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Analysis of Lymphocytes with T regulatory Phenotype in Kidney Allografts of Saint-Petersburg

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Hiermit erkläre ich, die Dissertation mit dem Titel "Analysis of Lymphocytes with T regulatory Phenotype in Kidney Allografts of Saint Petersburg" eigenständig angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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C4d ..... *The complement protein C4*
CD ..... *Claster of Differentiation*
DNA ..... *Deoxyribonucleic acid*
FACS ..... *Fluorescence-activated cell sorting*
FOXP3 ..... *Forkhead box P3*
HLA ..... *Human leucocyte antigen*
Ig ..... *Immunoglobulin*
IL ..... *Interleukin*
iTregs ..... *Induced regulatory T lymphocytes*
LC ..... *Lymphocytes*
MHC ..... *Major histocompatibility complex*
nTregs ..... *Natural regulatory T cells*
Pat ..... *Patient*
PBMC ..... *Peripheral blood mononuclear cells*
RNA ..... *Ribonucleic acid*
RT ..... *Renal transplantation*
TCR ..... *T cell receptor*
TGF-β ..... *Transforming growth factor β*
TH17 ..... *T helper 17*
Treg ..... *T regulatory lymphocytes*

1 Introduction

1.1 Immunological tolerance

One of the unique functions of the immune system is its ability to distinguish between self and non-self/foreign antigens. Taking into account the huge numbers of various foreign pathogens in the environment, there is a need for fast and effective immune protection, while at the same time it is very important to have an immune system that prevents pathological autoimmune attacks due to inadequate differentiation of self-antigens. 100 years ago, Paul Ehrlich already claimed the existence of regulatory mechanisms that protect the body from autoimmune reactions (Steinman and Nussenzweig 2002). The ability of the immune system to recognize, but to actively ignore self-antigens was later named immunological tolerance. With respect to the involved mechanisms, immune tolerance nowadays is differentiated into central and peripheral functions of tolerance.

1.1.1 T-cell maturation in the thymus and central T-cell immunological tolerance

Central T-cell immunological tolerance is formed during T-lymphocyte maturation in the thymus. T-cell development in the human thymus starts during the embryonic stage. Progenitor T-cells early migrate from the fetal liver into the thymus due to chemotactic signals of chemokines such as of CXC-motif chemokine 12 (CXCL12) and CC-chemokine ligand 25 (CCL25) (Savino et al. 2012; Gossens et al. 2009). These chemokines are produced by specific thymic epithelial cells (Gossens et al. 2009) and are recognized by specific receptors on the thymocyte progenitor cells - CXC-motif chemokine rezeptor 4 (CXCR4), C-C chemokine receptor type 9 (CCR9) and C-C-motif chemokin rezeptor 5 (CCR5) (Bromley et al. 2000).

Lymphocyte progenitor cells enter the thymus from blood through high endothelial venules at the cortico-medullary junction. Transmigration of other cells from blood is inhibited by a blood–thymus barrier consisting of three cell layers: the first cell layer is composed of specific endothelial cells of the post-capillary venules, the second one consists of macrophages and cells of the perivascular space, the third barrier cell layer is formed by thymic epithelial cells and their basement membrane. Transmigration of thymocytes is achieved by interaction of specific adhesion molecules such as L and P selectins, α4-integrins on the cell surfaces with their ligands such as fibronectin, vascular cell adhesion

protein 1 (VCAM-1) and mucosal vascular addressin cell adhesion molecule-1 (MadCAM-1) (Crisa et al. 1996; Savino et al. 2000; Cotta-de-Almeida et al. 2004). The adhesion molecule CD44, whose ligands are hyaluronic acid derivatives on endothelia, play an important role in cell migration into the thymus. After thymic immigration thymocytes lose CD44 cell surface expression (Rajasagi et al. 2009). Another adhesion molecule present in thymic venules - Vascular adhesion protein 1 (VAP-1) - is also important in pre-thymocyte migration processes (Salmi and Jalkanen 2006) of T-cell progenitors. Furthermore, thymocyte migration is dependent on the synthesis of hyaluronidase and collagenase enzymes, which participate in the inhibition of their uptake by macrophages (Bagriaçik and Miller 1999; Pilatte et al. 1990). The chemokine CCL25, known also as a thymus expressed chemokine (TECK), provides an important chemotactic force for the intra-thymic migration (Uehara et al. 2002) where thymocytes have to interact with thymic epithelia. This interaction is necessary for the initiation of thymocyte differentiation and maturation and is promoted by the adhesion molecules CD2 on the thymocyte cell membrane and CD58 on the epithelial cell membrane (Witherden et al. 1995). Also, participation of β1 and β2 integrins (lymphocyte function-associated antigen 1 (LFA-1), very late antigen-4 (VLA-4) and very late antigen-6 (VLA-6)) and their receptors (intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion protein 1 (VCAM-1)) in the interaction between thymocytes and cells of thymic epithelia was shown by Reiss and Engelhardt 1999 and Paessens et al. 2008.

In their development, thymocytes pass through several maturation stages from double negative to double and single CD4/CD8 positive T-cells. As double-negative (CD4-CD8-) cells, thymocytes enter the thymus via high endothelial venules at the medullary border and migrate toward the outer layers of the cortex. They extensively proliferate and start expressing specific diverse T-cell receptors (TCR). Only thymocytes with TCRs which specifically can recognize and bind MHC molecules on thymic epithelial cells are positively selected (Starr et al. 2003). As a result of positive selection, the cells of which TCRs can interact with MHC, presented on epithelial and antigen-presenting cells (APC) of the thymus, are selected (Klein et al. 2014). During these steps activation of genes, such as Notch, Runx-1, Gata-3, Ikaros, C-Myb, PU.1, E2A/HEB, TCF-1, RAG-1, RAG-2 (Turka et al. 1991; Spain et al. 1999; Ioannidis et al. 2001; Germain 2002; Bender et al. 2004; Egawa et al. 2007; Wojciechowski et al. 2007; Wang and Bosselut 2009; Yashiro-Ohtani et al. 2010; Yu et al. 2010; Taylor and Zimmermann 2013; Xu et al. 2013) was shown. TCR affinity for MHC leads to an increase in the expression of anti-apoptotic factor B-cell lymphoma 2 (Bcl-2), to the survival and further promotion (Sentmann et al. 1991; Linette

et al. 1994; Chao and Korsmeyer 1997) of the specific thymocytes. In addition, the immune-associated nucleotide-binding protein (IAN) gene family appears to play a role in thymocyte selection. Expression of IAN4 and IAN-5 genes is associated with the induction of the anti-apoptotic factors BCL-2 and BCL-XL (Nitta et al. 2006). These maturation stages are further characterized by the expression of CD3 as well as of the CD4 and CD8 co-receptors on the cell surfaces. The process begins in the deep layers of the cortex and ends in the medulla. After the double negative maturation stage, thymocytes reach the so called double positive CD4+CD8+ maturation stage and later differentiate into single CD4+ or CD8+ lymphocytes (LC) (Bosselut 2004). It is believed, that concomitant expression of transcription factors, such as T-helper inducing POZ-Kruppel factor (Th-POK) and GATA 3 binding protein (GATA-3), induces differentiation into CD4-expressing thymocytes (Wang et al. 2008; Gimferrer et al. 2011), whereas thymocyte selection-associated HMG box protein (TOX) and "runt related transcription factor 3 (RUNX3)" (Aliahmad et al. 2004) leads to the differentiation into CD8-expressing thymocytes. After going through positive selection thymocytes also may express CD69, CD5, CD27 and CD28 on their surfaces (Yamashita et al. 1993; Tarakhovsky et al. 1995). It was shown that only 10% of all double positive thymocytes pass through all these cortical differentiation stages.

At the end of these maturation processes and after completion of TCR gene rearrangement and positive selection two populations of T-lymphocytes are formed: γδ T-cells, which immediately leave the thymus and αβ T-cells, which are subjected to further development and negative selection (Burtrum et al. 1996; Joachims et al. 2006) in the medulla.

During negative selection, potential autoreactive T-cell clones with strong recognition of self-peptide MHC complexes are induced to undergo apoptosis. In this process of negative selection dendritic cells expressing a major histocompatibility complex (MHC) molecules complexed to peptides of diverse self-antigens on their surface are involved (Klein et al. 2014). It has been shown that apoptosis of cells with high-affinity TCR is triggered by activation of Nur77 gene (Matuszyk 2009). Cells with moderate affinity for autologous "MHC-peptide" complexes migrate into peripheral organs of the immune system. The phenomenon of negative selection was first postulated by the Australian immunologist Burnet in 1957 (Burnet 1957). He was awarded the Nobel Prize in Physiology or Medicine for "discovery of acquired immune tolerance".

For a long time it was unknown how thymic antigen-presenting cells (APC) could present self-antigens from organs outside of the thymus for the generation of self-tolerance. The

discovery of the gene Aire, whose activation in thymic medullary dendritic cells results in the expression of many organ specific self-antigens (Anderson et al. 2002) solved this scientific problem. Mutation of this gene leads to the development of the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy syndrome (APECED). AIRE-expressing APC have been also described outside of the thymus. These cells with high expression of MHC II and with CD80lo, CD86lo, EpCAMhi, CD45lo expression can also be found in secondary lymph organs and are thought to directly inactivate effector CD4+ cells and thus represent a regulatory tolerizing dendritic cell population (Gardner et al. 2013).

During negative selection, almost 50 percent of the thymocytes having passed positive selection die. Nevertheless, fair numbers of potentially auto-reactive T-cells are not eliminated which emigrate from the thymus into the peripheral blood. This could be due to the existence of a threshold level for the TCR affinity to self-antigens which allows mature potentially auto-reactive thymocytes to survive and escape into the pool of mature peripheral T-cells. Other researchers believe that within thymus not all peripheral self-antigens are presented in the negative selection process of medullary thymocytes. Autoreactive cells which could avoid negative selection in thymus are subjected to control in the peripheral blood in order protect from self-aggression and autoimmunity.

1.1.2 Peripheral immunological T-cell tolerance

Post-thymic mechanisms able to inactivate auto-reactive immune cells in the peripheral blood and tissues are part of regulation of peripheral tolerance (Goodnow et al. 2005; Zhou et al. 2008).

Peripheral tolerance is based on active and passive mechanisms. Passive mechanisms include ignorance of autoantigens by the immune system due to low concentration or isolation of the immune system from specific self-antigens (immune privileged sites/organs). The following important active mechanisms of tolerance have been described: Induction of anergy of autoreactive T-cell clones (Jenkins et al. 1987), differentiation of tolerizing antigen presenting cells with the capacity to directly or indirectly modulate T-cell differentiation and T-subset generation and immune response suppression via regulatory T-cells (Sakaguchi et al. 2009)

Mechanism of peripheral tolerance which relates to induction of clonal T-cell anergy after prolonged antigen stimulation (Webb et al. 1990; Jones et al. 1990; Kawabe and Ochi 1991) may be associated with activation-induced cell death (Janssen et al. 2000; Krueger et al.

2003). Anergy of autoreactive T-cells can be also induced by antigen presenting dendritic cells lacking appropriate co-stimulatory molecules for full T-cell activation. Jenkins et al. 1987 showed, that co-cultivation of T-lymphocytes with chemically fixed APC that were unable to present co-stimulatory molecules on their surface, induced anergy of T-cells. The relevant co-stimulatory molecules were described in details later (Lenschow et al. 1993; Kündig et al. 1996; Goodnow et al. 2005; Walker 2017). A major role in the activation of T-cells play CD28 and "inducible T-cell co-stimulatory receptor (ICOS)". An inhibitory activity on T-cell activation, however, is promoted by cytotoxic T-lymphocyte-associated Protein 4 (CTLA-4), programmed cell death protein 1 (PD-1) and "B- and T-lymphocyte attenuator (BTLA)" receptors (Chambers et al. 2001; Salomon and Bluestone 2001; Peggs et al. 2008). Ligands for CD28 and CTLA-4 receptors are CD80 and CD86 molecules, they are variably expresses on APC. In mice it first was demonstrated that blockade of B7-1 (CD80) as well as of B7-2 (CD86) leads to a reduction of T cell responses (Linsley et al. 1992). Both T-cell molecules CD28 and CTLA-4 are constitutively expressed on circulating lymphocytes, whereas CD80 and CD86 are up-regulated on activated APC (Sansom 2000; Vasu et al, 2003). The interaction of CD28 and CD80/86 is a central mechanism of T-cell activation. In the absence of an inflammatory milieu CD86 is weakly, whereas CD80 is not at all expressed on APCs (Hathcock et al. 1994). Interaction of auto-reactive T-cells with tissue-tolerizing APCs lacking CD80/86 expression in the absence of an active inflammatory process leads to permanent T-cell anergy (Jenkins et al. 1987; Falb et al. 1996; Bretscher 1999). Interaction of auto-reactive lymphocytes with APC matured at sites of inflammation and expressing CD80 and CD86 leads to lymphocyte activation, proliferation and differentiation into effector cells. These cells could participate in triggering autoimmunity (Vasu et al. 2003; Vasilevko et al. 2002).

Immunological tolerance is also influenced by the presence of specific T-cell populations with suppressive activity on immune responses. These lymphocytes, capable of also preventing the occurrence of autoimmune diseases in mice were called regulatory T-cells.

1.2 Regulatory T-cells

Regulatory T-lymphocytes (Treg) are a heterogeneous group of T-cells of various origin, the main function of which is to maintain the immunologic equilibrium and prevent autoimmune diseases. Due to their ability to regulate the activity of other cell populations (primarily, auto-reactive effector T-cells and activated APCs) they monitor immune responses against self-antigens. Also, an important role of regulatory T-cells in the

engraftment and tolerance of organ transplants as well as in suppression of immune reactivity against altered self-antigens of tumors is being discussed. Tregs today are differentiated into natural (terminal differentiation within the thymus) and peripheral induced Tregs (undergoing terminal differentiation in the peripheral blood).

Back in the early 70s of the last century, Gershon described suppressive functions of T-cell subsets (Gershon et al. 1970). It was shown, that the removal of the thymus in newborn mice led to the development of severe autoimmune diseases, which could be prevented by CD4+ T-cell infusions from another genetically identical mouse (Kojima et al. 1981). Therefore, the researchers concluded, that CD4+ T-cells might be involved in the mechanisms of tolerance and protection against autoimmune diseases. Later, Sakaguchi et al. 1995, first described interleukin 2 Receptor-CD25 and later the transcriptional factor FOXP3 (forkhead box P3) as specific markers of regulatory T-cells (Sakaguchi 2003). Deletion of CD4+CD25+ T-cells in mice induced the development of autoimmune disease and their replacement provided the regression of the disease. Moreover, transfer of CD4+CD25+ T-cells prevented the development of skin graft rejection in mice (Sakaguchi et al. 1995). In 2001, Shevach et al. claimed that CD25 (IL-2 receptor) is highly expressed on Tregs different to other activated CD25+ T-helper cells. Bennett et al. 2001 showed that mutation of the transcription factor FOXP3 in mice is associated with severe lympho-proliferative disease («Scurfy» mice). In humans, FOXP3-mutation is related to dysfunction of Tregs and development of multi-organ autoimmune disease. The disease called IPEX-syndrome is X-linked inherited (Barzaghi et al. 2012).

GITR (Shimizu et al. 2002), CTLA4 (Read et al. 2000), selectin L, as well as CCR7 (Szanya et al. 2002) also were described as further phenotypic markers of Tregs. Low expression of CD127 (IL-7 receptor) was shown to characterize activated regulatory T-cells specifically (Shklovskaya et al. 2006). CD127 as cell surface marker thus could be applied for specific in vitro selection of Tregs populations.

Regulatory T-cells can exert their suppressive functions on immune responses by diverse direct interactions with effector cells. In mice, it was shown that Tregs can directly destroy antigen-presenting cells and effector T-cells by perforin- and granzyme B-dependent signaling pathways (Gondek et al. 2005). Binding of CD80 and/or CD86 on APC to CTLA4 of Tregs can induce the expression of the enzyme indoleamine 2,3-dioxygenase which results in suppression of immune effector cells via the induction of a tryptophan catabolism (Mellor et al. 2004, Fallarino et al. 2003). Deaglio et al. 2007 suggested that also purinergic signaling pathways are involved in immune suppressive activities of Tregs. They

showed that co-expression of CD39/nucleoside triphosphate diphosphohydrolase-1 together with CD73 on regulatory T-cells leads to an accumulation of extracellular adenosine, which can modulate and polarize T-cell responses by interaction with adenosine receptors (Typ 1 purinogenen Adenosin Rezeptor A2A). Indirect suppressive effects of Tregs can be carried out by specific cytokines (von Boehmer 2005). Suppressive cytokines such as IL-10 and TGF-β produced by Tregs (Ghiringhelli et al. 2005; Peng et al. 2004) inhibit the expression of CD80 and CD86 on antigen-presenting cells and thus down-regulate antigen presentation followed by T-cell anergy due to the lack of sufficient co-stimulation (Gorelik and Flavell 2002).

1.2.1 Natural regulatory T-cells

Natural regulatory T-cells (nTregs) undergo their terminal differentiation within the thymus. Mechanisms of nTregs maturation within the thymus are not fully understood. Kerdiles et al. 2010 demonstrated the importance of Foxo transcription factors in the induction of the expression of FOXP3. Cheng et al. 2013 shown that the differentiation of single CD4+ thymocytes in direction of regulatory T-cells is determined by the appearance of CD3 and of high affinity IL-2 receptor on the cell surface of still immature thymocytes before the double CD4+CD8+ differentiation stage. Zorn et al. 2006 showed that IL-2 is able to activate FOXP3 through a STAT-dependent mechanism in vivo. In addition, the importance of transcription factors Smad3 and NFAT for activation of FOXP3 gene was shown by Tone et al. 2008. FOXP3 gene expression and maturation of nTregs is further driven by thymus epithelial cells producing TSLP (Thymic stromal lymphopoietin) (Jiang et al. 2006). Another important receptor in the development of nTregs is CD28, as CD28-deficient mice lack development of nTregs (Salomon et al. 2000).

nTregs stably express FOXP3 as major characteristic due to complete demethylation of the TSDR site (Floess et al. 2007; Baron et al. 2007). Maturation of regulatory T-cells in thymus is related to the resistance of thymocytes with high affinity TCR for self-MHC complexes to apoptosis. It is believed that notably the expression of FOXP3 renders these thymocytes resistant to apoptosis during negative selection. Selection of Tregs was named agonist selection. It takes place within the thymus and is common for Tregs cells only (Jordan et al. 2001). At the end of thymic differentiation, Tregs start to express CTLA-4, GITR, PD-1 and to migrate out of the thymus into the periphery where they constitute 4-5% of CD4+ cells in the peripheral blood (Sakaguchi et al. 2008).

1.2.2 Induced regulatory T-cells

Besides natural regulatory T-cells another heterogenous group of induced Tregs which mature in the peripheral blood from naive T-cells was described (Bach 2003; Roncarolo and Levings 2000).

In animal studies, it was shown that induction of peripheral Tregs represents an independent process as it can also occur in the absence of thymic nTregs (Jeker et al. 2012). Induction and differentiation of naive T-cells (CD4+CD25-FOXP3-) to regulatory T-cells (CD4+CD25+FOXP3+) occurs under influences of IL-2 and TGF-β cytokines (Horwitz et al. 2008; Lan et al. 2012; Curotto de Lafaille and Lafaille 2009). A significant increase of FOXP3+ cell numbers in blood of melanoma patients after treatment with IL-2 was described by Malek and Bayer 2004 and Cesana et al. 2006. TCR and IL-2 signaling pathways activate the transcription factors "signal transducer and activator of transcription 5" (STAT5), "nuclear factor of activated T-cells" (NFAT) and "cAMP response element binding protein"/"activating transcription factors" (CREB/ATF) which after binding to the promoter and enhancer activate the transcription of the FOXP3 gene (Zheng et al. 2010; Sakaguchi et al. 2010; Tone et al. 2008; Kim et al. 2007). TGF-β activates SMAD-signaling pathways through which either a direct effect on FOXP3-enhancer or activation by E2A and Id3-dependent pathways is executed (Tone et al. 2008; Maruyama et al. 2011). In addition, was shown that "programmed death-ligand 1" (PD-L1)-signaling pathway can increase the number of induced Tregs, its effect on Tregs is carried out by suppression of cellular signaling pathways such as phospho-Akt, mTOR, S6 and ERK2, and is accompanied by activation of "phosphatase and tensin homolog" (PTEN) cell signaling (Francisco et al. 2009).

