether CD4^{+} T cell-mediated immune response is altered in *ItgamItgax*$^{-/-}$ mouse line, we transferred OVA-specific CD4^{+} T cells (OT-II cells) into the knockout and WT mice one day before the immunization. On the next day, we immunized studied mice with OVA with the addition of polyI:C to stimulate DCs and 7 days later evaluated the proliferation of OT-II cells in the spleen as well as the activation of CD4^{+} T cells based on the expression of the activation markers CD25 and CD44 (Fig. 8A). Here, we focused on cDC2s because this cell type expresses both CD11b and CD11c, compared to cDC1s, and influences CD4^{+} T cell immune response.

We observed no significant difference in the proliferation of OT-II cells between *ItgamItgax*$^{-/-}$ and WT mice (Fig. 8B) as well as in their activation based on CD44 and CD25 expression (Fig. 8C-F), which brings us to the conclusion that lack of CD11b and CD11c does not influence T_h immune response after OVA/polyI:C stimulation.

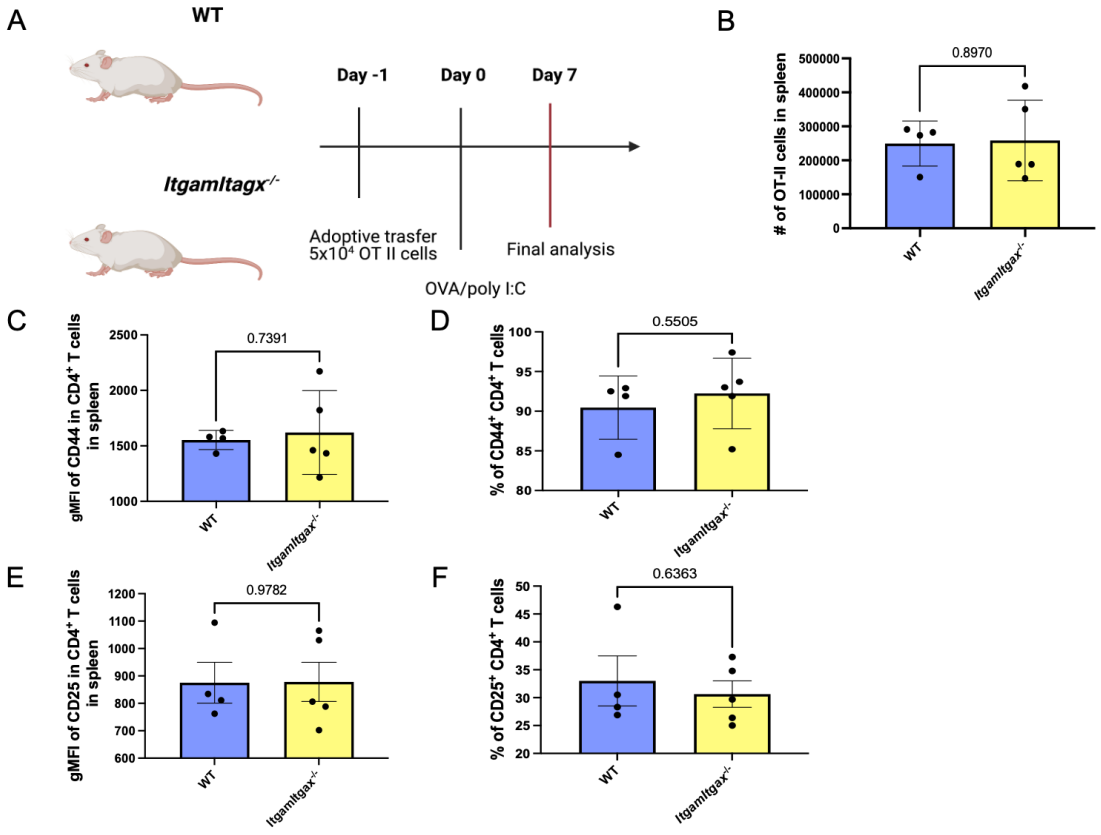


Figure 8. Comparison of the immune response of *ItgamItgax*$^{-/-}$ and WT mice. (A) Experimental

setup, (B) a count of OT-II cells in the spleen evaluated by flow cytometry, (C and D) expression of CD44 in $CD4^+$ T cells, (E and F) expression of CD25 in $CD4^+$ T cells. Data are presented as mean ± SEM and analyzed using an unpaired t-test. gMFI, geometric mean fluorescent intensity.

3.2 *Itgam* and *Itgax* in crescentic glomerulonephritis

Dysregulation of the complement system was shown in several studies to be responsible for the onset and progression of chronic kidney diseases. One of the proposed drugs for the treatment of C3 glomerulopathy is the monoclonal antibody Eculizumab. The antibody targets specifically C5a and it has already been approved by FDA. Unexpectedly, in the clinical studies, injections of Eculizumab did not result in a significant improvement in the condition of patients [67]. However, blocking C3 convertase as well as receptors that recognize C3 and products of the C3 convertase can be a more efficient strategy in the treatment of patients suffering from kidney diseases that are characterized by the dysregulation of the complement system. Despite this, no effective medicines that target this part of the complement cascade have been approved yet for the treatment of glomerulonephritis and we still lack studies on this topic. Hence, we asked whether targeting CR3 and CR4 could result in attenuation of one of the types of glomerulonephritis, which is crescentic glomerulonephritis, using our newly generated *ItgamItgax*$^{-/-}$ mouse line.

3.2.1 *Itgam* and *Itgax* genes are upregulated in crescentic glomerulonephritis

First, using publicly available data from global RNA-sequencing of the kidneys of patients suffering from different types of glomerulonephritis we evaluated the expression of *Itgam* and *Itgax* in their glomeruli.

Results obtained during the analysis showed that both *Itgam* and *Itgax* genes were significantly upregulated in glomeruli of patients suffering from diabetic nephropathy, IgA nephropathy, systemic lupus erythematosus and rapidly progressive glomerulonephritis (Fig. 9A and B), which confirms the connection between kidney glomerular diseases and changes of the expression of the studied genes.

Since *Itgam* and *Itgax* were upregulated in patients with RPGN, we decided to investigate whether in the murine model of nephrotoxic nephritis, a widely studied model of RPGN, the same effect could be observed. Ten days after injection of nephrotoxic

sheep antiserum specific for murine glomerular components, the kidneys of mice were collected and relative expression of *Itgam* and *Itgax* was measured by qRT-PCR (Fig. 9C). As expected, both genes were significantly upregulated in murine kidneys, which indicates the relevance of these genes also in a murine model of the disease (Fig. 9D-H).

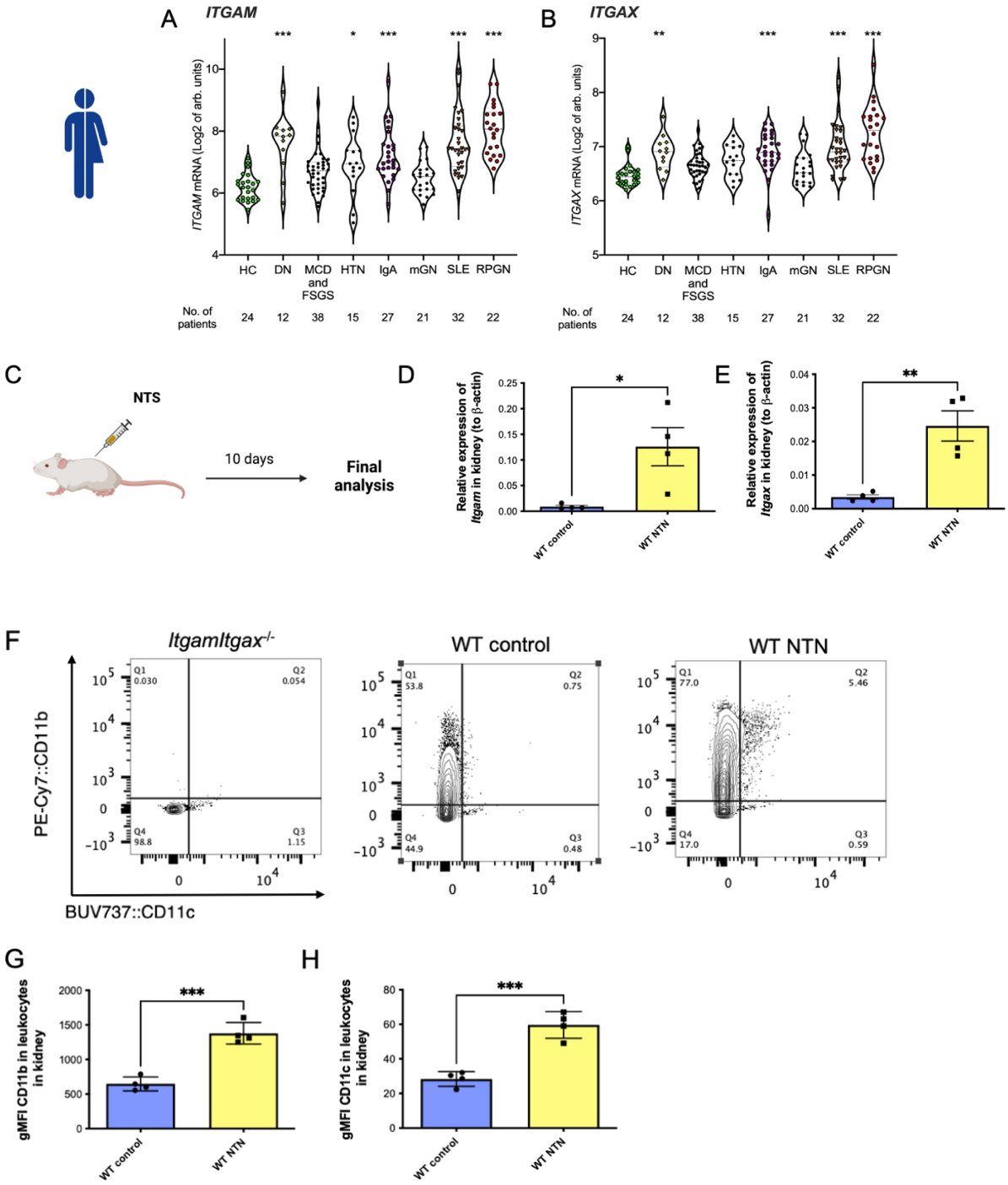


Figure 9. *Itgam* and *Itgax* genes are upregulated in glomerular diseases. (A, B) Expression of *Itgam* and *Itgax* in the glomeruli of patients, (C) experimental plan, (D and E) relative expression of *Itgam* and *Itgax* in kidneys of WT mice measured via qRT-PCR and represented as ∂C_t values, (F-H) gMFI of CD11b and CD11c in leukocytes. NTS, nephrotoxic serum; NTN, nephrotoxic nephritis; HC, healthy control; DN, diabetic nephropathy; MCD, minimal change disease; FSGS, focal segmental glomerulosclerosis; HTN, hypertension; mGN, membranous glomerulonephritis; SLE, systemic lupus erythematosus; RPGN, rapidly progressive glomerulonephritis. Data are presented as mean ± SEM and analyzed using one-way ANOVA or an unpaired t-test. *P<0.05, **P<0.01, ***P<0.001. Analysis of *Itgam* and *Itgax* expression in patients was performed by prof. dr. med. Sibylle von Vietinghoff.

3.2.2 Nephrotoxic nephritis is attenuated in *ItgamItgax*$^{-/-}$ mice

To investigate the exact impact of CD11b and CD11c on the progression of NTN, we induced the disease by injection of NTS into *ItgamItgax*$^{-/-}$ and WT mice. After 10 days post-injection, we obtained kidneys, urine, and serum for the evaluation of kidney damage during the disease (Fig. 10A).

Our results indicate that a lack of *Itgam* and *Itgax* improves the outcome of the mice as evidenced by decreased crescents' formation. Crescents are formed due to the high proliferation of parietal epithelial cells as well as immune cells and are the main hallmarks of the disease (Fig. 10B). Albumin-to-creatinine ratio (ACR) was also significantly lower in knockout mice in comparison to WT animals (Fig. 10C). Concentration of NGAL (another common marker of renal damage) showed a tendency toward a decrease in *ItgamItgax*$^{-/-}$ mice (Fig. 10E). However, level of creatinine in serum remained the same in WT and knockout animals (Fig. 10D). We also failed to identify differences in the severity of fibrosis between WT and *ItgamItgax*$^{-/-}$ mice as evidenced by data obtained via histological analysis of samples stained with Sirius red (Fig. 10F) as well as relative expression fibrosis-related genes: *Col1a1* and *Col3a1* (Fig. 10G and H).

