Monocytes and Macrophages in Solid Organ Transplantation

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Chapter 1

General Introduction

General Introduction

Kidney transplantation and rejection

The treatment of choice for patients with organ failure is transplantation [1]. In 1954 the first kidney transplantation was performed with acceptable allograft survival rates [2]. Without immunosuppression, immune cells of the patient will recognize the transplanted organ as foreign leading to rejection of the graft. Discovery of cyclosporine in the late seventies; the drug that suppresses this aggressive immune response, largely contributed to the success of organ transplantation. Treatment with cyclosporine significantly decreased the incidence of acute rejection episodes leading to acceptable graft and patient survival rates. In the 1990s, the introduction of more specific drugs further improved the outcomes after organ transplantation. Chronic kidney graft failure is the leading cause of transplant loss [3]. An important feature of rejection is deterioration of kidney function reflected by increased creatinine levels; i.e. decreased levels of estimated glomerular filtration rate and proteinuria. Kidney biopsy remains the golden standard diagnostic tool to confirm rejection and provide information that guides treatment [4]. Kidney transplant rejection can be subdivided into different categories that are based on histomorphology using Banff criteria (Figure 1) [5-12]. First, antibody mediated rejection (ABMR) which is mediated by antibodies against HLA and non-HLA molecules, endothelial-cell antigens, and ABO blood-group antigens on endothelial cells and red blood cells [13] of which there is; acute/active ABMR and chronic active ABMR. For diagnosis of acute/active or chronic/active ABMR three histomorphological features must/should be present; histologic evidence of acute tissue injury, evidence of current/recent antibody interaction with vascular endothelium and serological evidence of donor specific antibodies (DSAs) [11]. Second, T-cell mediated rejection (TCMR) of which there are also different grades; acute TCMR type IA (significant interstitial inflammation with foci of moderate tubulitis), acute TCMR type IB (significant interstitial inflammation with foci of severe tubulitis), acute TCMR type IIA (mild intimal arteritis with or without interstitial inflammation and tubulitis), acute TCMR type IIB (severe intimal arteritis with or without interstitial inflammation and tubulitis) and acute TCMR type III (transmural arteritis and necrosis of smooth muscle cells with lymphocytic inflammation). Chronic TCMR is defined as arterial intimal fibrosis with mononuclear cell infiltration with formation of neointima [11].

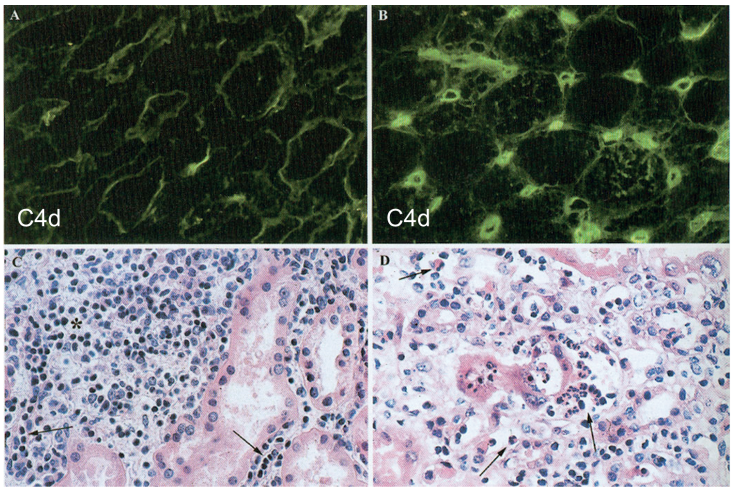

Figure 1. In the abovementioned figure a case of acute cellular rejection is displayed showing negative C4d staining (A) and high cellular infiltrates (C). In comparison with a case of acute antibody mediated rejection with strong C4d positive staining and mononuclear cell infiltrates in peritubular capillaries (D). Picture is adapted from the renal pathology education presentation by dr. Marian Clahsen van Groningen (Pathologist, Erasmus MC, Rotterdam, The Netherlands).

Heart transplantation and rejection

Also after heart transplantation rejection of the graft limits its long-term survival, to which both acute cellular rejection and ABMR (noncellular, vascular, humoral) significantly contribute [14-17]. For the diagnosis of rejection, heart transplant patients receive surveillance endomyocardial biopsies that are most frequently performed in the first months after transplantation. The International Society of Heart and Lung transplantation has a standardized nomenclature grading system for cardiac biopsies, which was introduced first in 1990 and revised in 2004. However, sampling error leads to underestimation of the severity of rejection [18-21]. The diagnosis of ABMR is based on the following histologic features; myocardial capillary with intravascular macrophage accumulation and positive immunofluorescence within the capillaries for immunoglobulins (IgG, IgM and/or IgA), complement (C4d, C3d, and or C1q), and CD68 macrophage staining [22]. For acute rejection three grades are defined; Grade 1R (mild, interstitial and/or perivascular infiltrate with up to one focus of myocyte damage), Grade 2R (moderate, two or more foci of infiltrate with associated myocyte damage and Grade 3R (severe, diffuse infiltrate with multifocal myocyte damage, with or without edema, hemorrhage, or vasculitis) (Figure 2) [21].

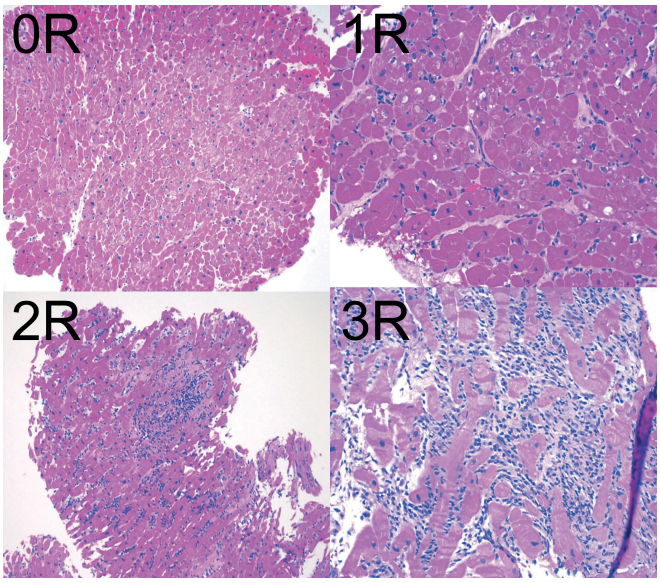

Figure 2: The illustrations are adapted from Stewart et al. JHLT, 2005.

Novel mechanisms involved in allograft rejection: The role of monocytes/macrophages

The contribution of monocytic cells to organ graft rejection has been attracting increasing attention. This cell population has been recognized as important players in both acute and chronic rejection responses. Monocytes arise from myeloid precursor cells in primary and secondary lymphoid organs such as the bone marrow and execute functions in innate immunity [23, 24]. These cells are the precursors of macrophages and essential for the first line of defense against pathogens. Monocytes can be distinguished into 3 subsets based upon CD14 (LPS co-receptor) and CD16 (Fcγ receptor III) expression. Three circulating monocyte subtypes can be identified: the $CD14^{++}CD16^{-}$ (classical monocytes), $CD14^{++}CD16^{+}$ (intermediate monocytes) or $CD14^{+}CD16^{++}$ (non-classical monocytes) [25, 26]. Classical monocytes are able to migrate in response to infection or tissue damage, and are released in blood in a CCR2-depended manner [27]. Intermediate and non-classical monocytes are considered as inflammatory cells, and the non-classical monocytes, specially, are known for their patrolling and crawling patterns [28]. CD16+ monocytes can produce pro-inflammatory cytokines such as TNF-α and IL-1β. CD16+ monocytes are also associated with development of atherosclerosis in chronic kidney disease patients [29]. In the transplantation setting, we documented a shift towards proinflammatory CD16+ monocytes in stable kidney transplant recipients that was already present at the time of transplantation and remained during the first 6 months, despite immunosuppressive regime and improvement of kidney function [30]. Macrophages have the capability of differentiating into a variety of phenotypes in response to the microenvironment [31]. In transplantation, macrophages are involved in allograft

damage, tissue remodeling, and have immunoregulatory and/or immunosuppressive effects [32]. Macrophages can also be subdivided into classically (M1) and the alternatively-activated (M2) subtypes. In response to IFN-γ and after engagement of toll-like receptors (TLRs), M1 macrophages display a pro-inflammatory phenotype by expressing high levels of the co-stimulatory molecules CD80 and CD86, inducible nitric oxide synthase (iNOS), and by production of many pro-inflammatory cytokines like TNF-α, IL-1 and IL-6. M2 macrophages display an anti-inflammatory phenotype and are activated in response to IL-4 or IL-13. These M2 cells express cell surface markers such as the mannose receptor CD206, the high affinity scavenger receptor for the hemoglobin-haptoglobin complex CD163, arginase-1 and the anti-inflammatory cytokine IL-10. This macrophage subset is mainly involved in wound healing and tissue remodeling [33-36].

The first report about the presence of macrophages in rejecting kidney allografts was published in 1958 [37]. Macrophage infiltration has been associated with both acute antibody mediated rejection and acute cellular rejection [38, 39]. It is also been described that infiltration of CD68+ macrophages is associated with acute rejection in human kidney and heart allografts [40, 41]. Importantly, intravascular macrophage accumulation in the endomyocardial capillaries is now one of the most important diagnostic criteria for antibody mediated rejection [21]. The study of Grimm et al. showed that infiltration level of activated macrophages discriminates between clinical and subclinical rejection in kidney allograft patients [42]. Presence of abundant numbers of macrophages are associated with poor graft outcome in kidney and in heart transplantation. Intragraft presence of macrophages is also associated with the development of fibrosis [41, 43] in which M2 macrophages play a dominant role [44, 45]. Currently, immunosuppressive drugs; i.e. agents that inhibit the anti-donor response, can induce CD163+ M2 macrophages that express high levels of mRNA coding for pro-fibrotic cytokines such as TGF-β1[46, 47]. CD163+ overexpression also correlates with kidney function as measured by serum creatinine levels at 1 year after transplantation [48]. Finally, CD16+ monocyte infiltration with smooth muscle like characteristics was described during chronic allograft dysfunction, which suggests the important role of monocytes in remodeling including neointima formation and formation of profibrotic matrix through TGF-β expression [49].

Novel mechanisms involved in allograft rejection: microRNAs

The expression of many genes including those coding for genes involved in rejection responses are controlled by microRNAs (miRNAs) which are endogenous, short non-coding RNAs [50]Antisense. As a consequence, miRNAs have been widely investigated in transplant patients to determine if by the measurement of these small molecules rejection can be diagnosed or even predicted. Particularly, because the early and non-invasive detection of rejection in transplant patients is warranted. Sui et al. was the first who identified 20 miRNAs in tissue, which were associated with acute rejection in kidney transplant recipients.

This first potential use of miRNA analysis as a diagnostics tool boosted the field and led to multiple studies reporting about the outcomes of miRNA studies [51]. A next article reported that 10 miRNAs were under expressed and 7 miRNAs overexpressed in acute rejection biopsy samples compared to allograft tissue without histological signs of rejection. This observation showed that miRNAs studies can be used as a tool to better diagnose rejection and to determine the mechanisms involved [52]. In a biomarker discovery study, miR-210 and miR-10b were identified as specific urinary biomarkers of acute cellular rejection. Compared to the urine from stable kidney transplant patients, miR-210 was expressed at lower levels, while miR-10b was highly expressed during acute rejection [53]. Scian et al. were the first to report on a miRNA signature for chronic allograft dysfunction (CAD) with interstitial fibrosis/tubular atrophy (IF/TA). Five miRNAs (miRNA-142-3p, miR-204, miR-107, miR-211 and miR-32) were differentially expressed and correlations between tissue samples and subsequent urine samples were found [54]. In a small cohort of IF/TA biopsies obtained from transplant patients and normal transplant tissue, differential miRNA expression was analyzed. In this study, miR-21, miR-142-3p/5p and miR-223 were expressed higher in IF/TA biopsies whereas miR-30 family members were higher expressed in normal biopsies [55]. More recently, Wilflingseder et al. performed a large miRNA profiling study with 65 renal allograft biopsies and described 4 other miRNAs being associated with acute cellular rejection: miR-150, miR155, miR-663a and miR-638. In antibody mediated rejection, six upregulated miRNAs were identified including miR-21 and miR-182 [56]. Note that miR-150 is critical in regulation of B cell development and lymphopoiesis, whereas miR-155 is important in CD8+ T cells effecting the regulation of CD8+ T cell responses [57]. Controlled and mechanistical studies need to be performed in order to further unravel the role of these molecules in the pathogenesis of rejection.

Aims and outline of this thesis

It only has been recently appreciated that cells of the innate immune system such as monocytes and macrophages are major players in acute and chronic immunity in organ transplantation. These cells contribute to antigen processing, antigen presentation, costimulation, production of pro- and anti-inflammatory cytokines, and tissue repair or remodeling. The overarching objective of this thesis is to shed light on the role of monocytes and macrophages in various types of rejection in both kidney and heart transplantation. More explicitly, we aimed to provide evidence that the balance between pro- and anti-inflammatory monocytes/macrophages can influence the outcome of the anti-donor immune response in terms of fibrosis and long-term graft function. We aimed to characterize the local distribution of divers monocyte and macrophage subsets in different types of rejections in order to search for specific features that could signify the type of rejection. Furthermore, the impact of the currently given immunosuppressive drugs on myeloid lineage is largely unknown. Therefore, a literature study was performed to gain an overview

on the current knowledge and new immunotherapeutic challenges. To better understand the mechanisms of rejection, we performed a miRNA study. To these ends, we analyzed both biopsy and blood specimens. In detail, the following aims were investigated:

- ✓ To determine and correlate monocyte-macrophage profiles in endomyocardial tissue with corresponding serial blood samples in heart transplant recipients undergoing acute cellular rejection and the relationship with fibrosis– **Chapter 2**
- ✓ To determine whether compartmental differences in distribution of monocyte-macrophage subsets between different types of rejection are present. Infiltration of subsets were correlated to serum creatinine and eGFR at the time of biopsy, 3, 6 and 12 months pre and post-rejection – **Chapter 3**
- ✓ To investigate whether there are essential differences in pretransplant monocyte subset composition and whether monocytes could serve as a biomarker to predict kidney allograft rejection – **Chapter 4**
- ✓ To determine if microRNAs could discriminate between different histopathological subtypes of kidney allograft rejection – **Chapter 5**
- ✓ To investigate whether human monocytes obtained from renal transplant recipients and healthy individuals possess the capacity to produce IFN-γ – **Chapter 6**
- ✓ To discuss the effects of currently prescribed immunosuppressive drugs on monocyte - macrophage features and the future challenges – **Chapter 7**

In **Chapter 8** and **Chapter 9** the above mentioned research will be summarized and discussed.

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Chapter 2

CD16+ monocytes and skewed macrophage polarization towards M2 type hallmark heart transplant acute cellular rejection

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Abstract

Background

During acute heart transplant rejection, infiltration of lymphocytes and monocytes is followed by endothelial injury and eventually myocardial fibrosis. To date, no information is available on monocyte-macrophage related cellular shifts and their polarization status during rejection. Here, we aimed to define and correlate monocyte-macrophage endomyocardial tissue profiles obtained at rejection and time-points prior to rejection, with corresponding serial blood samples in 25 heart transplant recipients experiencing acute cellular rejection. Additionally, 33 healthy individuals served as control.

Material and Methods

Using histology, immunohistochemistry, confocal laser scan microscopy and digital imaging expression of CD14, CD16, CD56, CD68, CD80 and CD163 was explored to define monocyte and macrophage tissue profiles during rejection. Fibrosis was investigated using Sirius Red stainings of rejection, non-rejection and one-year biopsies. Expression of co-stimulatory and migration-related molecules on circulating monocytes, and production potential for pro- and anti-inflammatory cytokines were studied using flowcytometry.

Results

At tissue level, striking CD16+ monocyte infiltration was observed during rejection ($p<0.001$). Significantly more CD68+CD163+ M2 macrophages were documented during rejection compared to barely present CD68+CD80+ M1 macrophages. Rejection was associated with severe fibrosis in 1-year biopsies ($p<0.001$). Irrespective of rejection status, decreased frequencies of circulating CD16+ monocytes were found in patients compared to healthy individuals. Rejection was reflected by significantly increased CD54 and HLA-DR expression on CD16+ monocytes with retained cytokine production potential.

Conclusions

CD16+ monocytes and M2- macrophages hallmark the correlates of heart transplant acute cellular rejection on tissue level, and seem to be associated with fibrosis on the long-term.

Keywords

Heart, Transplantation, Macrophages, Monocytes, Rejection

Introduction

During acute cellular heart transplant rejection, infiltration of lymphocytes and monocytes is followed by endothelial injury, structural tissue damage and eventually myocardial fibrosis [1]. Not only T-cells but also monocyte-/macrophage-lineage cells are abundantly present in the rejecting heart [2, 3]. To date, the rejection related monocyte–macrophage subset profiles in both tissue and blood compartment are not yet defined in heart transplant recipients. It is also unclear whether rejection-related changes of monocyte-macrophage subsets on tissue level are reflected in circulation.

Expression of CD14 (LPS co-receptor) and CD16 (Fcγ receptor III) define three phenotypically and functionally distinct human monocyte subsets: CD14++CD16- (classical), CD14++CD16+ (intermediate), and CD14+CD16++ (non-classical) monocytes [4]. The CD16+ monocytes are considered pro-inflammatory due to production of pro-inflammatory cytokines, like TNF-α and IL-1β compared to classical monocytes [4, 5]. Higher expression of HLA-DR, CD86 and CD54 (ICAM-1) distinguish CD16+ monocytes from the classical ones.

Monocytes are important in many inflammatory diseases. Coronary artery disease patients have higher numbers of monocytes compared to healthy cohorts [6, 7]. Local biphasic monocyte accumulation was observed following acute myocardial infarction [8, 9]. The monocytes, located in the infarct border zone during the inflammatory phase after infarction, consisted mainly of CD14+CD16- cells, whereas during the proliferative phase the monocytes in the infarct core showed comparable percentages of CD14+CD16- and CD14+CD16+. Different macrophage populations are also observed following myocardial infarction with pro-inflammatory cells early on followed by reparative macrophages [8].

Related to the state of activation, macrophages can be functionally grouped into two main classes: the M1 (pro-inflammatory) and the M2 (anti-inflammatory), although an increasing number of different phenotypes with intermediate and contrasting features have been described recently [10]. Classically, the M1 macrophages can be induced after stimulation of monocytes with IFN-γ, and mainly have phagocytic, anti-microbial and pro-inflammatory functions [11]. On the other hand, M2 macrophages are induced after monocyte-stimulation with modulating factors such as IL-4/IL-13, immune complexes, or glucocorticoids, and are phenotypically characterized by expression of CD163, CD206, and/or CD204. These macrophages exert anti-inflammatory functions by production of IL-10 and TGF-β [12]. M2 macrophages can also produce matrix metalloproteinases contributing to extra cellular matrix turnover and fibrosis [13].

As different monocyte and macrophage subset phenotypes are functionally different in induction and/or maintenance of inflammation or fibrosis, it is important to investigate their role in relation to heart transplant rejection. This information will eventually help identifying key cell types, molecules and markers, which can serve as diagnostic biomarkers of rejection,

and/or as targets for rejection treatment. Here, we aimed to define and correlate monocyte/macrophage profiles in tissue and circulation using endomyocardial biopsies obtained at rejection and time points prior to rejection, and their corresponding serial blood samples in 25 heart transplant recipients experiencing acute cellular rejection. Next, we wondered whether these cellular shifts were associated with structural graft damage and fibrosis. Additionally, blood profiles of non-rejecting heart transplant recipients were compared with 33 healthy individuals using a cross-sectional approach.