It is known that lipid sphingosin 1-P can suppress TGF-β signaling and decrease the activity of induced regulatory T-cells (iTregs) (Chi 2011). Also it was shown that activation of the phosphoinositide-3-kinase/serine/threonine-specific protein kinase AKT (PI3K-AKT) signaling pathway inhibits FOXP3 gene expression in the early induction phase of iTregs. Activation of this pathway does not influence stable FOXP3 gene expression in nTregs which is associated with FOXP3 gene demethylation (Haxhinasto et al. 2008). Constitutive over-expression of AKT leads to autoimmune diseases in animal models, possibly by altering the generation of Tregs populations (Garza et al. 2000; Rathmell et al. 2003). PI3K-AKT signaling pathway is also linked to mTOR cell signaling, as AKT partially is exerting its effect on FOXP3 via mTorC1 and 2 targets. Several publications indicated a correlation between application of mTOR inhibitors (rapamycin) and the

number of Tregs (Qu et al. 2007; Battaglia et al. 2005). It was shown that rapamycin partially could counteract AKT inhibition of FOXP3 induction and thereby possibly exert protective effects on Tregs preservation and generation (Haxhinasto et al. 2008).

Experimental studies have shown differences in the generation and the maturation of thymic nTregs and peripheral iTregs. However, the specific differences in the activation of Tregs signature genes and the expression of characteristic differentiating surface molecules still are incompletely known.

1.2.3 Difference between natural and induced regulatory T-cells

Tregs include nTregs and iTregs and represent specific subsets of CD4+CD25+ T-cells with suppressive regulatory activity on innate and adaptive immune responses. nTregs are generated in the thymus, stably express FOXP3 and mostly carry TCRs with high affinity for self-MHC. Thymic development of nTregs is dependent on TCR stimulation together with CD28 co-stimulation (Salomon et al. 2000). It has been reported that nTregs mainly exert suppressive functions through cell-cell contact-dependent mechanisms and thus are able to directly trigger cytolysis and apoptosis in cellular targets (Chattopadhyay and Shevach 2013). iTregs differentiate in the periphery from CD4+ T-effector or memory cells after stimulation by allo-, self- or altered self-antigens in the presence of cytokines such as IL-2, IL-10, IL-4, TGF-ß. iTregs vary in their FOXP3 expression and are suggested to mainly exert suppressive effects on T-effector and antigen-presenting cells through the production of anti-inflammatory cytokines such as IL-10 and TGF-ß (Wang and Zheng 2013). nTregs react antigen un-specifically, whereas iTregs seem to be mostly antigen-specific. nTregs seem to differ from iTregs in a more stable expression of FOXP3. It has been shown that stability of FOXP3 expression is regulated at the epigenetic level. In the first intron of FOXP3 gene, the existence of a specific demethylated DNA region (Treg-specific demethylated region, TSDR) (Baron et al. 2006, 2007) was shown to be associated with the Treg phenotype. Complete demethylation of the TSDR region is characteristic for nTregs constantly expressing FOXP3 and less common for iTregs with variable FOXP3 transcription (Floess et al. 2007; Baron et al. 2007).

Tregs show in vitro plasticity with a differentiation capability into pro-inflammatory TH17-cells in the presence of IL-6 and IL-1ß (Zheng et al. 2008). Lan et al. 2012 proposed that iTregs are more resistant to this trans-differentiation than nTregs. Zheng et al. 2008 claimed that nTregs even could differentiate into T-helper 1, T-helper 2 and T-helper 17 cells under the influences of specific cytokines in their surroundings. In contrast, Chen et

al. 2011 also demonstrated differentiation instability of iTregs in vivo, which had been observed by Floess et al. 2007 in vitro. Recently different autoimmune diseases have been linked to an impaired balance of Treg/TH17-cell frequencies and function. A shift to TH17 as pro-inflammatory antagonists of Tregs has been reported in several autoimmune diseases.

Currently, there are no specific markers or transcription signatures which allow to distinguish iTregs from nTregs. Knowledge of specific markers could help to determine their precise frequencies and functional activities in peripheral blood and tissues. Recently expression of the transcription factor Helios was claimed to be specific for nTregs (Sugimoto et al. 2006; Thornton et al. 2010). Helios binds to the FOXP3 gene promoter and promotes stable maintenance of FOXP3 expression (Getnet et al. 2010). However, Helios is also expressed during activation and proliferation of other T-cell populations (Akimova et al. 2011). Moreover, Gottschalk et al. 2012 and Zabransky et al. 2012 reported that iTregs also express Helios which thus cannot be used to differentiate between the Tregs populations. Similar observations were reported for neuropilin 1 (Nrp1) as another possible marker of nTregs (Yadav et al. 2012; Milpied et al. 2009).

Thus, no single of the so far proposed phenotypic markers can be used to accurately determine the heterogeneity and frequencies of regulatory T-cells. In present analyses, a combination of different markers mostly is used to follow-up function and differentiation of Tregs.

1.2.4 Other populations of FOXP3+ lymphocytes

Although FOXP3 has been accepted as characteristic transcription signature gene of Tregs there is an increasing body of evidence of a transient activation related FOXP3 expression in other T-cell populations. Recently, expression of FOXP3 was also detected in non-hematopoietic cells, particularly in epithelial cells of different tissue origins FOXP3 was also found to be expressed in different epithelial and non-epithelial tumor cells (Wang et al. 2010; Karanikas et al. 2008). Transcription of FOXP3 also has been described in rare B-lymphocytes, of both healthy people (Noh et al. 2010) and patients with various oncohematological diseases (Felcht et al. 2012). The role of FOXP3 in these cells remains unexplored.

Manrique et al. 2011 reported about FOXP3+ macrophages in lymphoid organs such as thymus, liver, spleen, lymph nodes, but later had to retract publication because of strong criticism of the reliability of the results.

1.3 The importance of regulatory T-cells in kidney transplantation

Major key players in renal transplantation immunology are CD4+ and CD8+ T-lymphocytes which recognize alloantigens and are responsible for cell-mediated graft rejection reactions, activation of humoral alloimmune responses and participation in inflammatory pathways with development of acute as well as chronic tissue damage and fibrosis. Clinical transplant tolerance in the patient has been achieved when normal graft function and histology could be maintained without immunosuppressive therapy for at least one year. In renal transplantation tolerance to the grafted organ is rarely spontaneous and probably actively mediated by the involvement of antigen-specific Tregs capable of suppressing allo-responses.

The interests in Tregs, in their clinical and prognostic use in renal transplantation and their role in immune regulation of allograft rejections are increasing worldwide. There are many publications showing the importance of regulatory T-cells in animal models of transplantation (Benghiat et al. 2005; Joffre et al. 2008; Miyajima et al. 2011). Engraftment of skin grafts transferred from male to female mice were successful with no signs of rejection. However, removal of CD25+ T-lymphocytes before transplantation in the skin recipient mice led to rejection reactions (Benghiat et al. 2005). Also, depletion of CD25+ T-lymphocytes in mice undergoing heart transplantation resulted in acute graft rejection (Schenk et al. 2005). Joffre et al. 2008 showed that the transfer of ex-vivo expanded donor antigen specific CD4+CD25+FOXP3+ Tregs to mice after bone marrow transplantation and subsequent transplantation of skin or heart prevented both acute and chronic rejection reactions. In most settings of animal transplantation models a significant role of Tregs in organ transplantation could be demonstrated. In spite of several clarifying experimental studies it is not an easy task to prove a tolerance mediating role of Tregs also in human organ transplantation.

Brouard et al. 2007 searched for transcription signatures in transplanted patients with operational tolerance to an allograft. Using diagnostic microarray technology (microarray analysis) the authors discovered 49 genes, including FOXP3 and genes regulated by TGF-β to be transcribed in association to tolerance induction and maintenance. The authors suggested that these genes may be used as prognostic biomarkers of transplant outcomes. In cross-comparative analyses by Louis et al. 2006 the number of circulating Tregs in healthy individuals, transplanted patients with chronic rejection and transplanted patients without anamnestic history of rejection episodes were evaluated. The authors reported about fewer peripheral Tregs in patients with chronic rejection than in healthy patients.

Frequencies of Tregs in patients tolerant to the allograft were about the same as in the healthy control group. Another research group presented results of a retrospective study, which included 17 kidney graft recipients, on Tregs infiltrations of the allografts and their correlation to rejection episodes and graft prognosis. This study suggested that increased Tregs infiltrates in the transplant could be associated with favorable graft outcomes (Martin et al. 2007). Similarly, in another retrospective study of 46 renal transplant biopsies with signs of borderline rejection it was observed that higher levels of FOXP3 mRNA transcripts could be only detected in the renal tissues of grafted patients (n=25) with stable renal function (serum creatinine level). In patients with reduced levels of FOXP3 transcripts, acute cellular rejection reactions were reported in subsequent biopsies (Mansour et al. 2008). Bestard et al. 2011 published observations of a retrospective study on 37 transplanted patients with histological signs of subclinical rejection in graft biopsies taken 6th month after transplantation. The absolute numbers of Tregs and the Tregs/total T-cell ratios were higher in patients with better renal function at 2 and 3 years after transplantation and cases with better 5-year graft survival. The authors suggested that higher ratios of Tregs in graft biopsy infiltrates could be the limiting factor for protection of rejection reactions. Similar results were published by Xu et al. 2012. They also showed that in patients with histological borderline changes in graft biopsies increased levels of FOXP3-transcripts correlated with a better 5-year graft survival and better renal function. The authors concluded that, in addition to the morphological evaluation of the graft according to Banff classification, especially in case of borderline rejection reactions, differentiation of infiltrating lymphocyte populations in kidney transplants as well as evaluation and enumeration of Tregs is mandatory for transplantation prognosis. In a retrospective study of 67 transplant biopsies Zuber et al. 2009 assumed that Tregs also could suppress acute immune responses and promote pro-inflammatory reactivity with a shift into chronic immune processes. In addition, a correlation between increased numbers of Tregs in chronic transplant infiltrates and better graft survival was demonstrated.

On other hand, interesting results were demonstrated by Muthukumar et al. 2005 in prospective study, which included 36 transplanted patients experiencing acute rejection reaction episodes. The transplanted patients with acute rejection showed higher levels of FOXP3 mRNA transcripts in the urine than the patients without acute rejection. Nevertheless, the elevated levels of FOXP3 transcripts in the graft correlated with better transplant outcomes. Also in one prospective study with 35 patients reported by Aquino-Dias et al. in 2008 was showed that acute renal rejection episodes were significantly correlated with elevated levels of FOXP3 transcription in blood, kidney tissues and urine of

transplanted patients. Similar, Veronese et al. 2007 reported on an association of high FOXP3 expression levels in the graft with acute cell-mediated rejection reactions, but they saw no correlation between Tregs with phenotype CD4+FOXP3+ in graft biopsies and kidney function at the time of the biopsies. Taflin et al. 2010 casted doubt on the diagnostic value of Tregs in the graft, as they did not see any correlation of CD4+FOXP3+ Tregs presence and morphological changes of the graft. No correlation between presence of Tregs in the graft and kidney function at 12 and 24 months post-transplantation was found by Kollins et al. 2011 in another retrospective study of 55 patients.

At present, quite contrary views on the prognostic and/or diagnostic relevance of Tregs in renal transplantation have been published.

In 2011 an international project "One Study" has been launched, which intends to develop ex vivo selected and expanded Tregs for clinical application and transfusion in transplanted patients. Major aims of the study are a better functional and phenotypic characterization of Tregs, clinical safety and efficacy studies of Tregs in transplant rejection. Many different methods of standardization of Tregs isolation have been tried, including specific cell sorting using flow cytometry, magnetic beads (CliniMACS) (Hoffmann et al. 2006; Peters et al. 2008; Putnam et al. 2009; Di Ianni et al. 2009; Issa et al. 2010; Nadig et al. 2010). Instability of FOXP3-antigen expression was recognized and confirmed as a major issue in the selection and expansion of regulatory T-cells. Hoffmann et al. 2006 reported about the problem of instability of FOXP3-antigen expression in cell culture in detail, and also suggested to use CD45RA+ as cell surface marker to select stable populations of Tregs, probably, nTregs.

The Project "One study" is still not completed, a clear answer to the importance of Tregs in kidney transplantation by this research group has not yet been formulated.

Although a large number of publications implicate Tregs in the pathogenesis and in the immune mechanisms of acute and chronic rejection of clinical renal transplants, their precise role and function in the affected graft still remains incompletely understood. Nevertheless, the therapeutic potentials of Tregs are very promising from experimental studies and justify their further analysis. This theoretical background also served as motivation for our comparative clinical study of Tregs in blood and renal biopsies after transplantation.

2 Aim of study

The aim of the study was to detect and characterize regulatory T-cells in the peripheral blood and in the kidney transplant biopsies of 53 patients after allogeneic kidney transplantation. Transplantation and clinical follow-up, immunosuppressive monitoring and treatment of the patients had been performed in St. Petersburg. Tregs evaluation and detection in renal biopsies was performed at the Dept. Nephrology and Rheumatology, University Clinics, Goettingen. FACS analysis of peripheral lymphocytes with Tregs phenotypes was performed in St. Petersburg. We intended to evaluate frequencies and dynamics of regulatory T-cells at two different time points after transplantation in patients with and without acute/chronic rejection reactions and correlate their frequencies in tissues and blood with morphological signs of renal damage related to cell- and/or antibody–mediated rejection reactions and thus wanted to get further insight into their clinical diagnostic and prognostic significance. Specifically, we also established immunohistological double staining procedures for the precise detection of CD4+FOXP3+ Tregs in paraffin-embedded renal biopsies.

3 Material and methods

3.1 Clinical analysis

3.1.1 Patients characteristics

This study included 53 renal transplant recipients who had received a renal transplantation (RT) from a living (n=34) or a deceased donor (n=19) between 2009 and 2011 at St. Petersburg State Medical University named after I. P. Pavlov. The study protocol had been approved by the local Ethic Committee in St. Petersburg. Documented clinical data and characteristics of the transplanted patients are summarized in Table 1.

Table 1:Characteristics and clinical data of the transplanted patients

Variable	Patients
Recipient gender	23 males /30 females
Recipient age (year±SD)	35,7 ± 11,8 years range 15-59 years, 95% -CI
Type of graft donor (LD/DD)	34/19
HLA-mismatches [1]	3,7 ± 1,3 (max. range 0-6 mismatches)
Crossmatch reaction	negative (n=53)
Time of cold ischemia of the transplant, hours	8,9 ± 6,5 range 1-23
RRT before Tx (+/-, number of patients)	45/8
Duration of RRT before transplantation, months	15 (mean) 25%-75%: 7,4-34,3 min-max: 1,4-170,4
Primary renal disease:	
• morphologically verified glomerulonephritis	32% (n=17)

• morphologically undifferentiated glomerulonephritis	24,5% (n=13)
• diabetic nephropathy	24,5% (n=13)
• renal/urinary tract (UT) abnormalities	7,5% (n=4)
• genetic kidney disorders	5,7% (n=3)
• polycystic kidney disease	3,8% (n=2)
• tubulointerstitial nephritis	2% (n=1)

[1-] 45 from 53 patients were tested

M- male, F-female, LD-living donor, DD- deceased donor; CI-confidence interval; RRT- renal replacement therapy

The following characteristics and clinical data of the graft donor were documented in the study as listed in Table 2:

Table 2: Characteristics of the donor/graft

Age
Living related/deceased donor
HLA-A, B, DRB1 phenotype
CMV serology
Time of cold ischemia

3.1.2 Immunosuppressive therapy regimens after grafting

Immediately after transplantation, all patients received a pre-adaptation immunosuppressive therapy which consisted of an anti-CD25 monoclonal antibody (basiliximab) for inhibition of T cell protein induction and activation for up to 2 months (Prescribing information, Basiliximab Novartis) in combination with a calcineurin inhibitor (cyclosporine or tacrolimus) of which the effective serum levels were regularly controlled 12 hours after drug administration. In addition, mycophenolate (CellCept, Myfortic) and Prednisolone (starting dose of 500 mg tapered to a maintenance dose of 5 mg after one

month) were first given and continued together with the calcineurin inhibitor as maintenance triple immunosuppressive therapy at least during the first year after grafting.

3.1.3 Records of graft function of the patients

For evaluation of graft function, serum creatinine and serum urea levels as well as the glomerular filtration rate at the time of the first biopsy, at a second biopsy and at the endpoint of the observation time were documented.

Serum creatinine and serum urea levels were determined with the help of an automatic biochemical analyzer using standard methods. Glomerular filtration rate was estimated according to the MDRD formula (https://www.niddk.nih.gov/health-information/health-communication-programs/nkdep/lab-evaluation/gfr/estimating/Pages/estimating.aspx):

$$eGFR\ (mL / min / 1.73\ m^2) = 186 \times (Serum\ Creatinine)^{-1,154} \times (Age)^{-0.203} \times (0.742\ if\ Female)$$

3.2 Histopathology

3.2.1 Renal biopsies

Regular protocol biopsies (n=76) performed in the routine clinical survey of the patients after transplantation were used in this study. Additional punctures of the allografts selectively induced for this study were not done. Regular protocol biopsies of the graft were performed at 3, 6, 12, 24, 36 months after the transplantation. In cases of a suspected rejection episode as well as for the control of the effectiveness of immunosuppressive therapy adjustments during rejection, additional diagnostic biopsies (n=16) outside of the protocol were done which also could be included in this study.

Renal biopsies were performed after informed consent of the patient by closed puncture under ultrasound guidance after local anesthesia. No complications in any case were recorded. Each biopsy was immediately controlled by a pathologist for adequacy of the obtained tissue and the need for further punctures. Immediately after removal all biopsies were fixed in 0,4% paraformaldehyde for 24 hours and later embedded in paraffin.

All biopsies were subjected to routine histological, immunohistochemical and immuno-fluorescence analysis.

3.2.2 Presence of allograft rejection

In general, differential diagnosis of transplant rejection was routinely based on clinical as well as on laboratory data and histological signs of rejection. Histological diagnoses of kidney transplant rejection based on the morphological criteria of the Banff classification are presented in Table 4.

Degree of rejection was scored as Grade 1 to 3 according to the Banff criteria and progression evaluated in a subsequent biopsy according to the development of the grade of histological rejection signs. In detail, for evaluation of the dynamics of rejection lesions no morphological changes indicating rejection in the first and second biopsy were scored as 0; if changes in the first biopsy reverted to a normal status (no change) in the second biopsy then rejection was scored as 1. Similarly; score 2 was given to dynamic improvement of rejection reactions grade 2 in the first biopsy to 1 in the second. Constant rejection signs of rejection in the first and second biopsy were scored "3". Score 4 and 5 were given to cases of rejection grade 0 or 1 in the first biopsy and grade 1 i.e. 2 in the second biopsy. Later this five stages were defined in three subgroups: with signs of worsening or progression of alteration, no changes and signs of improvement.

Table 3: Abbreviations of the morphological criteria of graft rejection according to the Banff classification 1997 (Update 2007)

T	tubulitis
V	vasculitis
I	Infiltration or mononuclear inflammation
G	glomerulonephritis
Ci	interstitial fibrosis
Ptc	peritubular capillaritis
C4dptc	C4d deposition in peritubular capillaries
C4dglom	C4d deposition in glomeruli
Ct	tubular atrophy
Cg	glomerulopathy
Mm	increase of mesangial matrix
Cv	fibrosis of the intima of capillaries

Ah	arteriolar hyalinosis
Pvi	perivenular infiltration

Rejection of the allograft tissue was assessed according to the criteria of the Banff classification from 1997 (Update 2007) (Gaber et al. 1997; Racusen et al. 1999; Solez et al. 2008, Bhowmik et al. 2010; Sis et al. 2010) and presented in Table 4.