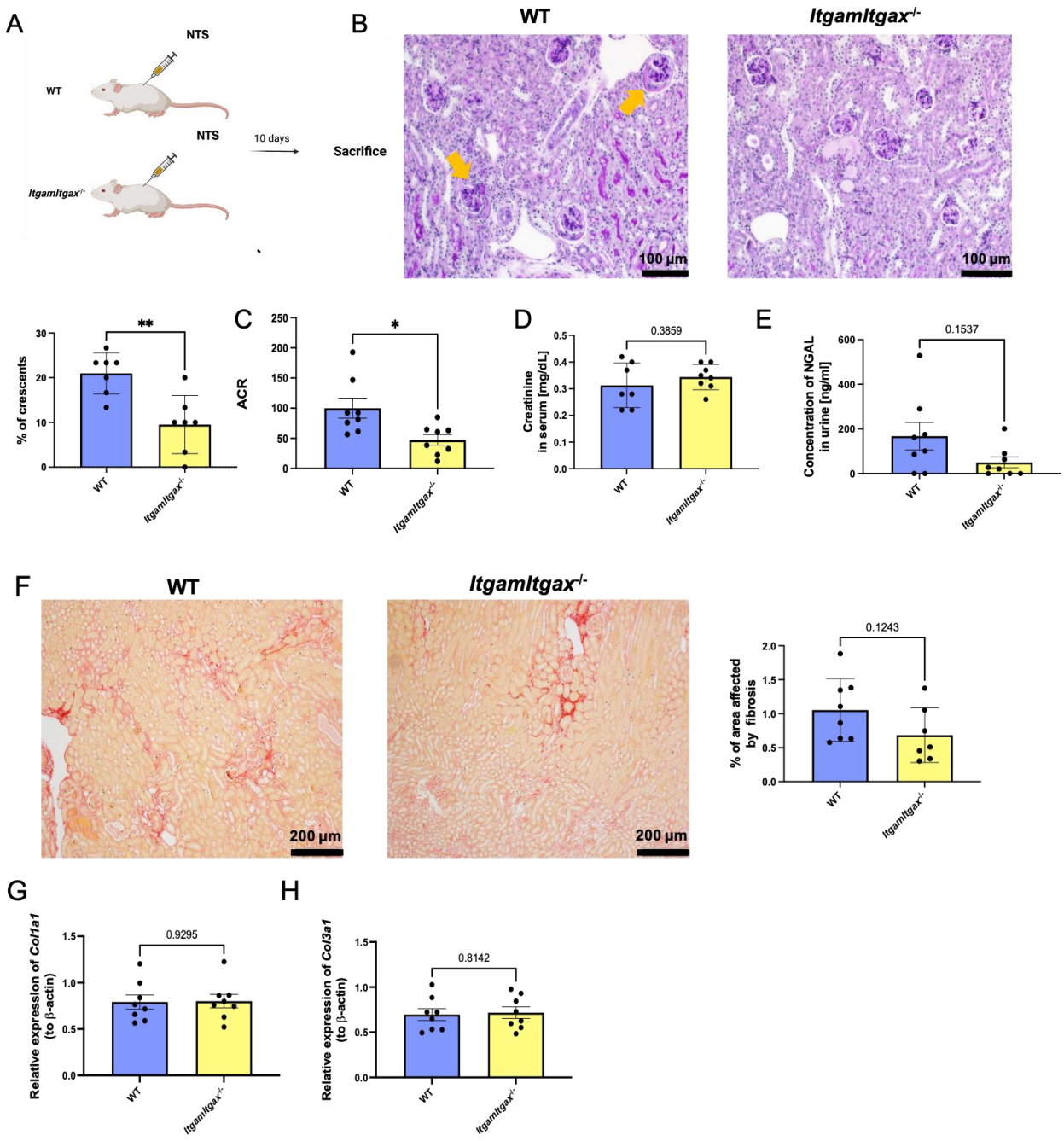


Figure 10. **Nephrotoxic nephritis is attenuated in *Itgamltgax*$^{-/-}$ mice**. (A) Experimental plan, (B) representative periodic acid–Schiff–stained glomeruli and percentage of crescentic glomeruli, (C) ACR, (D) creatinine in serum, (E) NGAL in urine measured via ELISA, (F) representative Sirius red-stained renal sections and percentage of area affected by fibrosis, (G and H) relative expression of *Col1a1* and *Col3a1* in kidneys of WT mice measured by qRT-PCR and represented as ∂C_t values. The percentage of crescents was analyzed by prof. dr. med. Ulf Panzer from the University Hospital in Hamburg. Performed together with dr. Fernando Magdaleno Verduzco. Data are presented as

mean ± SEM and analyzed using an unpaired t-test. ACR, albumin-to-creatinine ratio; NTS, nephrotoxic serum. *P<0.05; **P<0.01.

In summary, the presented data suggest that upregulation of *Itgam* and *Itgax* is pathological in the progression of crescentic glomerulonephritis, and therapy targeting both genes could be beneficial in the treatment of the studied disease.

3.2.3 Selective decrease in the inflammation in *ItgamItgax*$^{-/-}$ mice during NTN

The next step of the project was to check whether the immune response to nephrotoxic serum is attenuated in *ItgamItgax*$^{-/-}$ mice, which could explain the better outcome of these animals after induction of nephrotoxic nephritis. To evaluate the immune response of studied animals, we performed a flow cytometric analysis of immune cell populations as well as an analysis of the expression of inflammatory genes in the kidneys of the diseased mice 10 days post-NTS injection.

Flow cytometric analysis revealed a significant decrease in the cell number of cDC2s and CD4^{+} T cells in knockout mice compared to WT animals, which could suggest decreased inflammatory response in their kidneys (Fig. 11B and G). The abundance of other types of immune cells in kidneys was not changed between *ItgamItgax*$^{-/-}$ and WT mice after NTN induction (Fig. 11A, C, D, E, and H). This indicates that the main players in the development of NTN are, indeed, cDC2s and CD4^{+} T cells.

Analysis of the relative expression of several genes encoding inflammatory cytokines in the kidneys of the diseased mice did not reveal any significant differences between *ItgamItgax*$^{-/-}$ and WT animals (Fig. 11I-M), although the tendency toward a decrease in the expression of IL-23 in the samples from knockout mice was observed (Fig. 11J).

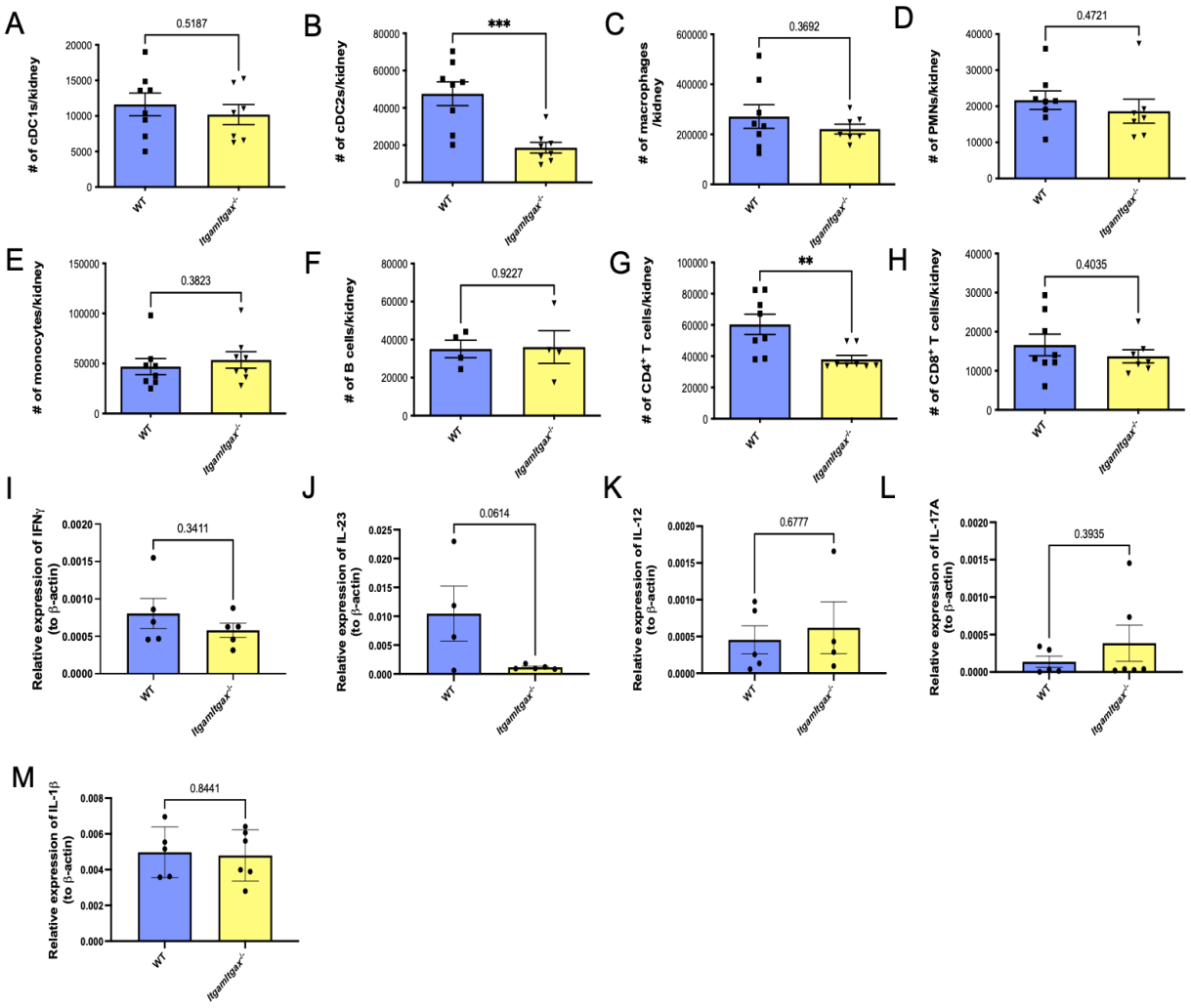


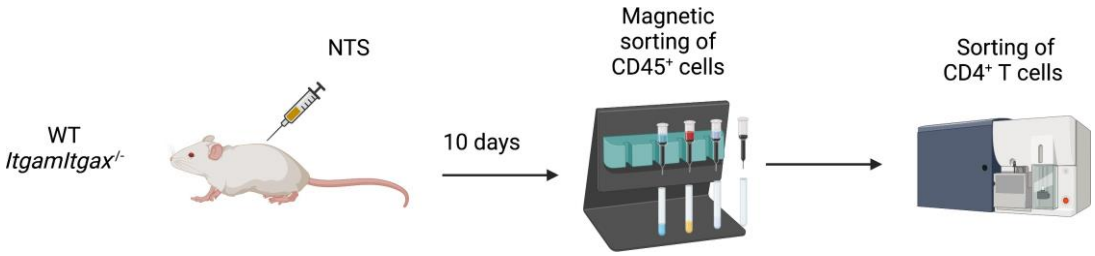
Figure 11. **Inflammation is lower in *ItgamItgax*$^{-/-}$ mice**. (A-H) A count of immune cells in the kidney evaluated by flow cytometry, (I-M) relative expression of inflammatory cytokines in kidneys measured via qRT-PCR and represented as ∂C_t values. Data are presented as mean ± SEM and analyzed using an unpaired t-test. NTS, nephrotoxic serum; DCs, dendritic cells; PMNs, polymorphonuclear leukocytes or neutrophils. **P<0.01, ***P<0.001.

3.2.4 Nephritic WT mice are characterized by selective activation of IL-1β signaling in CD4$^+$ T cells compared to *ItgamItgax*$^{-/-}$ animals

Since in the previous experiments, we could not identify significant differences in the expression of inflammatory mediators in the whole kidneys, we decided to perform 3'mRNA sequencing of CD4$^+$ T cells of nephritic WT and *ItgamItgax*$^{-/-}$ animals. We induced NTN in the mice as described before and after 10 days sorted CD4$^+$ T lymphocytes for further sequencing.

Obtained data revealed that the most significant differences were in the expression of genes related to IL-1 signaling. In the CD4$^+$ T cells isolated from the kidneys of *ItgamItgax*$^{-/-}$ nephritic mice expression of such genes as *Il1r2*, *IL1b, Irak3,* and *NLRP3* was significantly decreased compared to the WT group.

A



B

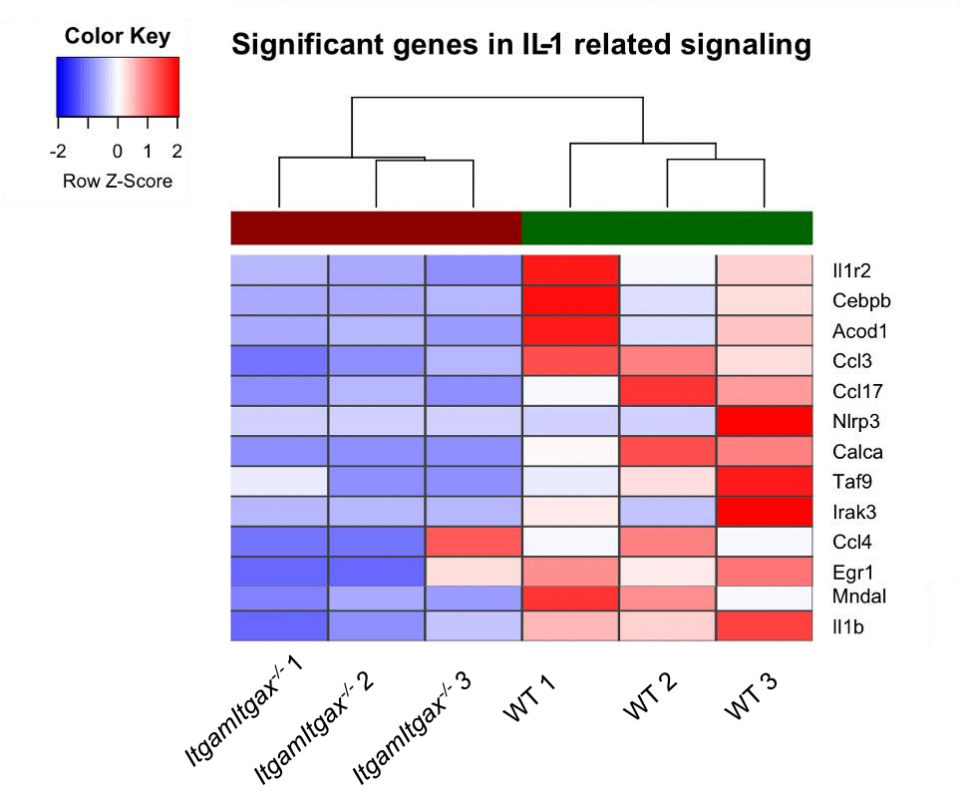


Figure 12. **Upregulation of IL-1 related genes in nephritic WT mice compared to *ItgamItgax*$^{-/-}$ animals**. (A) Experimental plan, (B) heatmap of differentially expressed genes in CD4$^+$ T cells of WT and *ItgamItgax*$^{-/-}$ animals. Data were analyzed by dr. Jian Li.

Presented results show that *Itgam* and *Itgax* selectively influence the induction of signaling pathways in CD4$^+$ T lymphocytes toward IL-1-related pathways.

3.2.4 Conventional dendritic cells type 2 uptake less antigen in nephritic *ItgamItgax*$^{-/-}$ mice

Since both CD11b and CD11c are abundant on cDC2s and can participate in phagocytosis of opsonized particles, we asked whether there is a difference in antigen uptake between *ItgamItgax*$^{-/-}$ and WT cDC2s. We did not take into consideration cDC1s in this experiment because these cells do not express CD11b and are not engaged in priming $CD4^+$ T cells [23].

To answer the question discussed above, we induced NTN in *ItgamItgax*$^{-/-}$ and WT mice and after 10 days injected them i.v. with fluorescently labeled OVA. We used OVA since it is known that DCs in kidneys can capture low-molecular weight antigens that are filtered in glomeruli [87]. Thirty minutes after the OVA injection we collected kidneys and measured via flow cytometry geometric mean fluorescent intensity of AF647 in cDC2s as well as the percentage of cDC2s which took up the antigen (Fig. 13A).