Material and Methods

Patient characteristics

Twenty-five heart transplant recipients underwent protocol surveillance biopsies within the first year after transplantation at the Erasmus University Medical Center (Rotterdam, The Netherlands). Peripheral blood mononuclear cells (PBMC) were collected serially in time: at a time point that the protocol biopsy showed no rejection, and subsequently at a time point of biopsy-proven rejection (Median time±SEM between both time points: 3±1.4 weeks). Additionally, biopsies obtained at 1 year post rejection were used for Sirius Red staining (Median time±SEM between rejection and post rejection time points: 56±12.5 weeks). Of note, no rejection episodes occurred between the rejection time point and 1 year post-rejection. Histopathological features were scored according to 2011 and 2005 International Society for Heart and Lung Transplantation guidelines in order to diagnose acute antibody mediated rejection and acute cellular rejection, and to grade endomyocardial biopsies as non-rejection (0R) or rejection (2R) [14, 15].

All studied endomycardial biospies (n=50) showed acute cellular rejection (2R according to 2005 ISHLT classification system) with no signs of histopathologic and immunopathologic evidence of acute antibody mediated rejection. All biopsies were C4d negative. Intravascular macrophages and neutrophils as well as signs of endothelial injury like swelling and denudation with congestion and/or hemorrhage were absent. No serologic evidence of anti-HLA antibodies could be detected using Luminex technique.

This study was performed according to the tenets of the Declaration of Helsinki and approved by the Medical Ethical Committee of the Erasmus MC. All patients signed written informed consent. Table 1 lists the transplantation characteristics, and the clinical and immunological features of this cohort. In addition, blood samples were collected from 33 healthy individuals (age: median + range: 51 (25-73); male: 42%) and used as control.

All patients were treated with horse or rabbit anti-thymocyte globulin (hATG or rATG) as induction therapy in combination with maintenance calcineurin inhibition (Prograft® or Neoral®), mycophenolate mofetil (Cellcept®), and steroids; the dose schedule was adjusted according to the local standard protocol.

Table 1. Clinical and immunological characteristics of heart transplant recipients

Characteristics	Recipients (n=25)
Age (median (year), range)	46 (15-64)
Gender (% male)	64%
Primary disease (number of patients, %)	
Cardiomyopathy	16 (64%)
Ischemic heart disease	9 (36%)
Induction therapy	
Horse-antithymocyte globulin	68%
Rabbit-antithymocyte globulin	32%
Maintenance therapy	
Cyclosporine/prednisone/mycophenolate mofetil	56%
Tacrolimus/prednisone/mycophenolate mofetil	44%
HLA mismatches total (median, range)	
Class I mismatches	3 (2-4)
Class II mismatches	2 (1-2)
Ischemia (median (min), range)	170 (137-250)

Phenotype, activation and co-stimulatory molecule status of monocytes

In order to investigate monocyte phenotype, activation status and co-stimulatory molecules (HLA-DR and CD54), PBMC were collected from whole blood using Ficol gradient. Labeling and flow cytometric assessment were performed as described before [16, 17]. Monocytes were identified based on forward/sideward scatter, lack of expression of CD3, CD20 and CD56 and expression of CD14 and CD16 (Figure 1A).

Intracellular cytokine production

PBMC were incubated overnight with 10 ng/ml LPS (Sigma-Aldrich) in the presence of golgiplug (1:1000, Becton Dickinson) after pre-stimulation with IFN-γ for 2 hours. The cells were then incubated with conjugated primary antibodies in phosphate buffered saline containing 0.5% bovine serum albumine for 30 minutes. The antibodies used were CD3-PE, CD14-Pacfic Blue, CD16-PE-Cy7, CD20-PE, CD56-PE (all Biolegend) at 4˚C and were incubated with EDTA for 15 min followed by incubation with FACS permeabilizing solution 2 (BD Biosciences) for 15 min. Next, conjugated antibodies to TNF-α-Percp-Cy5.5, IFN-γ-APC-Cy7, IL-1β-FITC, IL-6-APC, and IL-10-APC and their respective isotype controls (all Biolegend) were added to determine intracellular cytokine production. The cells were washed and analyzed using flow cytometry (FACSCanto II, BD Biosciences) and FACSDiva software [17].

Immunohistochemical staining

Immunohistochemistry was performed by an automated staining system (Ventana Benchmark ULTRA, Ventana Medical Systems, USA) using horseradish peroxidase (HRP) with brown chromogen (3,3'-Diaminobenzidine) as enzymatic label. Tissue sections were incubated with antibodies against CD14 (clone 7, mouse-monoclonal IgG2a, 1:100, Leica Biosystems, Newcastle), CD16 (clone sc-20052, mouse-monoclonal IgG1, 1:400, Santa Cruz Biotechnology, Dallas, Texas, USA), CD56 (clone 123C3, mouse-monoclonal, Ventana, ready to use, Tucson, AZ, USA), CD68 (clone KP1, mouse-monoclonal, ready to use, DAKO, Carpentaria, CA, USA), CD80 (clone 37711, mouse-monoclonal IgG1, 1:50, R&D systems, Minneapolis, USA) or CD163 (clone EDHu-1, mouse-monoclonal, IgG1, 1:400, AbD Serotec, Raleigh, USA). Two pathologists independently scored these endomyocardial biopsies (EBMs).

Immunofluorescence staining

Tissue sections were incubated with primary mouse-monoclonal CD14 IgG2a antibody overnight at 4° C. Secondary goat-anti-mouse IgG2a Alexa Fluor 488 (Invitrogen) was applied and incubated for 1 hour at room temperature (RT). After washing steps, the second primary antibody mouse-monoclonal CD16 IgG1 was added for one hour. Next the secondary goat-anti-mouse IgG1 Alexa Fluor 555 (Invitrogen) was applied for another one hour at RT. Slides were covered with anti-fading mounting medium containing DAPI (Vectashield, UK) and stored at 4°C until evaluation.

To distinguish CD16 expressing monocytes from the infiltrating CD16+ NK-cells and CD68+ macrophages, CD14/CD16, CD56/CD16 and CD68/CD16 double stainings were performed as described above. Double staining with CD68 (1:1600, clone KP1, mouse-monoclonal, DAKO, Carpentaria, CA, USA) and CD80 (clone 37711, mouse-monoclonal IgG1, 1:50, R&D systems, Minneapolis, USA) were used to characterize M1-type macrophages. For M2 macrophages, double stainings were performed with CD68 and CD163 (clone EDHu-1, mouse-monoclonal, IgG1, 1:400, AbD Serotec, Raleigh, USA) mAbs. Specific controls are used as displayed in supplementary figure 2.

Sirius Red staining

In brief, following deparaffinization slides were rehydrated by passage through decreasing ethanol series, 5 minutes predifferentation step using 0,2% fosformolybdeen-acid followed by 45 minutes incubation with 0,1% Sirius Red solution. Slides were analyzed using polarization method. Representative pictures were made under polarized light and positive stained area was analyzed by ImageJ software.

Image analyses and Laser Scanning Confocal Microscopy

EMB samples were scored using ImageJ IHC analysis software [18]. Analyses were performed blinded to the clinical source using scanned Nanozoomer Digital Pathology files. Images of the entire biopsy sample (mean size range: 3,4-3,6 mm) were analyzed at 10x objective magnification.

Confocal microscopy was performed using LSM-700 laser scanning confocal microscope (Carl Zeiss). The entire biopsy samples were scored counting the absolute number of cells as for CD16+CD56-, CD16+CD56+, CD16-CD56+, CD14+CD16-, CD14+CD16+ or CD14-CD16+ using 40x magnification.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 6. Statistical significance was evaluated by Mann-Whitney-U test, t-test, and one-way ANOVA. A p-value of <0.05 was considered statistically significant.

Results

CD16+monocytes are significantly decreased and CD16- monocytes are significantly increased in peripheral blood in heart transplant recipients independent of rejection status

We first aimed to investigate how the monocyte subset composition in heart transplant recipients would relate to that of healthy individuals. Although the absolute numbers of monocytes were similair, the percentages of classical CD14++CD16- monocytes (NR: %92±7,5, R: %90±4,5 (median±IQR)) were significantly higher ($p<0.05$, $p<0.001$), and the percentages of intermediate CD14++CD16+ (NR: %4±1,5, R: %4±3,5 (median±IQR)) and non-classical CD14+CD16++ monocytes (NR: %3±3,5, R: %3±3,5 (median±IQR)) were significantly lower in heart transplant recipients than healthy controls (%83±5,25, %5,5±3,5, %9±4,75 (median±IQR)) ($p<0.001$) (Figure 1B-D). Moreover, no subset differences could be detected between non-rejection and rejection time points.

Circulating CD3+T cell frequencies were significantly increased during rejection as compared with non-rejection time points which is consistent with previous findings [19] ($p<0.001$) (Supplementary Figure 1).

CD16+ monocytes are significantly increased in rejecting endomyocardial biopsies

Serial EMBs were stained using double immunofluorescence labeling with CD14 and CD16. Co-localization and membranous staining of CD14 and CD16 is shown in rejected tissue (Figure 1H). Absolute numbers of stained cells were counted using 20x magnification field by confocal microscopy. Considering the fact that mean surface area of the total biopsies ranged between 3.4-3.6 mm^2, there was no need to correct the data for the size of biopsies. Absolute numbers of CD14+CD16- monocytes were significantly higher during rejection compared to non-rejection biopsies ($p<0.05$). Although usually minor subsets in peripheral blood, both CD14+CD16+ and CD14-CD16+ subsets were prominantly increased during rejection in tissue compared to the prior non-rejection time point ($p<0.001$, Figure 1E-G).

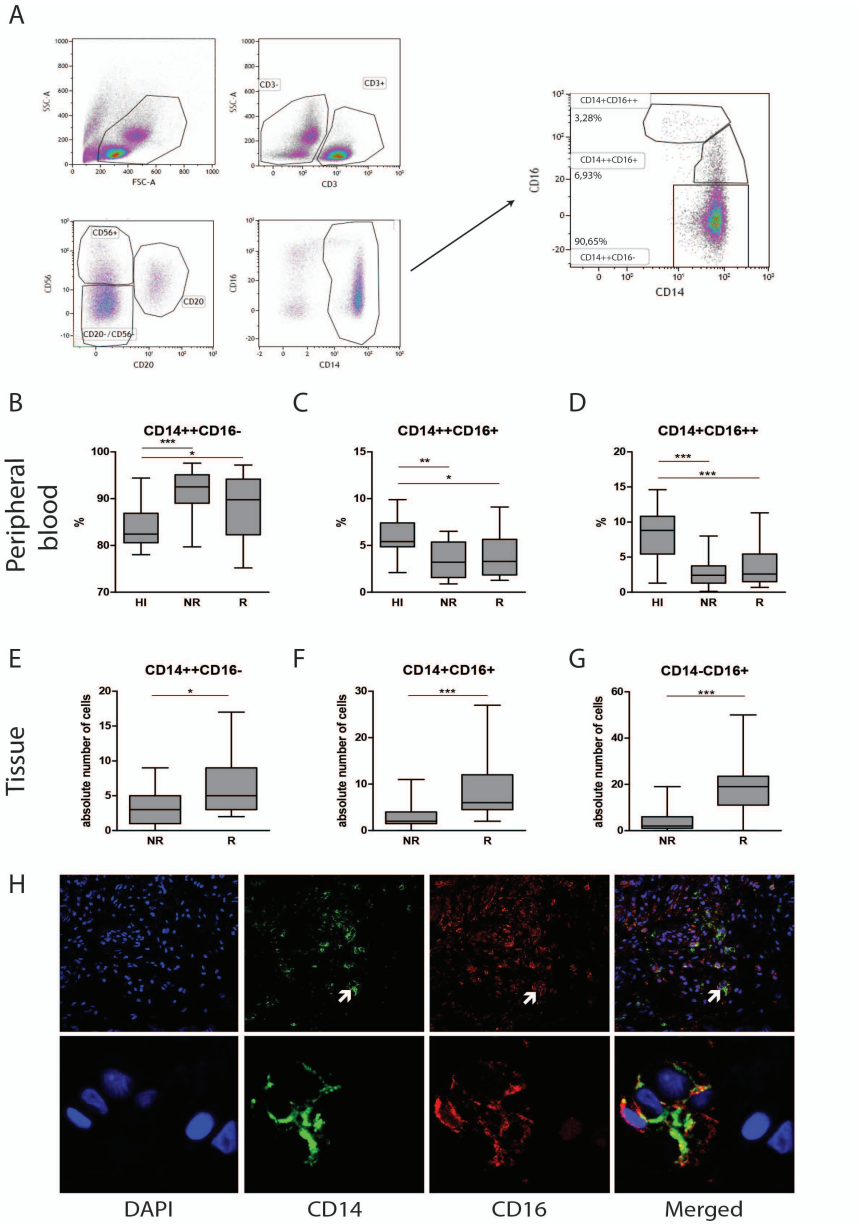

Figure 1. Contrasting monocyte subsets in blood and tissue during heart transplant rejection.
Representative monocyte subset FACS gating strategy is depicted (A). Blood: The percentage of (B) classical CD14++CD16-, (C) intermediate CD14++CD16+ and (D) non-classical CD14+CD16++ monocytes in healthy individuals (HI; n=33) and heart transplant recipients (n=25) at time points no rejection (NR) and rejection (R) are presented as median±IQR. Tissue: The absolute numbers of (E) CD14+CD16-, (F) CD14+CD16+ and (G) CD14-CD16+ monocytes in heart transplant biopsies at time points non-rejection (NR; n=25) and rejection (R; n=25) are given. A representative overview of double immunofluorescence stainings of rejected endomyocardial tissue is shown; (H) CD14 (green) and CD16 (red) at 10x magnification. Detailed co-localization (H) of CD14 (green) and CD16 (red) is shown at 63x magnification. Absolute numbers of cells are depicted. * p<0.05, ** p<0.01, *** p<0.001.

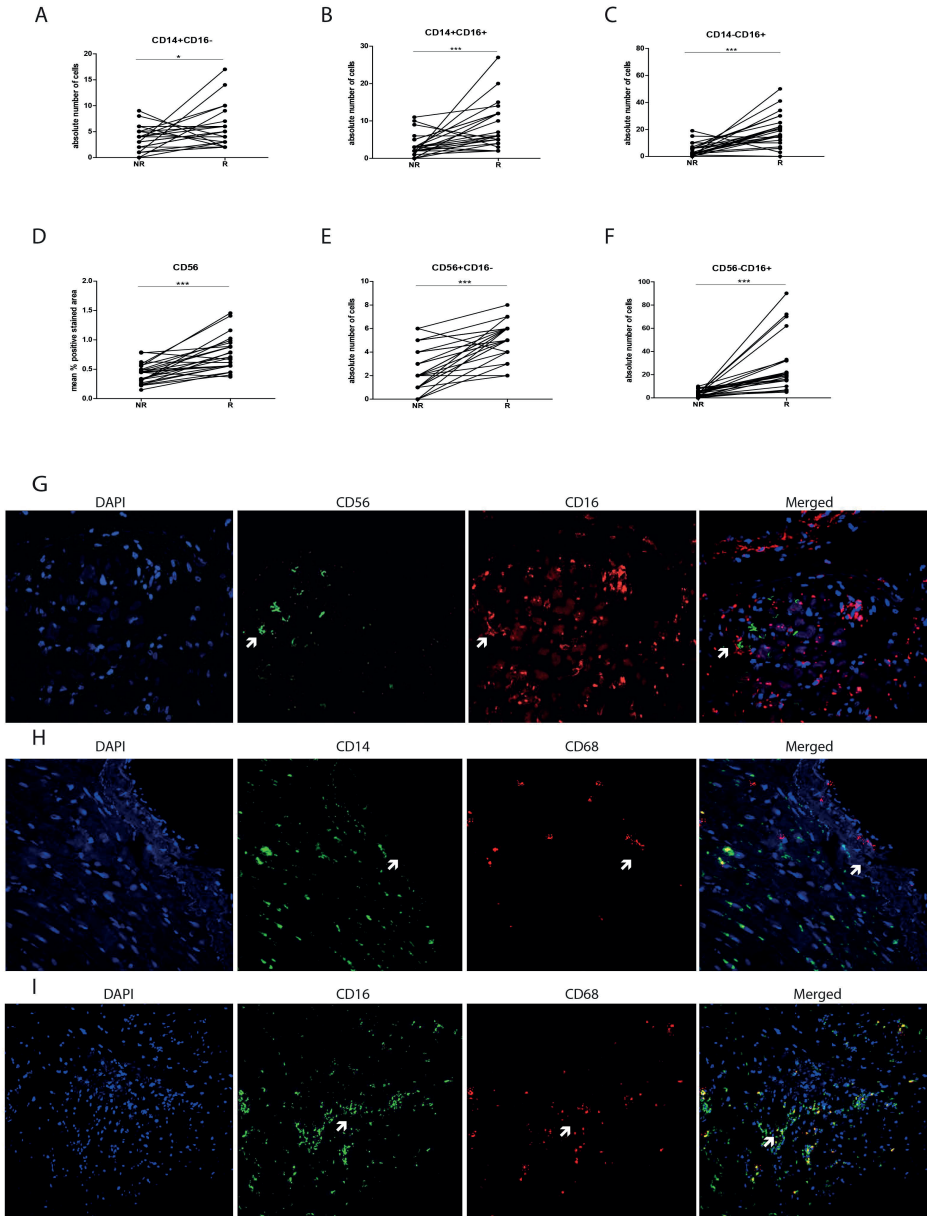

Figure 2. CD16+ cells in graft tissue are monocytes and accumulate upon rejection.

The expression of CD14 and CD16 was analyzed in biopsies at two time points; non-rejection (NR; n=25) and rejection (R; n=25) and compared intra-individually. The absolute number of cells is depicted for (A) CD14+CD16-, (B) CD14+CD16+ and (C) CD14-CD16+. The expression of (D) CD56 was measured using immunohistochemistry, analyzed by ImageJ and depicted as mean % positive stained area. The absolute numbers of cells of (E and F) CD56+CD16- and CD56+16+ were determined. A representative overview of rejecting endomyocardial tissue stained for (G) CD56 (green) and CD16 (red) at 20x magnification is shown. A representative overview of rejecting endomyocardial tissue stained for (H) CD14 (green), (I) CD16 (green) and CD68 (red) at 20x magnification is given. * $p<0.05$, *** $p<0.001$.

To verify whether CD16+ cells were either CD14+monocyte or CD56+NK-cell or CD68+ macrophage, double immunofluorescence stainings were performed using CD16/CD56, CD14/CD68 and CD16/CD68 labeling (Figure 2G-I). Individual biopsy analyses showed hardly any co-localization between CD56 and CD16 staining (Figure 2G). Intra-individual analysis of the monocyte subsets showed high numbers of CD16+ expressing cells at rejection (Figure 2A-C). Although the simultaneously present CD56+NK cells were very low in absolute numbers at rejection compared to non-rejection time points, this difference appeared to be statistically significant between these two time points (Figure 2D). The absolute numbers of infiltrating CD56-CD16+ cells were significantly higher compared to CD56+CD16- within rejecting tissue ($p<0.001$, Figure 2D-F). Similarly, representative confocal images could hardly show co-localization between CD14 and CD16 positive cells with CD68 surface-expression using CD14/CD68 and CD16/CD68 double stainings of all biopsies (Figure 2 H-I). Based on these data, the CD14-CD16+ tissue pool can be considered as monocytes.