Table 4: Histopathological diagnosis of renal allograft rejections according to Banff classification

1. Normal	
2. Antibody-mediated rejection $C4d^+$, presence of circulating antidonor antibodies, morphologic evidence of tissue injury, such as:	**Acute antibody-mediated rejection (AMR)** Type I: ATN-like minimal inflammation Type II: capillary and/or glomerular inflammation (ptc/g >0) and/or thromboses Type III: transmural arteritis (v3)
	Chronic active AMR glomerular double contours and/or peritubular capillary basement membrane multilayering and/or interstitial fibrosis/tubular atrophy and/or fibrous intimal thickening in arteries
3. Borderline changes	no intimal arteritis foci of tubulitis (t1-t3) and minimal interstitial infiltration (i0-i1) or interstitial infiltration (i2-i3) and mild tubulitis (t1)
4. T-cell mediated rejection	**Acute T-cell mediated rejection (TCMR)**

	IA: significant interstitial infiltration (i2-i3) and foci of moderate tubulitis (t2) IB: significant interstitial infiltration (i2-i3) and foci of severe tubulitis (t3) IIA: mild or moderate intimal arteritis (v1) IIB: severe intimal arteritis III: transmural arteritis and/or arterial fibrinoid change and necrosis of medial smooth muscle cells with accompanying lymphocytic inflammation (v3)
	Chronic active T-cell mediated rejection arterial intimal fibrosis with mononuclear cell infiltration in fibrosis, formation of neo-intima = "chronic allograft arteriopathy"
5. **Interstitial fibrosis and tubular atrophy**, no evidence of any specific etiology	Grade I: mild (< 25% of cortical area) Grade II: moderate (26-50% of cortical area) Grade III: severe (> 50% of cortical area)

In this study 22 from 92 studied biopsies had histological signs of borderline rejection (n=9), T-cell mediated rejection (n=5) or antibody mediated rejection (n=8). Because of the small number of biopsies, the grouping of the biopsies to the definitive rejection diagnosis also based on clinical data of the patients was not carried out.

3.3 Clinical study design

All patients (n=53) were retrospectively studied during an observation period of up to 41,3±17,6 months (min-max 4-72 months, 95% -CI) post-transplantation. End-point of follow up in the study was set to September 2013. Three patients died during this post-transplantation period (n=2 because of cardiovascular events; n=1 because of acute kidney insufficiency). Three cases of graft loss more than one year after transplantation were observed. For this study, two biopsies from each patient were selected at 4,0 months

(median) (25%-75%: 3-7,2; min-max: 0,6-39,4; n=53) and at 12,8 months (median) (25%-75%: 10,3-21,5; min-max: 1,9-52,8; n=39) after grafting. Thus, a total number of 92 biopsies could be included into the immunohistological analyses of regulatory T-cells in the renal tissues in correlation to morphological signs of graft rejection

In addition, frequencies of Tregs were evaluated in peripheral blood of 29 transplanted patients and 5 healthy individuals. Frequencies of regulatory T-cells in blood were compared to their occurrence in the allograft. All blood samples for FACS analysis were taken from April to June 2010 when the patients had survived 12,1 ± 9,0 months on average after grafting.

3.4 Immunological methods

3.4.1 HLA-typing

Molecular HLA-typing was performed in three loci: HLA-A, B, DR. A Sequence Specific Primer Kit (Kit Protrans HLA (SSP) for low-resolution genotyping based on an HLA-allele-specific amplification and subsequent differentiation and detection of the amplicons in a gel-electrophoretical analysis was applied. The typing was performed by the HLA-Laboratory of the Hematological Clinic, St. Petersburg Medical University of I. P. Pavlov. Results of HLA typing of the recipients and donors were registered and used for optimal donor/recipient HLA-matching. Complete match in 6/6 alleles was present in only 1 patient /donor pair. All others exhibited at least 2 HLA mismatches.

3.4.2 Immunohistochemistry

92 renal biopsies were investigated by indirect double immunohistochemical labelling for presence of regulatory T-cells in correlation to signs of rejection.

Immediately after removal all biopsies were fixed in 0,4% paraformaldehyde for 24 hours before they were dehydrated in ethanol chloroform and thereafter embedded in paraffin. 3-5 µm thick tissue sections were cut from the paraffin blocks using a standard microtome.

Before immunohistological staining the paraffin sections were deparaffinized and rehydrated with two immersions in xylene (for 10 minutes each time) and in a graded series of ethanol (for 5 minutes each time). Thereafter they were washed in destilled water for 5 minutes. Antigen retrieval was routinely performed by immersing the sections in sodium citrate buffer (pH 9,0, DAKO) and heating in a microwave for 10 min. For double

immunohistochemical staining of the tissues the Double Stain EnVision System kit (DAKO, Jena, Germany) for formalin-fixed, paraffin-embedded tissue sections were used.

Dual Endogenous Enzyme Block (from Kit) was used to inhibit endogenous alkaline phosphatase, peroxidase and pseudo-peroxidase activity.

On each slide two different antigens were bound and labelled with specific antibodies. The following ready-to-use antibodies were applied: polyclonal anti-CD4 (rabbit, Emelca Bioscience, Nederland), polyclonal anti-CD3 (rabbit, Dako, Denmark), polyclonal anti-CD20 (rabbit, Emelca Bioscience, Nederland), monoclonal anti-CD14 (rabbit, Dianova, Germany) and a purified monoclonal antibody against FOXP3 (mouse, eBioscience, Nederland). Applied dilutions of the antibodies are listed in Table 5.

Table 5: Antibody dilution

Antibody	Dilution (in PBS)
Rabbit anti-human (polyclonal) ready-to-use AKs: CD3/CD4/CD20 Rabbit anti-human monoclonal ready-to-use AK: CD14	1:2
Mouse anti-human (monoclonal) purified FOXP3-AK	1:100

Lymphocytes and monocytes were first differentiated by surface labelling with one of the antibodies against CD3/CD4/CD14/CD20 (rabbit anti-human polyclonal/monoclonal) followed by incubation with a polymer complexed to peroxidase conjugated goat anti-rabbit IgG. Before staining with the first antibody, any endogenous peroxidase activity was quenched by incubation of the specimen with a peroxidase blocking reagent of the kit for five minutes. Thereafter the reaction was visualized by incubation with diaminobenzidine tetrahydrochloride (DAB+ Chromogen) as enzymatic substrate.

After 5 min of incubation with Dual Endogenous Enzyme Block reagent, the sections were further labelled with the monoclonal antibody against the intracellular transcription factor protein FOXP3 in a wet chamber for 12 h at +4°C. Binding of this antibody was visualized in an enzymatic reaction with a goat anti-mouse alkaline phosphatase IgG complex and Permanent Red – Chromogen as substrate. After three final rinses in PBS the sections were finally mounted in mounting medium (Immu-Mount, USA) and cover-slipped. Each immunohistochemical staining experiment included negative controls in which the first or second specific antibody or both specific reagents were omitted for the control of unspecific binding and cross-reactivity of the detection reagents.

All stained slides were analyzed on an inverted microscope (Olympus BX43, Hamburg, Germany) with the use of CellSens Dimension software (Olympus, Hamburg, Germany) for on each section labelled cells were recorded. Using optical grid of Software ScanScope (Aperio, Bristol, UK) on each section labelled cells within 10 fields of vision and average numbers of positive cells were calculated from that for each slide.

3.4.3 Immunofluorescence staining

Indirect immunofluorescence staining of the kidney biopsies was evaluated as further technique to assure and verify adequate FOXP3 labelling. Due to high background labelling of the paraffin embedded, dewaxed and antigen retrieved tissues only selected biopsies could be evaluated and included in the analyses. Before staining, paraformaldehyde fixed and paraffin embedded biopsies were treated according to the above described immunohistochemical protocol of deparaffinization, rehydration and antigen retrieval. Before application of the first antibody each slide was incubated for 30 min with a blocking solution (bovine serum albumin 1%, Sigma-Aldrich, Germany) to reduce unspecific antibody binding. Samples were incubated with polyclonal goat anti-human IgG CD4 (R&D Systems, USA) and mouse anti-human IgG FOXP3 (eBioscience) as first specific antibody for about 12 hours at 4°C and with a second antibody in dilution 1:200 donkey anti-mouse 594, donkey anti-goat 488 (Invitrogen, Carlsbad, Californien, USA for 60 minutes at room temperature. Thereafter the slides were washed in PBS and embedded with a Mounting Medium containing DAPI ("Vectashield", Vector, USA) for nuclear staining. Bound fluorescence was evaluated on a Zeiss confocal laser scanning microscope (Fluoview FV-1000, Olympus, Hamburg, Germany) with the use of Software CellM 2.8 Built 1235, Olympus, Hamburg, Germany and corresponding filter for FITC and RED.

3.4.4 Flow cytometry

29 transplanted patients (14 with grafts from a related donor, 15 with grafts from a deceased donor) were investigated for Tregs frequencies in peripheral blood at one-time point between 180 to 720 days (average 353 days, 95% CI: 246-459) post-transplantation. Peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation from 20ml of blood drawn from each patient. Cells were counted and viability evaluated after two washes with washing buffer (BD Biosciences). They were again centrifuged at 1000 rpm for 5 minutes and cell concentration adjusted to 10^6 cells/ml. Thereafter 10^6 cells were incubated with 10µl of the FITC/APC conjugated first antibodies (CD4 (Clone RPA T4), CD25 (Clone M-A251) for 20 min in the dark. Thereafter cells were washed with BD

washing buffer and centrifuged. After thorough washing with BD washing buffer, cells were fix with buffers A (incubation for ten minutes in the dark) and permeabilized with buffers C (incubation for 30 minutes in the dark). Both buffers were included in the applied FOXP3 staining kit (Human FOXP3 Buffer Set 560098, BD Biosciences, USA). After further washings and centrifugation, cells were incubated with 10µl of the PE conjugated FOXP3 antibody (Clone 259D/C7, BD Pharmingen, USA) for 30 min in the dark. Thereafter the cells were again washed and analyzed on a flow-cytometer (BD Aria, USA) using FACSDiva software (BD Bioscience).

For the determination of CD4+ CD25+^{high}FOXP3+ T-cells, lymphocyte populations were first gated in the FSC/SSC plots of the analyzed PBMC followed by evaluation of the CD4+ subset. Electronically gated CD4+ cells were analyzed for the expression of the CD25 antigen and subsequently gated CD4+CD25+ cells analyzed for the expression of FOXP3.

3.5 Statistics

For statistical analysis, the STATISTIKA Update 12 software was used. Cell Counts are presented as absolute numbers or percentages of lymphocyte populations. For comparison of cell frequencies in different patient groups, both parametric and non-parametric methods were applied. Differences between the groups were calculated using paired and unpaired Student t-test, Wilson test, Mann-Whitney test, Kolmogorov-Smirnov test, Kruskal-Wallis test depending on the data type. In order to assess the relationship between defined parameters Pearson and Spearman correlation analyses were applied. Also, ANOVA method was used.

The results were considered significant when the P value was below 0,05.

4 Results

4.1 Specific lymphocyte subsets in peripheral blood of patients after kidney transplantation (Tx) and of healthy individuals

4.1.1 Phenotypic characterization

Peripheral blood mononuclear cells (PBMC) of 29 patients and controls (n=5) were analyzed for presence of regulatory T-cells. First, total leucocyte and lymphocyte counts were determined in peripheral blood samples and further sub-classified by fluorescence-activated cell scanning (FACS) analysis of specific differentiation antigens and transcripts. Results of the analyses are presented in Table 6.

Table 6: Different lymphocytes subsets in blood of transplanted patients and healthy individuals

PBMC of patients (n = 29)				PBMC of healthy individuals (n = 5)		
Cells	Mean	Min	Max	Mean	Min	Max
Leucocytes, 10^9/l	7,0	2,3	10,8	5,9	5,5	6,3
Lymphocytes, %*	27,4	13	49	31	30	33
Lymphocytes, 10^9/l	1,8	0,7	3,7	1,86	1,65	2,07
CD4+cells, % **	43,2	26,6	64	36,5	34,3	38,7
CD4+cells, x10^9/l	0,8	0,3	1,64	0,67	0,64	0,7
CD25+cells, %**	12,3	0	24	16,2	16	16,4
CD25+cells, x10^8/l	1,8	0	3,7	3,0	2,6	3,4
FOXP3+cells, % **	0,9	0,1	2,1	6,9	5,2	8,8

FOXP3+-cells, $x10^6$/l	16,8	0,5	28,6	133,5	85,1	182
CD4+CD25+-cells, %**	11,8	0	30	11,7	11,6	11,9
CD4+FOXP3+-cells, % **	0,6	0,1	1,85	2,8	1,75	3,88
CD25+FOXP3+-cells, % **	0,4	0	1,45	2,0	1,5	2,5
CD4+CD25+ high FOXP3+-cells, % #	0,8	0	2,7	4,3	2,4	6,2
CD4+CD25+high FOXP3+-cells, x 10^6/l	5,7	0	27,5	29,5	15,1	44,3

* - Percentages of lymphocytes of the total leucocytes in peripheral blood

** - Percentages of cells of the total lymphocyte counts

- Percentages of CD4+CD25+^{high}FOXP3+ cells of the CD4+ T-cell populations

The table shows total leucocyte and lymphocyte counts as well as percentages of CD4 positive T-cell subpopulations determined by FACS analysis of PBMC of patients after kidney transplantation (n=29) in comparison to healthy individuals as controls (n=5): lymphocytes were electronically gated in forward/side scatter plots of PBMCs and analyzed for the percentage of cells positively labelled with fluorescence conjugated monoclonal antibodies directed against specific differentiation antigens (also see Materials and Methods).

A highly significant correlation ($r_{pearson}$ = 0,87; p<0,001) was found between the relative numbers of CD4+ cells showing FOXP3 positivity and the absolute values of CD4+CD25+FOXP3+cells in peripheral blood (Figure 1) confirming dependency of the Tregs numbers on CD4+ FOXP3+ cell frequencies and test accuracy.

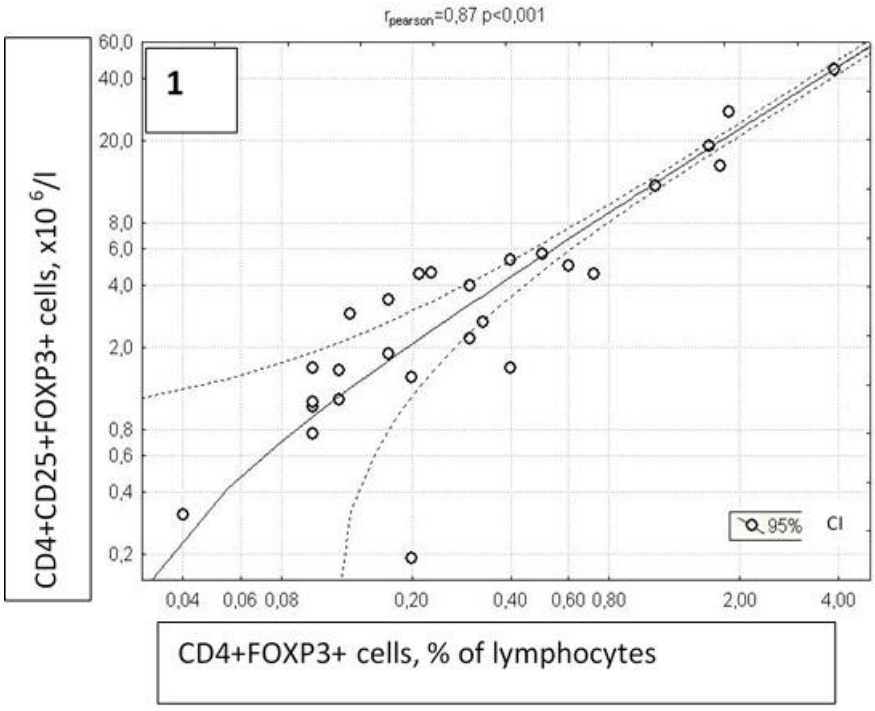


Figure 1: Correlation between percentage of CD4+FOXP3+ cells and absolute numbers of Tregs in blood. Scatter plot, both axis are logarithmic.

4.1.2 Influences on the determination of Tregs

Monoclonal antibodies given to patients after transplantation as prophylactic strategy of rejection episodes could be expected to alter T-cell subpopulations by deletion or down-regulation of the specific differentiation antigens to which they bound on the cell surface. In this study, the anti-CD25 (IL-2Rα) Mab given twice after transplantation (first and fourth day post grafting) appeared to influence detection of Tregs. Seven of all transplanted patients analyzed shortly after grafting showed an extremely low amount of CD25+ lymphocytes. All other patients investigated later than three months after transplantation revealed normal amounts of peripheral CD25+ lymphocytes in comparison to healthy controls. This finding suggested specific short-time down regulation of CD25 only, since the amount of peripheral CD4+FOXP3+ lymphocytes did not differ significantly in all patients irrespective of the time period studied. Thus, CD25 labelling did not appear to allow reliable detection of CD4+CD25+FOXP3+ regulatory T-cells in the early post transplantation period (<three months).

In the lymphocyte gate, the percentages of CD4+CD25+FOXP3+ cells related to the total CD4+ cell counts greatly varied in the analyzed patient group and were significantly lower on average in comparison to those of the healthy individuals (Figure 2). This could also be seen for the absolute values of these cell populations. In addition, also absolute values and

percentages of FOXP3+ cells were reduced on average in comparison to the normal controls

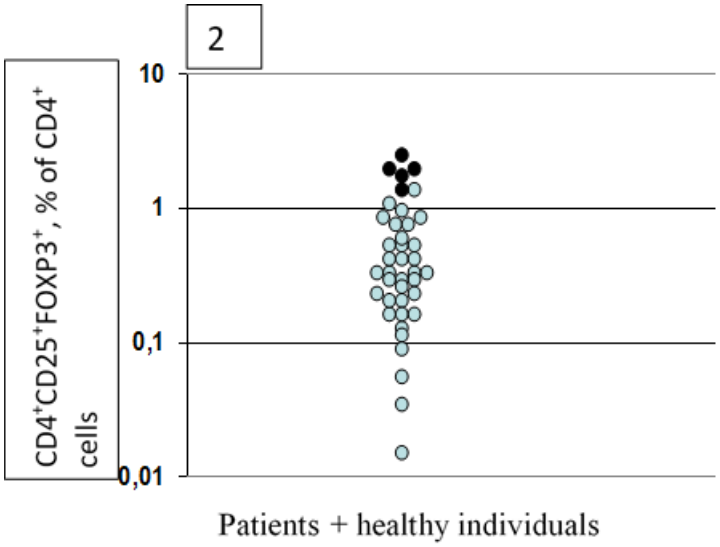


Figure 2: Reduced values of Tregs in blood of transplanted patients

Percentages of CD4+CD25+FOXP3+ cells with respect to the total CD4+ cell counts: Comparison of transplanted patients (n=29, white circles) to healthy individuals (n=5, black circles)

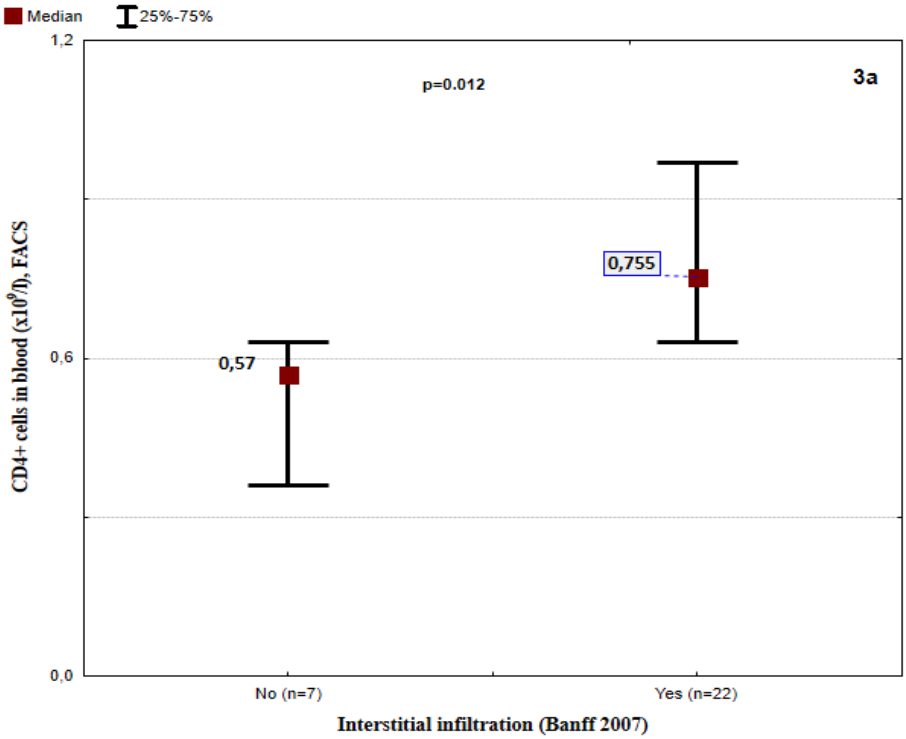
4.1.3 Association of CD4+ and FOXP3+ T-lymphocytes in peripheral blood of patients after kidney transplantation with histological signs of graft rejection in renal biopsies (Banff ´97/ Update 2007)

Banff classification has been developed to standardize diagnosis of graft rejection based on defined morphological changes in the allograft kidneys. Banff classification of morphological criteria associated with rejection include defined indicators such as tubulitis, vasculitis, fibrosis of the intima of capillaries, interstitial fibrosis, C4d deposition, tubular atrophy, glomerulopathy, perivenular infiltration, arteriolar hyalinosis, increase of mesangial matrix, peritubular capillaritis. Each indicator was scored on a scale from 0 to 3: 0-absence, 1-mild, 2-moderate, 3-severe in this study. The histopathological classification of renal allograft rejection according to the current Banff classification is shown in Table 4 (see Material and Methods)

For simplicity of analysis, patient samples were categorized into two groups with (scoring >=1) or without (scoring 0) signs of rejection reactions and evaluated for the relationship between peripheral and tissue regulatory T-cells. This analysis aimed at further insight into

the diagnostic relevance of peripheral Tregs detection for the prediction of rejection reactions and transplant prognosis.