Obtained results showed a significant decrease in the antigen uptake by *ItgamItgax*$^{-/-}$ cDC2s. (Fig. 13B), which could be due to the decreased phagocytic capacity of this type of dendritic cell.

Previous studies have also shown that β_2-integrins influence the expression of DCs activation markers. To check whether this also applies to our model, we measured by flow cytometry the expression of CD80 and CD86 on cDC2s since they are known markers of activation of DCs.

Results obtained during the experiment showed that *ItgamItgax*$^{-/-}$ cDC2s exhibit no differences in the expression of both activation markers compared to WT cells as evidenced by the similar geometric mean fluorescent intensity of CD86 and CD80 between both groups of animals (Fig. 13C and D).

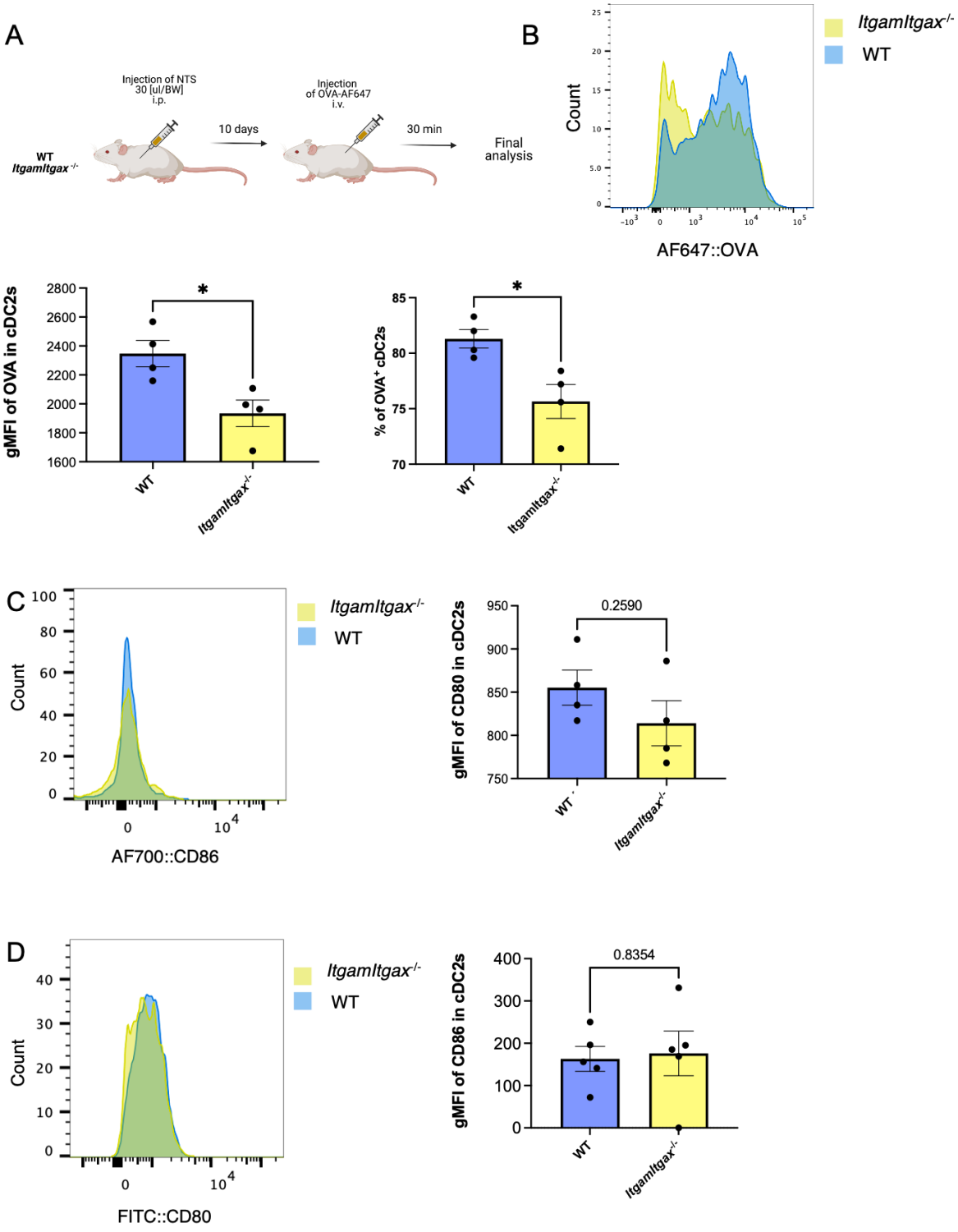


Figure 13. Conventional dendritic cells type 2 of *Itgamltgax*$^{-/-}$ mice uptake less antigen but are not less activated compared to WT cells. (A) Experimental plan, (B) gMFI of AF647 in cDC2s, indicating the amount of OVA taken up per DC and percentage OVA^{+} cDC2s, (C and D) activation of DCs represented as gMFI of CD86 and CD80. Data are presented as mean ± SEM and analyzed using an unpaired t-test. BW, body weight; NTS, nephrotoxic serum; gMFI, geometric mean fluorescent intensity. *P<0.05.

3.2.5 *ItgamItgax*$^{-/-}$ dendritic cells induce lower proliferation of CD4^{+} T cells

Since we observed a decrease in the number of CD4^{+} T cells in *ItgamItgax*$^{-/-}$ mice 10 days after NTN induction, we asked whether lack of CD11b and CD11c can impair the ability of DCs to induce proliferation of this type of T cells.

To answer this question, we isolated splenic DCs from *ItgamItgax*$^{-/-}$ and WT mice. Afterward, we stimulated cells with OVA with the addition of mouse complement serum, which contained active complement components. After 2.5 h of incubation, dendritic cells were washed and OT-II cells were added to the cell culture. Three days later we evaluated the proliferation of OT-II cells via flow cytometry (Fig. 14A). As controls, which allowed us to determine whether the effect that we observe is dependent on complement factors, we used co-culture of OT-II cells with DCs incubated with OVA and heat-inactivated mouse complement serum.

The results obtained during the experiment revealed that the proliferation of OT-II cells was more efficient when they were co-cultured with WT DCs compared to the ones co-cultured with *ItgamItgax*$^{-/-}$ DCs (Fig. 14B). This can mean that CD11b and CD11c play an important role not only in antigen-uptake by DCs, but also further stimulation of CD4^{+} T cell proliferation by dendritic cells. However, we did not notice significant differences between the proliferation of OT-II cells co-cultured with DCs incubated with OVA and heat-inactivated mouse complement serum.

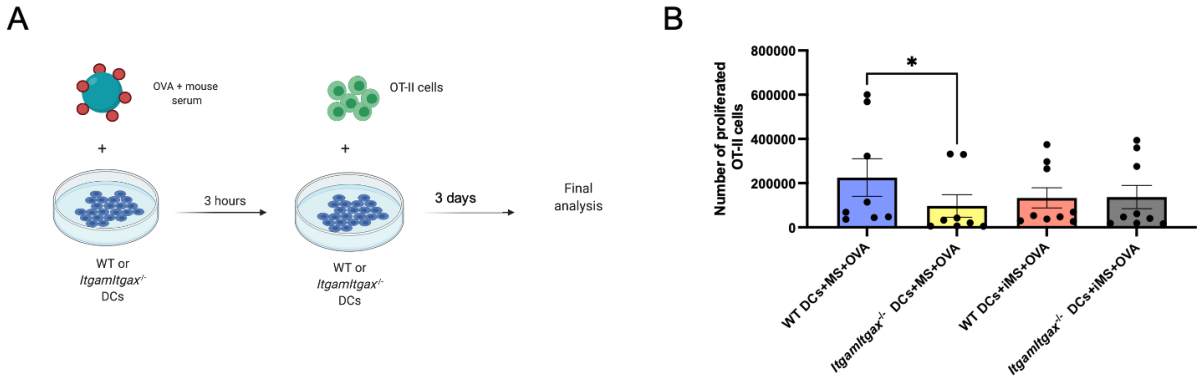


Figure 14. Dendritic cells of *ItgamItgax*$^{-/-}$ mice induce the proliferation of CD4^{+} T cells less efficiently. (A) Experimental plan, (B) OT-II cell number after 3 days of incubation with DCs, showing the ability of DCs to induce proliferation of CD4^{+} T cells after stimulation with OVA. Data

are presented as mean ± SEM and analyzed using an unpaired t-test. DCs, dendritic cells; MS, mouse serum; iMS, inactivated mouse serum; OVA, ovalbumin. *P<0.05.

3.2.6 Complement depletion with cobra venom factor in WT-NTN mice partially improves their condition

The next step was to check whether the lack of recognition of complement factors in *ItgamItgax*$^{-/-}$ mice by CR3 and CR4 is responsible for the attenuation of NTN in the studied mouse line. To answer this question, we decided to inject mice with CVF. CVF is a protein naturally present in cobra venom. The structure of CVF closely resembles the structure of C3. Injection of this compound leads to the depletion of complement proteins due to constant activation of the complement system. This subsequently results in the inability of the organism to synthesize more complement factors [92].

CVF was injected into the mice every other day starting from day -1 before NTS injection. After 10 days post-NTN induction, we collected kidneys and urine for the evaluation of renal damage (Fig. 15A).

Obtained results show that repeated injections of CVF did not reduce crescents formation in WT mice (Fig. 15B), although ACR was significantly reduced in WT animals injected with CVF (Fig. 15C). Again, we did not find differences in the values of creatinine in the serum between all studied groups (Fig. 15D). Surprisingly, *ItgamItgax*$^{-/-}$ mice treated with CVF were characterized by increased ACR compared to the untreated animals (Fig. 15C). Concentration of NGAL was decreased in all groups of mice compared to WT-NTN animals (Fig. 15E).

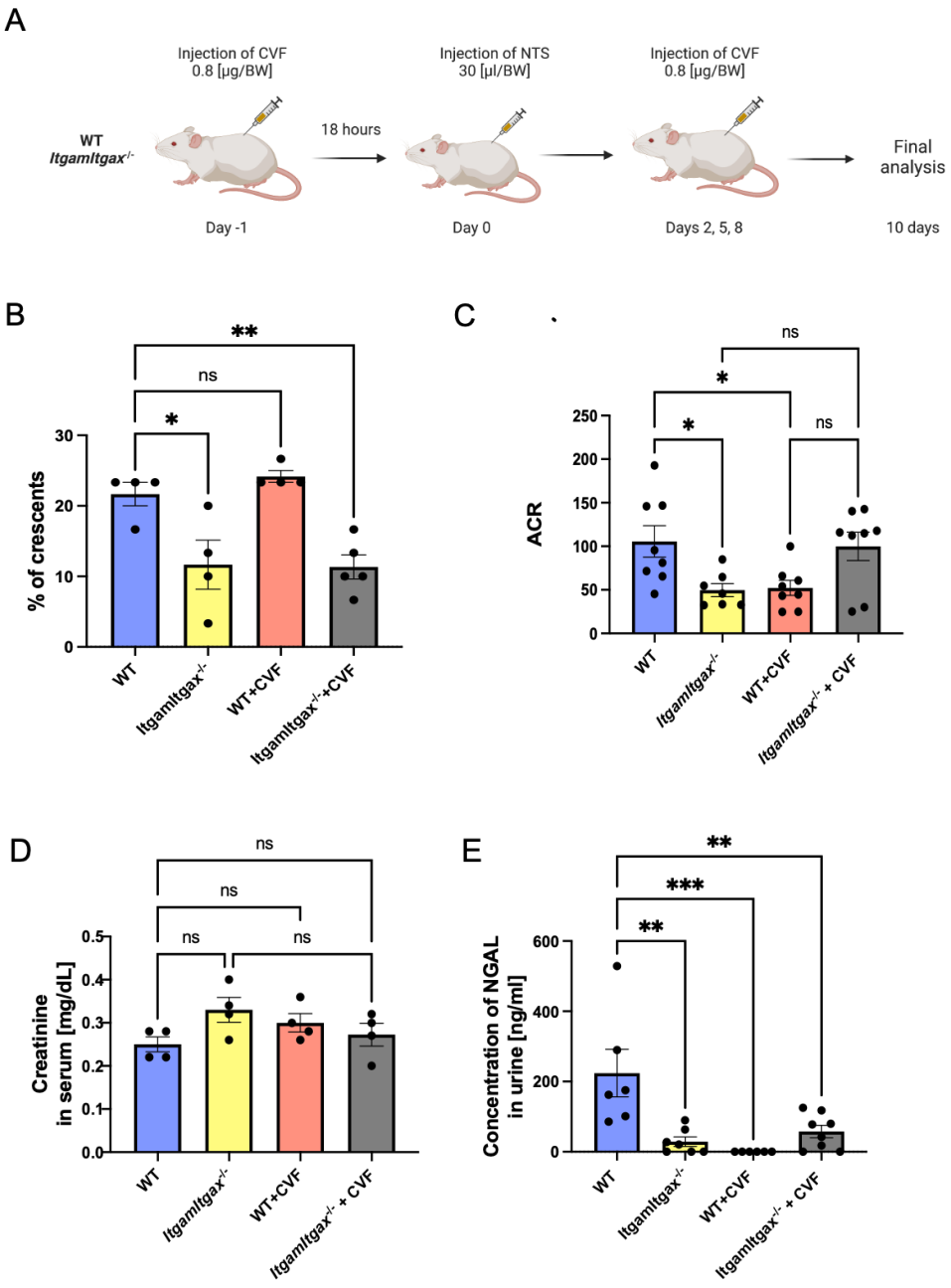


Figure 15. **Nephrotoxic nephritis is partially improved by the treatment of mice with CVF.** (A) Experimental plan, (B) percentage of crescentic glomeruli, (C) ACR, (D) creatinine in serum, (E) NGAL in urine measured by ELISA. The percentage of crescents was analyzed by prof. dr. med. Ulf Panzer from the University Hospital in Hamburg. Data are presented as mean ± SEM and analyzed using one-way ANOVA. ACR, albumin-to-creatinine ratio; CVF, cobra venom factor; NTS, nephrotoxic serum; BW, body weight. *P<0.05; **P<0.01, ***P<0.001.

3.2.7 Immune cell composition is not affected by treatment with CVF

To evaluate the immune response of studied animals, flow cytometric analysis of the populations of immune cells in the nephritic mice 10 days post-NTS injection was performed.