Rejection is reflected by increased expression of HLA-DR and CD54 by CD16+ peripheral blood monocytes

To compare monocyte subsets at functional level, expression of co-stimulatory and migration-related molecules by CD16- and CD16+ blood monocyte populations were studied in ten patients at non-rejection and rejection time points. The HLA-DR expression level was significantly increased on CD16+ monocytes compared with CD16- monocytes at both non-rejection and rejection time points (Figure 3A). During rejection, HLA-DR expression by CD16+ monocytes was even significantly higher as compared to non-rejection time point before. CD16+ monocytes express CD54 at a higher degree compared to CD16- monocytes and this CD54 expression was, although statistically not significant, enhanced during rejection (Figure 3B).

Pro-inflammatory cytokine production potential of monocytes in heart transplant recipients is preserved and independent of rejection status

To explore the cytokine production capacity of monocytes in ten heart transplant recipients, we preferred to investigate production of pro-inflammatory cytokines IL-1β, IL-6, TNF-α and IFN-γ and anti-inflammatory cytokine IL-10 by in vitro experiments after LPS stimulation, and compared this with healthy individuals (Figure 3C) because localizing cytokine expression on tissue level is generally considered to be associated with a high rate of false positive and false-negative results. The percentage of IFN-γ producing cells was higher in healthy individuals compared with heart transplant recipients (P:0.112), whereas the production potential of IL-1β, IL-6, TNF-α and IL-10 was similar between heart transplant recipients and healthy individuals. These findings indicate that, despite the use of potent immunosuppressive drugs in heart transplant recipients, monocytes still remain capable of cytokine production, oftentimes at a similar level as in healthy individuals.

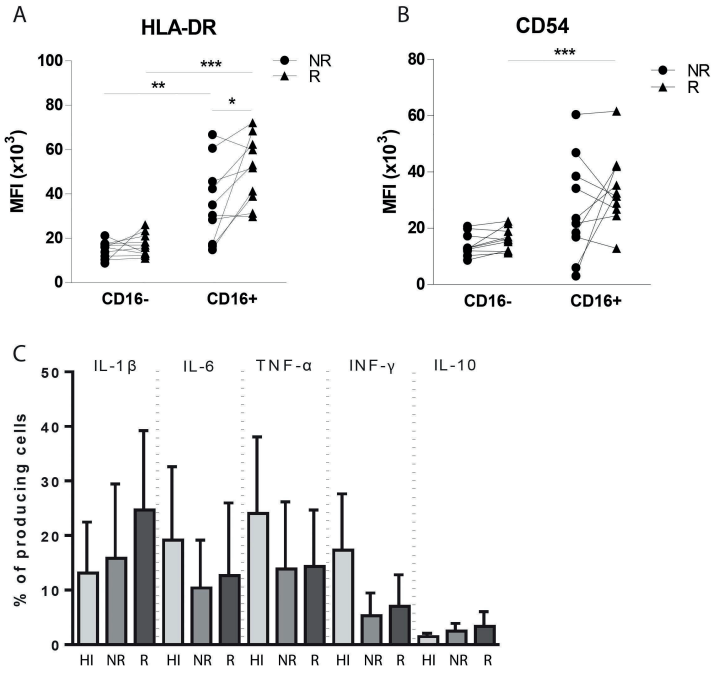

Figure 3. Phenotypic and functional characteristics of circulating monocytes in heart transplant recipients compared with healthy individuals

Expression levels of (A) HLA-DR, (B) CD54 (ICAM-1) are increased in CD16+ monocyte pool during rejection compared to non-rejection. The percentages of IL-1β, IL-6, TNF-α, IFN-γ, IL-12, and IL-10 -producing monocytes of both healthy individual group (n=9-14) and heart transplant recipients (n=10) are shown at both NR and R time points after LPS stimulation (C) (Mean±SEM) * p<0.05, ** p<0.01, *** p<0.001.

M2 macrophages increase in rejecting endomyocardial tissue compared to non-rejection time-point

To explore the type of tissue-infiltrating macrophages, CD68, CD80 and CD163 expression was tested using immunohistochemistry, and quantified using ImageJ analysis. A significantly increased presence of CD68+ macrophages was detected in rejected tissue compared to non-rejected tissue in both grouped and intra-individual analysis (p<0.001, Figure 4A and D). To investigate whether these macrophages are of M1 or M2 origin, adjacent immunohistochemical and double immunofluorescence staining were performed using CD68+CD80 (M1) and CD68+CD163 (M2) combinations. Confocal microscopy was used to show co-localization (Figure 4K). CD80 was hardly expressed by CD68+ macrophages in the endomyocardial tissue (Figure 4B and I). Expression increased, albeit not significantly, with rejection, but levels of CD80-expressing cells remained low. In contrast, the vast majority of CD68+ macrophages co-expressed CD163 in biopsies of both rejecting and non-rejecting tissue (Figure 4C, -F, and -K) showing a significant increase upon rejection.

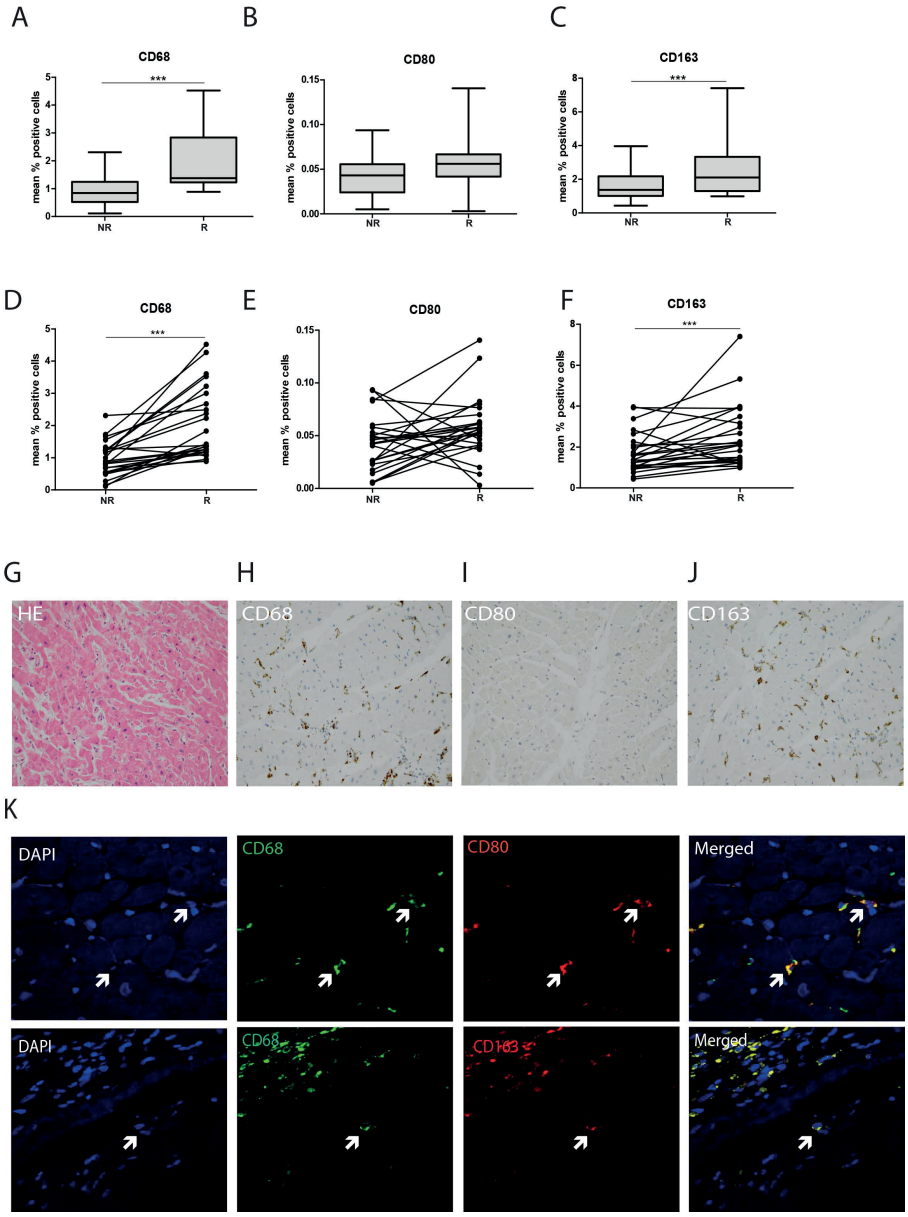

Figure 4. M2-type macrophages predominate in transplanted endomyocardial tissue and increase upon rejection. The expression of (A) CD68, (B) CD80 and (C) CD163 was measured using immunohistochemistry, analyzed by ImageJ and depicted as mean % positive stained area. The expression of (D) CD68, (E) CD80 and (F) CD163 was analyzed intra-individually at rejection and non-rejection time points. Representative histological and immunohistochemical images are shown at 20x magnification (G) HE, (H), CD68, (I) CD80 and (J) CD163. Co-localization of (K) CD68 and CD80 (M1 macrophage), and CD68 with CD163 (M2 macrophages) is shown at 40x magnification *** $p<0.001$.

Severe persistent fibrosis at rejection which is irreversible over time

To investigate the association between the detected cellular shifts and the degree of fibrosis, the positive Sirius Red stained area at non-rejection, rejection and approximately one year post-rejection was measured. A significantly increased degree of fibrosis was found at rejection compared to non-rejection time point persisting at 1-year post rejection ($p<0.001$: Figure 5B). Fibrosis was mainly localized in interstitium and the perivascular areas showing focal collagen accumulation (Figure 5A).

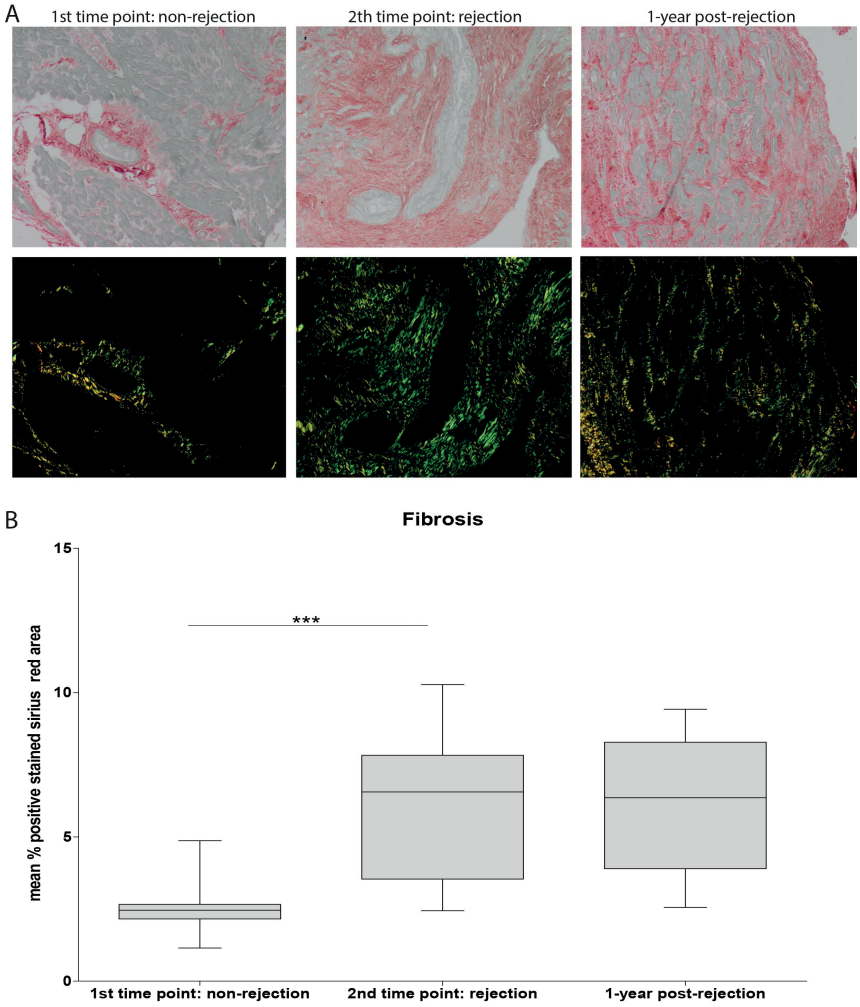

Figure 5. Severe fibrosis at rejection which is irreversible over time.

Sirius Red staining was used to assess fibrosis. Representative histological images for non-rejection time point (NR; 1st time point), Rejection time point (R; 2nd time point) and one year post-rejection time point (1-year post rejection, 3rd time point) are shown at 20x magnification (A). Fibrosis is quantified as mean%positive stained Sirius Red area (B) (Mean±SEM) *** $p<0.001$.

Discussion

Here, we present an in-depth analysis of peripheral blood and tissue monocyte/macrophage profiles of 25 heart transplant recipients experiencing acute cellular rejection. We found contrasting monocyte subset profile in blood and tissue during rejection with prominent presence of CD16+ monocytes and M2 macrophages at the tissue level. Increased classical monocytes and simultaneously decreased fractions of CD16+ monocytes signify the monocyte subset composition in heart transplant recipients compared to healthy individuals. No numerical differences were noted between rejection and non-rejection conditions. However, rejection was reflected by a significantly increased expression of HLA-DR and CD54 within the circulating CD16+ monocyte pool pointing towards a higher activation grade, antigen presentation potential, and increased migratory capacity of the activated monocytes towards the graft. In line, at tissue level, significantly more CD16+ monocytes, especially of CD14-negative phenotype were detected. Also significantly more CD68+CD163+ M2 macrophages were documented during rejection. CD68+CD80+ M1 subtype remained a minute subset. The finding of significantly increased fibrosis at rejection, which was also persistently detectable in one year biopsies together with the accumulation of CD16+ monocytes and M2 macrophages, indicates an association between these cellular shifts in induction of the prolonged damage to the heart transplant tissue.

On tissue level, we detected significantly higher frequencies of CD16+ monocytes in the rejecting heart tissue. We showed that CD16+ tissue-infiltrating cells are monocytes as hardly co-localization with CD68 and CD56 could be detected ruling out macrophage or NK cell phenotype as the cell source. M2 macrophages accumulate increasingly in tissue during rejection suggesting that the presence of CD16+ monocytes, with a presumed pro-inflammatory nature, and anti-inflammatory IL-10 producing M2 macrophages are parts of a micro-environmental balance within the endomyocardial tissue. Higher tissue macrophage frequencies are known to predict worse graft outcome [20]. Future research is needed to investigate monocyte-macrophage profiles during acute antibody mediated rejection.

In figure 6 we attempt to visualize a model based on our data. It is tempting to think that preferentially CD16+ monocytes will leave circulation and enter the graft at transplantation, causing vasculopathy in due time [21]. Rejection results in an even higher influx of activated CD16+ monocytes producing pro-inflammatory cytokines. At the same time, a counterbalanced predominance of anti-inflammatory M2 macrophages contribute to the remodeling and fibrosis of the damaged heart tissue [22].

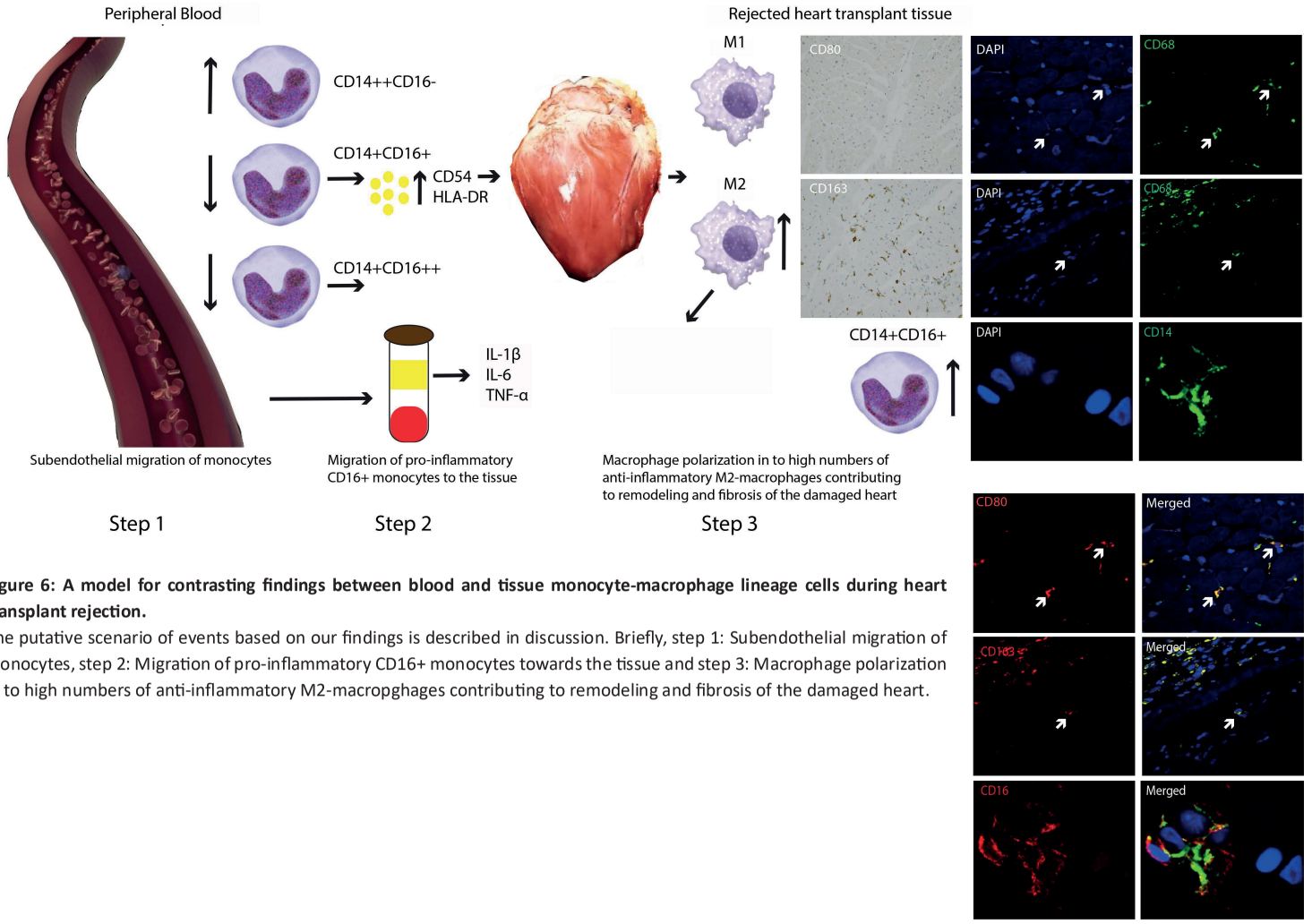

Figure 6: A model for contrasting findings between blood and tissue monocyte-macrophage lineage cells during heart transplant rejection.

The putative scenario of events based on our findings is described in discussion. Briefly, step 1: Subendothelial migration of monocytes, step 2: Migration of pro-inflammatory CD16+ monocytes towards the tissue and step 3: Macrophage polarization in to high numbers of anti-inflammatory M2-macropghages contributing to remodeling and fibrosis of the damaged heart.