No statistically significant association between peripheral Tregs with full phenotype and histological signs of rejection reaction was found. However, T-cell populations with a partial Tregs phenotype (CD4+ lymphocyte subsets, FOXP3+ lymphocyte sets, CD4+FOXP3+ lymphocyte subsets), which included Tregs in addition to different other T-cell subsets in peripheral blood of patients were associated with histological signs of graft dysfunction and rejection diagnosed according to the Banff classification in 1997 and its update in 2007. First, interstitial cell infiltrates in kidney allograft biopsies were found to be associated with statistically significant higher absolute numbers of peripheral CD4+ T-cells. Also significantly higher percentages of CD4+ T-cells related to all peripheral blood lymphocytes were seen in the group with rejection reaction induced glomerulitis (Figure 3a and 3b).



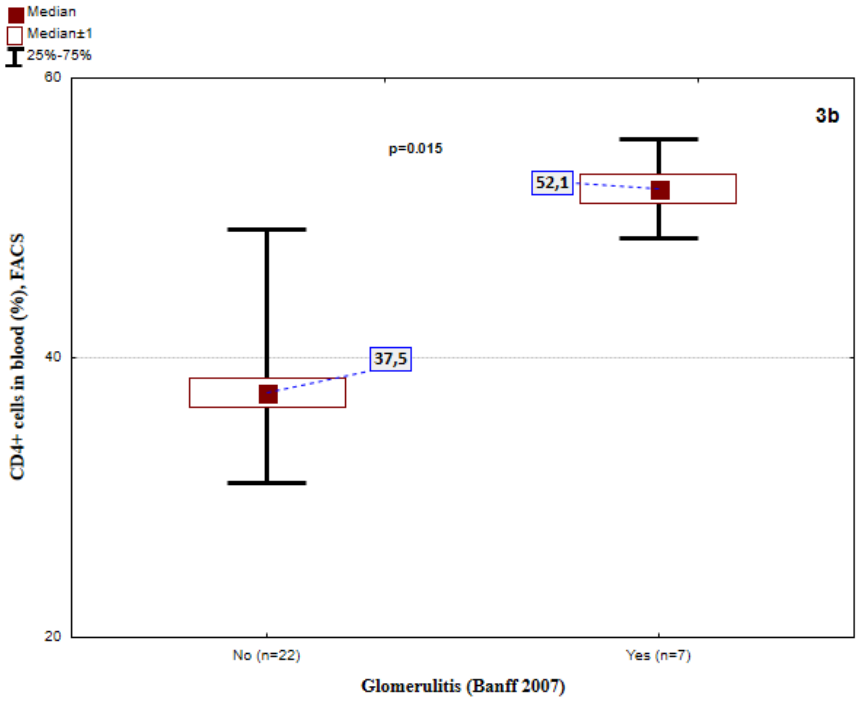


Figure 3: Comparison of CD4+ cells values in blood of transplanted patients with/without histological signs of rejection. Ratio of CD4+ cells to all lymphocytes in peripheral blood and their association with presence or absence of histological sign of rejection in concomitant renal biopsies of the same patients: 3a) interstitial infiltration; 3b) glomerulitis. Mann-Whitney Test, n- number of biopsies

A statistically significant higher amount of absolute numbers of FOXP3+ cells in peripheral blood appeared to be also associated with the presence of peritubular capillaritis and glomerulitis (Figure 4 a, b).

A similar relation was also observed for the percentages of peripheral CD4+FOXP3+ cells and the same histological rejection reactions in the renal biopsies (Figure 5 a, b).

Interestingly, both pathological renal changes are broadly acknowledged morphological signs of humoral mediated rejection reactions in the allograft.

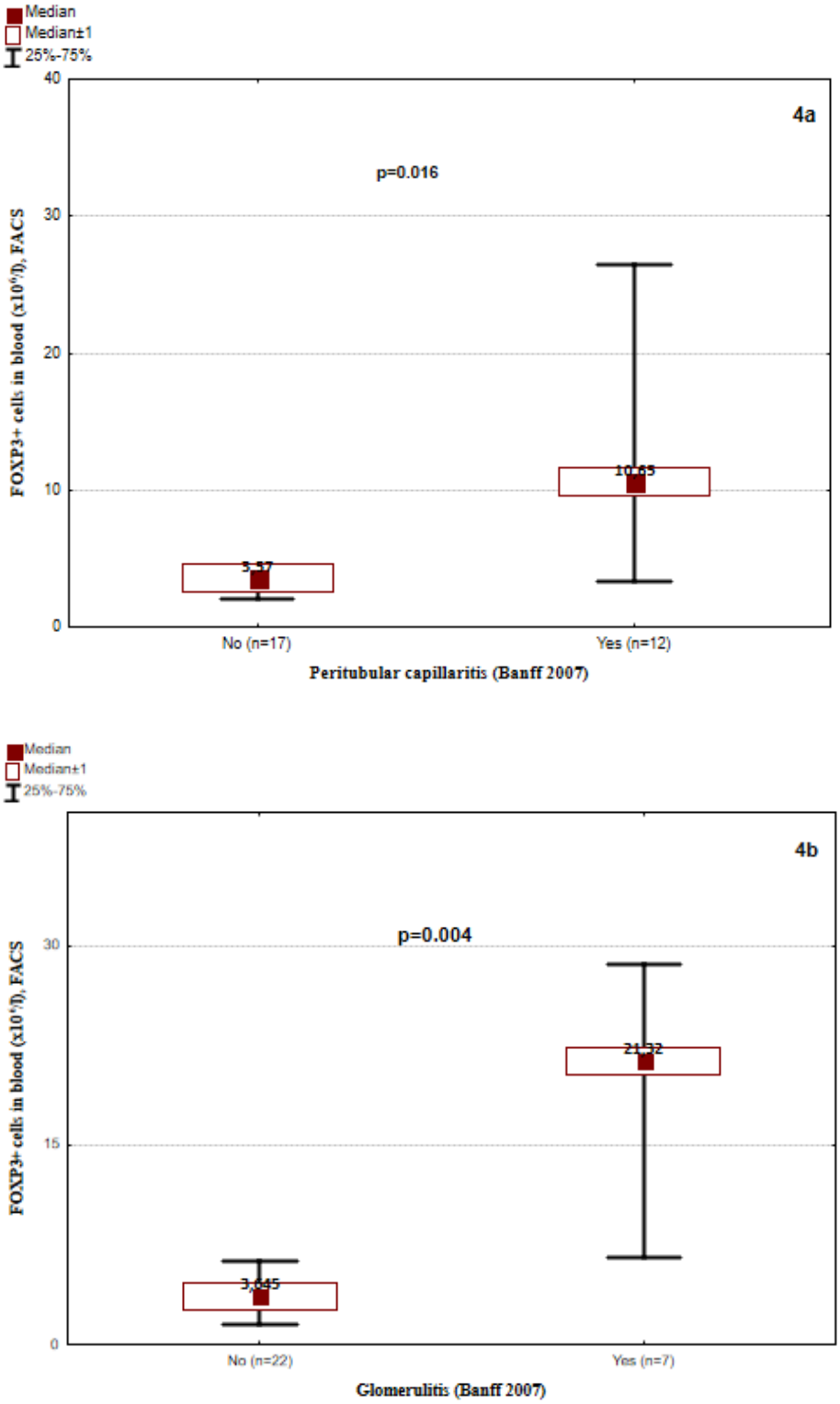
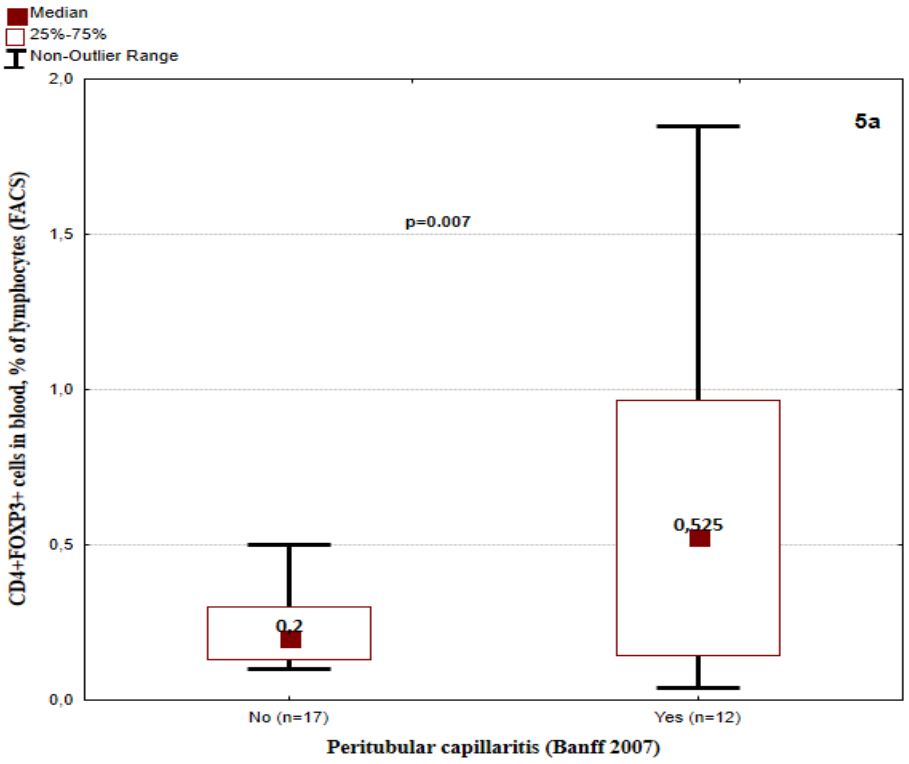


Figure 4: Comparison of FOXP3+ cells values in blood of transplanted patients with/without histological signs of rejection. Absolute count of FOXP3+ cells in peripheral blood and their association with the presence or absence of histological signs of rejection in concomitant renal biopsies of the same patients: 4a) peritubular capillaritis; 4b) glomerulitis. Mann-Whitney Test, n-number of renal biopsies



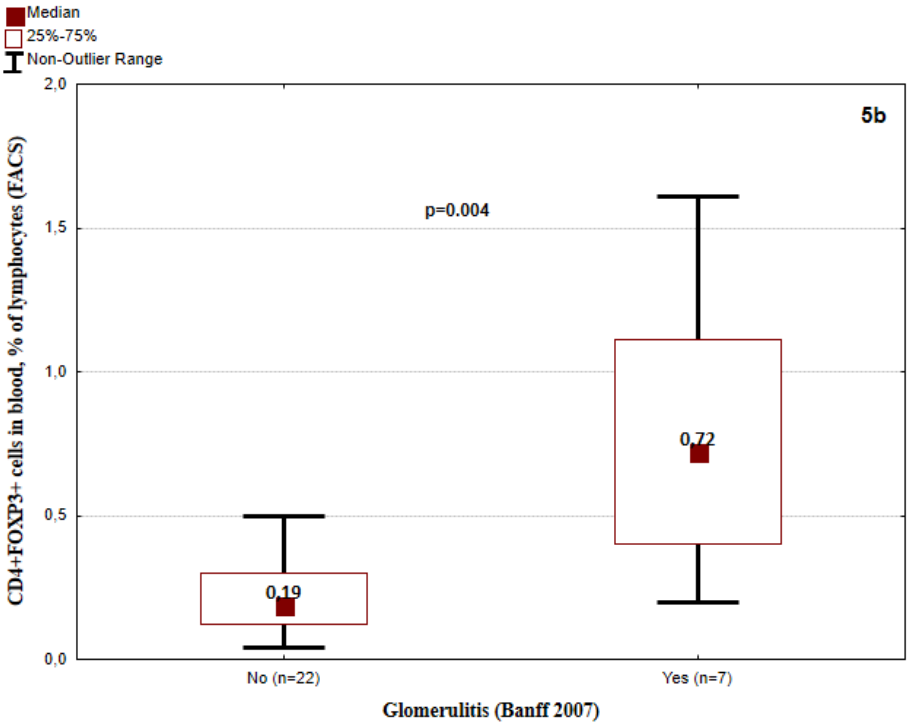
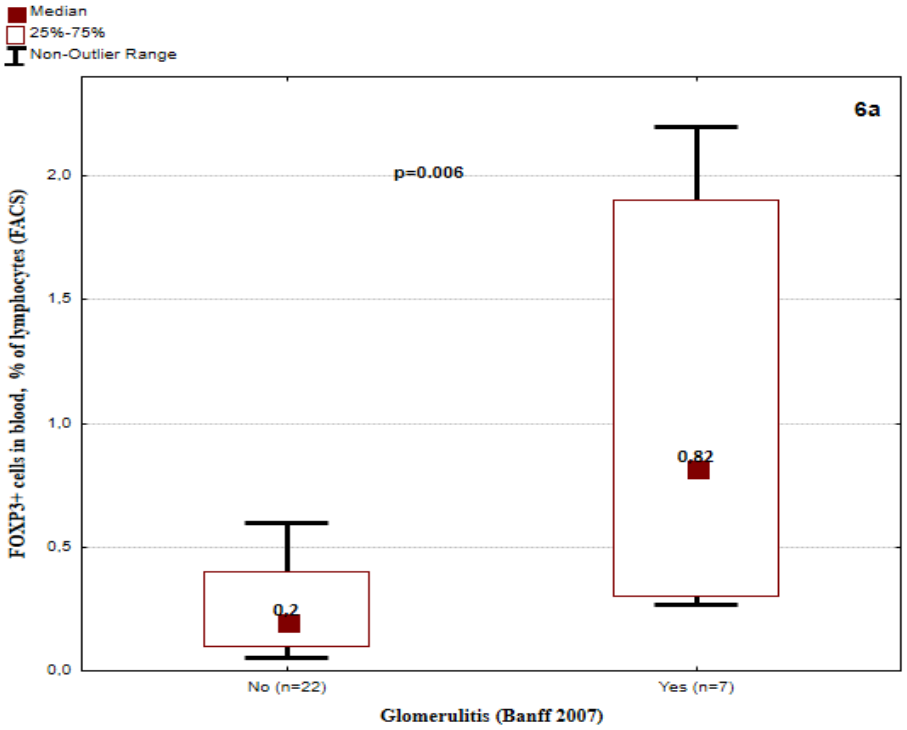


Figure 5: Comparison of CD4+FOXP3+ cells values in blood of transplanted patients with/without histological signs of rejection. Ratios of CD4+FOPX3+ cells to absolute lymphocytes counts in peripheral blood and their association with presence or absence of histological signs of rejection in concomitant renal biopsies: 5a) peritubular capillaritis; 5b) glomerulitis. Mann-Whitney Test, n-number of biopsies

The patients with signs of rejection reactions in the allograft, such as glomerulitis and tubulitis, showed significantly higher percentages of FOXP3+ cells related to absolute

lymphocyte counts in peripheral blood in comparison to the patient group without these signs of rejection (Figure 6 a, b)



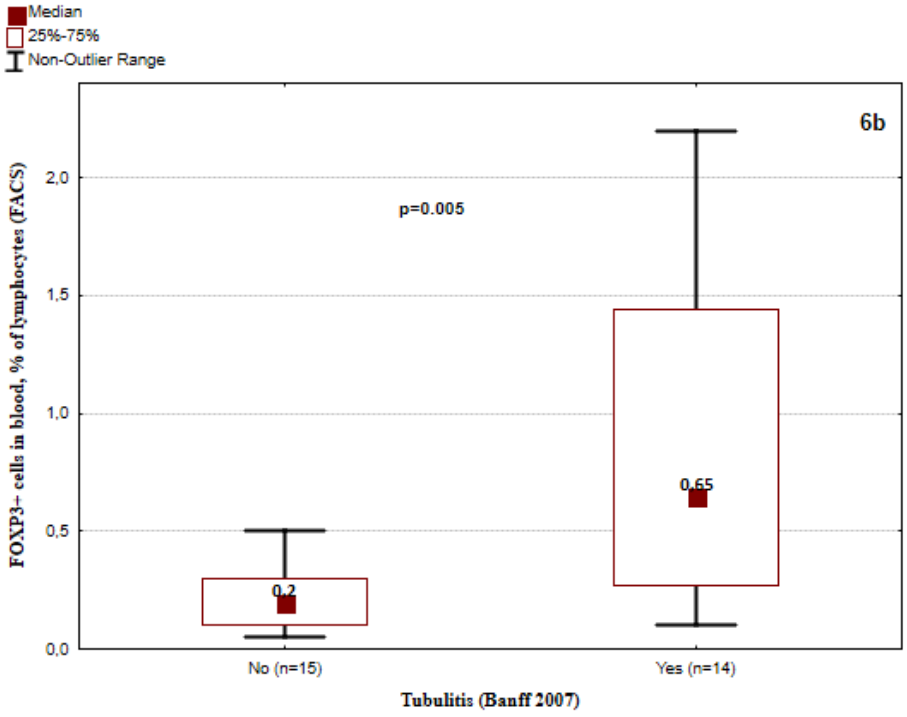


Figure 6: Comparison of FOXP3+ cells ratios to all lymphocytes in blood of transplanted patients with/without histological signs of rejection. Ratios of FOPX3+ cells to absolute lymphocytes counts in peripheral blood and their association with presence or absence of histological signs of rejection in concomitant renal biopsies: 6a) glomerulitis; 6b) tubulitis. Mann-Whitney Test, n-number of biopsies

Significant covariation between signs of allograft rejection and the number of lymphocytes with Tregs phenotype was also observed in a Spearman rank correlation. The ranked percentages of CD4+ cells labelled for FOXP3+ in the peripheral circulation were correlated with the ranked variable of tubulitis score in the respective biopsies after transplantation (Table 7).