We did not observe significant differences in the abundance of immune cells in the kidneys of the mice injected with CVF compared to the untreated WT animals as well as knockout mice treated with the drug (Fig. 16A-G). *ItgamItgax*$^{-/-}$ mice showed a decrease in the number of cDC2s and CD4^{+} T cells as has been already demonstrated in section 3.2.3 (Fig. 16B and F). Also, we observed an increase in the number of the PMNs in the kidneys of *ItgamItgax*$^{-/-}$ mice treated with CVF compared to the untreated knockout group (Fig. 16D). This finding can explain increased albuminuria in this group of mice.

Obtained data suggest that partial amelioration of NTN in WT mice after treatment with CVF is caused by another immune-mediated mechanism compared to the one responsible for the attenuation of the disease in *ItgamItgax*$^{-/-}$ animals.

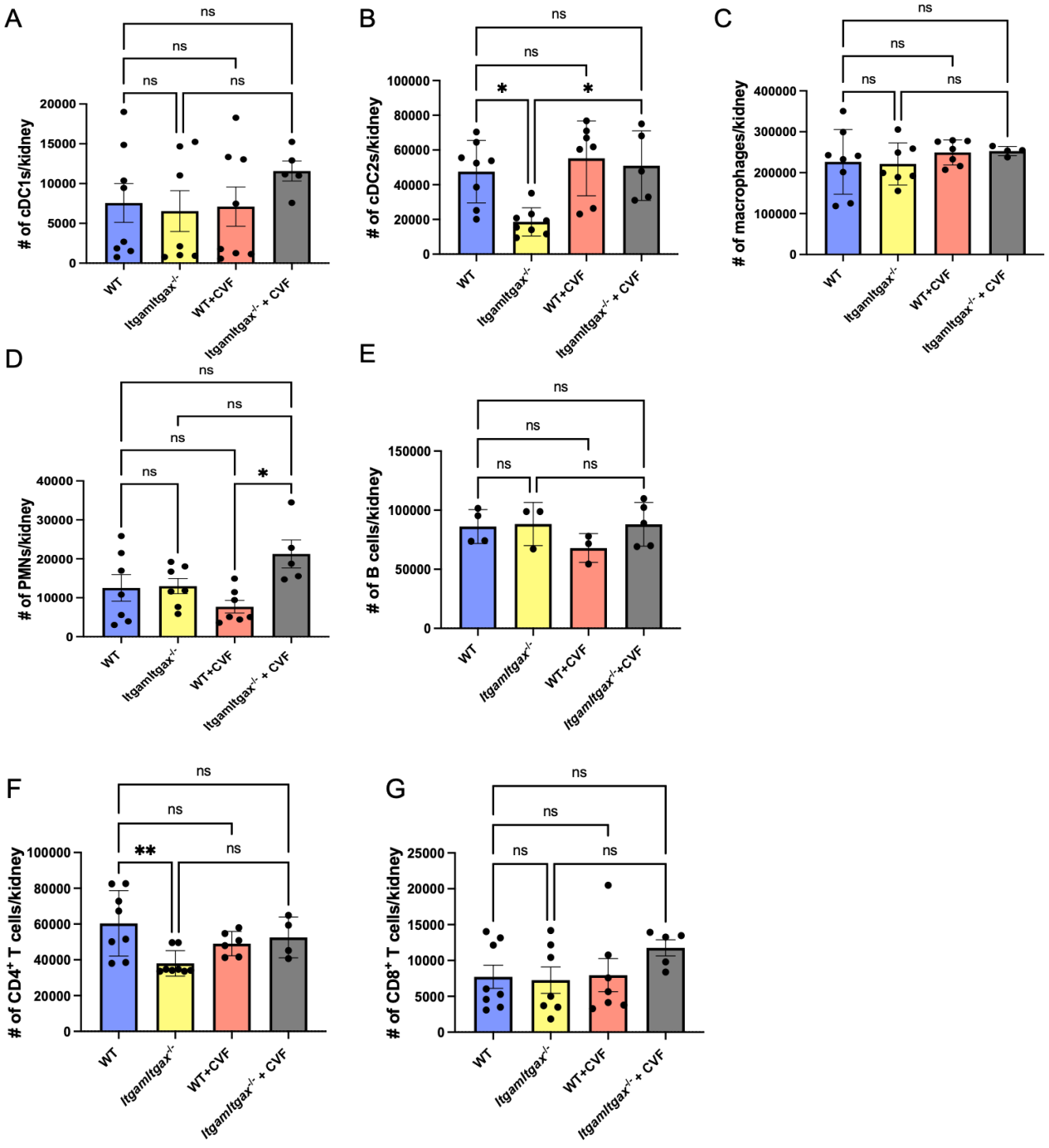


Figure 16. The composition **of the immune cells does not change upon CVF treatment**. (A-G) the number of immune cells in the kidney was measured via flow cytometry. Data are presented as mean ± SEM and analyzed using one-way ANOVA. CVF, cobra venom factor; NTS, nephrotoxic serum; DCs, dendritic cells; PMNs, polymorphonuclear leukocytes or neutrophils. *P<0.05, **P<0.01.

3.2.8 C5aR is upregulated on PMNs in *ItgamItgax*$^{-/-}$ mice

To explain the increase in the count of neutrophils in the kidneys of *ItgamItgax*$^{-/-}$ animals treated with CVF, we measured the expression of C5aR on these cells. C5aR recognizes anaphylatoxin C5a, which can lead to the chemoattraction of PMNs to the site of inflammation [93]. Also, due to the mode of action of CVF, which is a constant activation of the complement cascade, treatment with this compound can cause an increase in the presence of C5a in the blood [92].

To test this hypothesis, we measured via flow cytometry expression of C5aR in PMNs in WT, WT mice treated with CVF, *ItgamItgax*$^{-/-}$, and *ItgamItgax*$^{-/-}$ animals treated with CVF as well as the percentage of the cells positive for this receptor in all studied groups of animals.

Obtained results demonstrated that, indeed, the knockout of *Itgam* and *Itgax* leads to the increase of the expression of C5aR in neutrophils (Fig. 17B).

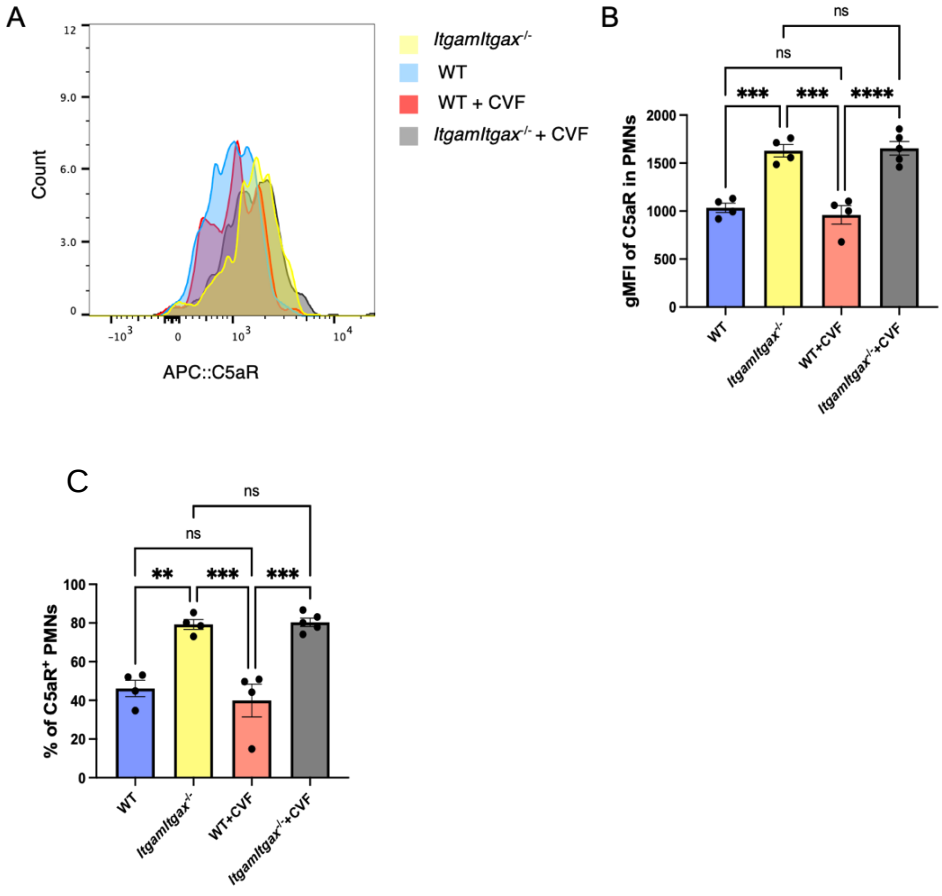


Figure 17. **PMNs of *ItgamItgax*$^{-/-}$ mice express higher levels of C5aR**. (A) histogram of C5aR in PMNs, (B) gMFI of C5aR in PMNs, indicating expression of the receptor on these cells, and (C) percentage of C5aR^{+} PMNs. Data are presented as mean ± SEM and analyzed using one-way ANOVA. CVF, cobra venom factor; gMFI, geometric mean fluorescent intensity; PMNs, polymorphonuclear leukocytes. **P<0.01, ***P<0.001, ****P<0.0001.

3.3 *Itgam* and *Itgax* in pyelonephritis

Previous studies showed that β_2-integrins, especially CD11b, are important for the mobilization of leukocytes during pyelonephritis. Dysregulation of this process leads to the increase of bacterial load in the kidneys of the infected mice [94]. On the other hand, mice that are deficient in C3 show signs of attenuation of pyelonephritis due to the impaired phagocytosis of bacteria by PTECs in kidneys, which can lead to the intracellular survival of *E. coli* inside PTECs and recurrent infections [79].

Based on the studies mentioned above, we decided to look at whether CD11b and CD11c can be important for the progression of bacterial pyelonephritis.

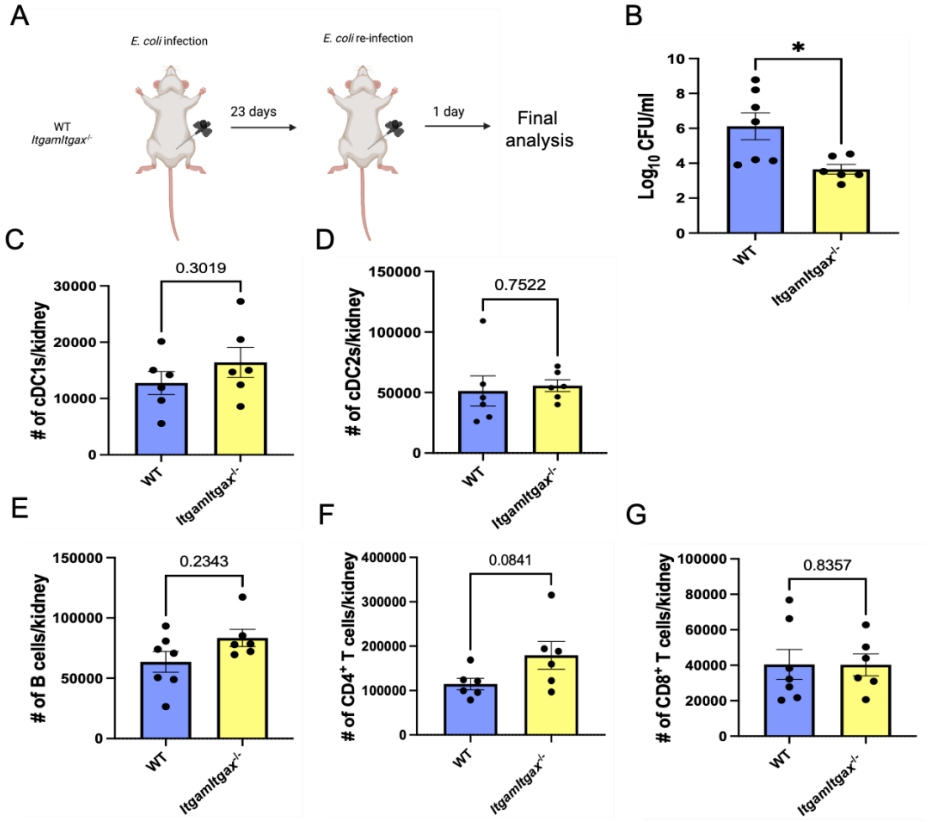
3.3.1 Pyelonephritis is attenuated in *ItgamItgax*$^{-/-}$ mice without changes in adaptive immune response

Since CD11b and CD11c are engaged in migration and adhesion of leukocytes [14], our initial hypothesis was that due to the lack of these integrins, DCs would migrate faster to the renal-draining lymph nodes and induce adaptive immune response more efficiently in *ItgamItgax*$^{-/-}$ mice. This would lead to better clearance of the infection.

To test this hypothesis, we infected WT and *ItgamItgax*$^{-/-}$ mice with UPEC using the previously described model of pyelonephritis [86]. After 23 days, we re-infected mice with the same strain of *E. coli* to boost the production of antibodies. On the next day, we sacrificed mice and evaluated bacterial load in the kidneys to determine the severity of the infection (Fig. 18A). We also checked for the immune response in kidneys and renal draining lymph nodes, to see whether it was affected by lack of CD11b and CD11c on leukocytes during the infection as well as to determine whether the migration of DCs in the case of *ItgamItgax*$^{-/-}$ mice to the renal-draining lymph nodes was more efficient in *ItgamItgax*$^{-/-}$ mice compared to WT animals. To investigate the production of antibodies in response to *E. coli* infection in studied animals, we collected plasma from the mice and performed ELISA using UV-inactivated *E. coli* to coat the plate.

Obtained results revealed significantly decreased bacterial load in the kidneys of knockout mice compared to WT animals (Fig. 18B). But there were no significant differences in the population of both types of DCs, B, and T cells in kidneys as well as in the renal draining lymph node (Fig. 18C-G and J-N). Production of IL-17A and IFNγ was also not impaired in *ItgamItgax*$^{-/-}$ mice as evidenced by the same values of gMFI of these cytokines obtained during flow cytometric analysis (Fig. 18M and N). No significant differences in the titers of anti-*E. coli* antibodies in plasma were also observed (Fig. 18O).

Based on the results below, it can be concluded that the lack of CD11b and CD11c is protective against pyelonephritis. However, the better outcome of the *ItgamItgax*$^{-/-}$ mice is not mediated by the adaptive immune response.