In conclusion, although the numbers of included patients in this explorative study are limited, fibrosis is investigated by Serius Red stainings and not by cardiac MRI with delayed gadolinium pre- and post-rejection, to our knowledge, this is the first report on *matched serial* blood samples and endomyocardial biopsies at time points prior to rejection and at rejection. Here, we showed that CD16+ monocytes and M2- macrophages hallmark the correlates of acute cellular rejection on tissue level and seem to be associated with fibrosis after heart transplant rejection on the long-term. The elucidation of the molecular mechanisms underlying these cellular shifts may lead to discovery of new molecular biomarkers indicating the immunological graft status, and may help finding new molecular targets for specific immunotherapy.

Acknowledgements

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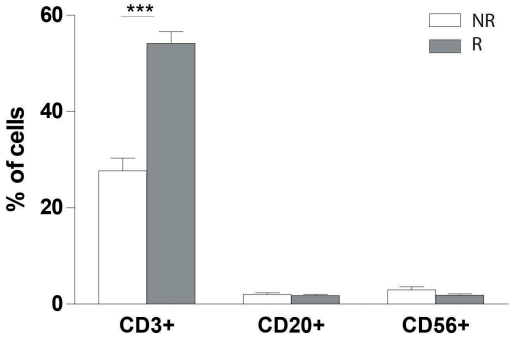
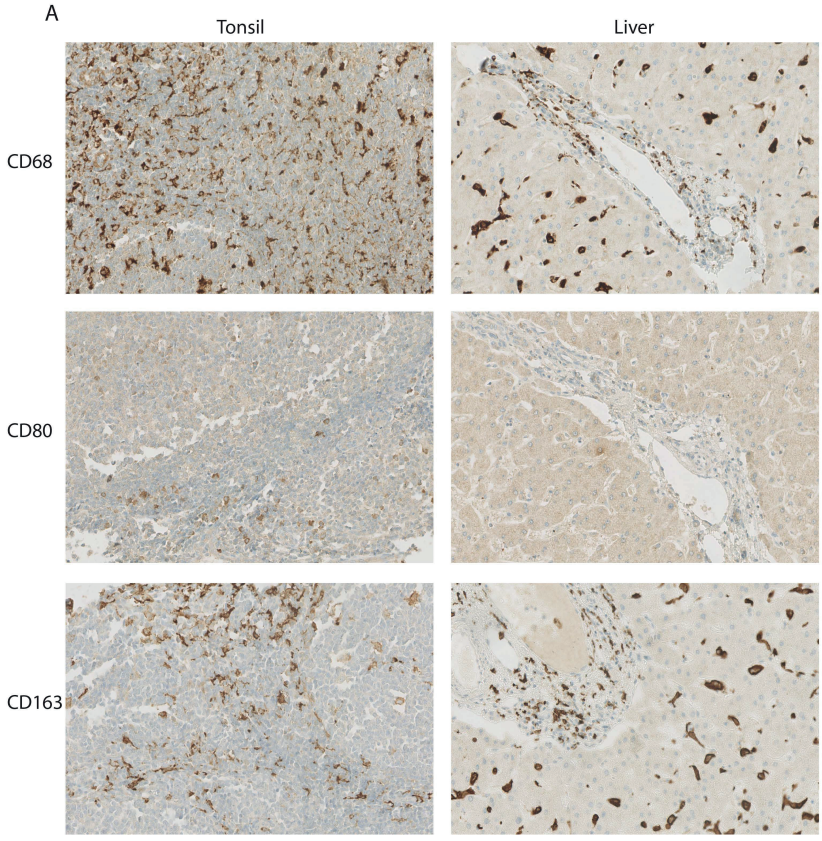
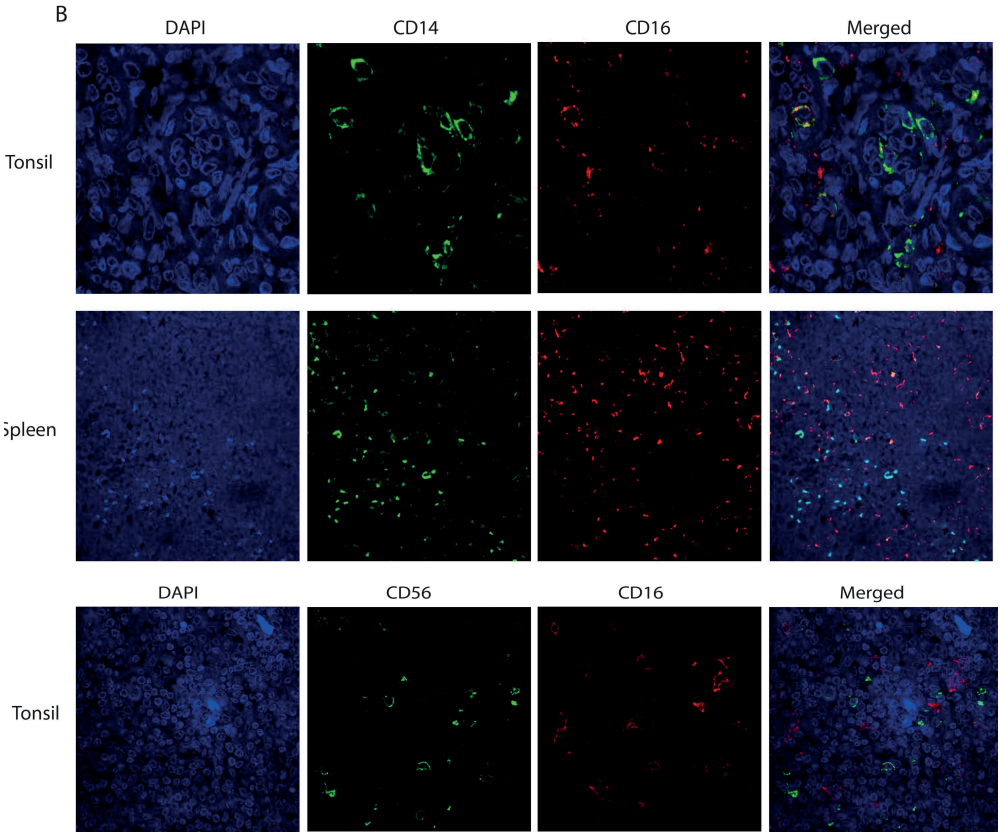
Supplementary Materials



Supplementary Figure 1. Peripheral blood leukocyte profiles in heart transplant recipients compared to healthy individuals
Percentages of CD3+, CD20+, and CD56+ in circulation showed a significantly increased number of CD3+ T cells during rejection. *** p<0.001.



Supplementary Figure 2. Control tissue stainings.



Supplementary Figure 2. Control tissue stainings (*continued*)

Tonsil and liver tissue: positive controls for CD68, CD80 and CD163 immunohistochemical stainings (A) Tonsil and spleen: positive controls for CD14+CD16 immunofluorescence stainings (B). The replacement of the secondary antibody by PBS was used as negative control.

Chapter 3

Glomerular and perivascular kidney transplant infiltration by monocytes and CD68+CD163+ macrophages as a hallmark of clinical rejection; a compartmental subset infiltration analysis

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Abstract

Monocytes-macrophages have crucial roles in kidney transplant rejection. In this study, compartmental differences in the distribution of monocyte-macrophage subsets between different types of rejection were investigated. 46 kidney transplant biopsies were studied with Banff 2015 diagnosis of chronic, active, antibody-mediated rejection (c-aABMR, n=9), acute, active ABMR (a-aABMR, n=8), acute T cell-mediated rejection type I and II (aATCMRI: n=6, aATCMRII: n=8), and 15 protocol biopsies from recipients with stable kidney allograft function. Infiltrating monocytes were characterized by double immunofluorescent staining with CD14 and CD16. Type 1 and 2 macrophages were identified using immunohistochemistry as follows: CD68+CD80+ (M1) and CD68+CD163+ (M2). Results of immunohistochemistry were correlated to renal function at the time of biopsy and 3, 6 and 12 months post-rejection. The presence of CD68+CD163+ macrophages was significantly associated with both the occurrence of rejection and worse transplant function after rejection ($p=<0.002$, $p<0.010$). With regard to compartmental infiltration, glomerular monocyte infiltration and CD14+ perivascular monocyte distribution signified c-aABMR in comparison to a-aABMR, whereas perivascular infiltration by CD68+CD163+ macrophages was correlated to a-aABMR. Comparing a/a ABMR and aATCMRI and II, we found significantly more glomerular CD68+CD163+ macrophage infiltration in a-aABMR ($p=<0.001$), perivascular infiltration of mainly CD16+ monocytes ($p=<0.001$) and CD68+CD163+ macrophages ($p=<0.001$) in aATCMR II, and abundant glomerular CD16+ monocyte infiltration in aATCMR I ($p=<0.01$). Altogether, the presence of glomerular and perivascular monocytes and CD68+CD163+ macrophages in the tissue is a hallmark of clinical rejection independent of histopathological Banff assignment.

Introduction

As early as in 1958 monocytes and macrophages were next to the T cells recognized as the dominant cell types infiltrating acutely rejecting grafts, however, this cell lineage was further neglected in transplantation research [1-4]. Emerging evidence reveals a crucial role for monocyte-macrophage lineage in the pathogenesis of acute and chronic (antibody) rejection. Immune regulation, antigen processing and presentation, inflammation, cytotoxicity, phagocytosis, response to injury and tissue remodeling comprises the broad spectrum of monocyte-related functions [5, 6]. CD68+ mononuclear cells is also associated with the degree of kidney transplant dysfunction [7]. Futhermore, glomerular and peritubular monocyte infiltration in acute rejection kidney biopsies could predict worse graft outcome [8-10]. It is shown that interstitial infiltration including macrophages is regarded as an independent predictor of worse graft outcome during acute cellular rejection [8, 11]. Recent studies showed that more macrophages are present in biopsies with positive C4d staining in peritubular capillaries compared to those with no C4d positivity [12-14]{Mannon, 2012 #44}. Another study analysed the renal function of 78 kidney transplant recipients experiencing T-cell mediated rejection (TCMR) and found that monocyte infiltration was associated with deterioratation of kidney transplant function [7].

We previously showed that there is a skewed shift towards circulating pro-inflammatory CD16+ monocytes in stable kidney transplant recipients, and that this was still so during the first 6 months post-transplant [15]. These monocytes were capable of IFNγ production [5]. Furthermore, we have shown that pre-transplant numbers of CD16 + monocytes can serve as an early biomarker to predict acute rejection. Also, rejection-related shifts in CD16+ monocyte composition between circulation and the rejecting kidney tissue were observed. An increased number of pretransplant CD16+ monocytes in blood was associated with a significantly higher risk of acute rejection [16].

Diagnosis of rejection relies upon histopathological lesions of an active alloimmune response consisting of immune cell infiltration of different renal tissue compartments. These lesions are non-specific [17-20]. Banff consensus guidelines have led to a clear improvement of diagnostic accuracy [21-23]. However, medical management of clinical rejection depends on both clinical judgment and histopathological assessment (24-26). Treatment decisions in case of discrepant biopsies are often made based on solely clinical presentations [24]. In line, the heterogeneity of clinical expression of ABMR has been a matter of debate sinds many years [21]. To reduce these disagreements between clinical and histopathological assignments, our field can benefit from incorporation of molecular classifiers into the Banff grading system. At the same time the question arises whether our evaluation steps take into account the different immune cell types dominating the plethora of tissue damage. As long as other immune cells like monocytes and macrophages are not considered, we will still face an incomplete evaluation of rejection and may miss diagnostic and therapeutic opportunities.

Based on our previous findings, we hypothesized that there are significant compartmental differences in the distribution of monocyte-macrophage subsets between different types of rejection which may influence the functional outcome of kidney transplants, and may signify distinctive histopathological features specific for different rejection types. We aimed to study monocyte-macrophage compartmental infiltration in renal allograft biopsies showing different Banff categories. We used double immunofluorescent staining and immunohistochemistry techniques to identify monocyte and macrophage subsets on a single cell level in the renal allograft and relate these to serum creatinine and eGFR at the time of biopsy and 3, 6 and 12 months post indication biopsy.

Materials & Methods

Study population

The compartmental infiltration by monocytes-macrophages was studied in 46 for cause kidney transplant biopsies with Banff 2015 categories [21] of either acute T cell mediated rejection type I or II (aTCMRI: n=6, aTCMRII: n=8), acute, active ABMR (a-aABMR, n=8), chronic, active antibody-mediated rejection (c-aABMR, n=9) and in 15 protocol biopsies from recipients with a Banff catergory I and stable kidney allograft function. The biopsies were evaluted by a renal pathologist according to the Banff 2015 classification [21]. Clinical and transplantation related characteristics were collected, including serum creatinine concentrations and corresponding eGFRs according to MDRD formula [25] at the time of biopsy and 3, 6 and 12 months post-biopsy (Table 1). Stable biopsies were obtained from the study of Shuker et al. with approved medical ethical review board number 2010-080 [26]. All patients signed written informed consent. Tissue samples are used according to the Dutch Code of Conduct 2011 and according to the decleration of Helsinki.

Immunohistochemistry, Sirius Red staining and Double-immunofluorescence

These techniques were performed as previously described (29).

Image analysis

Detailed compartmental analyses of kidney biopsy samples stained for CD68, CD80, CD163 was performed using ImageJ IHC software [27]. Slides were investigated in a blinded fashion to the clinical information using scanned Nanozoomer Digital Pathology files. Images of the entire biopsy sample (mean size range: 8.5-10.5 mm) were studied at 20x objective magnification for mean area precentage positively stained. Selections were made to analyze all glomeruli and the average area percentage positively stained was calculated. Perivascular compartment analysis was done by selection of all arteries excluding peritubular capillaries. Tubulointersitial compartment surface percentage was calculated by excluding glomeruli and arteries of the cortical tissue.

Table 1. Baseline characteristics

Baseline Characteristics	Stable grafts (N=15)	Rejection (N=31)	P-value
Age, mean±SEM, years	53.47±3,6	46.55±2,8	0.154
Gender Recipient: Male, N (%)	11 (73,3)	20 (64,5)	0.560
Ethnicity: N (%)			0.623
Caucasian	12 (80)	24 (77,4)	
Black	2 (13,3)	4 (12,9)	
Asian	1 (6,7)	1 (3,2)	
Other	0 (0)	2 (6,5)	
Primairy kidney disease: N (%)			0.166
Hypertensive nephropathy	8 (53,3)	16 (51,8)	
Diabetic nephropathy	2 (13,3)	2 (6,5)	
Polycystic kidney disease	3 (20)	1 (3,2)	
IgA nephropathy	1 (6,7)	2 (6,5)	
Other	1 (6,7)	10 (32)	
Creatinine level (µmol/l), mean±SEM			
12 months before biopsy	252,66 ± 26,23	239,58 ± 42,73	0.840
6 months before biopsy	290,86 ± 29,02	345,77 ± 57,58	0.525
3 months before biopsy	306,53 ± 36,90	351,09 ± 57,74	0.612
at the time of biopsy (protocol/indication)	131,81 ± 8,86	402,38 ± 58,05	**0.002**
3 months after biopsy	125,42 ± 7,69	272,16 ± 34,00	**0.005**
6 months after biopsy	125,85 ± 6,71	294,32 ± 43,90	**0.011**
12 months after biopsy	127,41 ± 8,5	230,25 ± 22,38	**0.003**
eGFR (ml/min), mean±SEM			
12 months before biopsy	11,80 ± 0,81	37,93 ± 3,73	**<0.001**
6 months before biopsy	11,93 ± 0,88	28,06 ± 3,73	**0.005**
3 months before biopsy	9,80 ± 0,65	27,87 ± 3,84	**0.002**
at the time of biopsy (protocol/indication)	50,26 ± 4,31	21,16 ± 2,54	**<0.001**
3 months after biopsy	52,66 ± 4,13	30,70 ± 3,56	**0.001**
6 months after biopsy	51,53 ± 3,89	29,70 ± 3,55	**0.001**
12 months after biopsy	51,2 ± 3,90	32,70 ± 3,43	**0.002**
CMV seropositive: N (%)	13 (86,7)	20 (64,5)	0.123
EBV seropositive: N (%)	15 (100)	28 (90,3)	0.222
VZV seropositive: N (%)	14 (93,9)	31 (100)	0.153
HCV seropositive: N (%)	0 (0)	1 (3,2)	0.493
Time on dialysis: mean±SEM	0,53±0,165	2,77±0,395	**<0.001**
Type of dialysis prior to transplantation: N (%)			**0.014**
No dialysis	8 (53,3)	3 (9,7)	
Hemodialysis	3 (20)	15 (48,4)	
Peritoneal dialysis	4 (26,7)	13 (41,9)	
Previous kidney transplantation: N (%)	0 (0)	13 (41,9)	**0.002**
Living kidney donation: N (%)	15 (100)	25 (80,6)	0.070
Delayed Graft function: N (%)	1 (6,7)	7 (22,6)	0.190
Maintenance Therapy: N (%)			0.109
tacrolimus/prednison/cellcept	15 (100)	31 (100)	
Basiliximab Induction therapy: N (%)	12 (80)	25 (80,6)	0.594
Peak current PRA %, median (IQR)	0 (0-17)	0 (0-54)	0.707
Peak historical PRA %, median (IQR)	4 (0-57)	2 (0-98)	0.324

Baseline Characteristics	Stable grafts (N=15)	Rejection (N=31)	P-value
Total number of HLA mismatches: mean±SEM			
A	1,13±0,165	0,94±0,139	0.395
B	1,20±0,175	1,23±0,129	0.908
DR	1,27±0,206	1,16±0,115	0.631
Total	3,60±0,412	3,32±0,247	0.546
Return to dialysis after 1 year: N (%)	1 (3,22)	15 (48,4)	**0.008**

Abbreviations: SEM (standard error of the mean), eGFR (estimated glomerular filtration rate), CMV (cytomegalovirus), EBV (Epstein-Barr virus), VZV (Varicella-zoster virus), HCV (Hepatitis C virus), PRA (Panel Reactive Antibody) and HLA (human leukocyte antigen).

Laser Scanning Confocal Microscopy

Confocal microscopy was performed using LSM-700 laser scanning confocal microscope (Carl Zeiss). The entire biopsy samples (mean size range: 8.5-10.5 mm) were evaluated for CD14+CD16-, CD14+CD16+ or CD14-CD16+ cells using 40x magnification. Absolute number of CD14+CD16-, CD14+CD16+ or CD14-CD16+ cells were double blind counted. Immunohistochemical stainings for overall macrophages, M1 and M2 macrophages were analyzed using ImageJ software [27, 28].

Statistical analysis

Differences between means were analyzed using either Student's t-test or the Mann-Whitney U test. Univariate analyses was used when multiple groups were compared with Bonferroni correction for multiple comparisons. Multivariable analyses were performed to examine the relation of monocytes and macrophages with different Banff categories of rejection in a multinomial logistic regression. Covariates included donor age, the number of previous transplantations, the presence of HLA-DSA, current PRA and the total number of HLA mismatches. Two-sided p-values <0.05 were considered statistically significant. Statistical testing was performed using RStudio software version 0.99.441 as well as graphpad prism software version 5.01.

Results

Patient and histopathological baseline characteristics

The clinical characteristics of the renal transplant recipients are shown in table 1. The median post-transplantation follow up for stable, a-aABMR, aTCMR I and aTCMR II was 9 months (IQR 0.2-13). All indication biopsies were obtained within the first year after transplantation except for those showing c-aABMR (median: 61 months, IQR range: 44-91). Banff 2015 classification of indication biopsies and donor specific antibody (DSA) status at the time of rejection are given in table 2. Clinical analyses based on Banff categories showed significant inferior transplant function as measured by serum creatinine and eGFR at the time of biopsy and 3 , 6 and 12 months post-biopsy in the rejection groups as compared to stable controls (p=<0.01, supplementary table 1). Compared to patients with stable graft function, patients with biopsy proven rejection had a longer pretransplant dialysis time (p=<0.001) and included a higher percentage of patients with multiple previous kidney transplantations (p=0.004). Percentage current or historical PRA and the number of HLA mismatches were similair between the groups. Graft outcome as defined by return to dialysis one year posttransplant was significantly inferior in rejection group compared to those with stable grafts (p=0.008).