Furthermore, the higher the proportions of CD3+ T-cells simultaneously expressing CD25 and CD4 in the peripheral blood the larger were the interstitial cell infiltrates in the corresponding renal biopsies (Table 7).

Glomerulitis and peritubular capillaritis as characteristic morphological signs of humoral allograft rejection were also found in positive rank correlation with the percentages of CD4+FOXP3+ of CD3+ T-cells in peripheral blood (Table 7). However, a negative correlation between the absolute counts of peripheral CD4+CD25+FOXP3+ cells and the extent/severity of C4d deposits in peritubular vessels (PTV) was observed (Table 7). C4d deposition has been reported as an immunomorphological sign of complement activation during antibody-mediated rejection (Murata and Baldwin 2009).

Table 7: Correlation between peripheral FOXP3+ cells and morphological changes in the corresponding renal transplant biopsies. Spearman rank correlation, n=29

	Spearman rank correlation analysis				
Morphological changes in RT (Determinants of rejection score)	Frequencies of lymphocyte subsets (%) in peripheral blood				Absolute values
	FOXP3+/ total lymph.	CD4+CD25+ / total lymph.	CD4+FOXP3+ / total lymph.	CD4+CD25+ FOXP3+ / CD4+ cells	CD4+CD25+ FOXP3+, 10^6/l
Tubulitis	0,39*	ns	ns	ns	ns
Interstitial infiltration	ns	0,44*	ns	ns	ns
C4d deposition in PTC	ns	ns	ns	ns	-0,5*
Peritubular capillaritis	0,46*	ns	0,43*	ns	ns
Glomerulitis	0,53*	ns	0,53*	ns	ns

ns-not significant; * significance p<0,05; lymph. – lymphocytes, PTC-peritubular capillaries, RT–renal transplant

Kidney transplant rejection was determined by a decrease of kidney function (i.E eGFR) and the presence of Banff histological criteria (see also "Materials and Methods"). The results of a Spearman rank correlation analysis presented in Table 7 indicated that the presence of specific CD4+CD25+FOXP3+ T-cell subsets (%) in peripheral blood were not significantly correlated with the extent of any of the defined morphological Banff rejection signs in the allograft. This applied also to a correlation analysis where rejection reactions were grouped into those with morphological signs of either cellular or humoral reactions. Frequencies of FOXP3+ cells or CD4+FOXP3+ cells only correlated with variable signs of rejection reactions. Also when the clinically graded graft rejections were considered and correlated with the presence of blood T-lymphocytes with a Tregs phenotype, no statistically significantly correlations could be seen. However, a significant correlation between the frequencies of CD4+ lymphocytes in peripheral blood and the number of T-cell rejection episodes during the observation period (41,3 ± 17,6 months, s. Methods 2.3.) was observed (Spearman's rank correlation coefficient 0,62; p<0.05)

4.2 Lymphocyte subsets in kidney allografts of patients after transplantation (Tx) and their correlation to morphological alterations of Banff defined rejection reactions

4.2.1 Detection of FOXP3+ lymphocytes in kidney allografts

4.2.1.1 Immunohistochemical staining of FOXP3+ lymphocytes

As regulatory T cells could be induced or recruited locally into renal tissues during rejection reactions, their frequencies in the allograft biopsies were evaluated and compared to their presence in peripheral blood of the same patient. For the detection of Treg cells in kidney allograft biopsies, a double immunohistological staining technique was developed for simultaneous labelling of cells carrying FOXP3+ and either CD3+ or CD4+ or CD20+ or CD14+ on paraffin embedded tissue as described in the 2.1 "Materials and Methods".

92 renal transplant biopsies, (22 with rejection reactions (9 biopsies with borderline changes, 13 biopsies with rejection), 70 without rejection) were analyzed for presence of FOXP3+ populations in cell infiltrates of the interstitial areas.

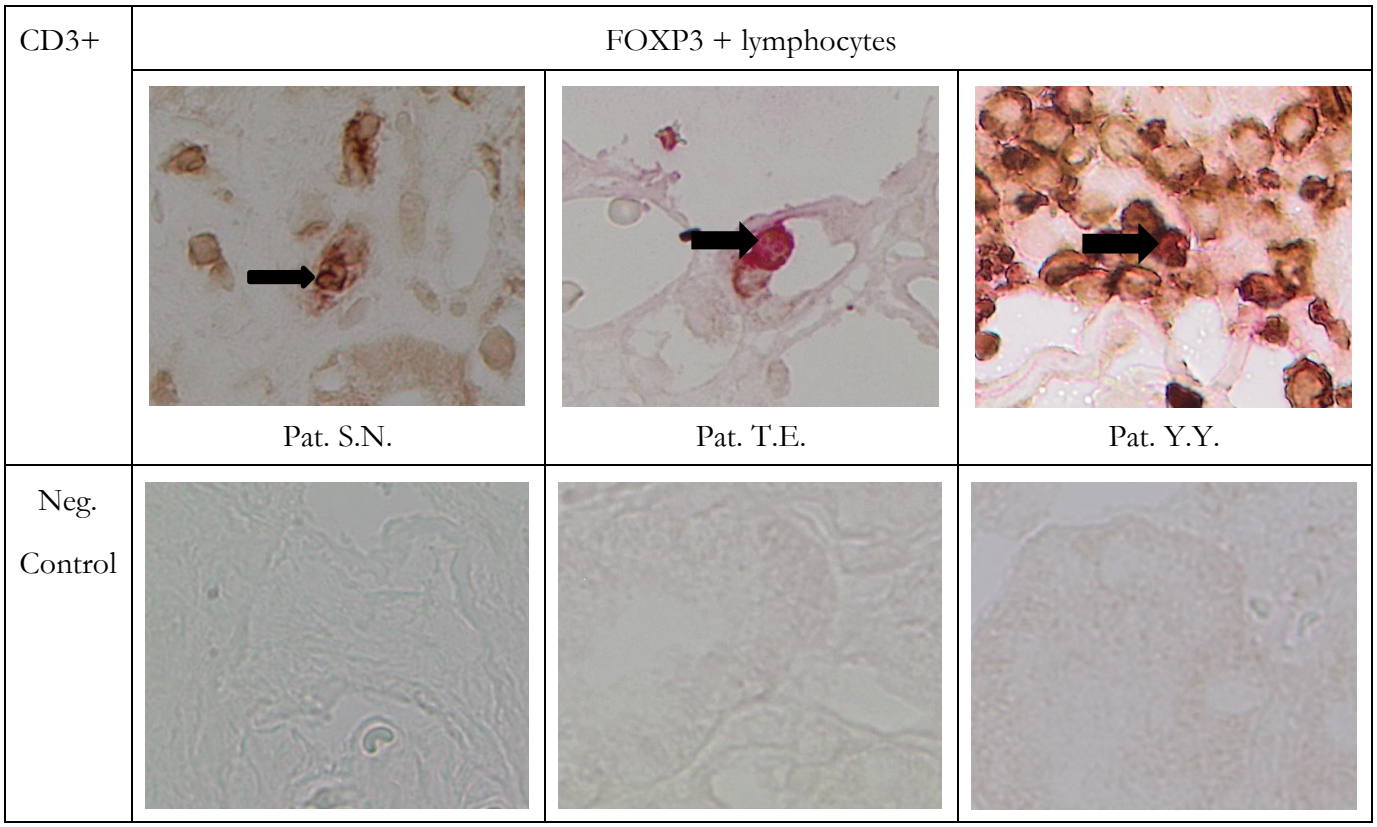


Figure 7: CD3+ cells in allograft biopsies. Representative examples of immunohistochemical analyses of FOXP3+ lymphocytes in paraffin sections of renal allograft biopsies after double labelling with the surface antigens CD3. Brown color indicates staining of the cell surface antigens CD3. Double positive cells are indicated by arrows. Original magnification 40x

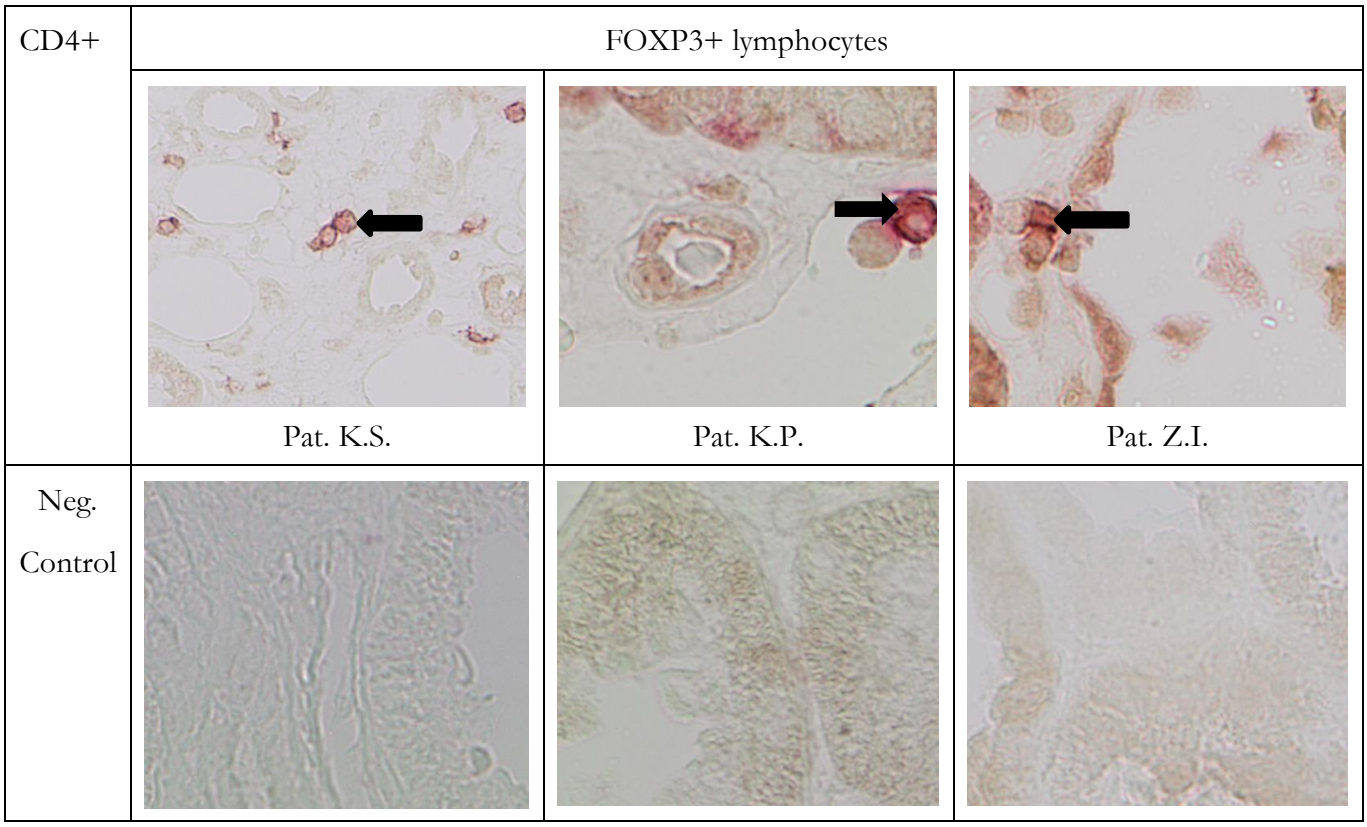


Figure 8: CD4+ cells in allograft biopsies. Representative examples of immunohistochemical analyses of FOXP3+ lymphocytes in paraffin sections of renal allograft biopsies after double labelling with the surface antigens CD4. Brown color indicates staining of the cell surface antigens CD4. Double positive cells are indicated by arrows. Original magnification 40x

Summarized statistical results of the characterization of CD3+, CD4+ and FOXP3+ cells in kidney allograft biopsies are presented in Table 8. Each cell population was counted after immunohistological staining of a paraffin section in ten standardized fields with the use of the scale access grid option. Using basic statistics, the median of the cell counts on all analyzed biopsies for each population was calculated from the means of the counted cells of each section. Descriptive statistics of the nonparametric results are given.

Table 8: Frequencies of different FOXP3+ T-lymphocytes in renal allograft biopsies, n=92

Cell Subsets	Cell Counts Median	25^{th} - 75^{th} percentiles of the median	Min-Max	Number of analyzed biopsies
CD3+ cells	40,5	20-99	1,3- 391	92
CD3+FOXP3+ cells	0,9	0,3-2,15	0-9,5	92
CD4+ cells	15,5	4,75-31	0,4-125	76
CD4+FOXP3+ cells	0,5	0,1-1,20	0-7,4	76

In biopsies of all the renal transplants FOXP3+ expression was found in CD3+, CD4+ at variable frequencies (Figure 7, Table 8). Double immunohistological labelling experiments with antibodies against FOXP3 and the B-cell differentiation antigen CD20 or the TLR4 co-receptor CD14 suggested that also single CD20+ lymphocytes or CD14+ myeloid-monocytic cells could express FOXP3 (Figure 8). FOXP3 expression has been reported to be restricted to the T-cell lineage and has been described to only occur in malignant cell types of the B cell lineage (Felcht M et al. 2012). CD14+ myelomonocytic cells expressing FOXP3 could belong to those subsets expressing also CD4 during early differentiation stages. Unfortunately, in this study technical limitations did not allow to further clarify this issue on isolated microdissected cells.

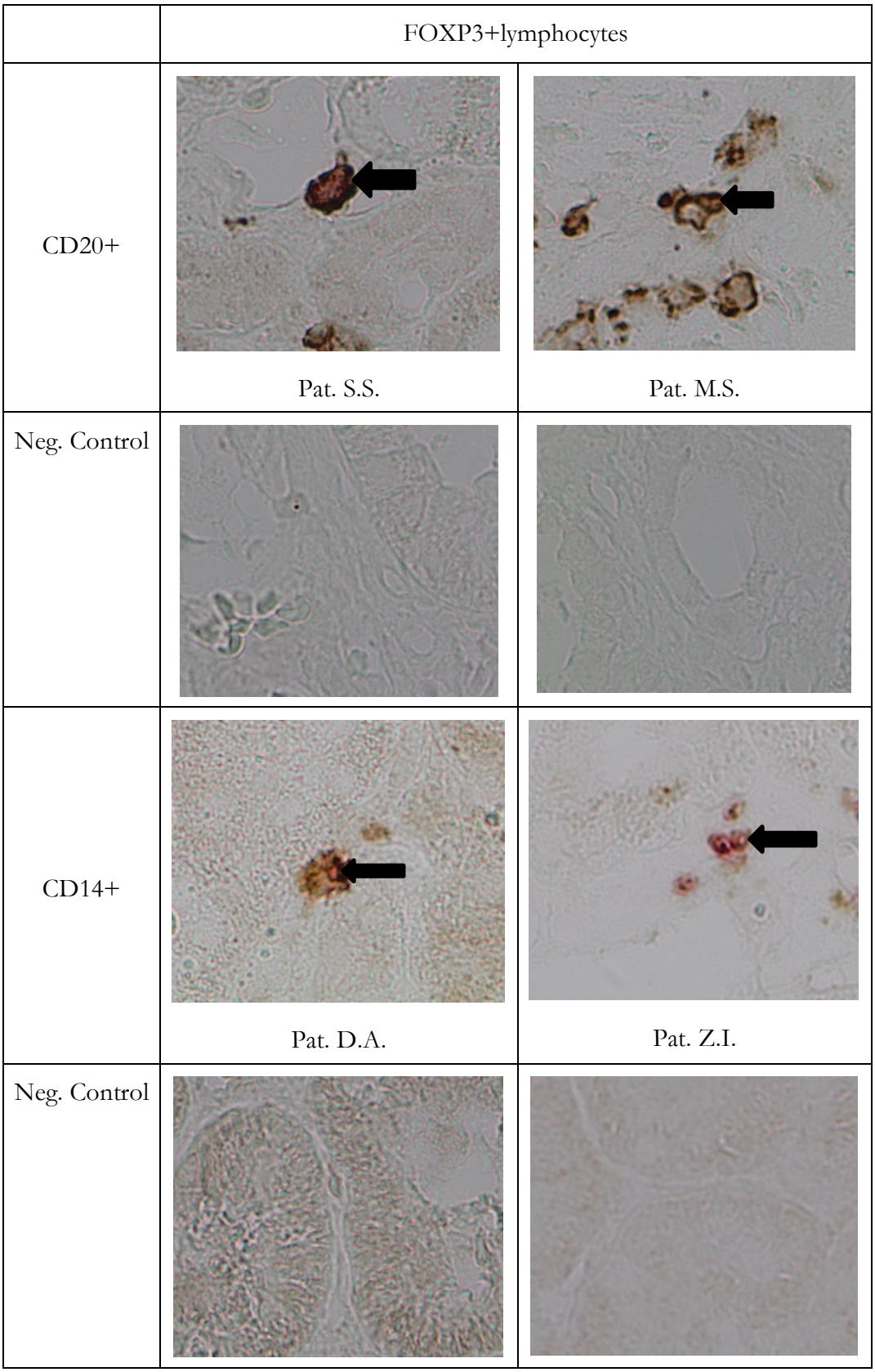


Figure 9: CD14+ cells and CD20+ cells in allograft biopsies. Representative example of an immunohistochemical experiment of FOXP3+ lymphocytes in paraffin sections of allograft biopsies after double labelling with antibodies against the surface antigens CD20 or CD14. Brown color indicates staining of the cell surface antigens; red color indicates

intra-nuclear/intra-cellular staining of FOXP3. Double positive cells are indicated by arrows. Original magnification 40x

4.2.1.2 Immunofluorescence staining of FOXP3+ lymphocytes

Furthermore, a comparative immunofluorescence labelling technique was evaluated to confirm presence of CD4+FOXP3+ cells (Figure 9). However, in spite of extended blocking procedures, a highly variable unspecific background immunofluorescence of the unstained renal tissues from the paraffin embedded biopsies hindered reliable counting and detection of T-cell subsets in these experiments. Therefore, T-cell subsets were evaluated and specified on the immunohistologically stained tissues only.

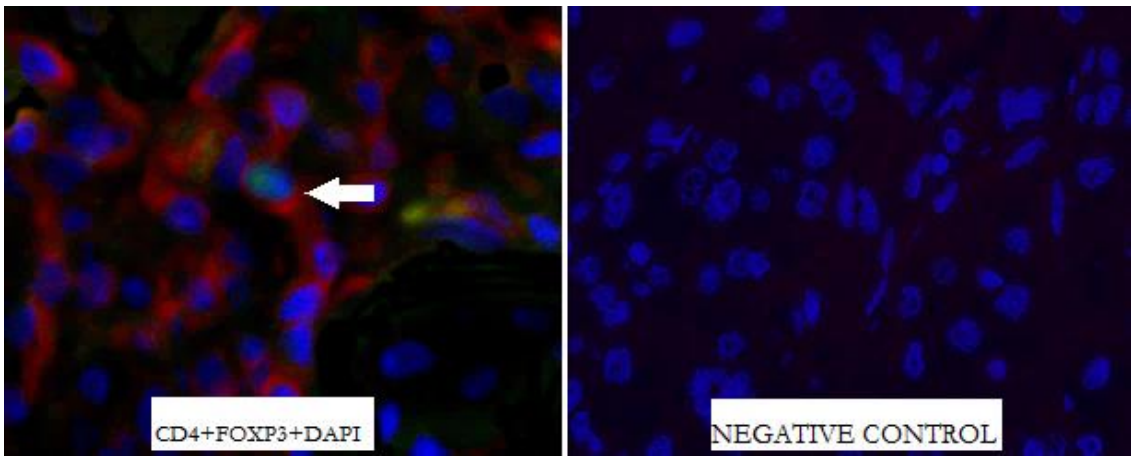


Figure 10: Immunofluorescence staining of CD4+FOXP3+ cells in allograft biopsies. Representative example of the detection of CD4+FOXP3+ cells in renal allograft biopsies: triple immunofluorescence staining of a paraffin section of a renal transplant biopsy with antibodies against FOXP3 (green-nucleus) and CD4 (red-cytoplasm/surface) and by DAPI (blue-nucleus); A single CD4+FOXP3+ cell is indicated by arrow. Original magnification 40x

4.2.2 Distribution of CD3+ and FOXP3+ lymphocytes in kidney allografts

For further analysis of the presence of regulatory lymphocytes the tested biopsies were divided into two groups: the first group included biopsies with confirmed rejection reactions such as borderline, cellular or humoral rejection (n=22), the second group included biopsies without rejection (n=70).