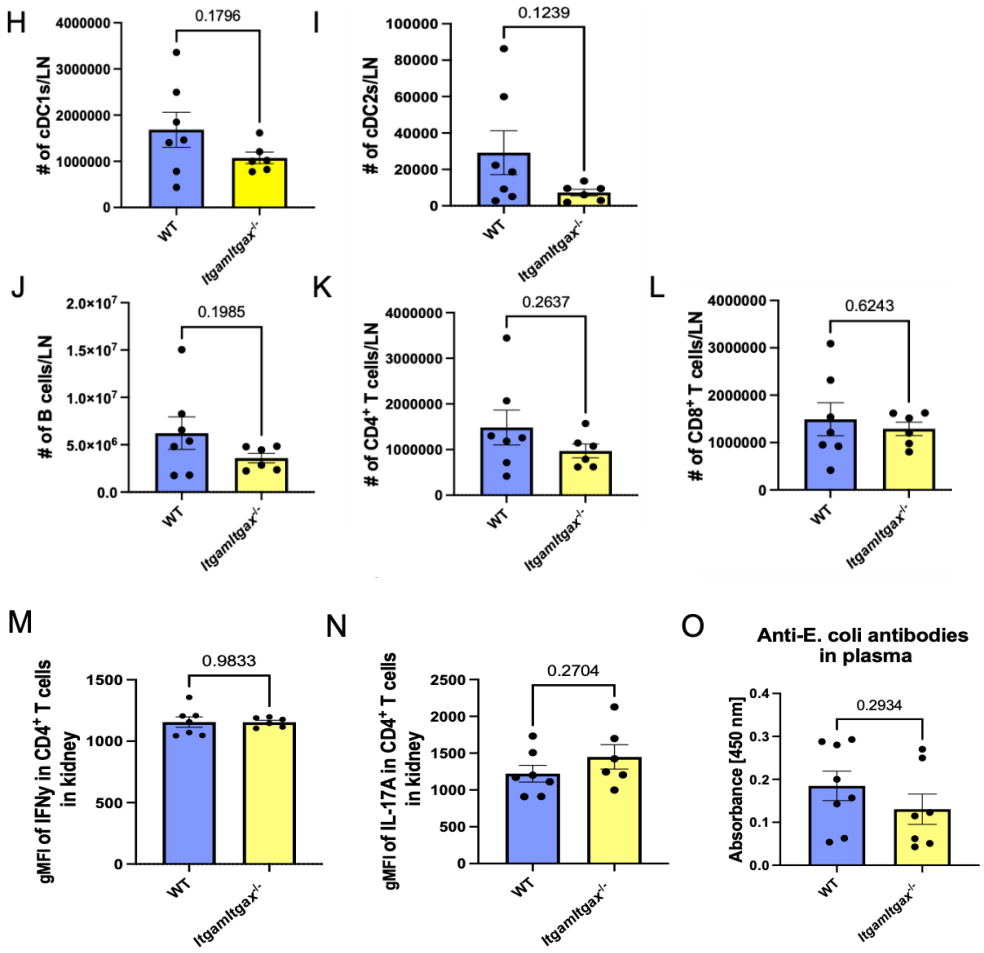


Figure 18. **Pyelonephritis is attenuated in *ItgamItgax*$^{-/-}$ mice without changes in the adaptive immune response**. (A) Experimental plan, (B) bacterial load in kidneys, (C-G) numbers of immune cells in kidney measured via flow cytometry, (H-L) numbers of immune cells in renal draining lymph node measured via flow cytometry, (M and N) production of IFNγ and IL-17A by CD4^{+} T cells in kidneys represented as gMFI, (O) titers of anti-*E. coli* antibodies measured by ELISA and represented through absorbance values. The experiment was performed together with dr. rer. nat. Katarzyna Jobin and dr. Cristina Martin-Higueras. Data are presented as mean ± SEM and analyzed using an unpaired t-test. CFU, colony forming unit; DC, dendritic cell; gMFI, geometric mean fluorescent intensity; LN, lymph node. *P<0.05.

3.3.2 Lack of CD11b and CD11c does not influence the course of pyelonephritis during the first stages of the infection

Since no significant changes in the adaptive immune response, which can explain better clearance of UTI in *ItgamItgax*$^{-/-}$ mice, were observed after 3 weeks post-infection, we decided to look into the earlier time point i.e., 2 days post-infection. Therefore, we infected WT and *ItgamItgax*$^{-/-}$ mice with UPEC as described above and after 2 days

collected kidneys to determine bacterial load by counting CFUs. Subsequently, we analyzed immune cell populations in the kidneys via flow cytometry (Fig. 19A).

In the course of the experiment, we did not observe significant changes in the clearance of the infection between *ItgamItgax*$^{-/-}$ mice and WT animals (Fig. 19B). Although no changes in the number of cDC1s, cDC2s, and macrophages were observed (Fig. 19C-E), the number of neutrophils was significantly increased in mice lacking CD11b and CD11c compared to WT animals (Fig. 19F).

Obtained results suggest that in the case of *ItgamItgax*$^{-/-}$ animals, PMNs are not efficient in the clearance of the infection, since an increase in their number did not influence bacterial clearance in kidneys on day 2 post-infection. Also, the lack of the studied β_2-integrins did not abolish the ability of neutrophils to migrate into the kidneys.

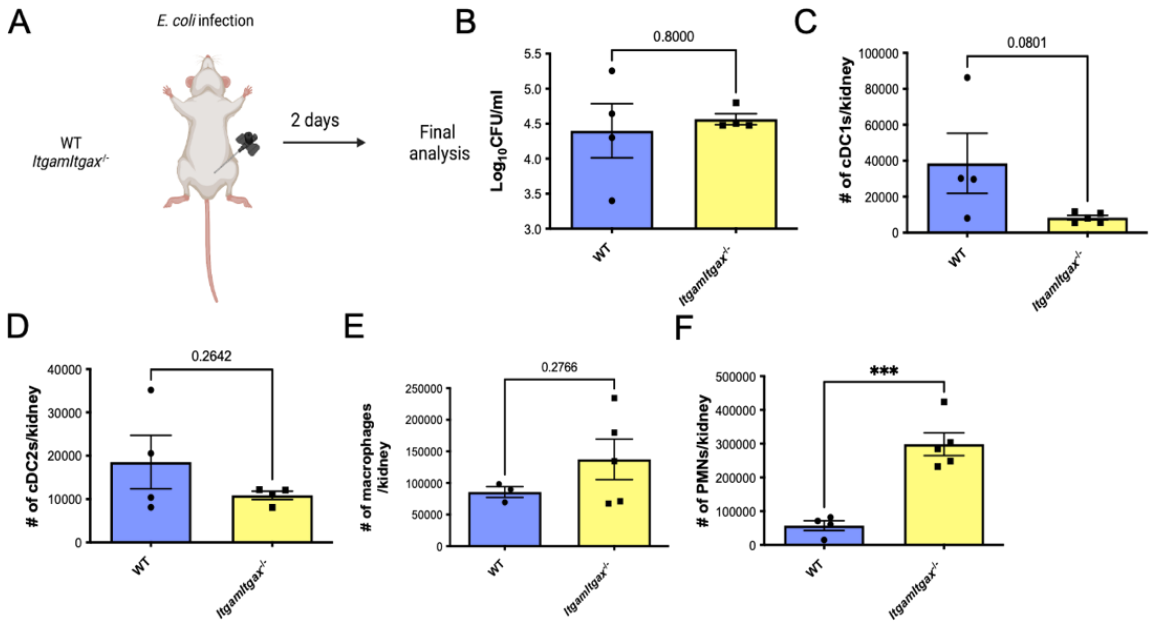


Figure 19. ***ItgamItgax*$^{-/-}$ mice and WT animals are characterized by the same severity of pyelonephritis despite the increase in PMN infiltration in the case of *ItgamItgax*$^{-/-}$ animals**. (A) Experimental plan, (B) bacterial load in kidneys, (C-F) numbers of immune cells in kidney measured via flow cytometry. Data are presented as mean ± SEM and analyzed using an unpaired t-test or Mann-Whitney test. CFU, colony forming unit; DC, dendritic cell; PMNs, polymorphonuclear leukocytes or neutrophils. ***P<0.001.

3.3.3 Neutrophils derived from *ItgamItgax*$^{-/-}$ mice show an impaired ability to phagocytose *E. coli*

Since we observed an increase in the number of PMNs in *ItgamItgax*$^{-/-}$ mice 2 days post-infection, we decided to look into the ability of these cells to phagocytose and kill bacteria.

To check the ability of neutrophils to phagocytose *E. coli*, we infected them with heat-inactivated GFP-expressing *E. coli*. After the incubation, we performed a flow cytometric analysis to assess how many neutrophils contain *E. coli* based on the GFP signal.

For the evaluation of the ability of neutrophils to kill bacteria, we again infected the cells with *E. coli*. After the incubation, we changed the medium to the one containing gentamycin to kill extracellular bacteria and incubated neutrophils for 1 h longer. Afterward, we lysed the cells and plated bacteria onto agar plates to calculate the number of viable intracellular bacteria (Fig. 20A).

Obtained results reveal that neutrophils isolated from *ItgamItgax*$^{-/-}$ mice have a lower capacity to phagocytose *E. coli* (Fig. 20B) as well as a lower number of viable intracellular bacteria (Fig. 20C). This means that β_2-integrins expressed on PMNs play an important role in the uptake of the bacteria.

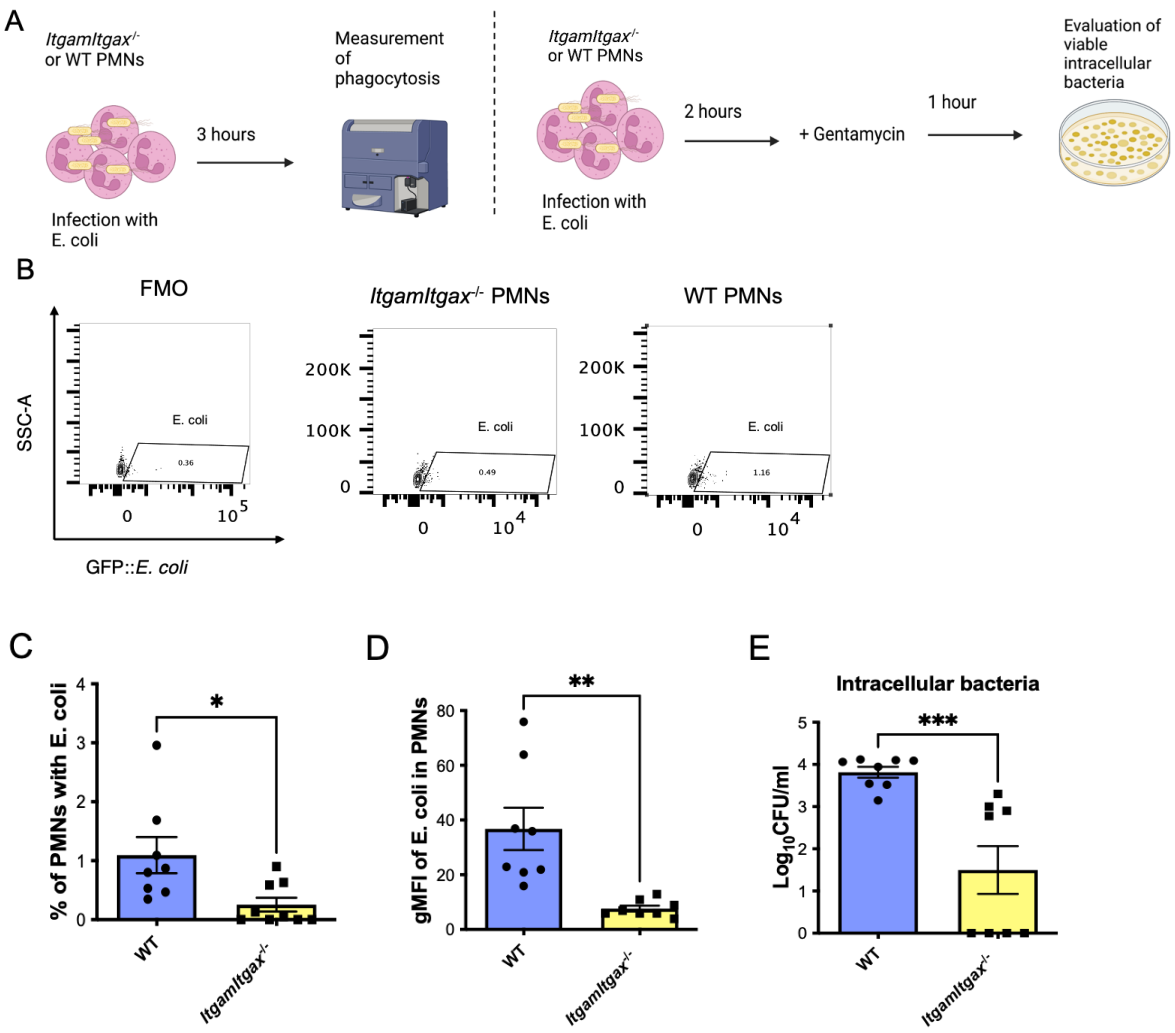


Figure 20. **Neutrophils isolated from *ItgamItgax*$^{-/-}$ mice are characterized by impaired phagocytosis of *E. coli***. (A) Experimental plan, (B) uptake of *E. coli*-GFP by PMNs measured using flow cytometry and represented as gMFI of GFP in PMNs (C) and the percentage of GFP^{+} PMNs (D), (E) number of viable intracellular bacteria. Data are presented as mean ± SEM and analyzed using an unpaired t-test. CFU, colony forming unit; FMO, fluorescence minus one; gMFI, the geometric mean of fluorescence intensity. *P<0.05; **P<0.01, ***P<0.001.

Taking into consideration the previous results, it can be concluded that in the case of *ItgamItgax*$^{-/-}$ animals, neutrophils do not play a significant role in the clearance of the infection since their inability to uptake bacteria as well as increased infiltration in the kidneys on day 2 post-infection did not affect the progression of pyelonephritis.

3.3.4 Pyelonephritis is attenuated in *ItgamItgax*$^{-/-}$ mice on day 7 post-infection with decreased inflammation

Since we observed attenuation of pyelonephritis on day 24 post-infection but lack of the differences in bacterial load in kidneys on day 2 post-infection between *ItgamItgax*$^{-/-}$ and WT mice, we decided to analyze the severity of pyelonephritis on day 7 post-infection to see whether at this time point the effect of the knockout of *Itgam* and *Itgax* will affect the course of the infection as well as the innate immune response.