Table 2. Banff 2015 categories

In suspicious for c-aABMR group : one of the patients had a c-aABMR (this patient had DSAs and C4d positivity). In the suspicious for a-aABMR group the patient with positive DSA showed no C4d positivity in the biopsy.

Histomorphology	C4d positivity	DSA
Suspicious for c-aABMR (N=8)	1/8	1/8 (DQ7 and CDC)
Suspicious for a-aABMR (N=9)	5/9	1/9 (DR3 and DR14)
aTCMR grade I (N=6)	0/6	1/6 (DR11)
aTCMR grade II (N=8)	0/8	2/8 (A29,B45,DR6 and B44 DR11)
No abnormalities (protocol biopsies: N=15)	0/15	1/15 (DQ9)

Abbreviations: c-aABMR (chronic active antibody mediated rejection), a/aABMR (acute active antibody mediated rejection), aATCMR(acute T cell mediated rejection), DSA (donor specific antibodies).

The overall presence of monocytes and CD68+CD163+macrophages is significantly associated with clinical rejection regardless of Banff category, an univariate analysis

The compartmental distribution of CD14+CD16- (classical), CD14+CD16+ (intermediate) and CD14-CD16+ (non-classical) monocytes was analysed by counting the absolute number of cells using confocal microscopy. Correction for biopsy size variation was applied according to international standard formula [28]. The compartmental distribution of CD68+, CD68+CD80+ and CD68+CD163+ macrophages was investigated by calculating the percentage area staining using ImageJ analysis (Figure 1A-D, Figure 2A-C).

We found significantly more area % staining of classical (median±SEM: 26 ± 4.4 cells), intermediate (10 ± 1.5 cells) and non-classical monocytes (31± 4.5 cells) in all biopsies demonstrating rejection as compared to stable grafts (6.4 ± 0.6, 3.0 ± 0.8, 1.5± 0.3 cells for classical, intermediate and non-classical monocytes, respectively). Intermediate monocytes were shown to be the preffered monocyte subset infiltrating the perivascular area (Figure 1D, arrows). As no colocalization of CD16+ cell surface expression with CD56 and/or CD68 could be found, we exclude the NK cells and macrophages as possible cell source of CD16+ cells and regard these CD16+ cells as monocytes [29].

With respect to macrophages, a significantly higher precentage of positive stained infiltration area by CD68+ (5.4% ± 0.9%) and CD163+ (5.1% ± 0.65%) macrophages was detected in all Banff catagories compared to protocol biopies of stable patients (0.74% ± 0.07%, 0.92% ± 0.02%) with a trend towards higher numbers of CD68+CD163+ macrophages in TCMRI/II (Figure 2A-B). Of note, CD80+ (0.07 %± 0.02%) macrophages were barely present (data not shown).

The overall presence of monocytes and CD68+ CD163+ macrophage infiltration is associated with clinical rejection regardless of Banff catagory; a multivariate analysis

Odds ratios of infiltrating classical (CD14+CD16-), intermediate (CD14+CD16+), and non-classical monocytes (CD14-CD16+) in different Banff catagories are shown in table 3. All monocyte subsets were present in rejection regardless of Banff catagory but not in stable grafts. This is in sharp contrast to graft infiltrating T-cells known to be present in protocol biopsies of patients with stable kidney function [30]. Other variables included: donor age, total number of HLA mismatches, number of previous transplantations, pretransplant donor-specific HLA antibodies (HLA-DSA), and historical peak panel reactive antibodies (PRAs).

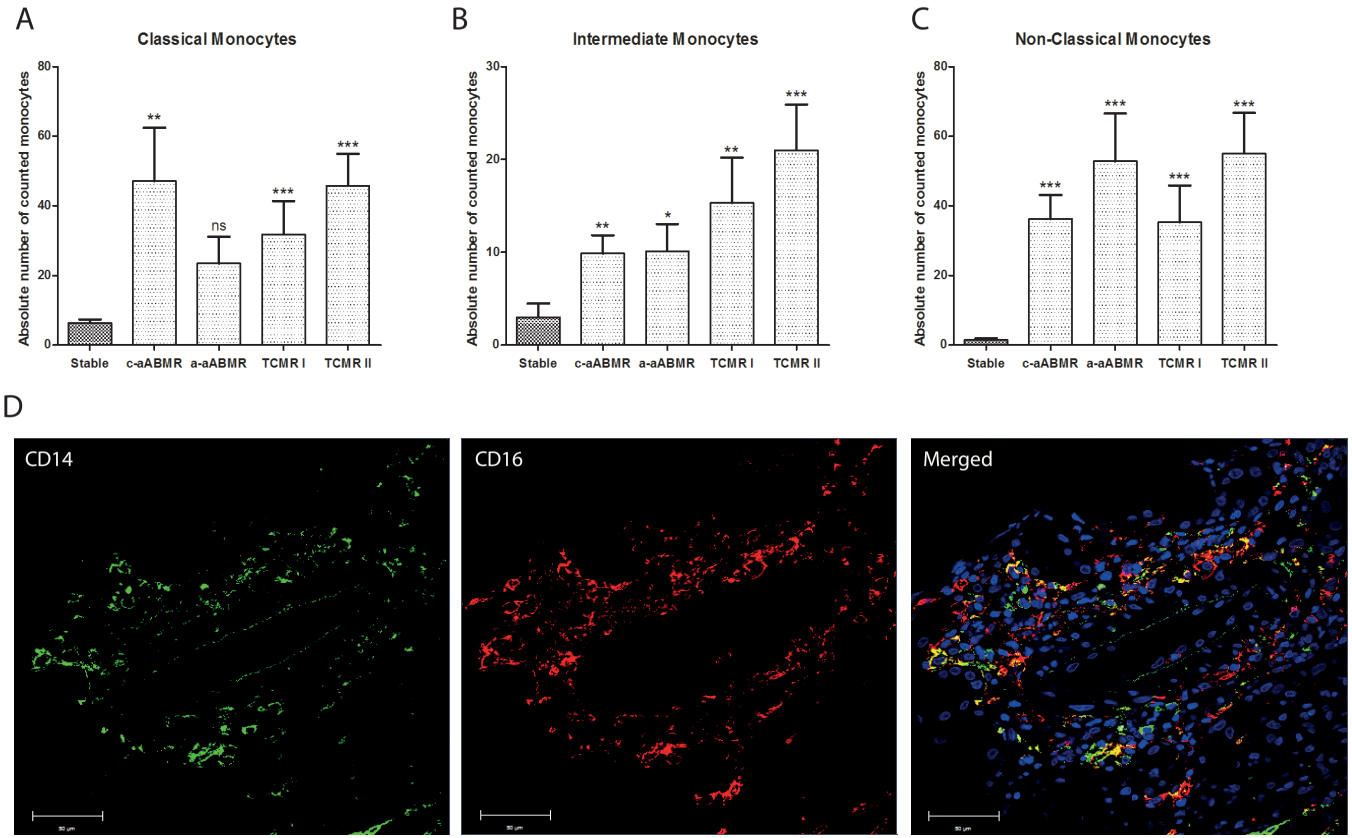


Figure 1. Monocyte infiltration is significantly associated with rejection regardless of Banff category

Classical (A), intermediate (B) and non-classical (C) monocytes are manually counted using confocal laser microscopy. Individual rejection grades are compared to stable kidney grafts. (D) represents a 20x magnification example of a ATCMRgrade II showing severe CD14+ (green) CD16+ (red) monocytes infiltration mainly located perivascular. Data are presented as Mean±SEM. * p<0.05, ** p<0.01, *** p<0.001.

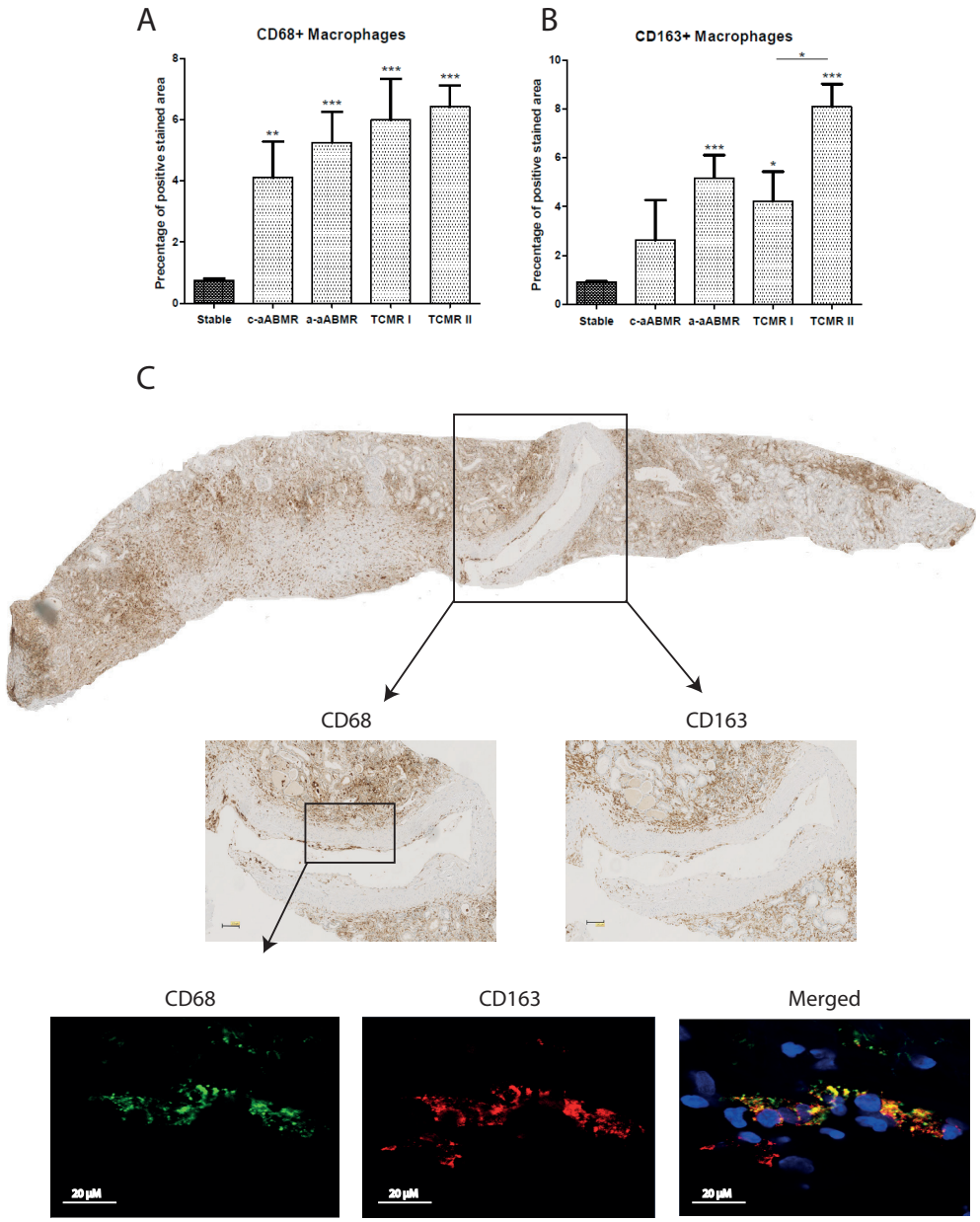


Figure 2. CD68+CD163+ macrophage infiltration is significantly associated with rejection regardless of Banff category.

CD68+ (A) and CD163+ (B) macrophages are analysed using ImageJ and expressed as percentage of positive stained area. Individual rejection grades are compared tostable kidney grafts. (C) represents a 5x magnification overview example of a ATCMRgrade II showing severe CD68+ infiltration mainly located perivascularly. CD68+ macrophages harboured mainly the M2 CD68+CD163+ subset as indicated by immunofluorescent double staining of CD68+ with CD163 showing a complete perivascular overlap (magnification 63x). Data are presented as Mean±SEM. * p<0.05, ** p<0.01, *** p<0.001.

The overall presence of CD68+CD163+ macrophages is independently associated with rejection regardles of Banff catagory compared to stable grafts (c-aABMR: OR: 21.34 (CI: 1.57, 289.16), p=0.021), (a-aABMR: OR: 25.20 (CI: 1.84, 344.19), p=0.016), (aATCMR I: OR: 27.66 (CI: 2.01, 381.15), p=0.013), (aATCMR II: OR: 29.13 (CI: 2.12, 400.64), p=0.012), showing that the CD68+ CD163+ macrophages are a hallmark of clinical rejection (Table 3).

Table 3. Monocytes and CD68+ CD163+ macrophage kidney graft infiltration is independently associated with rejection

Odds ratios of infiltrating classical (CD14+CD16-), intermediate (CD14+CD16+), non-classical monocytes (CD14-CD16+), macrophages (CD68+) and M2-macrophages (CD163+) in different Banff catagories show significant associations with rejection regardless of Banff category compared to stable grafts. Univariate analysis was used when multiple groups were compared with Bonferroni correction for multiple comparisons. Multivariable analyses were performed to examine the relation of monocytes and macrophages with different Banff categories of rejection in a multinomial logistic regression. Covariates included donor age, the number of previous transplantations, the presence of HLA-DSA, current PRA and the total number of HLA mismatches.

	c-aABMR HR (CI)	**P value**
Classical monocytes	1.21 (1.06, 1.38)	p=0.004
Intermediate monocytes	1.22 (1.03, 1.45)	p=0.023
Non-classical monocytes	1.74 (0.98, 3.09)	p=0.058
CD68	21.34 (1.57, 289.16)	p=0.021
CD163	1.87 (0.93, 3.73)	p=0.077

	a-aABMR HR (CI)	**P value**
Classical monocytes	1.17 (1.03, 1.33)	p=0.018
Intermediate monocytes	1.23 (1.03, 1.45)	p=0.019
Non-classical monocytes	1.77 (1.00, 3.15)	p=0.050
CD68	25.20 (1.84, 344.19)	p=0.016
CD163	2.42 (1.21, 4.86)	p=0.013

	TCMR I HR (CI)	**P value**
Classical monocytes	1.19 (1.04, 1.35)	p=0.010
Intermediate monocytes	1.30 (1.08, 1.55)	p=0.004
Non-classical monocytes	1.74 (0.98, 3.08)	p=0.059
CD68	27.66 (2.01, 381.15)	p=0.013
CD163	2.23 (1.10, 4.51)	p=0.026

	TCMR II HR (CI)	**P value**
Classical monocytes	1.21 (1.06, 1.37)	p=0.005
Intermediate monocytes	1.35 (1.13, 1.61)	p<0.001
Non-classical monocytes	1.78 (1.00, 3.16)	p=0.049
CD68	29.13 (2.12, 400.64)	p=0.012
CD163	3.20 (1.52, 6.75)	p=0.002

Abbreviations: OR (Odds Ratio), CI (Confidence Interval 95%)

HR: Hazard ratio, Covariates included: donor age, the number of previous transplantations, the presence of HLA-DSA, current PRA and the total number of HLA mismatches.

Glomerular and perivascular infiltration of all monocyte subsets and tubulointerstitial infiltration of CD16+ non-classical monocytes are associated with rejection, a compartmental analysis

Glomerular, perivascular and tubulointerstitial infiltration of monocytes was analysed manually by confocal microscopy. Higher numbers of all monocyte subsets were seen in the glomeruli of all Banff categories compared to stable grafts, in particularly during c-aABMR compared to a-aABMR and aATCMR I and aATCMR II ($p<0.01$, $p<0.001$) (Figure 3 A-D).

Perivascular infiltration was more pronaounced in all rejection subtypes compared to stable garfts, whereas increased amounts of classical monocytes were particularly seen in c-aABMR. Perivascular CD16+ monocytes were abundantly present during the aATCMR II subtype compared to stable ($p=<0.001$), and to other rejection subtypes ($p=<0.05$, $p=<0.01$) (Figure 3 E-H).

Tubulointerstitial infiltration showed higher counts of CD16+ monocytes in rejection catagories compared to stable grafts ($p<0.05$, $p<0.01$, $p<0.001$) (Figure 3 I-L). Nevertheless, monocytes were also present in tubulointerstitial compartments of stable grafts while no perivascular and glomerular monocyte infiltration was seen.

Monocyte and macrophage infiltration is associated with fibrosis

Kidney graft infiltration by classical monocytes (OR: 0.01 (CI: 0.01, 0.02), $p=0.001$), intermediate monocytes (OR: 0.02 (CI: 0.01, 0.02), $p<0.001$) and non-classical monocytes (OR: 0.03 (CI: 0.00, 0.05), $p=0.030$) is significantly associated with % area staining of Sirius Red in all Banff rejection catagories compared to stable grafts. In line, CD68+ macrophage (OR: 0.12 (CI: 0.03, 0.20), $p=0.006$) and CD163+ monocytes (OR: 0.08 (CI: 0.01, 0.16), $p=0.032$) tissue infiltration is also significantly associated with % area staining of Sirius Red in the rejection groups (Figure 4A-C).