Figures 9 and 10 show the distribution of CD3+ and CD3+FOXP3+ cells in biopsies with or without rejection. In this study, CD3+ and CD3+FOXP3+ cells were present in the allografts without rejection and in allografts with confirmed rejection. There was a tendency that the biopsies without confirmed rejection had a lower frequency of CD3+ T-cells within the tissues than those with rejection reactions (Figure 11 a, b). While the

absolute number of CD3+FOXP3+ T-cells did not differ in biopsies with and without rejection reaction (Figure 12 a, b). However, the percentage of FOXP3+ cells related to the absolute counts of CD3+ cells was lower in biopsies with confirmed rejection (Fig. 13 a, b).

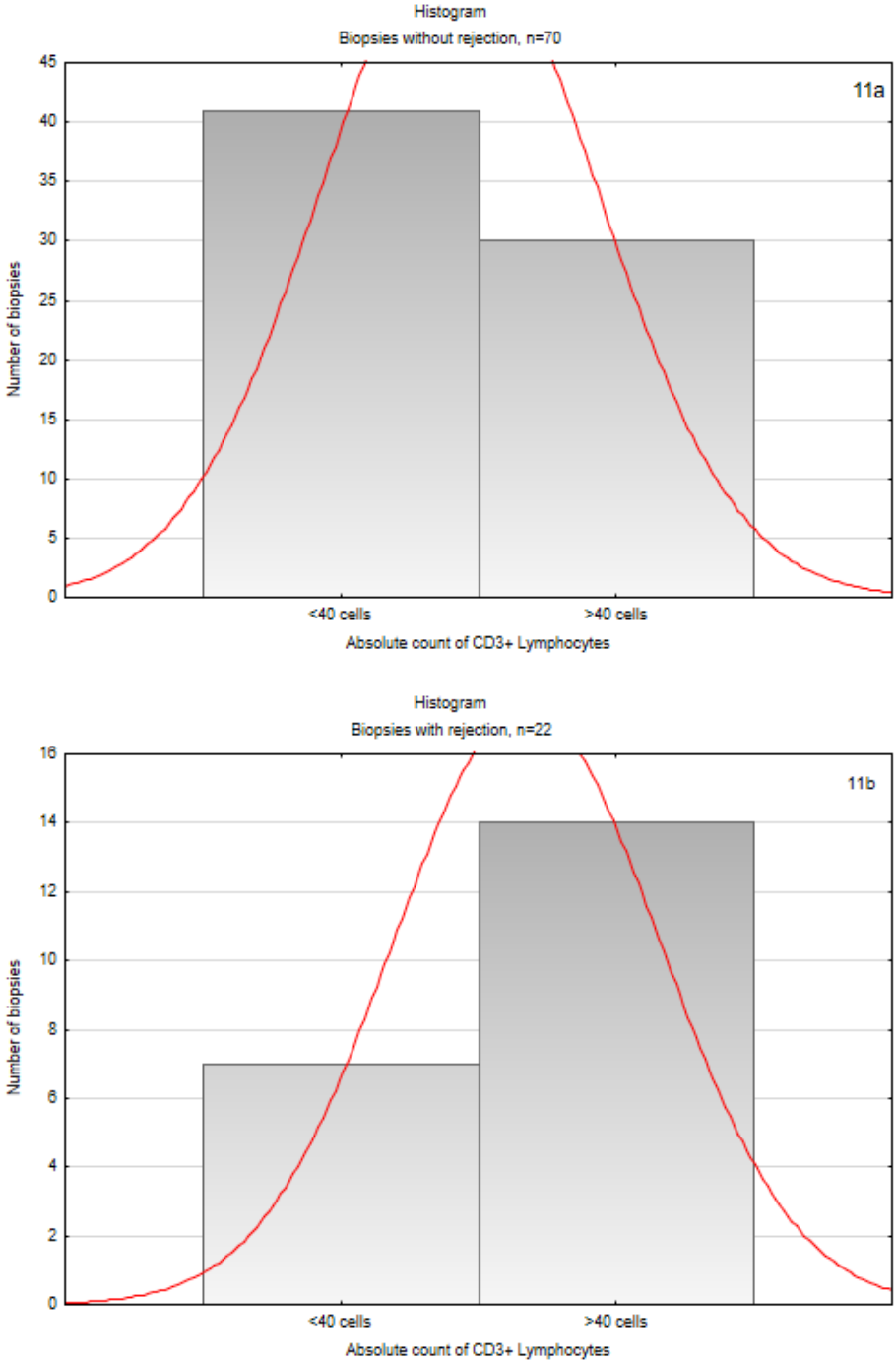


Figure 11: Distribution of CD3+ cells in allograft biopsies without/with confirmed rejection reaction: 11a) without rejection reaction, 11b) with rejection reaction; 40 cells - median of CD3+ lymphocytes

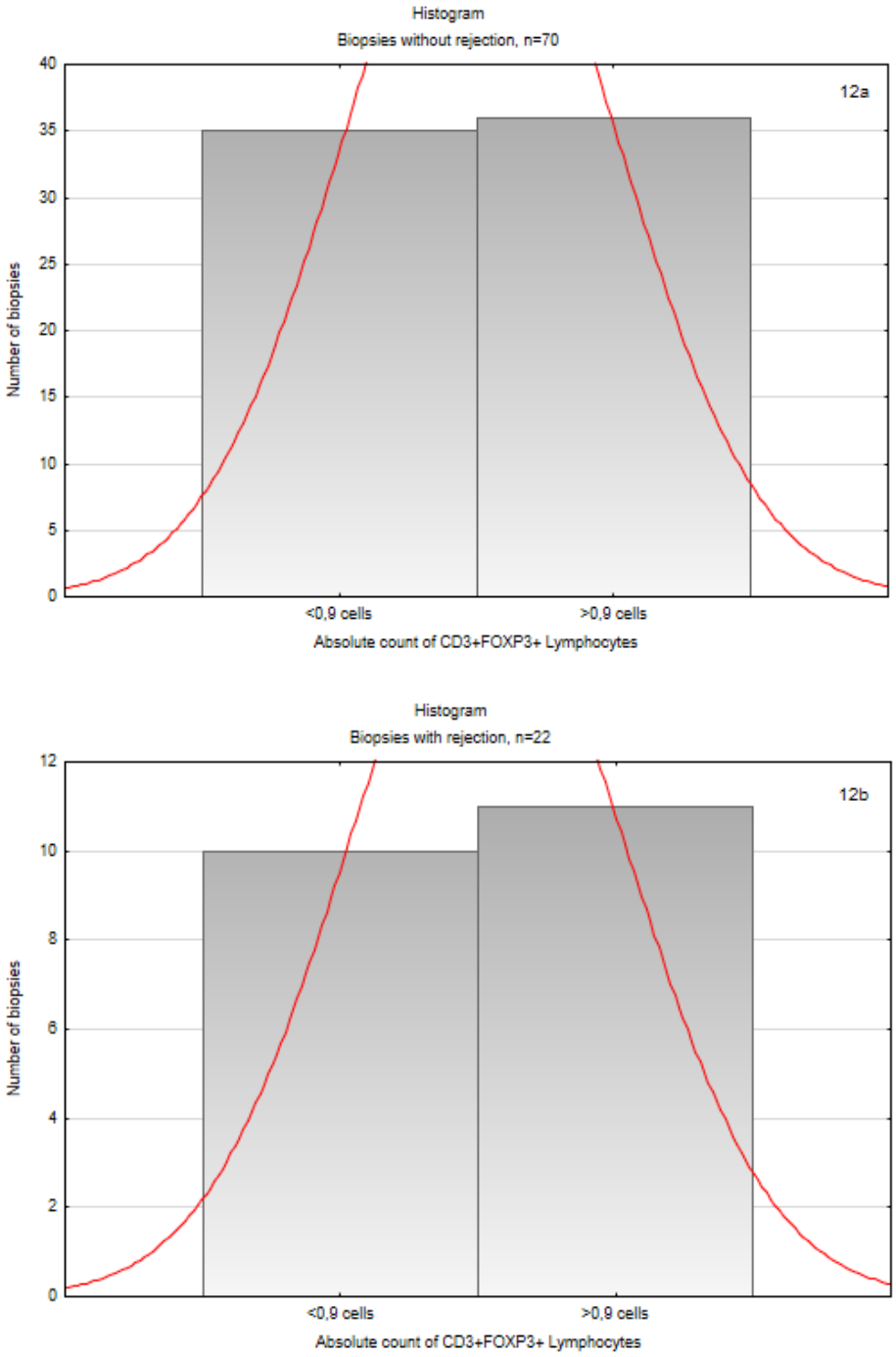


Figure 12: Distribution of CD3+FOXP3+ cells in allograft biopsies without/with confirmed rejection reaction: 12a) without rejection reaction, 12b) with rejection reaction; 0,9 - Median of CD3+FOXP3+ cells

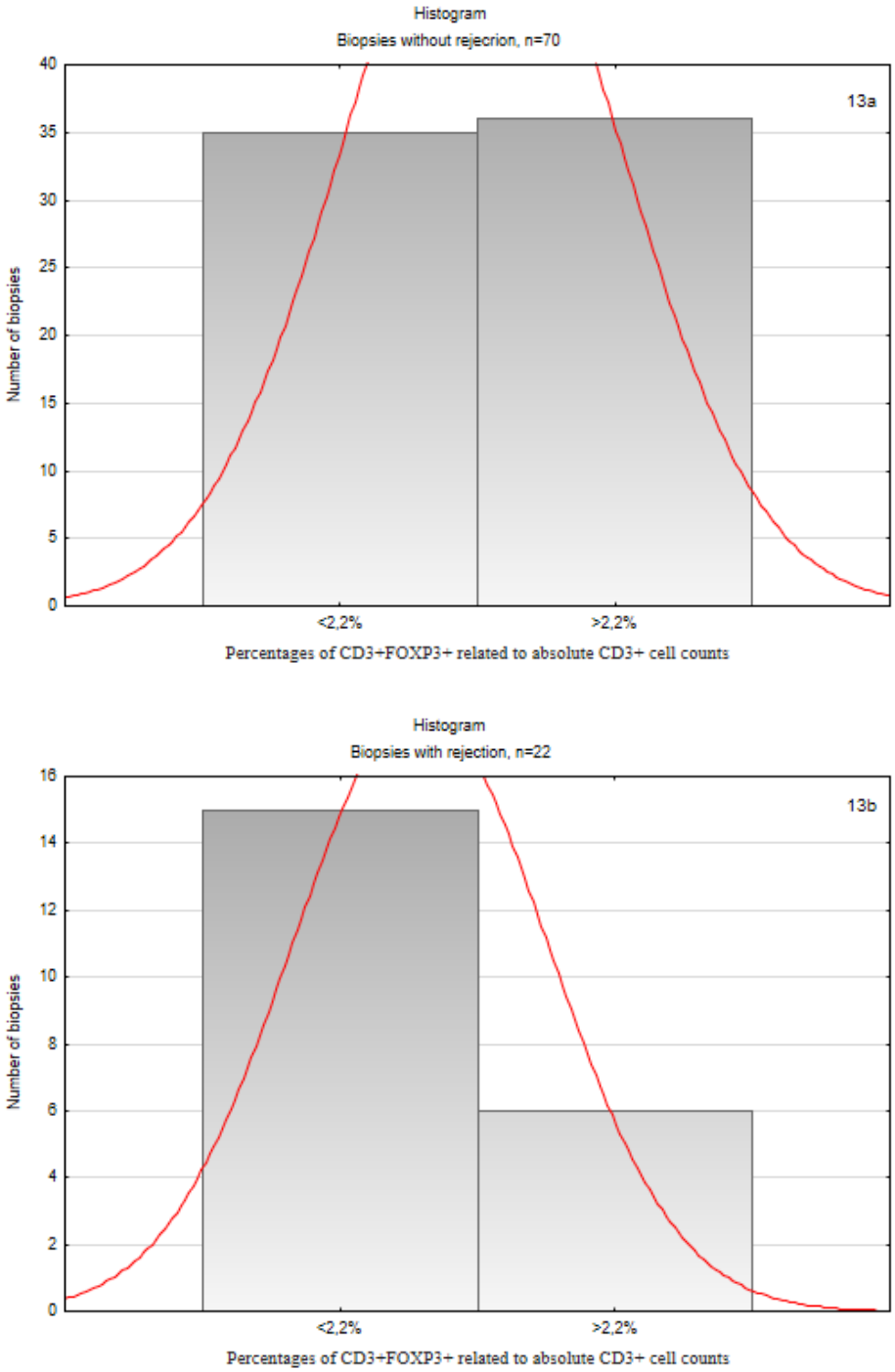


Figure 13: Distribution of ratios of CD3+FOXP3+ cells in allograft biopsies without/with confirmed rejection reaction. Percentages of CD3+FOXP3+ cells related to the absolute CD3+ cell counts in kidney allograft biopsies without (13a) and with (13b) confirmed rejection reaction; 2,2 – Median of percentages of CD3+FOXP3+ to absolute counts of CD3+ cells

In Figure 14 it is shown that absolute numbers of CD3+FOXP3+ cells in the transplant biopsies were weakly correlated to the frequency of CD3 positive cells irrespective of the presence of rejection reactions in the biopsies (Figure 12).

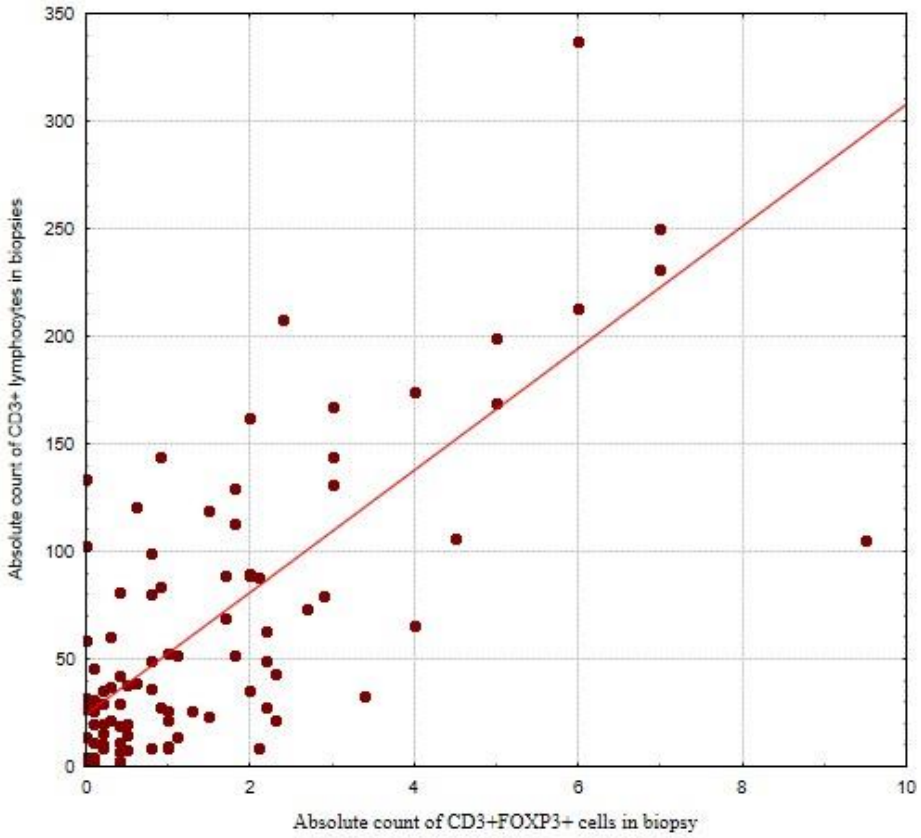


Figure 14: Positive linear correlation between CD3+ cells and CD3+FOXP3+ cells in allograft biopsies. Scatterplot, r=0,6; p<0.05; irrespective of rejection

A weak positive correlation between the CD4+ and CD14+ cells counts was also observed in the graft biopsies (Figure 15). This was suggestive that a fraction of the CD4+ cell populations could also belong to the myelomonocytic cell lineage expressing CD4 in addition to CD14 early during monocyte differentiation.

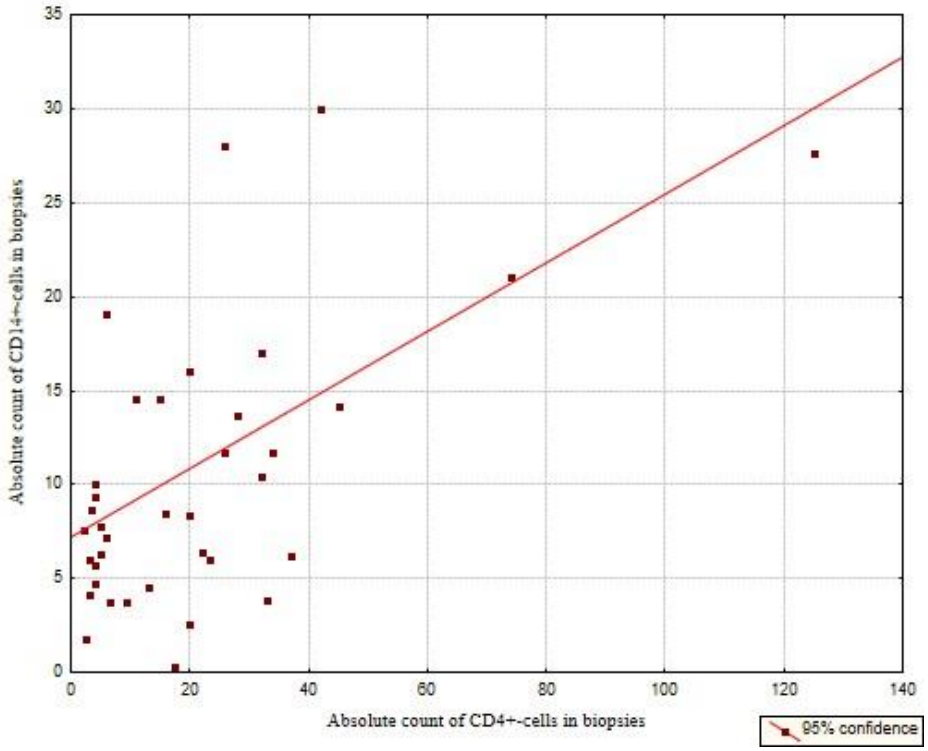
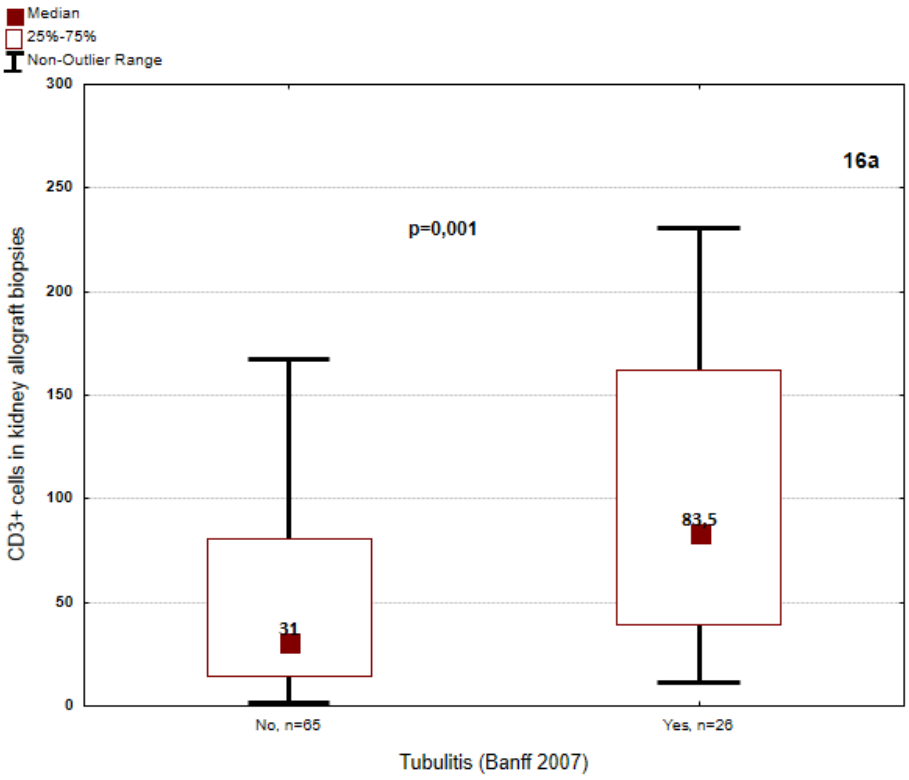


Figure 15: Positive linear correlation between CD4+ cells and CD14+ cells in allograft biopsies. Scatterplot, r=0,5, p<0,05; irrespective of rejection

4.3 CD3+ and CD3+FOXP3+ T-lymphocytes in allografts of patients after kidney transplantation: correlation with histological categories of graft rejection (Banff ´97/ Update 2007)

Further analysis revealed a significant association of the absolute numbers of CD3+, or of CD3+FOXP3+ cells with signs of rejection reactions such as tubulitis and glomerulitis in the same renal biopsies (Figure 16 a, b and Figure17a). In biopsies with higher scores of tubulitis and glomerulitis increased numbers of CD3+ and of CD3+FOXP3+ cells were present in comparison to those biopsies without rejection. Presence of signs of chronic graft changes (interstitial fibrosis and vascular fibrous intimal thickening) was associated with reduced numbers of CD3+FOXP3+ cells in biopsies with signs of rejection (Figure 17 b, c).