For this purpose, we infected WT and *ItgamItgax*$^{-/-}$ mice with UPEC and after 7 days collected kidneys for further analysis (Fig. 21A). CFU assay revealed that, indeed, mice without CD11b and CD11c had decreased number of bacteria in their kidneys (Fig. 21B). Flow cytometric analysis also showed a decrease in the number of cDC1s and cDC2s in the case of *ItgamItgax*$^{-/-}$ animals, suggesting lower inflammation in this mouse line, possibly due to the decrease in the bacterial load and beginning of the resolution of the infection (Fig. 21C and D). No significant differences in the number of macrophages and neutrophils between *ItgamItgax*$^{-/-}$ and WT mice were observed on day 7 post-infection (Fig. 21E and F).

Due to the decrease in the bacterial load in the kidneys of *ItgamItgax*$^{-/-}$ mice after 7 days of infection as well as lower infiltration of kidneys with several types of innate immune cells in the case of this mouse line, we also decided to look at whether expression of inflammatory cytokines is altered in these animals. For this purpose, we performed qRT-PCR analysis to check the relative expression of the genes encoding several important inflammatory cytokines such as TNFα, IL-6, IL-1β , and NLRP3. We observed a significant decrease in the expression of TNFα and IL-6 in the case of *ItgamItgax*$^{-/-}$ animals as well as a tendency toward a decrease in the expression of IL-1β and NLRP3, which suggests an almost complete resolution of the inflammation 7 days after pyelonephritis induction (Fig. 21G-J).

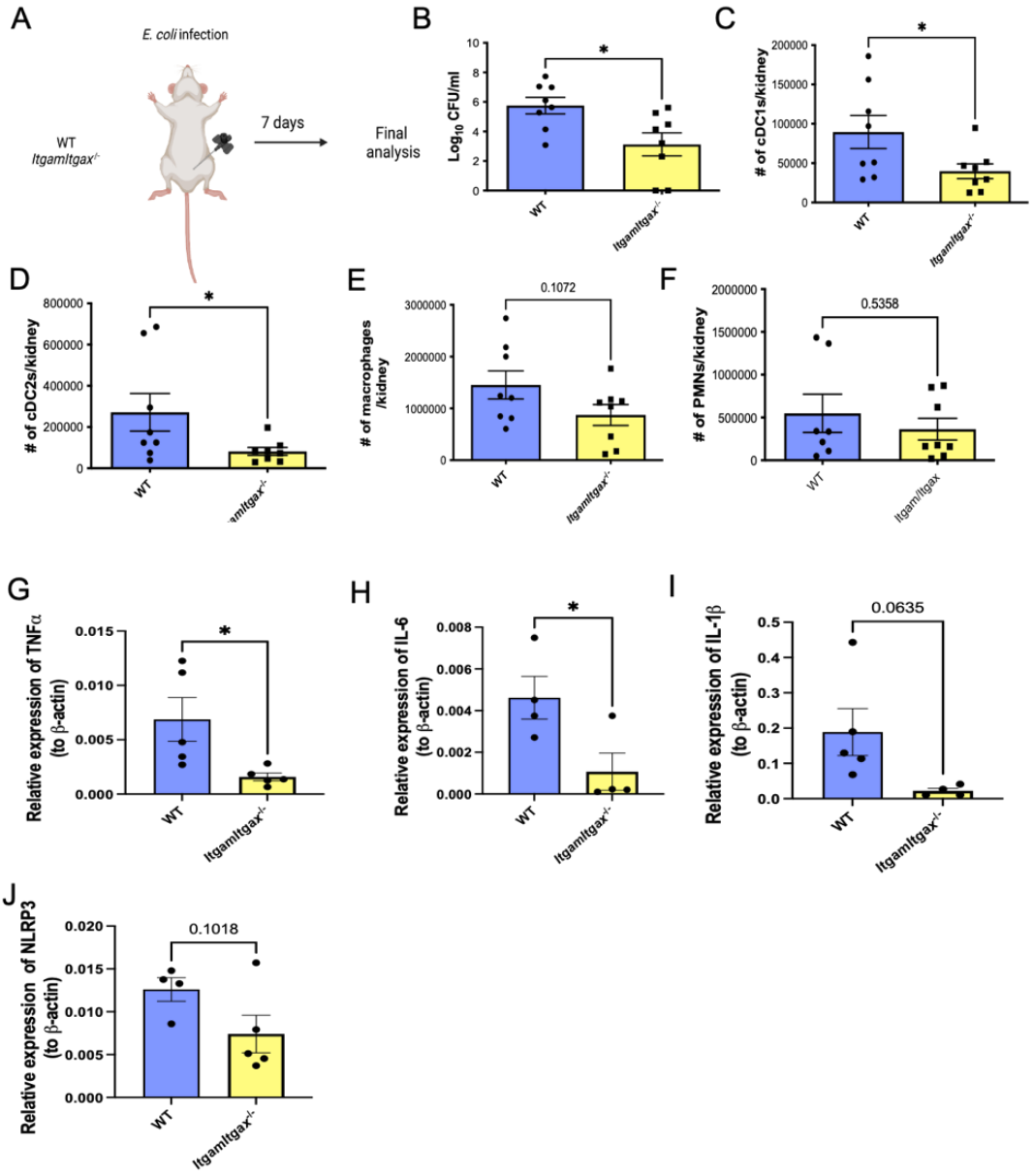


Figure 21. **Pyelonephritis is attenuated in *ItgamItgax*$^{-/-}$ mice on day 7 post-infection**. (A) Experimental plan, (B) bacterial load in kidneys, (C-F) the number of immune cells in the kidney measured via flow cytometry. (G-J) relative expression of inflammatory cytokines in kidneys measured via qRT-PCR and represented as ∂C_t values. Data are presented as mean ± SEM and analyzed using an unpaired t-test. CFU, colony forming unit; DC, dendritic cell; PMNs, polymorphonuclear leukocytes or neutrophils. *P<0.05.

3.3.5 Bone marrow-derived macrophages from *ItgamItgax*$^{-/-}$ mice contain less viable intracellular *E. coli*

Previous studies have shown that certain strains of uropathogenic *E. coli* isolated from patients with recurrent UTIs can survive inside macrophages without further delivery

to phagolysosomes, which allows bacteria to avoid anti-bacterial inflammatory response and enables further survival of the pathogen and its replication [95]. Also, β_2-integrins, especially CD11b, were proven to be important for the uptake of the microorganisms by macrophages and neutrophils [5].

Since in our previous experiments, we observed a decrease in the bacterial load in kidneys as well as almost complete resolution of inflammation in *ItgamItgax*$^{-/-}$ mice on day 7 after infection, we hypothesized that due to the impaired ability of macrophages without CD11b and CD11c to phagocytose bacteria, *E. coli* cannot utilize them as a niche for survival, which allows more efficient clearance of the infection in the case of the knockout animals.

To test this hypothesis, we isolated monocytes from the BM of WT and *ItgamItgax*$^{-/-}$ mice and differentiated them into macrophages. Afterward, we infected cells with UPEC. After the incubation, we washed the cells and added gentamycin to kill extracellular bacteria. Later, we lysed BMDMs and plated cell lysates onto agar plates to evaluate the number of viable intracellular bacteria (Fig. 22A).

As expected, *ItgamItgax*$^{-/-}$ macrophages harbored less *E. coli* (Fig. 22B), which might suggest an impaired ability of knockout macrophages to uptake bacteria. In this case, it can be beneficial and explain the better outcome of *ItgamItgax*$^{-/-}$ mice compared to WT animals.

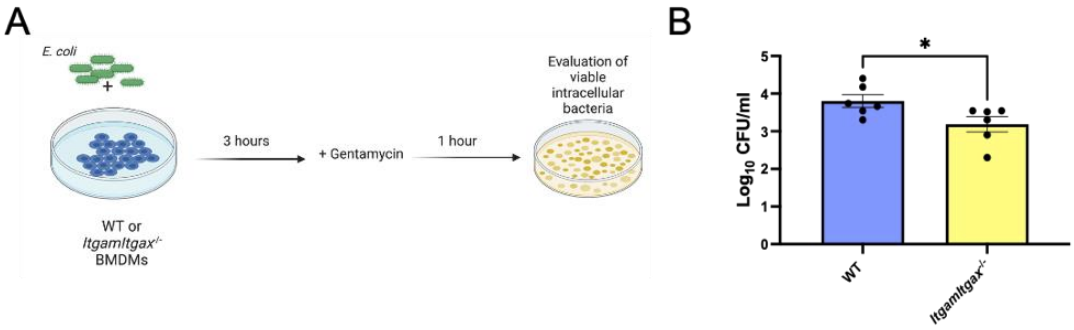


Figure 22. ***ItgamItgax*$^{-/-}$ BMDMs contain less viable intracellular *E. coli*.** (A) Experimental plan, (B) viable intracellular bacteria inside BMDMs. Data are presented as mean ± SEM and analyzed using an unpaired t-test. CFU, colony forming unit. *P<0.05.

4. Discussion

Integrins are one of the most important proteins needed for the phagocytosis of opsonized particles, migration of the cells, and induction of the signaling pathways necessary for the differentiation of the cells and their proliferation. Integrins consist of extracellular and intracellular domains. Their ability to perform certain functions is dependent on their conformational state. Bent-closed conformation means that these proteins are inactive [1]. The presence of chemokines and their recognition by certain receptors can result in the activation of the integrins. An example of this process is the recognition of CXCL1 by the receptor CXCR2, which leads to the change of the conformation of LFA-1 on PMNs to the extended-open state and ICAM-1-dependent arrest of the cells [96].

4.1 The *ItgamItgax*$^{-/-}$ mouse line is a more suitable model compared to CD18$^{-/-}$ mice to study effect of CD11b and CD11c during inflammatory diseases

Among the integrins which are highly expressed on myeloid cells such as DCs, monocytes/macrophages, and PMNs are CD11b and CD11c. In combination with CD18, they form CR3 and CR4, respectively. Both integrins can recognize several common ligands among which are iC3b, ICAM-1, and fibrinogen. Due to the fact mentioned above it is beneficial to study the effect of CD11b and CD11c on inflammation together [5]. Before it was not possible because of the lack of blocking monoclonal antibodies effectively targeting CD11c *in vivo* as well as due to the lack of a mouse line with a knockout of both integrins. To solve this issue, CD18$^{-/-}$ mice are often used since CD18 is a common subunit for both CR3 and CR4, as mentioned before. But this solution brings several other problems. CD18 forms heterodimers also with CD11a and CD11d. LFA-1 (heterodimer of CD11a and CD18) recognizes ICAM-1, ICAM-2, ICAM-3, ICAM-4, ICAM-5, and JAM-A [97]. Heterodimer CD11d/CD18 recognizes ICAM-1 too. It also binds ICAM-3 and VCAM-1 [98]. Because of this usage of CD18$^{-/-}$ animals will not provide enough information about the type of ligand that influences the progression of certain diseases the most. Another disadvantage of the CD18$^{-/-}$ mouse line is the fact that it is characterized by increased neutrophils count, splenomegaly, and reduced peripheral lymph nodes already in the steady state, which can affect the results of experiments [89]. In the case of our newly generated *ItgamItgax*$^{-/-}$ mouse line, we did not observe these effects (Fig. 5 and 6). CD18

is known to be important for the migration of T and B cells to LNs and forms heterodimers with other integrins that recognize ICAM-1. One of the studies showed that blockage of ICAM-1 and LFA-1 drastically decreased the ability of T cells to migrate into the draining LNs [99]. Similarly, B cells in ICAM-1$^{-/-}$ mice demonstrated lower motility and migration to LNs [100]. This decreased migration of B and T cells can explain the reduced size of peripheral LNs in CD18$^{-/-}$ mice. Since in *ItgamItgax*$^{-/-}$ animals LFA-1 and CD11d/CD18 are still present, migration of lymphocytes is not severely impaired in this mouse line and lack of CD11b and CD11c does not lead to changes in the size of LNs (Fig. 5A). An increase in the number of neutrophils was also not observed in *ItgamItgax*$^{-/-}$ mice (Fig. 6E, I and N) as it is described in the case of CD18$^{-/-}$ animals. It is suggested that due to the increased susceptibility to infectious diseases, mice deficient in CD18 are characterized by constant activation of bone marrow resulting in increased granulopoiesis. Myeloid hyperplasia in CD18$^{-/-}$ animals also results in splenomegaly [101], which is not the case for *ItgamItgax*$^{-/-}$ mice (Fig. 5C).

To check whether there is no underlying inflammation caused by CD11b and CD11c knockout, we performed LEGENPlex assay to measure the concentration of several cytokines (IFNγ, TNFα, IL-6, IL-17A, and IL-10) in the plasma of mice. As expected, we did not notice any significant differences between WT and *ItgamItgax*$^{-/-}$ mice, which means that this mouse line does not have underlying inflammation (Fig.7).

All of the above shows that *ItgamItgax*$^{-/-}$ mice can be a useful tool to study inflammatory diseases.

4.2 Proliferation and activation of CD4^{+} T cells are not different between WT and *ItgamItgax*$^{-/-}$ mice after OVA/polyI:C stimulation of DCs

To check how the knockout of *Itgam* and *Itgax* influences the immune response of CD4^{+} T cells, we transferred OT-II cells into WT and *ItgamItgax*$^{-/-}$ mice and then stimulated DCs with injections of OVA together with polyI:C. In this experiment we did not observe differences in the proliferation of OT-II cells as well as their activation based on CD44 and CD25 expression between WT and *ItgamItgax*$^{-/-}$ animals (Fig.8). The reason for this outcome can explain the fact that in healthy mice complement cascade is not highly activated because of strict regulation of the activation of the complement cascade, which

means that complement components are present in low concentration in murine blood [102]. This low activation of the complement system might not be enough for efficient opsonization of OVA and its further uptake by CR3 and CR4. Unopsonized OVA can be taken up through micropinocytosis or by mannose receptors. These mechanisms of uptake are not dependent on CR3 and CR4. That is why they can result in antigen-presentation of the same efficacy in WT and *ItgamItgax*$^{-/-}$ mice leading to a similar rate of the proliferation of OT-II cells.