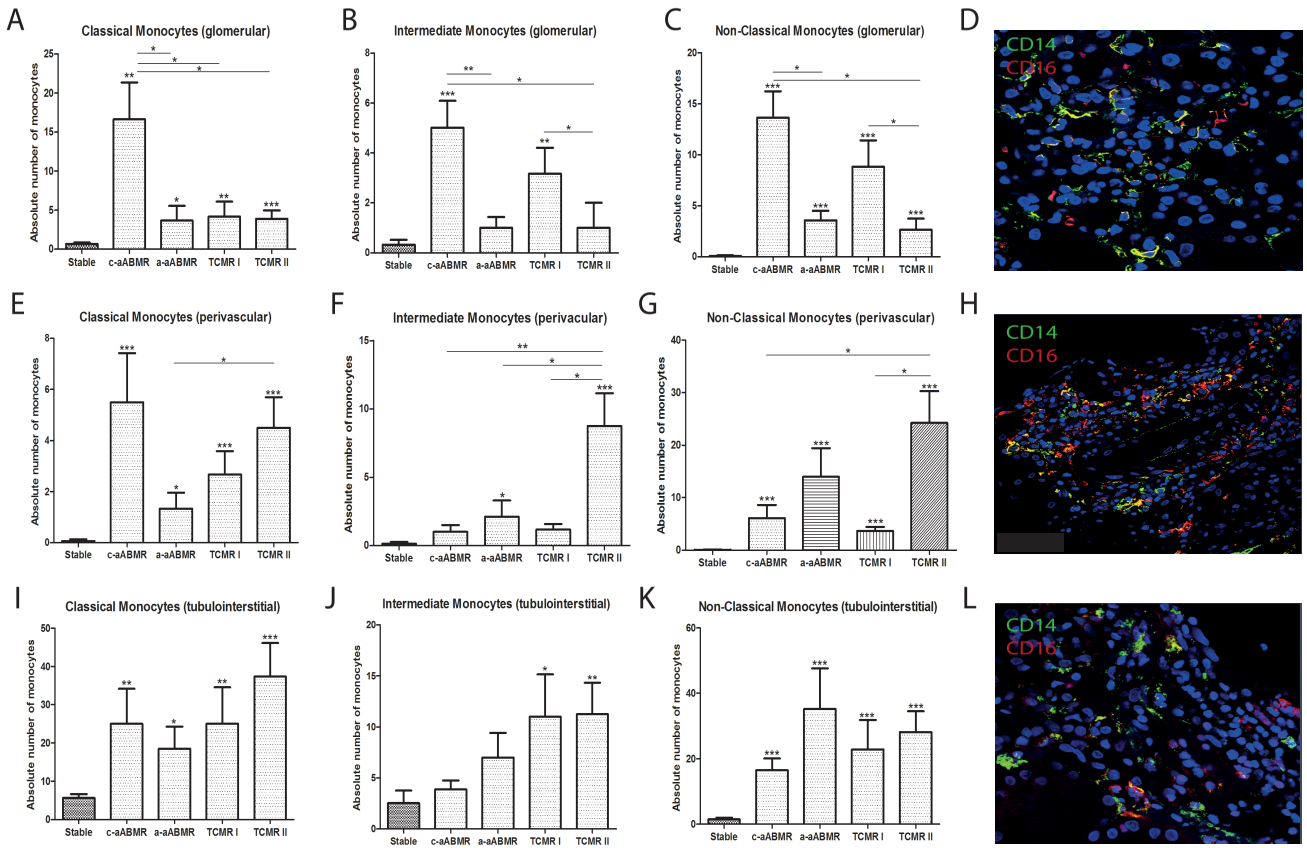


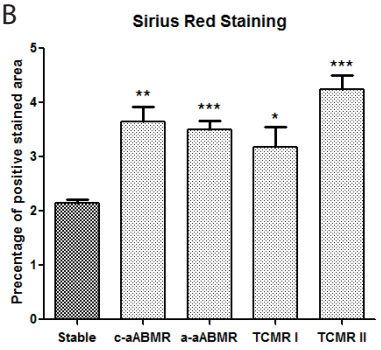
Figure 3. Glomerular and perivascular infiltration of all monocyte subsets and tubulointerstitial infiltration of CD16+ non-classical monocytes are associated with rejection

Monocyte subsets are analysed in glomerular (A-C), perivascular (E-G) and tubulointerstitial (I-K) compartment of different diagnostic Banff categories. (D) represents a double immunofluorescent image of CD14+CD16+ monocytes in a glomerulus, (H) represents CD14+CD16+ monocytes in perivascular compartment and (L) represents CD14+CD16+ monocytes in tubulointerstitial compartment. * p<0.05, ** p<0.01, *** p<0.001.

A

	Coefficient (95%CI)	p-value
Classical Monocytes	0.01 (0.01, 0.02)	p=0.001
Intermediate Monocytes	0.02 (0.01, 0.02)	p<0.001
Non-classical Monocytes	0.03 (0.00, 0.05)	p=0.030
CD68	0.12 (0.03, 0.20)	p=0.006
CD80	3.25 (0.51, 6.00)	p=0.020
CD163	0.08 (0.01, 0.16)	p=0.032

B



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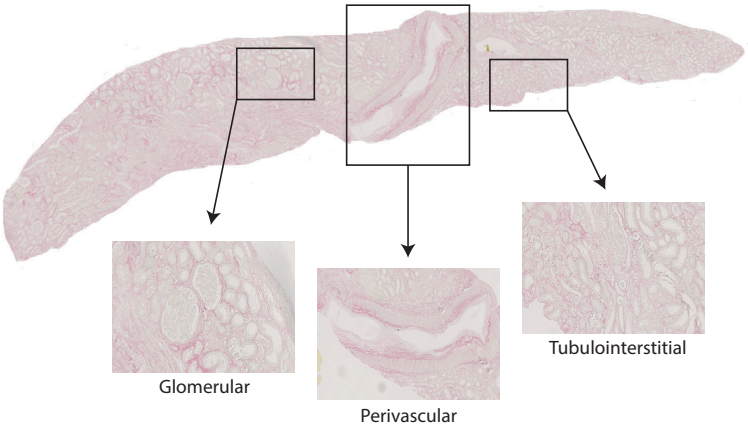

Figure 4. Monocyte and macrophage infiltration is associated with fibrosis
The presence of different monocyte and macrophage subsets were analysed in different Banff cataegories and correlated to the degree of fibrosis as measured by the percentage of positive stained Sirus Red area in these biopsies (A-B). (C) represents a 5x magnification overview example of a ATCMRgrade II showing moderate fibrosis. Fibrosis was strongly expressed in perivascular or tubulointerstitial area as shown with 20x magnification. Data are presented as Mean±SEM. * p<0.05, ** p<0.01, *** p<0.001.

c-a ABMR versus a-a ABMR , a compartmental subset analysis

Glomerular infiltration of all monocyte subsets was significantly higher in c-aABMR compared to a-aABMR (p<0.05, p<0.01) (Figure 3A-C and Supplementary figure 1A). Perivascular and tubulointerstital infiltration of all monocyte subsets showed no significant difference between c-aABMR and a-aABMR (Figure 3E-L and Supplementary figure 1B-C). No significant differences was found in glomerular and tubulointerstitial infiltration of macrophages, whereas perivascular infiltration of CD68+ CD163+ macrophages predominated in a-aABMR (p=<0.01) (Figure 5A-C).

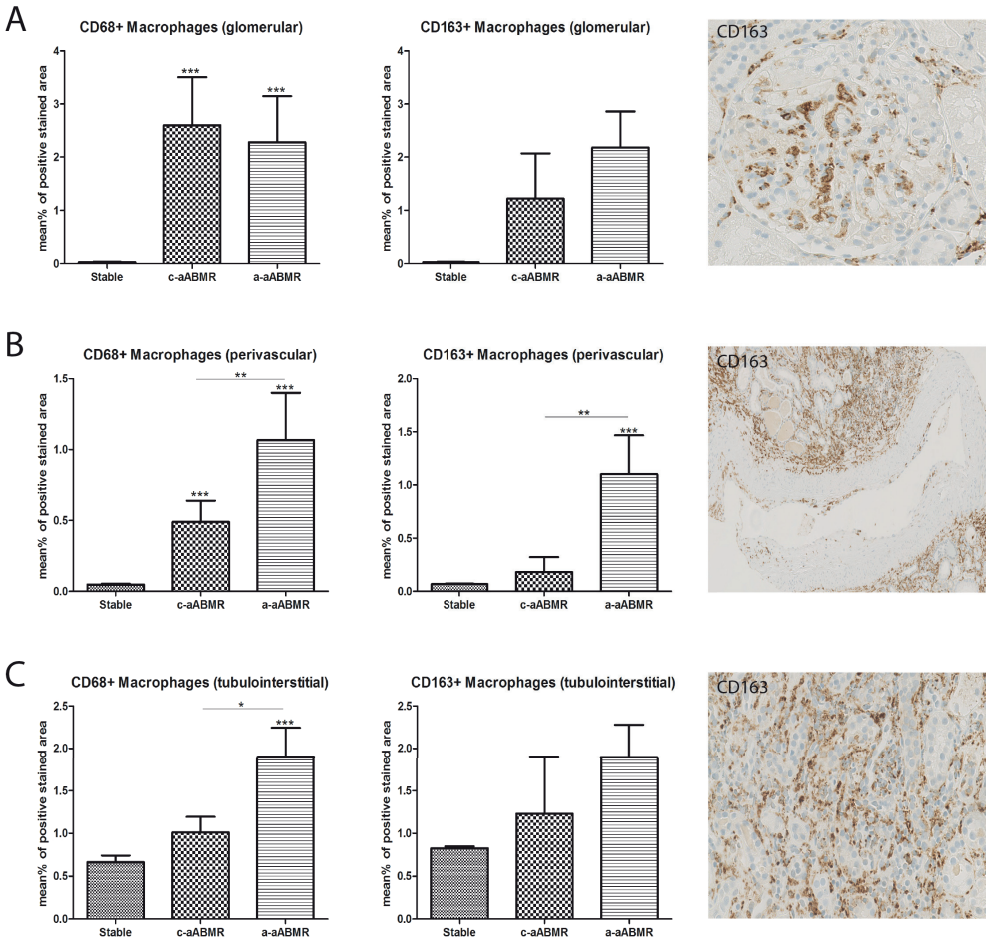

Figure 5. Perivascular infiltration of CD68+CD163+ macrophages signifies a-aABMR and not c-a ABMR
Percentage positive stained areas for glomerular CD68+ and CD163+ (A), perivascular CD68+ and CD163+ (B) and tubulointerstitial CD68+ and CD163+ (C) macrophages are analysed using ImageJ. Rejection groups are compared individually and with stable kidney grafts. (A-C) CD163+ staining represents a 40x magnification example of glomerular, perivascular and tubulointerstitial infiltration. Data are presented as Mean±SEM. * p<0.05, ** p<0.01, *** p<0.001.

a-a ABMR versus ATCMRI &ATCMRII , a compartmental subset analysis

Glomerular infiltration of CD16+ monocytes was significantly higher in aTCMR I compared to either aTCMR II or a-aABMR (p=<0.05) (Figure 3A-C and Supplementary figure 2A). Perivascular infiltration of CD16+ monocytes was significantly higher in aATCMR II compared to aATCMR I (p=<0.05) (Figure 3E-G and Supplementary figure 2B).Tubulointerstitial infiltration showed no significant difference between a-a ABMR and aATCMRI & aATCMRII (Figure 3I-K and Supplementary figure 2C). Glomerular infiltration of macrophages was highly present during a-aABMR compared to stable and aATCMRI/II (Figure 6A). Prominent

perivascular infiltration of CD68+CD163+ macrophages hallmarked aATCMRII (p=<0.001) (Figure 6B). Strikingly, significantly more infiltrating CD163+ macrophages were found in tubulointerstitial area during aATCMR II (p=<0.05) (Figure 6C). Glomerular CD16+ monocyte infiltration hallmarked aATCMR I as perivascular infiltration of CD16+ monocytes and CD68+CD163+ macrophages was pronounced in aATCMR II.

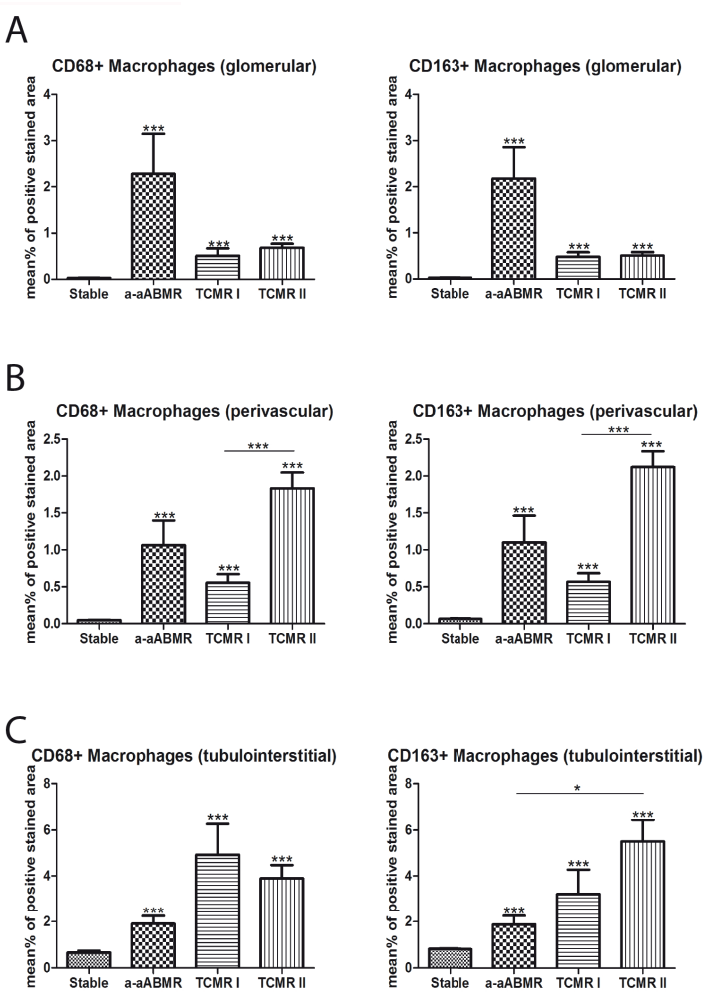

Figure 6. Perivascular CD68+CD163+ macrophages hallmarks aATCMRII

Area percentage positively stained for glomerular CD68+ and CD163+ (A), perivascular CD68+ and CD163+ (B) and tubulointerstitial CD68+ and CD163+ (C) macrophages are analysed using ImageJ. Individual rejection grades compared tostable kidney grafts. Data are presented as Mean±SEM. * p<0.05, ** p<0.01, *** p<0.001.

CD68+CD163+ macrophage infiltration is significantly associated with loss of kidney graft function in the long term

Classical, intermediate, and non-classical monocytes are not related to a decline in eGFR within the first 12 months after rejection (p>0.05). In contrast, CD68+ macrophage and CD163+ macrophage infiltration is significantly associated with an eGFR decline 12 months after biopsy proven rejection (-2.49 (-4.03, -0.95), p=0.002) (Figure 7A).

A

eGFR

	Coefficient (95%CI)	p-value
Classical Monocytes	-0.10 (-0.29, 0.09)	p=0.321
Intermediate Monocytes	-0.37 (-0.87, 0.13)	p=0.150
Non-classical Monocytes	-0.11 (-0.27, 0.06)	p=0.204
CD68	-2.49 (-4.03, -0.95)	p=0.002
CD80	-45.87 (-100.39, 8.65)	p=0.099
CD163	-1.87 (-3.29, -0.45)	p=0.010

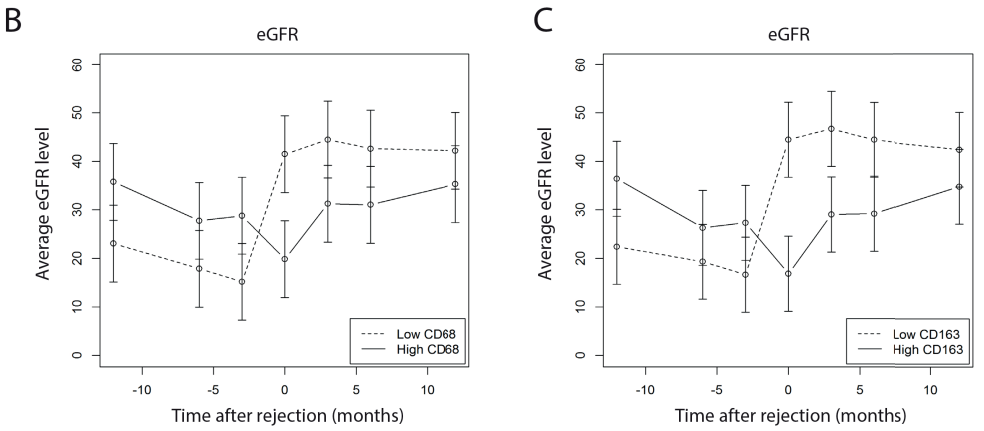

Figure 7. CD68+CD163+ macrophages are significantly associated with loss of long term renal allograft function
Classical, intermediate, and non-classical monocytes are not associated with a decline in eGFR 12 months after rejection, whereas CD68+ and CD163+ macrophage infiltration is (A). Graft outcome was analyzed by dividing the rejection group into CD68+ or CD163+ low group consisting of patients with a low CD68+ or CD163+ macrophage infiltration (below the median of 2.8 precentage positive area) and a CD68+ or CD163+ high group consisting of high presence of CD68+ or CD163+ macrophages (above the median of 2.8 precentage positive area) (B-C).

Rejection group was divided into subgroups based on precentage area positive staining by macrophage subsets: CD68+ low group consisting of patients with a low CD68+ macrophage infiltration (below the median of 2.8 precentage positive area) and a CD68+ high group consisting of high presence of CD68+ macrophages (above the median of 2.8 precentage positive area). The same strategy was used to subdivide the rejection group based on CD163+ macrophage infiltration (Figure 7B and C). Higher graft infiltration of CD68+CD163+ macrohages is significantly associated with loss of renal allograft function over time in all Banff rejection catagories.

Discussion

The overall presence of CD68+CD163+ macrophage is significantly associated with clinical rejection regardless of type of rejection. Importantly, we observed that the amount of CD68+CD163+ macrophages is clearly associated with significantly inferior graft function over time. As such, CD68+CD163+ macrophage can be regarded as an independent histopathological risk factor for rejection indicating inferior graft survival. Larger validations studies also including other changes not considered to be caused by rejection(for example BK nephropathy or recurrence of disease) are needed to confirm the clinical relevance of our findings. Furthermore, we show significant compartmental differences in the distribution of different macrophage and monocyte subsets between c/a ABMR vs. a-aABMR, and a/a ABMR vs. aTCMR I and II. These findings may have possible clinical implementation in typing and grading of rejection in relation to graft outcome. To date, the Banff grading system still needs to refine the diagnostic categories in a more specific way. Our compartmental analysis show that glomerular and perivascular infiltration of all monocyte subsets and tubulointerstitial infiltration of mainly CD16+ non-classical monocytes are associated with rejection. Considering the fact that monocyte lineage cells are also frequently present in interstitial compartment of protocol biopsies of stable cohort, we find that the interstitial compartmental is not discriminative for rejection. Compartemental infiltration showed specific patterns as for example glomerular monocyte infiltration during c-aABMR, prominent perivascular presence of CD68+ CD163+ in a/a ABMR and TCRII, and high perivascular CD16+ monocyte infiltration in aATCMRII.

The presence of a T cell infiltrate doesn't necessarily imply clinical rejection [31-35]. Here, we found that the presence of CD68+CD163+ macrophages is associated with clinical rejection regardless of Banff rejection category. At the moment, it is not routine standard practice to treat sublicincal rejection in all centers. Controversial results are published on benefitial outcomes of subclinical rejection treatment with steroids [32, 34, 36]. One can envision that a histopathological feature, such as CD68+CD163+ macrophage, which is directly linked to clinical rejection and worse graft outcome, would help to answer these questions.

Several findings of this explorative study are in line with previously reported research results. As we found high numbers of M2 type macrophages in aTCMR II, increased numbers of CX3CR1+ CD68+ macrophages were detected in acute tubuointerstitial and acute vascular rejection biopsies in comparison to normal protocol kidney graft biopsies. CX3CR1+CD68+ macrophage infiltrates were associated with an inferior graft outcome one year post-transplantation. CX3CR1 is a chemokine receptor which is mainly expressed on monocytes and macrophages [37]. Glomerular presence of monocytes and macrophages has been related to severe histopathological kidney graft injury with deleterious consequences for the graft function. Tinckam et al. described that a close correlation between peritubular C4d staining and glomerular monocyte infiltrates [38]. Moreover, cases of severe glomerular

endothelial injury associated with monocyte/macrophage-rich infiltrate in patients receiving alemtuzumab as induction therapy have been described [39-41]. A recent study showed that CD68+ macrophages are strongly linked to ABMR and T-cell mediated rejection. In this study, glomerular CD68 expression was reported as a surrogate marker for ABMR accompanied by higher Ki67 expression which is a cell proliferation marker, indicating that infiltrating macrophages act as ongoing triggers of alloimmune inflammation and subsequent graft injury [42].

Limitation of the presented proof of concept study is small numbers, but results are intriguing with a potential for clinical implementation in typing and grading rejection in relation to graft outcome. Addition of monocyte and macrophage markers to recently discovered and applied molecular microscope diagnostic system has the potential to assist in précising Banff histopathological categories (15). Further research on underlying mechanisms and compartmental specific distribution patterns of macrophages and monocytes will allow us to develop new therapeutic targets and specific treatments options in order to combat acute and chronic allograft injury.