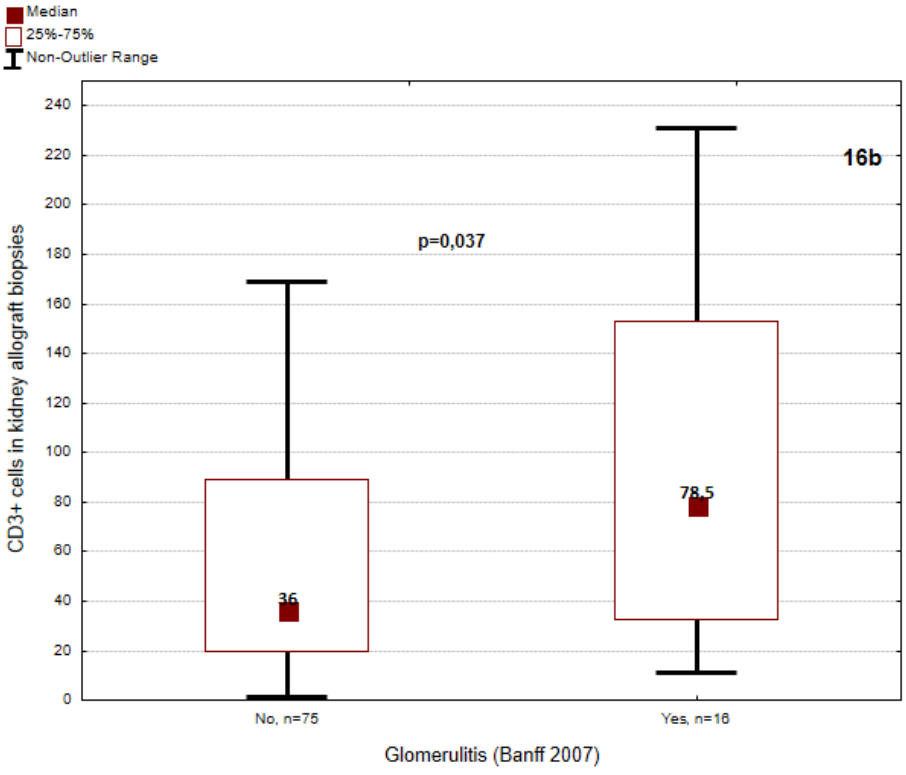
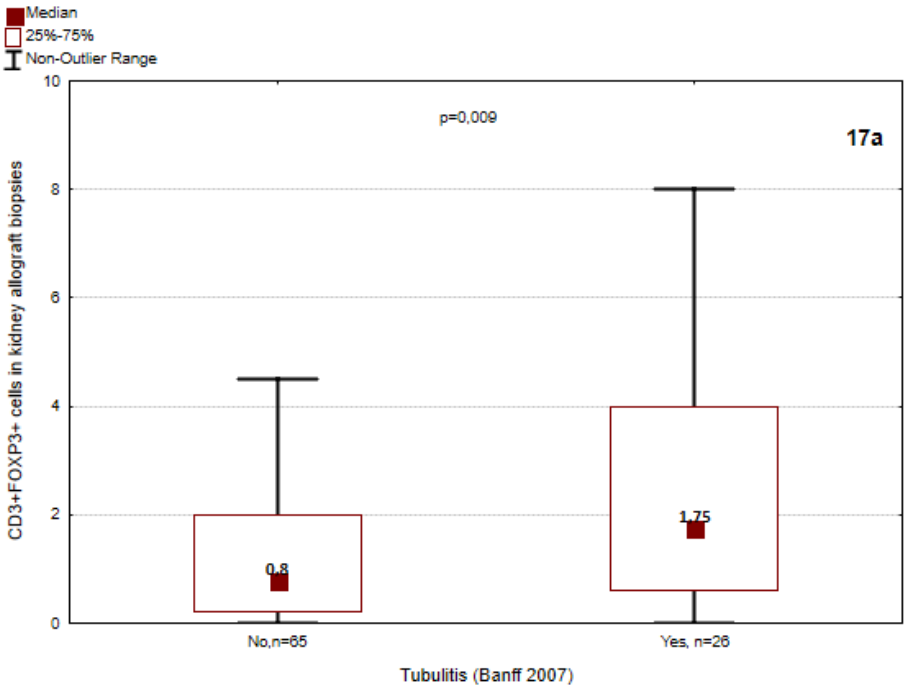
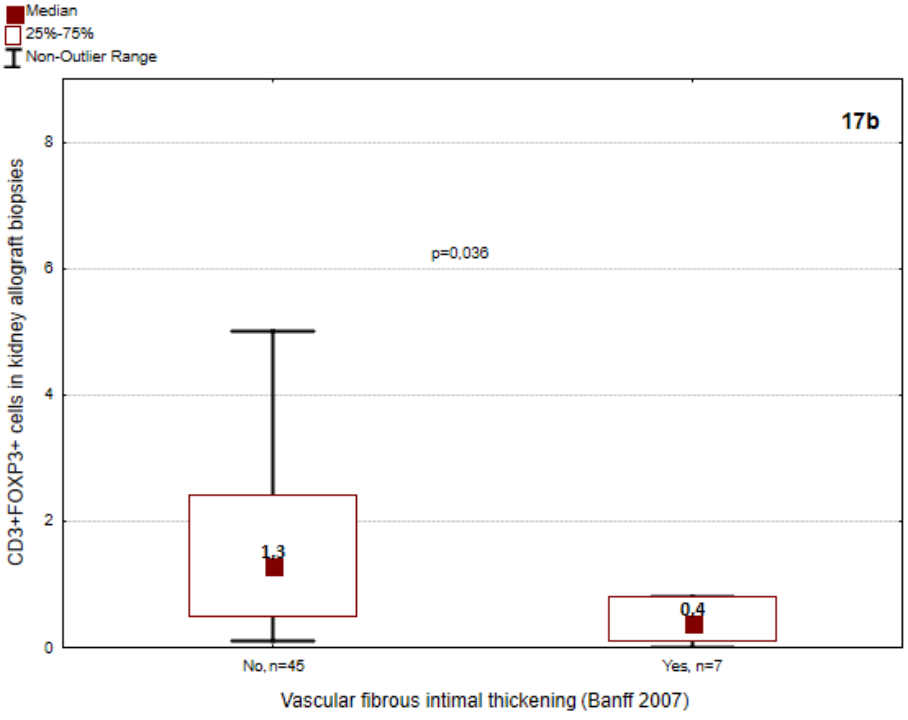


Figure 16: Comparison of CD3+ cells values in allograft biopsies with/without histological signs of rejection reaction. Association between absolute number of CD3+ cells in kidney allograft biopsies and Banff signs. Comparison of absolute cell values of CD3+ lymphocytes with presence versus absence of 16a) tubulitis and 16b) glomerulitis in the same renal biopsies. Mann-Whitney Test, n-number of biopsies


Median
25%-75%
Non-Outlier Range
p=0,009
17a
CD3+FOXP3+ cells in kidney allograft biopsies
10
8
6
4
2
0
0,8
1,75
No,n=65
Yes, n=26
Tubulitis (Banff 2007)


Median
25%-75%
Non-Outlier Range
17b
p=0,036
CD3+FOXP3+ cells in kidney allograft biopsies
8
6
4
2
0
1,3
0,4
No, n=45
Yes, n=7
Vascular fibrous intimal thickening (Banff 2007)

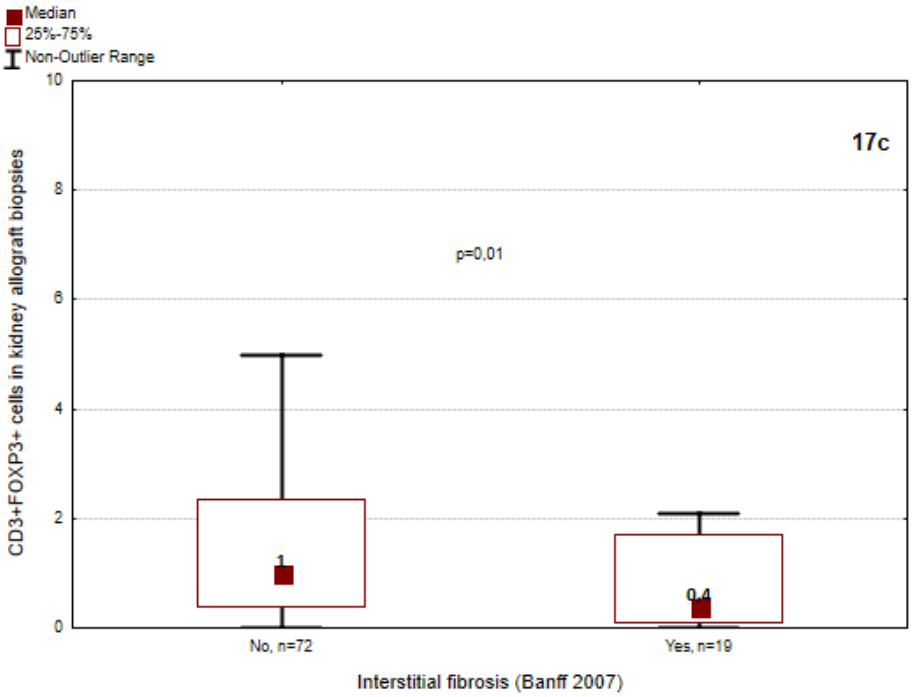


Figure 17: Comparison of CD3+FOXP3+ cells values in allograft biopsies with/without histological signs of rejection reaction. Association between absolute number of CD3+FOXP3+ cells in kidney allograft biopsies and Banff signs. Absolute cell numbers of CD3+FOXP3+ in kidney allograft biopsies associated with presence versus absence of tubulitis; Absolute cell numbers of CD3+FOXP3+ in kidney allograft biopsies associated with absence versus presence of 17b) vascular fibrous intimal thickening and 17c) interstitial fibrosis in the same renal biopsies. Mann-Whitney Test, n-number of biopsies

In contrast to many other studies that evaluated the allograft by biopsy at only one-time point after transplantation, graft alterations based on the diagnosis of Banff criteria were also evaluated over a period of observation time of 4-72 months (min-max) post-transplantation in this study. To evaluate progression of tissue lesions, only cases with at least 3 biopsies of the 53 allografts (patients) were selected in this study: 2 of these biopsies were included in the immunohistological analysis of this study, the third biopsy was evaluated in St. Petersburg by histological diagnostics only and used for the description of the changes in a database. The evaluation and scoring of the tissue lesions was carried out between the first and second biopsy (n=40) and the second and third biopsy (n=40). 12 biopsies had to be excluded from the analysis because of the lack of a detailed morphological description based on the Banff criteria.

The progression of renal injury was defined for each sign of Banff classification by worsening of at least one sign in the third subsequent biopsy on a rejection score of 0 to 5

with further division into subgroups without (0,1,2,3) and with (4,5) progression of morphological changes.

A detailed description of the progression scale is given in 3.2.2. (Materials and Methods). The data were analyzed using "Statistika" software for nonparametric data sets (Mann-Whitney Test and Spearman´s correlation).

When the cases with morphological changes were divided into groups with and without progression of signs, a significant difference between an increase of interstitial fibrosis and tubular atrophy and presence of CD3+FOXP3+ lymphocytes in renal allograft was seen. Statistically significant differences between transplant outcome with respect to the same morphological changes and regulatory T-cells (CD4+FOXP3+) were not found.

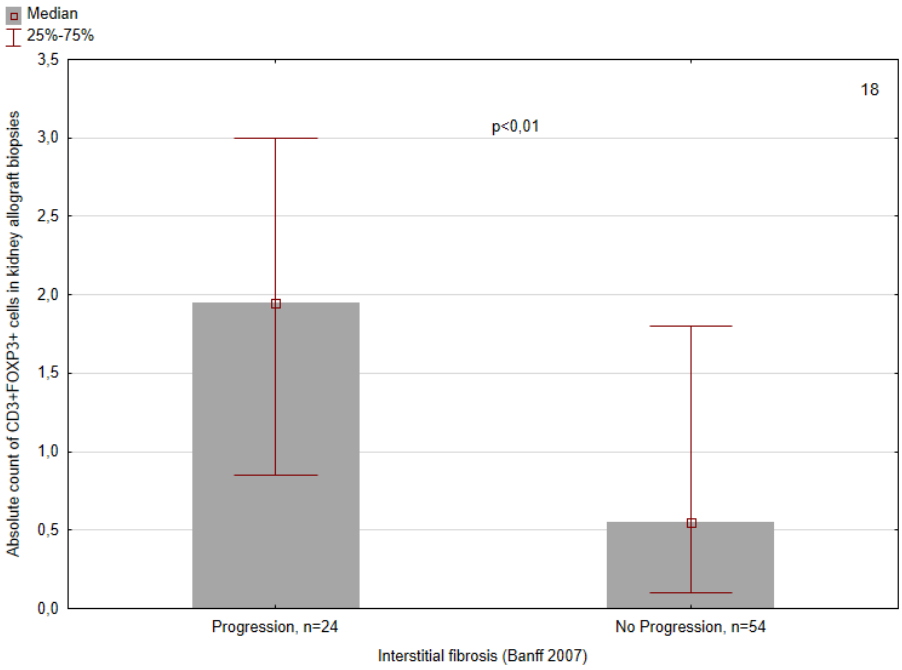


Figure 18: Association of CD3+FOXP3+ cells values in allograft biopsies and progression of interstitial kidney fibrosis. Association between absolute values of CD3+FOXP3+ cells and progression/lack of progression of interstitial kidney fibrosis in the same renal biopsies. Mann-Whitney Test, 2D Box Plot, n-number of biopsies samples

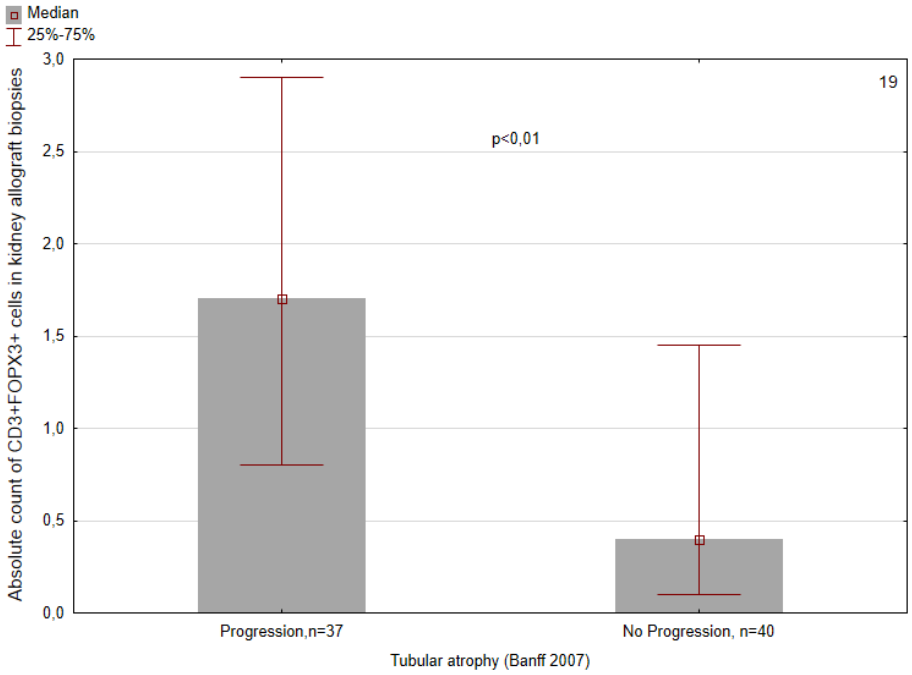


Figure 19: Association of CD3+FOXP3+ cells values in allograft biopsies and progression of tubular atrophy. Association between absolute cell counts of CD3+FOXP3+ and progression/lack of progression of tubular atrophy in the same renal biopsies. Mann-Whitney Test, 2D Box Plot, n-number of biopsies samples

5 Discussion

Kidney transplantation is one of the life-expanding therapeutic options for patients with end-stage renal disease (ESRD). Patients after kidney transplantation in comparison to patients on chronic dialysis have lower mortality and enhanced quality of life (Tonelli et al. 2011). Irrespective of pre-transplant risk factors, such as age, comorbidities of the patients or donor/recipient immunogenetic mis-matching conditions, the success of transplantation strongly relies on induction and maintenance of immune tolerance post-grafting provided by immunosuppressive therapies. Therefore, in most cases, lifelong (transplant viability long) immunosuppression has to be given to suppress acute and chronic rejection reactions. Immunosuppressive therapies, however, also frequently implicate adverse side effects, such as direct toxic damage to kidney tissue leading to a progressive decline of graft function. They also increase the risks of cardiovascular events, of infectious or tumor complications, of which a transplanted patient can die off even in the presence of a still functioning graft (Wolfe et al. 1999). Today in renal transplantation, it is still an important goal and everyday challenging clinical problem to optimally adjust immunosuppression that avoids rejection reactions without raising the frequency of drug complications. Therefore, recipients with an operational immune tolerance of the renal graft have to be confidently diagnosed for a safe reduction of immunosuppression and avoidance of harmful over-treatment. This affords in particular that episodes of acute or flares of chronic graft rejection reactions are early and reliably differentiated from side effects of immunosuppressive therapies.

In this respect, one of the foci of recent experimental and clinical research has been the validation of immune cells with regulatory potential on immune reactions to foreign antigens whilst support of tolerance to tissue self-components in animals and humans (Steinman and Nussenzweig 2002). Although many different immune cells with suppressive function have been characterized, regulatory T-cells were shown to be critical for tolerance maintenance against self-antigens in animals (Benghiat et al. 2005; Joffre et al. 2008; Miyajima et al. 2011). Also in transplantation models of animals, it could be shown that Tregs are relevant for the induction of a state of tolerance against alloantigens of the graft (Mansour et al. 2008; Bestard et al. 2011; Xu et al. 2012). Induction of tolerance was found to be correlated with a reduction of alloreactive effector T cells and an increase of Tregs in the graft as well as in the associated lymphoid tissues. Disparate results, however, exist with respect to the relevance of Tregs in human transplantation (Bestard et al. 2011). Particularly in kidney transplantation it is still a matter of debate, if rising numbers of Tregs after

grafting can be correlated with an operational immune tolerance of the transplant, whereas a decline of Tregs indicates an increased risk of rejection reactions. Most of the previous studies concentrated on single time-point analyses of Tregs either in blood or grafted tissues (Mansour et al. 2008; Batsford et al. 2011; Xu et al. 2012), but did not take a follow–up view of their local influences in the graft as well as of their peripheral origin in the blood.