4.3 *Itgam* and *Itgax* in crescentic glomerulonephritis

The next aim of the project was to investigate whether the expression of *Itgam* and *Itgax* is changed in several types of human glomerulonephritis. Analysis of the expression of these genes using publicly available data from global RNA-seq of glomeruli of patients with kidney diseases revealed that in most types of glomerular disorders, among which is RPGN, there is upregulation of both genes (Fig. 9A). This is in line with genome-wide studies of European and Asian individuals suffering from IgA nephropathy, where the authors showed that several single nucleotide polymorphisms (SNPs) in the *Itgam-Itgax* locus are associated with an increased risk of the development of the disease [103,104]. IgA nephropathy is characterized by a high accumulation of IgA antibodies in the kidneys. In rare cases, patients suffering from this disease can develop RPGN [105]. Other research projects associated with autoimmune disorders also discovered polymorphisms in *Itgam* and *Itgax* that predisposed to the development of SLE and systemic sclerosis [106,107]. This means that SNPs and overexpression of *Itgam* and *Itgax* are strongly associated with increased inflammation, which can be associated with the ability of CR3 and CR4 to recognize complement-coated antigens. Increased antigen uptake e.g., by DCs due to the upregulation of CR3 and CR4 can subsequently result in higher antigen-presentation capacities of these cells leading to increased activation of T cells and a further increase in inflammation. Also, diseases that were shown to be associated with SNPs in the *Itgam-Itgax* locus (IgA nephropathy, SLE, and systemic sclerosis) are characterized by abnormal activation of complement cascade [108–110] which points to the importance of the recognition of complement factors by CR3 and CR4 in the development and progression of autoimmune disorders.

In the next stage of the project, we wanted to confirm the upregulation of *Itgam* and *Itgax* in the murine model of crescentic glomerulonephritis known as NTN. To induce NTN, mice were injected with nephrotoxic sheep serum. NTS contains antibodies specific to murine cortex antigens. It deposits in glomeruli and afterward is taken up by DCs, which present antigens to T cells. The exact type of antigens which cause immune response leading to the development of NTN in mice is unknown [87]. After 10 days post-NTS injection, we examined murine kidneys for the expression of *Itgam* and *Itgax* both on RNA and protein levels. In both cases, we observed a significant increase in the expression of CD11b and CD11c in murine kidneys (Fig. 9B and C), which means that the NTN model is suitable for studies of the influence of both genes on the course of cGN.

The next step of the project was to examine the severity of NTN in WT and *ItgamItgax*$^{-/-}$ mice 10 days post-NTS injections. This time point was chosen because of the previous research data that showed that renal DCs are the most activated on this time point based on the expression of CD80 and CD86. Ten days after NTN induction, T_h1 and T_h17 immune responses are also present, which drives the disease progression. Also, 10 days after the NTN induction, kidney damage and albuminuria are observed [87]. Data obtained during the study showed that *ItgamItgax*$^{-/-}$ mice had attenuated symptoms of the disease as evidenced by a lower percentage of crescents, decreased ACR, and a tendency toward decreased NGAL concentration in urine (Fig. 10B, C, and E). Creatinine level in serum in WT and *ItgamItgax*$^{-/-}$ animals was similar (Fig. 10D). This can be explained by the fact that in mice 10 days post-NTS injection percentage of crescents was around 20%, which means that renal damage has not been severe yet and could not cause relevant changes in creatinine levels in serum. It is known that creatine level in serum correlates with glomeruli damage [111]. In moderate to severe cases of NTN in mice percentage of crescents should be around 40 to 50% [112]. We also did not observe any significant changes in the severity of fibrosis between WT and *ItgamItgax*$^{-/-}$ mice (Fig 10F-H). Fibrosis is the last stage of NTN development and occurs around 3 weeks post-NTN induction [57]. We performed a final analysis of the mice on day 10, which is a too-early time point for the assessment of fibrosis. Also, the percentage of the area affected by fibrosis in both groups of mice was only around 1.5%. To check the influence of *Itgam* and *Itgax* knockout on fibrosis progression during NTN, later time points should be investigated.

Interestingly, Chaves AJ *et al* by the usage of *Itgam*$^{-/-}$ mice for the investigation of complement-mediated immune complex glomerulonephritis showed that CD11b was protective during the disease. *Itgam*$^{-/-}$ mice had higher hematuria and percentage of crescents compared to WT animals. This finding is in contradiction to what we observed in *ItgamItgax*$^{-/-}$ mice. The differences between the outcomes of the experiments can be explained through the usage of different models of glomerulonephritis. In the study described above accelerated nephrotoxic nephritis (ANTN) model was used, which means that for the induction of the disease, authors injected NTS into the previously immunized mice with sheep IgG. Also, we performed experiments with *ItgamItgax*$^{-/-}$ animals and it is possible that signaling pathways induced by the activation of CD11b and CD11c can intercalate with each other leading to different effects. The worse outcome of *Itgam* $^{-/-}$ mice in that study was explained through the observation that macrophages deficient in CD11b secreted less inflammatory cytokines i.e., IL-1β and TNFα compared to WT cells after the treatment with LPS together with iC3b-coated guinea pig red blood cells [113]. However, other research groups observed that activation of CD11b leads to the stimulation of TLR4 signaling, which is induced by LPS, and subsequent upregulation of the expression of inflammatory cytokines [47]. Another study where *Itgam* $^{-/-}$ mice were utilized showed that these animals were protected against NTN as a result of impaired neutrophil adhesion and decreased infiltration of kidneys by this cell type [114].

When we looked deeper into the immune response in WT and *ItgamItgax*$^{-/-}$ mice during NTN, we figured out that *ItgamItgax*$^{-/-}$ mice had a lower number of renal cDC2s. However, the abundance of other types of myeloid immune cells i.e., cDC1s, macrophages, monocytes, and PMNs, was not changed between the groups (Fig11A, C, D, and E). This result can be explained by the lower migration and adhesion of myeloid cells to the tissues as a result of the lack of CD11b and CD11c, which can recognize ICAM-1 or fibrinogen (important molecules for migration and adhesion of the cells) [5,23]. However, the number of monocytes was the same in WT and knockout animals on day 10 post-NTN (Fig. 11E), which does not support the hypothesis discussed above. To confirm that migration of monocytes is not affected by *Itgam* and *Itgax* knockout, nephritic mice should be injected with fluorescently-labeled WT and *ItgamItgax*$^{-/-}$ monocytes. After 3 days from the adoptive transfer of the cells, the abundance of monocytes should be evaluated by flow cytometry. It is also possible that in *ItgamItgax*$^{-/-}$ mice inflammation has already started to resolve on day 10 post-NTN induction leading to a decrease in the count of

cDC2s. Another cell type that was significantly decreased in the kidneys of *ItgamItgax*$^{-/-}$ mice was CD4^{+} but not CD8^{+} T cells (Fig. 11G and H). cDC2s are known to prime CD4^{+} T cells and induce their proliferation and activation [23]. On the other hand, CD8^{+} T cells are primed by cDC1s. However, cDC1s do not express CD11b and their abundance in kidneys was similar between WT and *ItgamItgax*$^{-/-}$ mice. A decrease in the number of CD4^{+} T cells can be beneficial during NTN. Previous studies showed that these cells are pathogenic in the context of the disease, which was proven by the adoptive transfer of CD4^{+} T cells to the recipient animals, which promoted the disease progression[115].

When we looked into the expression of inflammatory cytokines (IFNγ, IL-12, IL-23, IL-17A, and IL-1β) in kidneys on day 10 post-NTN induction, we did not observe any significant differences between WT and *ItgamItgax*$^{-/-}$ mice. Only the expression of IL-23 showed a tendency toward a decrease in *ItgamItgax*$^{-/-}$ animals (Fig. 11J). IL-23 can be produced by cDC2s and it is important for the survival of T_h17 cells. T_h17 cells and IL-23 have already been shown to be detrimental in nephritic mice [58]. This means that low expression of IL-23 can explain the better outcome of knockout animals. However, we did not observe differences in the expression of IL-17A between both groups of mice. This result could be explained by the fact that the qRT-PCR analysis was performed using RNA from the whole kidneys, which means that it could be too diluted for the reliable evaluation of the expression of cytokines. In this case, a better approach will be to sort immune cells from kidneys before RNA isolation and perform gene expression analysis afterward.

In the previous experiments, we observed a selective decrease in the abundance of cDC2s and CD4^{+} T cells in *ItgamItgax*$^{-/-}$ animals compared to WT mice. However, we failed to identify significant differences in the expression of several inflammatory mediators. Because of this, we decided to do 3'mRNA sequencing of CD4^{+} T cells isolated from nephritic knockout and WT mice. The analysis of the experiment revealed that expression of the genes involved in IL-1-related signaling pathways was the most affected by *Itgam* and *Itgax* knock-out (Fig. 12). *Il1r, Irak1, Il1b,* and *NLRP3* genes were significantly downregulated in CD4^{+} T cells obtained from knockout animals compared to the cells isolated from WT mice. This effect is cell-specific since the total expression of IL-1β in the kidneys was similar between both groups of animals (Fig. 11M). Activation of NLRP3/ASC axes in DCs that leads to an increase in IL-1β production has already been shown to be detrimental during murine cGN. Mice deficient in NLRP3 and ASC (adaptor

protein used for the recruitment of caspase-1, which is needed for cleavage of inactive pro-IL-1β to its active form) had less severe symptoms of NTN [116]. Also, a previous study has shown that intrinsic synthesis of IL-1β, which is dependent on NLRP3 inflammasome, by human $CD4^+$ T lymphocytes led to decreased activation of this cell type as well as to downregulation of IFNγ expression [22]. IFNγ is one of the major cytokines involved in the progression of cGN [57]. However, we failed to identify differences in the secretion of this cytokine between WT and *ItgamItgax*$^{-/-}$ animals on day 10 post-NTN in our experiments. This can be explained by the usage of a too-early time point for the evaluation of the production of IFNγ. It will be useful to extend the course of the experiment to 14-21 days and then perform an analysis of the expression of this inflammatory mediator. To show that low activation of $CD4^+$ T cells is responsible for the attenuation of NTN in *ItgamItgax*$^{-/-}$ animals, it is be needed to transfer $CD4^+$ T lymphocytes from nephritic WT mice to *ItgamItgax*$^{-/-}$ animals with NTN and evaluate disease severity afterward. Also, it can be interesting to generate $CD4^+$ T cells with *Il1* and/or *NLRP3* knockout and perform the adoptive transfer of these cells to WT-NTN mice to see whether this can ameliorate cGN in the animals. It is also needed to perform 3'mRNA sequencing of cDC2s because this cell type influences the activation of $CD4^+$ T cells. It is also possible that differences in the functionality of cDC2s between WT and *ItgamItgax*$^{-/-}$ animals can influence the course of the disease by itself.

Since CR3 and CR4 are known to be engaged in the phagocytosis of iC3b-opsonized antigens [5], we wanted to confirm that cDC2s phagocytose fewer antigens also in our model of NTN. Uptake of the antigen, further processing, and presentation on MHC II together with costimulatory molecules and cytokines belong to three signals needed for the activation and differentiation of $CD4^+$ T cells [24]. For the evaluation of the antigen uptake, we injected nephritic mice with a fluorescently labeled antigen OVA. Afterward, we performed a flow cytometric analysis to evaluate OVA uptake by renal cDC2s. OVA is a low-molecular-weight molecule that can be filtrated by kidneys and then processed by DCs [87]. As expected, cDC2s deficient in CR3 and CR4 captured significantly less OVA compared to WT cells proving that these receptors are needed for antigen uptake (Fig. 13B).

Since one of the previous studies on NTN showed that $CD80^{-/-}$ mice had attenuated symptoms of cGN due to the decreased proliferation of $CD4^+$ T cells in the spleen and

increased apoptosis of this cell type [117], we also looked into the expression of this co-stimulatory molecule as well as CD86 (another marker of DCs activation) on cDC2s via FACS in nephritic *ItgamItgax*$^{-/-}$ and WT mice to see whether lack of these β_2-integrins plays a role in the regulation of the expression of co-stimulatory molecules. In the course of the experiment, we did not observe any significant differences in the expression of both CD80 and CD86 on day 10 post-NTN induction (Fig. 13C and D). This means that CR3 and CR4 are not needed for the expression of these molecules.

In one of the previous experiments, we were able to show that nephritic *ItgamItgax*$^{-/-}$ mice had fewer CD4$^+$ T cells in the kidneys. To check whether this observation was due to the low efficacy of the proliferation induction of CD4$^+$ T cells by *ItgamItgax*$^{-/-}$ DCs, we co-cultured previously stimulated with OVA and complement mouse serum DCs from knockout and WT mice together with OT-II cells. During the experiment, we were able to show that, indeed, DCs deficient in both integrins stimulate the proliferation of CD4$^+$ T cells less efficiently compared to WT cells (Fig. 14B). This result can be explained by the lower uptake of OVA opsonized with complement by *ItgamItgax*$^{-/-}$ DCs. However, culturing of OT-II cells with DCs stimulated with OVA and heat-inactivated mouse complement serum did not lead to a significant reduction in OT-II cell proliferation. In the future, it will be beneficial to isolate DCs from mice with NTN and then co-culture them with OT-II cells since complement factors outside the organism can become inactive, which can affect *in vitro* experiments. The finding discussed above is in line with the study, which shows that BMDCs from mice with 77His variant of CD11b, which has lower binding capacity toward iC3b, did not stimulate the proliferation of OT-II cells as efficiently as WT BMDCs[118]. Another study where C3$^{-/-}$ mice were used, also claimed that in this mouse line, the proliferation of CD4$^+$ and CD8$^+$ T cells during *L. monocytogenes* infection was diminished, partially due to the effect of C3 knockout on antigen uptake by DCs [119].