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Author contributions

Thierry PP van den Bosch contributed in design, collecting data, experimenting, analyses and writing. Marian C. Clahsen-van Groningen contributed in design, analysis, writing and reviewing. Dennis A. Hesselink contributed in collecting patients, writing and reviewing. Daan Nieboer contributed in statistical analyses and reviewing. Ewout W. Steyerberg contributed in interpretation and reviewing. Farhad Rezaee contributed in design and reviewing. Carla C. Baan contributed in writing and reviewing. Ajda T. Rowshani was responsible for the conceptualization and design, contributed in collecting data, experimenting, analyses, writing and reviewing.

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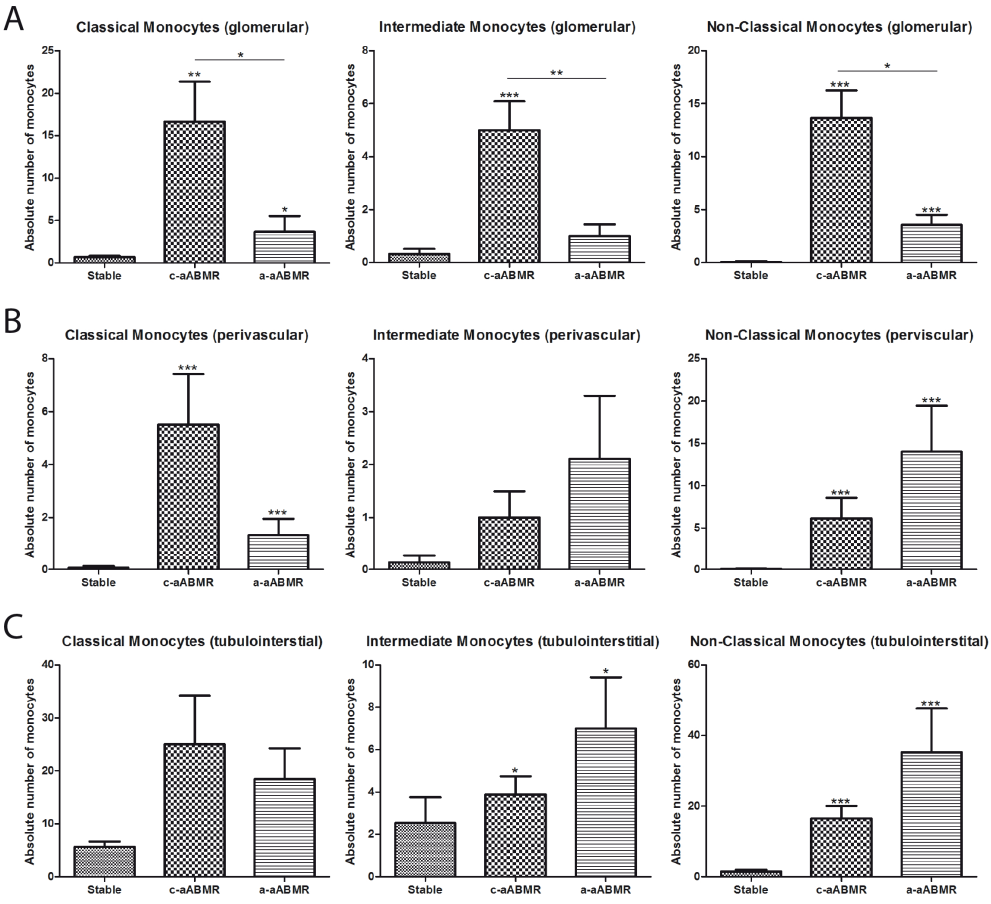
Supplementary Materials

Supplementary Table 1. Clinical characteristics for the different Banff categories and stable grafts

	Stable	c-aABMR	a-aABMR	TCMR I	TCMR II
Number of patients	15	8	9	6	8
Age, mean±SEM, years	53,46 ± 3,47	50 ± 6,6	39 ± 3,2	53 ± 8,0	50,5 ± 4,2
Gender Recipient: Male, n (%)	11 (73,33%)	6 (75%)	5 (55,55%)	3 (50%)	6 (75%)
Living kidney donation: n (%)	15 (100%)	7 (87,5%)	8 (88,8%)	5 (83,33%)	5 (62,5%)
Total no. HLA mismatches: mean±SEM	3,60 ± 0,39	2,75 ± 0,59	3,56 ± 0,29	3,83 ± 0,65	3,25 ± 0,49
Peak current PRA %, median (IQR)	0 (0-17)	0 (0-0)	0 (0-33)	0 (0-0)	0 (0-54)
Peak historical PRA %, median (IQR)	4 (0-57)	0 (0-13)	4 (0-96)	0 (0-10)	4 (0-98)
Delayed Graft function: N (%)	1 (6,66%)	2 (25%)	2 (22%)	1 (16%)	3 (38%)
Time point of biopsy postTX in days (mean±SEM)	83,86 ± 2,08	1975 ± 505 β	284 ± 259	111 ± 62	357 ± 128 α
Creatinine level (µmol/l), mean±SEM					
12 months before biopsy	252,66 ± 26,23	126,5 ± 7,99 §	262,88 ± 43,26	144,66 ± 18,93 §	397,62 ± 145,69
6 months before biopsy	290,86 ± 29,02	159,62 ± 11,18 §	615,44 ± 144,98 §	204,16 ± 36,07	334,75 ± 91,73
3 months before biopsy	306,53 ± 36,90	172,25 ± 11,81 §	668,88 ± 140,31 §	256,66 ± 51,53	243,25 ± 71,35
at the time of biopsy (protocol/indication)	131,8 ± 8,86	212,5 ± 25,24 α	548,88 ± 131,09 α	297,66 ± 76,76 α	506,00 ± 137,02 α
3 months after biopsy	125,4 ± 7,69	256,87 ± 48,30 α	211,55 ± 50,68 §	245,93 ± 108,13	373,37 ± 71,26 β
6 months after biopsy	125,8 ± 6,71	372,75 ± 115,50 α	218,55 ± 41,57 §	196,50 ± 66,58	374,5 ± 101,63 α
12 months after biopsy	127,4 ± 8,5	290,50 ± 31,26 β	190,88 ± 31,20 §	182,33 ± 49,60	250,25 ± 60,82 §
eGFR (ml/min), mean±SEM					
12 months before biopsy	11,80 ± 0,81	49,75 ± 4,68 β	23,11 ± 3,41 α	43,50 ± 5,76 β	38,62 ± 10,86 α
6 months before biopsy	11,93 ± 0,88	39,50 ± 3,63 β	12,00 ± 2,68	25,16 ± 5,38 α	36,87 ± 11,04 α
3 months before biopsy	9,80 ± 0,65	35,50 ± 3,48 β	10,88 ± 3,06	20,16 ± 5,04 α	45,12 ± 10,21 β
at the time of biopsy (protocol/indication)	12,33 ± 1,87	8,37 ± 1,85	7,00 ± 0,95 §	8,16 ± 1,86	6,5 ± 1,59
3 months after biopsy	52,66 ± 4,13	27,25 ± 4,32 α	41,22 ± 8,77	32,166 ± 8,10 §	21,25 ± 5,11 α
6 months after biopsy	51,53 ± 3,89	22,87 ±4,37 β	36,66 ± 8,02	35,00 ± 9,44	24,75 ± 6,30 α
12 months after biopsy	51,2 ± 3,90	21,00 ± 2,94 β	40,66 ± 8,27	36,66 ± 8,21	32,5 ± 5,62 §

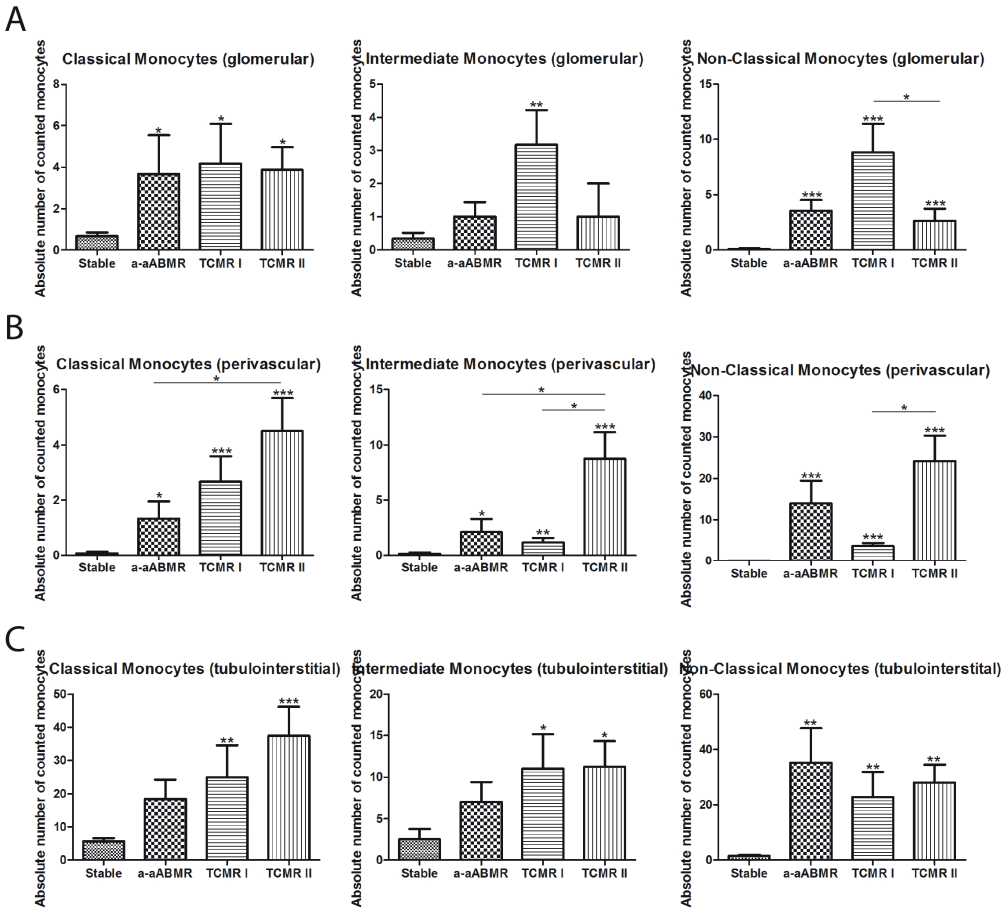
Significance levels are given as: § = p-value <0.05, α = p-value <0.01 and β = p-value <0.001.

c-aABMR:Chronic-active Antibody Mediated Rejection, a-aABMR: Active-acute Antibody Mediated Rejection, TCMR I: T-cell mediated rejection type 1, TCMR II: T-cell mediated rejection type 2



Supplementary Figure 1. Glomerular infiltration of all monocyte subsets and perivascular infiltration of mainly CD14+ monocytes denotes c-aABMR

Classical (A), intermediate (B) and non-classical (C) monocytes are manually counted in the glomeruli, perivascular or tubulointerstitial compartement using confocal laser microscopy. Individual rejection grades are compared to stable kidney grafts. Data are presented as Mean±SEM. * p<0.05, ** p<0.01, *** p<0.001.



Supplementary Figure 2. Glomerular CD16+ monocyte infiltration hallmarks ATCMRI and perivascular infiltration of CD16+ monocytes hallmarks ATCMRII

Classical (A), intermediate (B) and non-classical (C) monocytes are manually counted in the glomeruli, perivascular or tubulointerstitial areas using confocal laser microscopy. Individual rejection grades are compared to stable kidney grafts. Data are presented as Mean±SEM. * p<0.05, ** p<0.01, *** p<0.001.

Chapter 4

Pretransplant numbers of CD16+ monocytes as a novel biomarker to predict acute rejection after kidney transplantation; a pilot study

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Abstract

Acute rejection is one of the major immunological determinants of kidney graft function and survival. Early biomarkers to predict rejection are lacking. Emerging evidence reveals a crucial role for the monocyte-macrophage lineage cells in the pathogenesis of rejection. We hypothesized that higher pre-transplant numbers of proinflammatory CD16+ monocytes can predict rejection. The study cohort consisted of 104 kidney transplant recipients (58 no-rejections and 46 biopsy-proven rejections), and 33 healthy individuals. Posttransplant median±IQR follow up time was 14.7 (0.3-34) months. Pretransplantation blood samples were analyzed by flow cytometry for monocyte immunophenotypes. Groups were compared by Cox regression models for the occurrence of acute rejection. We documented a significantly increased absolute number of pretransplant CD16+ monocytes in patients who developed biopsy proven rejection after transplantation compared to no-rejections and healthy individuals (Hazard Ratio [HR], 1.60; 95% Confidential Interval [CI], 1.28 to 2.00; p<0,001 and HR, 1.47; CI, 1.18 to 1.82, p<0,001). In parallel, significantly less absolute numbers of CD16- monocytes were observed at pretransplant time point in rejectors vs non-rejectors (HR, 0.74; CI, 0.58 to 0.94; p<0,014). A higher pre-transplant number of CD16+ monocytes is significantly associated with a higher risk of acute rejection after kidney transplantation.

Introduction

Extensive research on short and long-term kidney graft fate has repeatedly shown that the occurence of rejection, regardless of the type and timing, significantly worsens the graft survival and function [1-3]. The golden standard diagnostic proof for acute rejection is renal biopsy which is an invasive, time consuming technique comprising a risk of bleeding and even graft loss [2]. At present, no clinically applicable biomarkers are available to predict rejection. As T cells are directly involved in the pathogenesis of rejection, a large body of data on T cell related biomarker research exists. Unfortunately, none of them could find their way to clinical practice as yet [4-6]. The low specificity of current diagnostic factors remain a major hurdle to be routinely used to discriminate differential diagnostic possibilities like rejection or infection.

Recenst advances in the field of molecular biology extending from genomics to proteomics and metabolomics have opened new possibilities to search for early rejection biomarkers in solid organ transplantation [7-9]. mRNA encoding cytotoxic proteins like granzyme B, and FOXP3 in urinary cells have been extensively investigated in prospective clinical trials [10-14]. Reversal of acute rejection could be predicted with 90% sensitivity and 73% specifity using urinary FOXP3 mRNA [12]. Urinary chemokines CXCL-9 and CXCL-10 were markedly elevated during acute rejection or BK infection [15, 16]. Sui et al. identified 12 upregulated and 8 downregulated miRNAs which are differentially expressed in acute rejection compared to normal kidney tissue [17]. Anglicheau et al. reported 10 upregulated miRNAs and 7 downregulated miRNAs in acute rejection biopsies compared to stable kidney graft tissue [18]. Increased levels of donor-derived cell-free DNA were found during acute rejection at a very early stage [19]. Suhre et al. found a composite mRNA signature consisting of 18S ribosomal RNA, CD3ε mRNA, and interferon-inducible protein-10 with a specificity of 84% and a sensitivity of 90% in diagnosing acute rejection [20]. Using micro-arrays, a five gene-set was classified to diagnose acute rejection with 91% sensitivity and 94% specificity which needs further validtion [21, 22]. A top-20 gene signature involving proliferation of B and CD4 T cells, and inhibition of CD14 monocyte related functions was shown to identify tolerant patients with almost 92% accuracy [23, 24].

Although, as early as in 1958 monocyte-macrophages were next to T cells recognized as the dominant cell types infiltrating acutely rejecting grafts, biomarker research in this area has lagged behind [25]. The heterogeneity and plasticity of the monocyte-macrophage-dendritic cell lineage have been obstacles to translate the functional relevance to diagnostic and prognostic clinical biomarkers. A new approach is to focus on the monocyte/macrophage lineage cells as emerging evidence reveals a crucial role for this cell line in the pathogenesis of rejection associated with worse outcome [26-28]. Monocyte-macrophage lieange cells have a number of roles in the rejection process: Immune regulation, antigen processing and presentation, inflammation, cytotoxicity, phagocytosis, response to injury

and tissue remodelling [29-31]. Glomerular and peritubular monocyte infiltration in acute rejection kidney biopsies could predict worse graft outcome [32-34]. In another study, increased infiltration of an activated population of macrophages could discriminate between clinical and subclinical kidney transplant rejection [35]. Furthermore, monocyte infiltration has been positively associated with the degree of kidney transplant dysfunction [36]. Circulating monocyte subsets can be characterized as classical (CD14++CD16-), intermediate (CD14++CD16+) and non-classical (CD14+CD16++). CD16+ monocytes are known to produce high amounts of TNF-α and IL-1β, and therefore are considered as highly pro-inflammatory [37-39]. CD16+ monocytes have unique functions in angiogenesis, production of reactive oxygen species and patrolling behaviour [37, 40-42]. Moreover, the intermediate CD16+ monocytes have been associated with the development of atherosclerosis in patients with chronic kidney disease [43-46].

We hypothesized that there are essential differences in pretransplant monocyte subset composition between patients with allograft rejection and with no rejection, in particular with regard to pretransplant proinflammatory CD16+ monocyte numbers. In that case, pretransplant numbers of CD16 + monocytes could serve as an early biomarker for acute rejection after kidney transplantation. To this end, we investigated the pretransplant monocyte immunophenotypes in a study cohort consisting of 104 kidney transplant recipients. Of the included patients, 58 developed biopsy proven rejection within the first 24 months after transplantation, and 46 patients remained free of any rejection episodes with a median±IQR follow up time of 14.7 (0.3-34) months. 33 healthy individuals served as control.

Methods

Population information

One hundred and four patients who had undergone renal transplantation between 2007 and 2012 were studied. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood using Ficoll-Hypaque density gradient (Lymphoprep™) and stored at −150°C until analysis. From this cohort 58 patients were selected who remained free of rejection and 46 patients who developed biopsy-proven acute rejection. 33 healthy individuals served as control. Additionally, we performed a pilot case-control study to investigate monocyte subset composition in blood, and to relate these findings to the same variables at tissue level in case of an acute rejection compared to 6 patients with stable graft function were served as control. To this end, the PBMC were serially obtained from 6 rejecting kidney transplant recipients at the time of transplantation and at the time of biopsy proven acute rejection (T-cell mediated rejecton type IA) . Six stable kidney transplant recipients served as control with blood samples obtained at exactly the same corresponding time points. Table 1 lists the transplantation characteristics and the clinical and immunological features of the cohort. 20% of no acute rejection group and 13% of rejection group received basiliximab

induction therapy No induction treatment is given in all other cases. All patients received same maintenance immunosuppressive regimen consisting of a calcineurin inhibitor; tacrolimus (Prograft®), Mycophenolate Mofetil (Cellcept®), and prednisolone. Prednisolone was tapered to zero according to local standard protocol during the first four months after transplantation if no rejection was detected. Delayed graft function post transplantation was defined as the need for continuing dialysis due to insufficient kidney transplant function. Rejection was diagnosed by for cause biopsies. No rejection was defined by a stable transplant function. We don't perform protocol biopsies at our centre. Biopsy proven acute rejection was defined according to the Banff criteria 2015. All patients signed written informed consent. The Medical Ethical Committee approved the study (Blood samples: MEC number 2010-080, MEC-2007-228, and patients with 6 rejecting biopsy samples: EudraCT 2010-018917-30).