This analysis was performed to correlate changes of Tregs in peripheral blood with their dynamics in kidney transplant biopsies taken at rejection events and according to the transplantation protocol in the follow-up of the grafted patients. All grafted patients were biopsied at least twice up to 36 months after transplantation. It was intended to evaluate the dynamics of Tregs in peripheral blood as well as renal tissue and to analyze their value as diagnostic/prognostic marker of rejection or tolerance of the graft. It could be shown that methodological problems of the detection of Tregs still hinder their precise tracking and evaluation, although there were signs of an association with specific rejection reactions. Nevertheless, the results support the view of possible contradictory influences of FOXP3+ T-cells on graft function in renal transplantation.

5.1 Tregs – limitations in their characterization

Regulatory T cells are a heterogeneous group of specialized T-cells that have the capability to down-regulate the proliferation and function of different other immune cells such as of monocytes, dendritic cells, B-lymphocytes, NK-cells and neutrophils. Thus, they are capable to modulate innate and adaptive immune responses. They have been claimed to account for 5-10% of the CD4+ T-cells in healthy individuals (Jonuleit et al. 2001). Tregs belong to CD4+CD25+ T-cell subsets and express the nuclear transcription factor FOXP3 (Sakaguchi et al. 2003) as more specific marker. We found a highly significant correlation of the relative numbers of CD4+FOXP3+ cells with the absolute values of CD4+CD25+FOXP3+ cells ($r_{pearson}$ 0,87) in peripheral blood of healthy and grafted individuals in our study. This indicated that changes in the larger CD4+FOXP3+ cell populations were correlated with similar alterations of the T-cell subsets also expressing CD25 as a sign of a full Tregs phenotype. Nevertheless, expression of CD25+ on peripheral CD4+ T-cells in immunosuppressed transplanted patients was much lower than in the healthy population of our analysis and similar to observations of previous studies of Stalder et al. 2003 and Böhler et al. 2009. The reduced number of CD25+ T-cells in this study appeared to be due to the induction therapy with a monoclonal antibody binding and blocking the IL-2 receptor alpha chain (anti-CD25). This induction therapy is generally

recommended as standard procedure in the first phase after grafting by the KDIGO clinical practice guidelines to prevent acute rejection reactions in kidney transplant recipients (Türk et al. 2010). Albeit the used analytical and therapeutic monoclonal anti-CD25 antibodies differed in epitope specificity, this therapeutic strategy could have influenced detection of CD25+ T-cells in this study. Treatment with anti-CD25 antibodies intends to inhibit the IL2-mediated activation of T-lymphocytes by donor/recipient mismatched alloantigens, a critical pathway of alloreactions in the acute cellular transplant rejection early after grafting. Nevertheless, duration and effectiveness of the IL-2R blockade in the individual transplanted patient is unknown and appraisal of its efficiency mostly depends on the clinical readout of a well-functioning graft. Thus, the directly tested peripheral decline of the CD25+ T-cells, which lasted up to 3 months at least after grafting, could have been related to the induction therapy, but also to specific down-modulation or blood emigration of Tregs together with the CD25+ T cell populations.

Baan and colleagues 2005 had shown in vitro that monoclonal anti-CD25Mab treatment may inhibit FOXP3 gene transcription and thus influence Treg suppressive activity. These observations did not correlate with in-vitro experiments of Game et al. 2005 who showed no influences of IL2RA blockade on the suppressive activity of human CD4+CD25+ T-cell populations. Nevertheless, we have to assume from these data and our observations that phenotypic FACS determination of Tregs as CD4+CD25+FOXP3+ T-cells in patients early after grafting and after an induction therapy with IL2R blockade may provide improper results due to methodological as well as clinical problems. This may also explain the variable observations of peripheral Tregs counts in our and previous studies (Baan et al. 2005).

Furthermore, not only the absolute values of CD4+CD25+FOXP3+ Tregs, but also the relative numbers of CD4+FOXP3+ T-cells were considerably lower in the blood of the transplanted patients than of the healthy control individuals (CD4+CD25+FOXP3+ 5,7 vs 29.5 x 10^6/l; CD4+FOXP3+, % 0,6 vs. 2,8) of this study. These results suggested that the aggressive systemic immunosuppressive therapy induced a general severe reduction of CD25+CD4+ T-cell populations after grafting. In our study, we also could not evaluate if the applied various immunosuppressive drugs differed in suppressive influences on Tregs, since all cases were treated similarly. Another risk factor for the development of an immunosuppressed status with low CD25+CD4+ T-cell counts in patients after transplantation could have been related to the duration of the hemodialysis as therapy of end-stage chronic kidney disease before grafting. Caprara et. al. 2016 had reported in a meta-analysis an association between the duration of the uremic state of patients on

hemodialysis and pre-transplant reduced counts of Tregs in the blood of the patients in comparison to healthy controls. Thus, pre-grafting influences on Tregs differentiation or depletion could have contributed to variations of the Tregs phenotypes and cell counts after transplantation and thus increased or lowered risks of activation of immune responses against alloantigens.

5.2 Tregs and their detection in the renal transplant tissues

Tregs include natural (n)Tregs induced in the thymus and induced in the peripheral tissues (i)Tregs. nTregs are thought to primarily reside in lymphoid tissues, whereas iTregs have been claimed to predominantly localize to non-lymphoid tissues and there participate in the regulation of local tissue-specific immune responses. iTregs can be further divided into Tr1 cells that mainly produce IL-10 and Th3 cells that predominantly secrete TGFß. So far markers which have total specificity for Tregs on the whole or allow to differentiate the various subsets are still missing. The best characterized subset of Tregs are the CD4+CD25+FOXP3+ Tregs which have been reported to play a major role in the establishment and maintenance of operational tolerance in animal models. Also in humans, Salama et al. 2003 showed that Tregs of this phenotype can be found in renal transplant recipients and may suppress alloreactivity to donor HLA alloantigens. On the other hand, graft recruitment of Tregs with a FOXP3+ phenotype has been also observed as part of allogeneic immune reactions (Dijke et al. 2008). This suggests that Tregs also could play a role in rejection reactions and kidney damage. In order to get insight into the role and influences of Tregs on graft-specific alloimmune responses in kidney transplantation, this study aimed at a comparison of peripheral Tregs frequencies with their occurrence in kidney transplants and correlation to renal injuries of cell- or antibody mediated rejection reactions.

For this purpose, immunohistochemical and immunofluorescence detection of FOXP3+ lymphocytes in human kidney tissue was established. Triple immunofluorescence staining of cellular infiltrates of allografts with established Tregs markers such as CD4, CD25 and FOXP3 proved to be difficult and not stably reproducible due to high unspecific background fluorescence of the available paraffin embedded renal tissues in spite of extensive blocking procedures. Therefore, we only used a double immunofluorescence labelling method with antibodies against the antigens CD4+ and FOXP3+ for the differentiation of Tregs in paraffin sections. Finally, in optimal characterization of T-cell populations expressing FOXP3+ cells was only achieved by double immunohistological staining with a PO-labelled antibody against FOXP3 and an ALP-labelled reagent either

against CD3, CD20 or CD14. This at least allowed to precisely localize FOXP3 expressing T-cells and evaluate their relative and absolute frequencies with respect to the general T-cell counts and other lymphocytic or monocytic cells. Nevertheless, transient expression of FOXP3+ cells in conventional TCR-activated effector T-cells (Wang et al. 2007) could not be excluded. Due to this lack of precision in phenotypic Tregs differentiation, correlations of peripheral to renal tissue Tregs had to consider could not be reliably performed. Interestingly rare cells expressing FOXP3 in cells carrying CD20 as B-cell marker were seen and supported observations of Felcht et al. 2012.

5.3 Tregs and specific rejection reactions in the grafts

Whereas B-cells with their production of alloantibodies are widely thought to be pathogenetically relevant in the induction of acute graft rejections (antibody mediate rejection reactions, ABMR), T-cell mediated immune reactions (TCMR) are suspected to predominantly mediate chronic rejection reactions. Besides recipient lymphocytes which can infiltrate the graft from recirculation in the peripheral blood, also donor resident lymphocytes particularly including memory CD8+ T-cells, CD4+ T-cells including Tregs, as well as diverse innate lymphoid cells transferred within the transplanted kidney could participate in rejection reactions (Mackay and Kallies 2017). In this study, a significant correlation of recipient peripheral T-cell counts with specific histological signs of graft dysfunction and damage evaluated on graft biopsies according to the BANFF criteria was observed. Notably, an increase of peripheral CD4+ cells was found to be significantly associated with renal interstitial T-cell infiltration and glomerulitis in the allografts. Also a rise of the peripheral FOXP3+ cells, as well as of the percentages of CD4+FOXP3+ T-cell counts per total lymphocytes was significantly associated with glomerulitis and peritubular capillaritis as signs of T-cell mediated graft damage. Nevertheless, no statistically significant correlations between clinically diagnosed or the standard Banff-graded graft rejections of the transplant biopsies and the counted peripheral T-lymphocytes with full Tregs phenotypes were observed. Similar data were reported by Aquino-Dias et al. 2008, Wang et al. 2011. These results indicate that an increase of peripheral T-cells with a Tregs phenotype cannot be easily and reliably directly related to rejection reactions in the allograft. Beside the previously mentioned ongoing difficulties in unequivocal differentiation of the CD4+ Tregs subset, these results may reflect variable participation of other, possibly also donor T cells temporarily expressing FOXP3 during activation in a local inflammatory process against viral or damaged self-antigens. Thus, the data of this study do not support a view that characterization of peripheral T-cell populations with a

CD4+FOXP3+CD25+ Tregs phenotype in blood and renal transplants could allow to distinguish recipients with and without rejection reactions. Although T-cell populations with a Tres phenotype could be evaluated in follow-up biopsies of the patients with acute and later chronic rejections, also no reliable prognostic conclusions could be drawn from the Tregs counts in rejecting and non-rejecting patients. Thus, the results of this study are in line with previous reports (Zuber et al. 2013; Salcido-Ochoa et al. 2012) that the diagnostic or prognostic role of FOXP3 expressing T-cells in renal transplantation may be influenced by further issues such as functional activities of FOXP3+ T-cells, effectiveness of immunosuppression and local immunity within the graft.

Nevertheless, in biopsies with confirmed rejection reactions an increased number of CD3+ T-cells interestingly was found. A similar phenotypic analysis of infiltrating lymphocytes in protocol renal biopsies had been described by Moreso et al. 2007. It was shown that the number of interstitial CD3+ T-cells increased in allografts with subclinical graft rejection in particularly associated with interstitial fibrosis/tubular atrophy. As we know from previous studies subclinical rejection, interstitial fibrosis and tubular atrophy are associated with poorer allograft survival (Isoniemi et al. 1992; Dimeny et al. 1995; Seron et al. 1997). Thus, we questioned whether characterization of CD3+ T lymphocytic infiltrates could predict outcome of rejection reactions or graft survival in follow-up biopsies of this study. Statistically significant associations were found between an increased number of CD3+FOXP3+ cells and the progression of interstitial fibrosis and of tubular atrophy, that, in turn, were associated with poor graft survival. Statistically significant differences between transplant outcome with respect to the same morphological changes and Tregs (CD4+FOXP3+) were not found. We therefore assume that the CD3+FOXP3+ T-cell populations most likely consisted to a greater extent also of activated effector lymphocytes and to a lesser extent of Tregs regulatory cells than the CD4+FOXP3+ T-cells. Again an association between specific morphological signs of renal tissue damage included in the Banff criteria of rejection, such as tubulitis and glomerulitis, and CD3+ FOXP3+ T-cells was observed. Similar results were shown by Bestard et al. 2008, Bunnag et al. 2008 and Veronese et al. 2007. In this case, Bestard et al. 2008 also used CD3 and FOXP3 as the only specific cell markers to define and evaluate regulatory T-cell populations. This further supports the view that in situ analysis of Tregs has to be improved to elucidate pathogenetic role and clinical relevance of Tregs in renal transplantation.

5.4 Tregs – future aspects in kidney transplantation

Recent in vitro analyses reported on CD161, CD127, Helios, CTLA-4, GARP as potential new cell markers which could allow to differentiate Tregs according to a correlated activation status, stable FOXP3 expression or suppressive activity (Zheng et al. 2006, Sugimoto et al. 2006, Shklovskaya et al. 2006, Shimizu et al. 2002). None of these cell markers yet allows to detect all or specific Tregs subsets with sufficient specificity that allows to apply peripheral Tregs frequencies and phenotypes as indicators of tolerance toward renal allografts (Alvarez Salazar et al. 2017). In healthy individuals, Tregs are claimed to be normally in homeostatic balance with TH17-cells and other effector T-cells to guarantee normal immune function (Abdulahad et al. 2011). Criteria for the induction, suppressive function and survival of Tregs in tolerated grafted tissues is still an enigmatic issue in humans. Thus, it is still unknown how pre-transplant immune disturbances and post-transplant immunosuppressive regimens influence their differentiation and function in kidney recipients. In spite of an increasing evidence that Tregs are involved in the development of transplant tolerance, their plasticity in response to immunosuppressive therapy, exact role and possible therapeutic potential also have to be further investigated in renal allograft recipients with and without rejection reactions. Major challenge of this goal will be to improve phenotypic characterization of Tregs in order to develop them as prognostic and therapeutic tools in transplantation.

6 Summary

T regulatory cells expressing FOXP3 as a characteristic transcription marker are thought to have an essential role in the development of self-tolerance in animals and humans. Their loss in animals with FOXP3 deficiency leads to a lethal autoimmune syndrome. In humans FOXP3 deficiency is also associated with polyendocrinopathy, immune dysregulation and X-linked syndrome. Tregs mediate self-tolerance but also tolerance to alloantigens by suppression of the differentiation of effector T-cells, by downregulation of adaptive and innate immune responses. In experimental renal transplantation, induction and activation of Tregs has been associated with reduction of rejection reactions and development of tolerance of allografts. In human organ transplantation, Tregs are also implicated in the establishment and maintenance of an operational allograft tolerance. Another view, however, suggests that Tregs could be also part of inflammatory processes during rejection reactions and tissue injury.

This study was performed to further elucidate the role of Tregs in kidney transplantation as indicators of ongoing immune responses. Therefore, Tregs in peripheral blood and renal biopsies were investigated in a clinical observation program of 53 transplanted patients from the University Clinic in St. Petersburg. Biopsies were taken shortly after grafting as well as regularly later in the follow-up and at episodes of clinically diagnosed rejection reactions. Peripheral blood lymphocytes were differentiated by FACS analysis with the use of specific antibodies against characteristic markers of Tregs, such as CD4, CD25 and FOXP3. Double immunohistochemical labelling techniques with antibodies against CD3, CD4, FOXP3 were applied on tissue sections of the graft biopsies to detect and enumerate Tregs in correlation to rejection reactions or rejection-related kidney tissue damage. The analysis questioned Tregs association to morphological Banff criteria of rejection reactions, their correlation in tissues to peripheral blood frequencies and their use as indicators of operational tolerance in patients with a well-functioning graft versus a state of alloreactivity and tissue damage in patients with progressing graft rejection. It was also intended to analyze if Tregs infiltrates in the allograft tissues could be used to modulate and adjust the immunosuppressive therapy in the individual patient.

In this study, frequencies of peripheral Tregs were significantly reduced in patients shortly after kidney transplantation in comparison to the healthy population. Peripheral cell counts of T-cells with CD4+CD25+FOXP3 full Tregs phenotype, however, did not show a statistically significant association with Banff-graded morphological rejection changes in the

graft. Analyses of peripheral cell populations with individual markers of regulatory T-cells, such as CD4 or FOXP3, nevertheless showed a correlation with specific signs of rejection reactions in the allografts. Also the numbers of CD3+FOXP3+ cells showed significant correlation with specific Banff-graded morphological signs of acute rejection reactions or with cellular-mediated renal damage in progressing rejection reactions. The absolute numbers of CD3+ T-cells increased in allografts with subclinical graft rejection associated with interstitial fibrosis or tubular atrophy. Overall the results indicated that an increase of peripheral T-cells with a Tregs phenotype could not be easily and directly related to rejection reactions in the allografts. The same appeared to apply to T-cells with Tregs phenotype in the renal grafts themselves. Although T-cell populations with a Tregs phenotype could be evaluated in follow-up biopsies of patients with acute and later chronic rejections, no reliable prognostic value of the FOXP3 expressing T-cells in rejecting and non-rejecting patients could be found. Like in previous studies, differentiation and enumeration of Tregs in blood and tissues turned out to be still a major theoretical and practical issue, as none of the present known cell markers of Tregs allowed to detect all and/or specific Tregs subsets with sufficient specificity. In addition, in the clinical setting, diverse immunosuppressive therapies could have influenced differentiation and activation of Tregs and account for variation in their specific cell counts. In spite of increasing experimental evidence that Tregs are involved in the induction and maintenance of transplant tolerance, their precise characterization and homeostatic differentiation balance with other CD4+ T-cells such as TH17 have to be further considered and elucidated in patients with and without biopsy-proven rejection reactions to elucidate their prognostic role in renal transplantation.

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Lebenslauf

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Ich bin in St. Petersburg, Russland, am 21.11.1984 geboren.

Meine Schulausbildung habe ich zunächst, von 09/1991 bis 06/2000, in der staatlichen Schule Wsevologsk und danach, von 09/2000 bis 06/2002, in der Medizinischen Schule St. Petersburg erfolgreich absolviert. Darauf folgte, von 09/2002 bis 06/2009, ein Medizinstudium in der Staatlichen Medizinischen Pavlov-Universität St. Petersburg, mit dem Abschluss „Humanmedizin". Schon während des Studiums begann meine wissenschaftliche Tätigkeit im Forschungsinstitut für Zytologie der Russische Akademie der Wissenschaften. Dort lernte ich in der Zeit von 10/2005 bis 08/2008 die Basistechniken von Zellenkultivierung und beschäftigte mich mit der Kultivierung der Nierenfibroblasten von Mäusen. Später, im Molekular-genetischen Labor für Transplantationsmedizin der Staatlichen Medizinischen Pavlov-Universität St. Petersburg erlernte ich die Techniken der Kultivierung von mesenchymalen Stammzellen.

Im 07/2008 absolvierte ich eine Famulatur in der Chirurgischen Klinik und Poliklinik für Transplantations- Thorax- und Gefäßchirurgie am Universität Klinikum Leipzig. Ein Jahr später konnte ich ebenfalls eine Famulatur in der Abteilung Nephrologie und Rheumatologie, Universitätsklinikum Göttingen, absolvieren und mich mit der Forschungslabor sowie klinischer Arbeit der Abteilung bekannt machen.

Danach begann meine Weiterbildung in der Inneren Medizin in der Klinik für Nierentransplantation, St. Petersburger Staatliche Medizinische Pavlov-Universität, wo ich als Arzt im Praktikum ein Jahr gearbeitet habe. Dort begann ich 2010 mit der Ausarbeitung der aktuellen Forschungsarbeit „Analysis of Lymphocytes with T regulatory Phenotype in Kidney Allografts of Saint-Petersburg". Im Oktober 2010 konnte ich meine klinische Weiterbildung und meine Doktorarbeit in der Abteilung für Nephrologie und Rheumatologie, im Rahmen einer Zusammenarbeit zwischen der Universität Göttingen und der Universität St. Petersburg, fortführen. Seitdem arbeite ich als Assistenzärztin der Klinik für Nephrologie und Rheumatologie an der Universitätsmedizin Göttingen.