Taking together the findings presented above, we can propose that attenuation of NTN in *ItgamItgax*$^{-/-}$ mice can be connected with the inability of cDC2s to efficiently take up opsonized antigen through CR3 and CR4 and further present it to CD4$^+$ T cells. This leads to the decreased proliferation of T_h cells. To prove that this effect is, indeed, dependent on the uptake of opsonized with complement factors antigens, we decided to deplete complement with CVF. CVF is a compound derived from cobra venom, which constitutively activates complement cascade leading to the inability of the organism to

synthesize more complement components [92]. However, after the experiment we did not see significant changes in the percentage of crescents between WT mice and WT animals injected with CVF (Fig. 15B). Unexpectedly, ACR was significantly increased in *ItgamItgax*$^{-/-}$ mice after treatment with CVF. At the same time ACR was reduced in WT mice treated with CVF compared to the untreated WT animals (Fig. 15C). When we looked into the infiltration of kidneys with major populations of immune cells by FACS, we observed that only untreated *ItgamItgax*$^{-/-}$ mice had decreased count of cDC2s and CD4^{+} T cells compared to other groups of animals (Fig. 16B and F). Another interesting finding was that the number of PMNs significantly increased in *ItgamItgax*$^{-/-}$ mice treated with CVF compared to the untreated knockout animals (Fig. 16D). PMNs are known to be pathogenic in kidney inflammatory disorders since they release MPO, which causes tissue damage [120]. An increase in the number of PMNs in *ItgamItgax*$^{-/-}$ mice that received CVF can explain the increase in albuminuria in this group. CVF is known to induce the formation of a lot of complement fragments among which is C5a [92,93]. C5a is an anaphylatoxin that attracts neutrophils through C5aR to the site of inflammation [93]. Due to the facts discussed above, we decided to measure the expression of C5aR on the surface of PMNs. Indeed, mice with *Itgam* and *Itgax* knockout had significantly higher expression of this receptor on their surface compared to WT groups (Fig. 17A and B). To confirm that recognition of C5a by C5aR leads to the worsening of NTN in *ItgamItgax*$^{-/-}$ mice after CVF treatment, the animals should be injected with monoclonal antibodies against C5a. Resolution of albuminuria in the animals treated with the antibodies will indicate that the detrimental effect of CVF on knockout animals is, indeed, dependent on C5a. The findings presented above show that treatment with CVF does not have the same effect as lack of CD11b and CD11c on the progression of NTN. To demonstrate the relevance of the recognition of complement factors in the attenuation of NTN in *ItgamItgax*$^{-/-}$ mice, it is needed to cross this mouse line with C3$^{-/-}$ animals. C3 has already been shown to be pathogenic in the case of the ANTN model since it promotes the recruitment of PMNs to the kidneys [121]. However, the role of C3 in the infiltration of other subsets of immune cells during cGN as well as the role of its fragment iC3b in the progression of the disease has not been investigated yet.

In conclusion, we have shown that the upregulation of *Itgam* and *Itgax* is associated with the development of several types of human glomerulonephritis among which is RPGN as well as with the progression of murine NTN. We have demonstrated

that CD11b and CD11c are pathogenic during NTN in mice since mice deficient in these integrins had lower ACR, percentage of crescents, and NGAL in urine. *ItgamItgax*$^{-/-}$ animals were characterized by lower infiltration of cDC2s and CD4^{+} T cells in kidneys on day 10 post-NTN induction. CD4^{+} T cells from nephritic *ItgamItgax*$^{-/-}$ mice had lower expression of the genes involved in IL-1-related signaling compared to this type of lymphocytes in WT animals. cDC2s of knockout mice had impaired ability to uptake antigens. They were also less efficient in the induction of the proliferation of CD4^{+} T cells, which can be associated with impaired phagocytosis of the antigen by knockout DCs and subsequent lower antigen presentation. However, *Itgam* and *Itgax* knockout did not influence the expression of co-stimulatory molecules i.e., CD80 and CD86 on cDC2s. The conclusion and the proposed role of CR3 and CR4 signaling in cGN are summarized in the figure below.

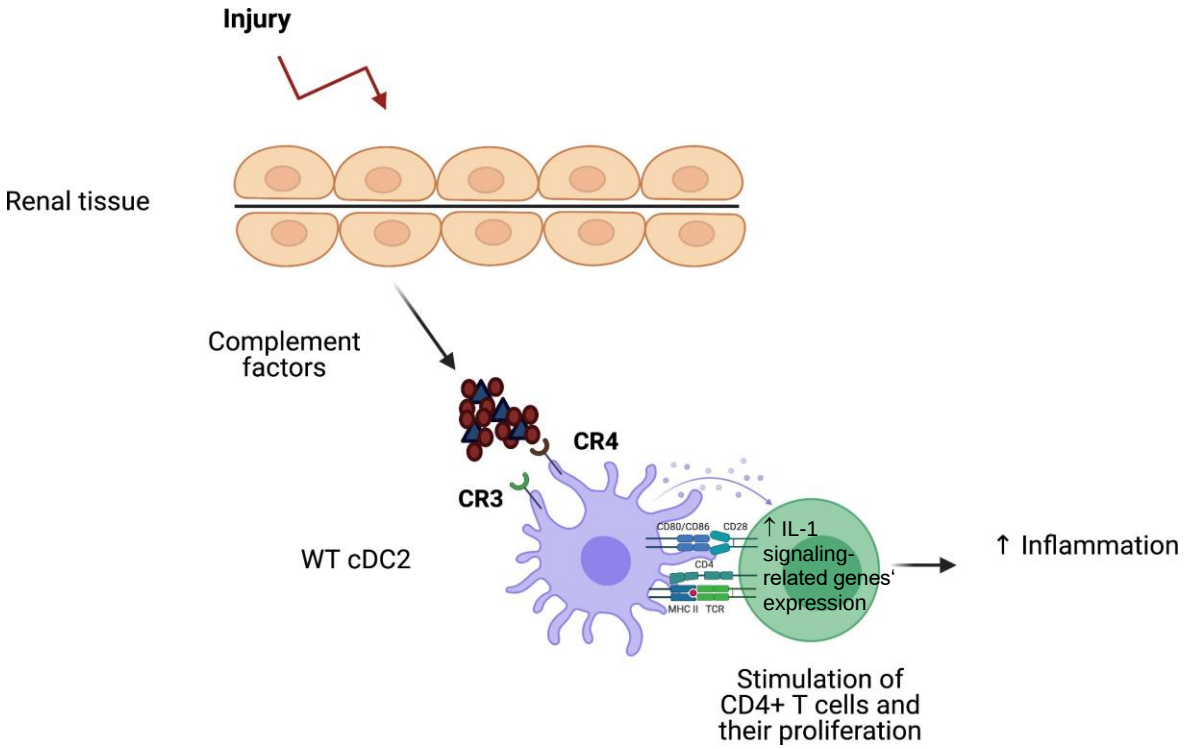


Figure 27. Role of CR3 and CR4 in crescentic glomerulonephritis. Due to the injury of renal tissue is activated complement system. Complement fragments then opsonize antigens and such opsonized particles are taken up by cDC2s via CR3 and CR4. This leads to increased antigen presentations by cDC2s, which could stimulate CD4^{+} T cells and increase their proliferation resulting in a selective increase in inflammation. The figure was created using BioRender.

The preceding results point out that targeting CR3 and CR4 can be a therapy for the treatment of RPGN. It is already known that Eculizumab, which is a monoclonal antibody against C5a, has a beneficial effect on the course of IgA nephropathy and C3 glomerulopathy in clinical trials [67,122]. However, more precise targeting of C3 and its fragments can have a more significant impact on the condition of patients suffering from complement-mediated glomerular disorders.

4.4 *Itgam* and *Itgax* in pyelonephritis

Pyelonephritis is one of the most common kidney infections, affecting every 12 to 13 people per 10 000 each year. However, current treatment strategies for the disease are not highly effective and a lot of cases of recurrent UTIs are constantly reported [123]. Another issue is that UTIs are mainly treated with antibiotics, which could lead to the appearance of antibiotic-resistant bacteria. Due to the facts mentioned above different treatment strategies for the disease should be developed [83]. During the project, we decided to investigate whether targeting CR3 and CR4 could be one of these strategies.

Our first hypothesis was that DCs of *ItgamItgax*$^{-/-}$ animals could move faster to the LNs due to their impaired adhesion to the tissues and induce adaptive immune response more efficiently. To investigate this question, we infected mice with UPEC and 23 days later re-infected them again. The experiment revealed that *ItgamItgax*$^{-/-}$ animals were, indeed, protected from pyelonephritis (Fig. 18B). However, we did not observe significant differences in the count of immune cells in both kidneys and renal-draining LNs between WT and *ItgamItgax*$^{-/-}$ animals (Fig. 18C-L). When we measured the production of cytokines by $CD4^+$ T cells as well as titers of the antibodies against UPEC in plasma, we also failed to identify significant differences between the groups (Fig. 18M-O). This is in the line with the study where mice deficient in β_2-integrins were infected with *T. muris*. Here the authors were able to show that DCs migration to LNs was not affected by the lack of β_2-integrins. Also, activation of T cells was not dependent on these integrins [13].

Since we observed a significant decrease in the bacterial load in the kidneys of *ItgamItgax*$^{-/-}$ mice without alteration in the adaptive immune response, we decided to look into the earlier timepoint of the infection, which was day 2 post-infection. However, at this

time point, we failed to observe better clearance of UPEC in knockout animals compared to the WT group (Fig. 19B). Interestingly, we could see a drastic increase in the number of PMNs in *ItgamItgax*$^{-/-}$ animals (Fig. 19F). Although it is known that CR3 plays an important role in the migration of neutrophils, the cells can also use LFA-1 for this purpose since it recognizes similar ligands to CR3 [124]. PMNs from *ItgamItgax*$^{-/-}$ mice can use LFA-1 to migrate to the site of infection, which can explain why the migration of PMNs was not impaired by the deficiency of CD11b and CD11c.

To see whether PMNs from *ItgamItgax*$^{-/-}$ animals can efficiently phagocytose UPEC, we performed *in vitro* experiment where we infected neutrophils isolated from WT and *ItgamItgax*$^{-/-}$ mice with the bacteria and then evaluated phagocytosis of the pathogen via flow cytometry as well as intracellular survival of UPEC using gentamycin protection assay. As expected, neutrophils from knockout animals had impaired phagocytosis. The number of live intracellular bacteria was also decreased in *ItgamItgax*$^{-/-}$ cells (Fig. 20B-E). CR3 and CR4 have already been shown to be important for the phagocytosis of complement-coated antigens, which explains the lower phagocytic capacity of knockout cells [16]. The observed increase in the infiltration of neutrophils on day 2 p.i. in *ItgamItgax*$^{-/-}$ animals can be a compensatory strategy as a result of the inability of knockout PMNs to efficiently take up bacteria.

Since on day 2 p.i. we failed to confirm a protective phenotype of *ItgamItgax*$^{-/-}$ mice in pyelonephritis, we decided to extend the time of the infection to 7 days. Final analysis of bacterial load in kidneys 7 days p.i. revealed a decrease in the CFUs in the kidneys of knockout animals (Fig. 21B). We also observed a decrease in the infiltration of kidneys with cDC1s and cDC2s (Fig. 21C and D). This can result from decreased inflammation in *ItgamItgax*$^{-/-}$ mice due to the beginning of the resolution of the infection. We also identified lower expression of several inflammatory cytokines among which were TNFα and IL-6 in *ItgamItgax*$^{-/-}$ animals (Fig. 21 G and H). Both cytokines are known to cause renal damage during pyelonephritis, which can result in increased BUN and creatinine levels in mice [76].

A previous study showed that phagocytosis of complement-opsonized UPEC by PTECs results in the intracellular survival of bacteria in these cells and the inability of immune cells to eliminate the pathogen [79]. Also, it was reported that *E. coli* can survive inside macrophages utilizing them as a niche for replication [95]. Due to the studies

mentioned above, we asked whether there are differences in the uptake of UPEC between WT and *ItgamItgax*$^{-/-}$ macrophages. We did not take into consideration PTECs since it is known that CD11b and CD11c are expressed only in immune cells [5]. To answer the question discussed above, we infected *ItgamItgax*$^{-/-}$ and WT BMDMs with UPEC and evaluated the intracellular survival of bacteria in both groups of cells. The results confirmed that, indeed, BMDMs deficient in CD11b and CD11c contained less viable intracellular bacteria compared to WT cells (Fig. 22B). However, evaluation of phagocytosis efficacy as well as the identification of the compartments of phagosome where UPEC resides inside macrophages via microscopy are still needed to validate this finding. It can be also useful to measure pH in the phagosomes after the infection of WT and knockout macrophages with *E. coli*. It is needed to isolate renal macrophages from already infected WT and *ItgamItgax*$^{-/-}$ mice and look for intracellularly survived UPEC too.

In summary, we were able to show that in the pyelonephritis model, knockout of *Itgam* and *Itgax* was beneficial since mice without CD11b and CD11c cleared bacteria faster compared to WT animals on day 7 p.i. The better outcome of *ItgamItgax*$^{-/-}$ animals is likely to be caused by the inability of their macrophages to efficiently phagocytose bacteria due to the lack of CR3 and CR4, which leads to the decreased number of viable intracellular UPEC. In this case, *E. coli* cannot use macrophages to evade the immune response. Although other studies claim that PMNs are the main effector cells responsible for fighting pyelonephritis[74], we did not observe a correlation between better clearance of bacteria and efficiency of phagocytosis of UPEC by neutrophils. Also, deficiency of CD11b and CD11c protected mice from overexpression of inflammatory cytokines (TNFα and IL-6), which are known to cause renal damage. Adaptive immune response and migration of DCs to the renal draining LNs were not affected by the lack of CD11b and CD11c. The findings discussed above point out the possibility to target CR3 and CR4 during the treatment of pyelonephritis. The summary of the following conclusions is presented in the figure below.

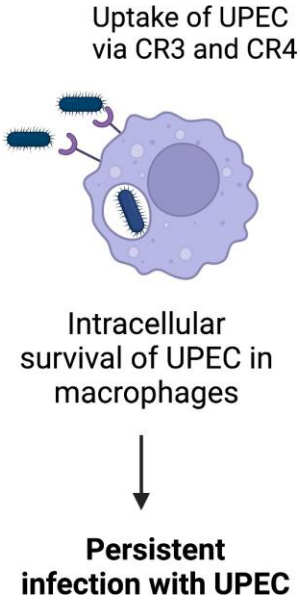


Figure 28. Role of CR3 and CR4 in the clearance of UPEC. UPEC uses macrophages as a niche for intracellular survival. The uptake of the bacterium by macrophages is dependent on CR3 and CR4. The figure was created using BioRender.

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1. Glowczyk I, Wong A, Potempa B, **Babyak O**, Lech M, Lamont RJ, Potempa J, Koziel J. Inactive Gingipains from *P. gingivalis* Selectively Skews T Cells toward a Th17 Phenotype in an IL-6 Dependent Manner. *Front Cell Infect Microbiol.* 2017 Apr 27;7:140. doi: 10.3389/fcimb.2017.00140.
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