Table 1. Baseline Characteristics of Kidney Transplant Recipients

	No Acute Rejection (N = 58)	Acute Rejection (N = 46)	P Value
Gender Recipient			0.828
Male (%)	75.5	70.83	
Age, mean±SEM, years	50±1,87	53±2,14	0.245
Ethnicity -- no. (%)			0.635
Caucasian	47/58 (81)	40/46 (87)	
Black	11/58 (19)	5/46 (11)	
Asian	0/58 (0)	1/46 (2)	
Primary kidney disease -- no. (%)			0.636
Diabetic nephropathy	10/58 (17)	7/46 (15)	
Polycystic kidney disease	10/58 (17)	6/46 (13)	
Glomerolonephritis	5/58 (9)	7/46 (15)	
Hypertensive nephropathy	17/58 (29)	16/46 (35)	
Reflux disease/chronic	1/58 (3)	4/46 (9)	
Other	15/58 (25)	6/46 (13)	
CMV seropositive -- no. (%)	37/58 (63)	24/46 (52)	0.155
Previous Kidney Transplantation -- no. (%)	5/58 (8)	11/46 (24)	0.053
Patients not on dialysis -- no. (%)	18/58 (31)	12/46 (26)	0.668
Time on dialysis -- mean (range) -- yr	1.0 (0-9)	1.0 (0-17)	0.068
Living kidney donation -- no. (%)	45/58 (77)	38/46 (82)	0.877
Delayed graft function -- no. (%)	5/58 (8)	10/46 (21)	0.075
Basiliximab Induction therapy -- no. (%)	12/58 (20)	6/46 (13)	0.403
Percent current PRA %, median (IQR)	0 (0-4)	0 (0-4)	0.395
Percent historical PRA %, median (IQR)	0 (0-0)	0 (0-48)	**0.013**
Positive DSA -- no. (%)	1/58 (1)	2/46 (1)	0.429
Total no. HLA mismatches -- mean±SEM	2.5±0.20	2.80±0.18	0.526
HLA class I mismatches	1.44±0.15	1.59±0.17	0.998
HLA class II mismatches	1.10±0.14	1.10±0.09	0.412
Outcome after 1 year -- no. (%)			**0.012**
Functional graft	55/58 (94)	39/46 (84)	
Back to RRT	2/58 (3)	9/46 (19)	

CMV: cytomegalovirus, RRT: renal replacement therapy, PRA: panel reactive antibody, IQR: interquartile range, HLA: human leucocyte antigen, DSA: Donor Specific Antibodies

Monocyte phenotype

Monocytes were identified based on forward/sideward scatter, lack of expression of CD3, and lack of expression of CD20 and CD56. Subsequently, they were characterized by the expression of CD14 and CD16 as described before (Supplementary Figure 1) [47].

CD14 and CD16 double immunofluorescence stainings of rejection-biopsies

Immunofluorescence stainings were performed using double indirect labeling. In brief, following fixation with acetone, slides were blocked with normal goat serum 10% (Jackson ImmunoResearch). Primary mouse-monoclonal CD14 IgG2a antibody was incubated overnight at 4°C. Secondary goat-anti-mouse IgG2a Alexa Fluor 488 (Invitrogen) was applied for 1 hour at room temperature (RT). To minimize non-specific binding, goat-anti-mouse Fab fragment (Jackson ImmunoResearch) was applied for 30 minutes at RT. After washing steps, the second primary antibody mouse-monoclonal CD16 IgG1 was added for one hour. Next, the secondary goat-anti-mouse IgG1 Alexa Fluor 555 (Invitrogen) was applied for another one hour at RT. Following washing steps, the slides were covered with anti-fading mounting medium containing DAPI (Vectashield, UK) and stored at 4°C until evaluation.

Confocal microscopy was performed using LSM-700 laser scanning confocal microscope (Carl Zeiss). The entire biopsy samples (size range: 4-5 cm) were evaluated for presence of CD14+CD16-, CD14+CD16+ or CD14-CD16+ cells using 40x magnification.

Statistical analysis

Differences between means were analyzed using the Student's t-test or Mann-whitney U test as appropriate. Both univariate and multivariate analysis were performed to study the relation between the absolute numbers of different monocyte immunophenotypes at pretransplant time point and the occurrence of biopsy proven acute rejection (Table 2). Univariate analysis was performed when multiple groups were compared, and corrected using Bonferroni test for multiple comparisons. Multivariate analysis was performed using other immunological variables and covariates to examine the relation with acute rejection in Cox proportional hazards regression analysis. Covariates used include donor age, total number of HLA mismatches, number of previous transplantations, pre-transplant donor-specific HLA-antibodies (HLA-DSA), and historical peak PRA. Crude and adjusted hazard ratios (HRs) for rejection-free time were calculated using Cox proportional hazards regression. Two sided P values <0.05 were considered statisitically significant. Statistical analysis was performed using the RStudio software version 0.99.441.

Table 2. Hazard Ratios for the absolute number of monocytes in relation to acute rejection

	Univariable OR (95% CI)	P value	Multivariable OR (95% CI)α	P Value
Absolute number of Classical Monocytes (per 150/µl)	0.74 (0.58, 0.94)	0.014	0.73 (0.57, 0.94)	0.016
Absolute number of Intermediate Monocytes (per 50/µl)	1.60 (1.28, 2.00)	<0.001	1.63 (1.28, 2.07)	<0.001
Absolute number of Non-classical Monocytes (per 50/µl)	1.47 (1.18, 1.82)	<0.001	1.46 (1.16, 1.85)	0.002
Absolute number of CD16+ Monocytes (per 50/µl)	1.27 (1.03, 1.57)	0.013	1.26 (1.01, 1.57)	0.038

α Multivariable Cox Regression analysis with covariates included were number of HLA mismatches, age, delayed graft function, previous transplantation, and current PRA percentage. OR: Odds Ratio, CI: Conficence Interval, HLA: human leucocyte antigen, DSA (donor specific antibodies), PRA: panel reactive antibody

Results

Kidney transplant recipient characteristics

The clinical and immunological characteristics of the kidney transplant recipients are shown in Table 1. Posttransplant median±IQR follow up time was 14.7 (0.3-34) months. The median age for the group with no rejection or the group with rejection was 50 or 53 years, respectively. 77% were living donor kidneys while in the rejector group 82% of kidneys were from living donors (p=0.87). Total numbers of human leukocyte antigen (HLA) mismatches were not different between both groups (p=0.52). Both groups showed no significant difference in the numbers of previous transplantions (p=0.05). No statistically significant difference was noted with regard to the occurrence of delayed graft function between two groups (p=0.07). Historical peak percent panel reactive antibody (PRA) was significantly higher in rejectors (p=0.01). All observed acute rejection episodes occurred within the first 24 months after transplantation with the majority of rejection incidence within the first year posttransplant (44/46). Rejection were classified according to the latest Banff 2015 classification (Supplementary Table 1.). Patients with loss of graft function were returned to dialysis (p=0.03).

High pretransplant numbers of CD16+ monocytes are associated with biopsy proven acute rejection

At pretransplantation, significantly higher absolute numbers (Figure 1A) and precentages (Figure 1B) of both intermediate CD14++CD16+ and non-classical CD14+CD16++ monocyte subsets were detected in the rejector group compared to non-rejectors and healthy individuals (p=<0.05). Concordantly, the absolute numbers of classical (CD14++CD16-) monocytes were significantly lower in rejectors compared to non-rejectors (p=<0.001) and healthy controls (p=<0.01). These findings may refer to a higher pro-inflammatory set point in the monocytic system at pretransplant time point in kidney transplant recipients who develop acute rejection after transplantation during the follow up time.

The hazard ratios of absolute numbers of intermediate (1.60, CI: 1.28 to 2.00; p<0.001) and non-classical monocyte subsets (1.47, CI: 1.18 to 1.82, p<0.001) indicate that higher pretransplant absolute numbers of CD16+ monocytes significantly increase the risk of rejection, and is associated with significantly shorter rejection-free survival time (p=<0.001). Conversely, classical monocytes showed a hazard ratio of 0.74 (CI: 0.58 to 0.94; p<0.014) pointing towards an inverse relationship between the absolute number of classical monocytes and the rejection-free survival time; the higher the number of classical monocytes the lower the risk of acute rejection.

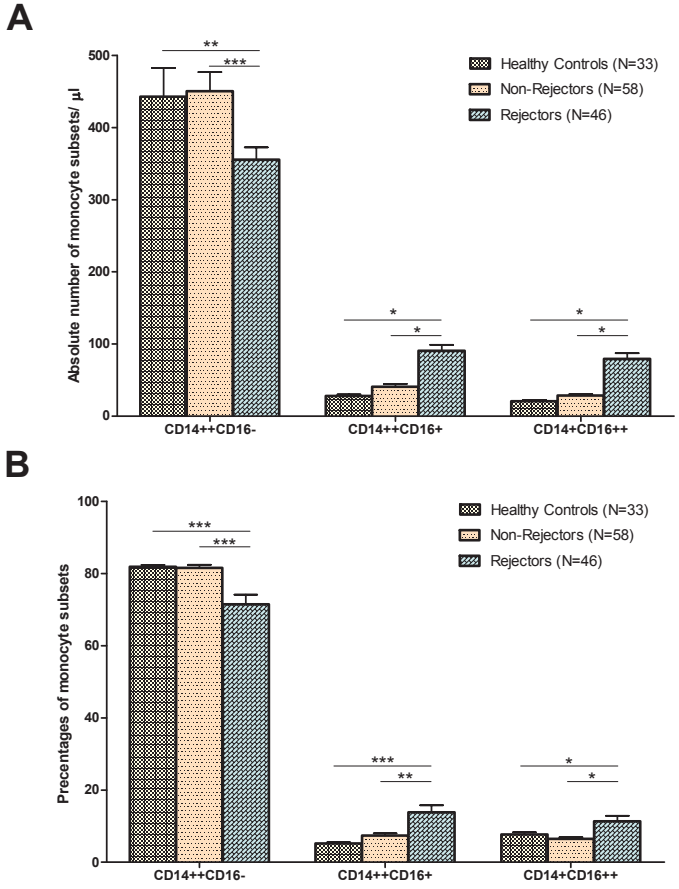

Figure 1. Higher absolute numbers of pretransplant intermediate and non-classical CD16+ monocytes are significantly associated with rejection

(A) Absolute pretransplant numbers of monocyte subsets in rejectors, non-rejectors and healthy individuals. (B) FACS percentages of pretransplant monocyte subsets in rejectors, non-rejectors and healthy controls. Posttransplant median±IQR follow up time of 14.7 (0.3-34) months. *p<0.05, **p<0.01, ***p<0.001.

Higher absolute pretransplant numbers of CD16+ monocytes are associated with shorter rejection free survival while higher absolute numbers of classical monocytes are associated with longer rejection free survival

Kaplan-Meier acute rejection free survival curves were extracted for tertiles of classical (Figure 2A), intermediate (Figure 2B) and non-classical monocytes (Figure 2C). Differences between survival curves were analyzed using log-rank test between the highest and the lowest tertile. A higher cumulative rejection incidence was associated with the lowest tertile of absolute numbers of classical monocytes in pretransplant patient's sample, while higher tertiles relate were associated with a lower rejection risk (Chisq=6.1, p=<0.05). In contrary, higher tertiles of absolute numbers of both intermediate (Chisq=9.6, p=<0.01) and non-classical (Chisq=6.4, p=<0.05) CD16+ monocytes in pretransplant patient samples were associated with a significantly higher rejection risk.

Transplant tissue infiltration by CD16+ monocytes at rejection; a pilot case-control study

We studied subset composition of circulating monocytes in patients experiencing biopsy proven acute cellular rejection at two time points; i.e. at the time of transplantation and at the time of biopsy (before start of treatment). As controls, blood samples were obtained from age and sex matched non-rejecting stable patients at the same corresponding time points. Figure 3A representative demonstration of monocytes subsets in patients with rejection (n=6) and patients with no-rejection (n=6) at pretransplantation and rejection-matched time points. In the group with no rejection, no numerical differences were detected in monocyte subsets between these two time points. Whereas, in the group with rejection the CD16+ monocytes were significantly increased in pretransplant specimens as compared with monocytes from patients with no rejection. Intriguingly, frequencies of CD16+ monocytes were significanly decreased in peripheral blood at the time of rejection (p=<0.05) pointing towards the possibility of migration of pro-inflammatory CD16+ monocytes to the rejecting kidney. In line, extensive tubulo-interstitial infiltration of CD14+CD16+ monocytes was detected in graft tissue during rejection (Figure 3B).

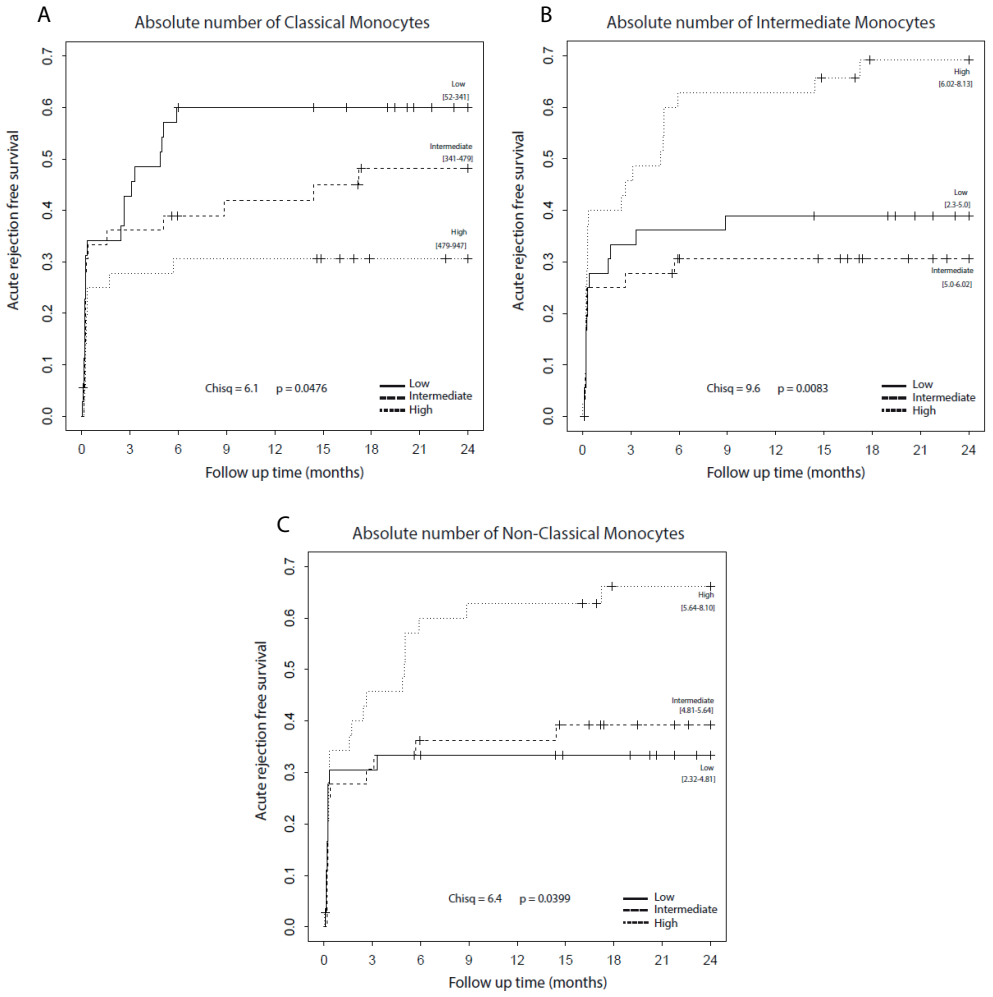

Figure 2. Higher absolute pretransplant numbers of CD16+ monocytes are associated with higher rejection risk while higher absolute numbers of classical monocytes are associated with lower rejection risk

Kaplan Meier survival curves for acute rejection free survival and the incidence of acute rejection after kidney transplantation on stratification for the absolute numbers of classical (Chisq=6.1 p=<0.05), intermediate (Chisq=9.6 p=<0.01) and non-classical monocytes (Chisq=6.4 p=<0.05). Absolute numbers of classical monocyte subsets were subdivided into tertiles (high [479-947/µl], intermediate [341-479/µl] and low [52-341/µl]). Absolute numbers of intermediate monocyte subsets were subdivided into tertiles (high 6.02-8.13/µl], intermediate [5.0-6.02/µl] and low [2,3-5,0/µl]). Absolute numbers of non-classical monocyte subsets were subdivided into tertiles (high [5.64-8.10/µl], intermediate [4.81-5.64/µl] and low [2.32-4.81/µl]). Differences between survival curves were analyzed using log-rank test between the highest and the lowest tertile.

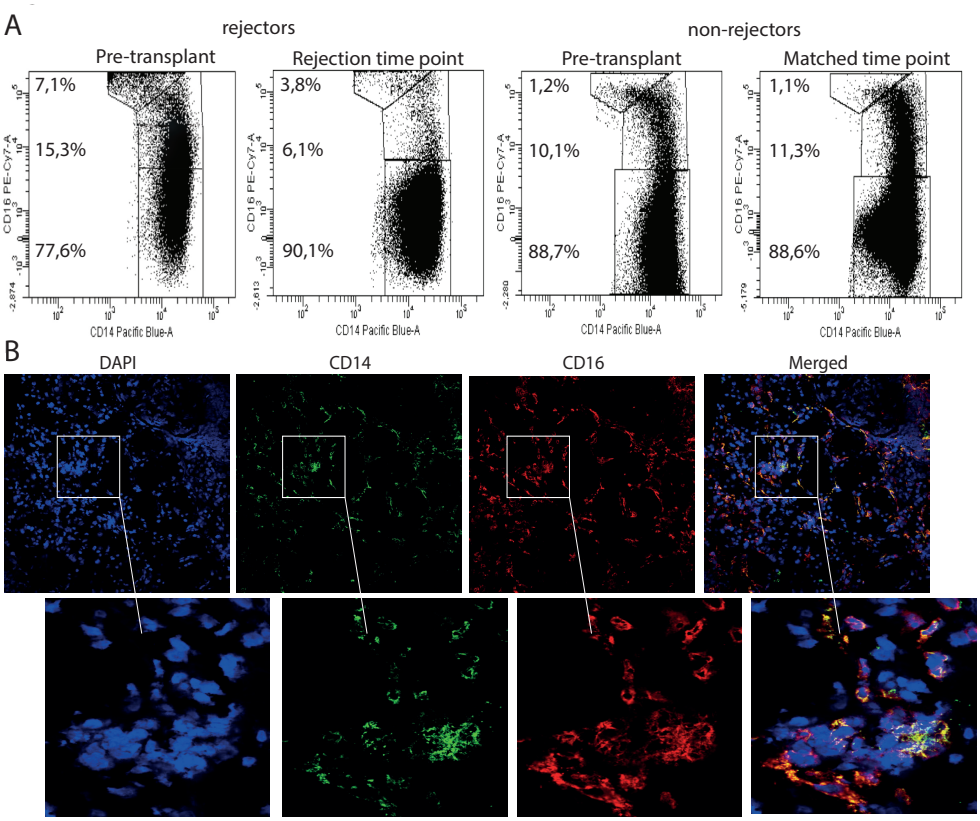

Figure 3. Transplant tissue infiltration by CD16+ monocytes at rejection; a pilot case-control study

(A) Representive FACS figure showing the difference in CD16+ monocyte subsets between non-rejectors and rejectors at pretransplantation and at rejection-matched time points. (B) Overview of double immunofluorescency stainings analyzed with confocal laser scan microscopy showing large tubulo-interstitial infiltrates of CD14+CD16+ monocytes at respectively 10x and 40x magnification.

Discussion

A major clinical challenge is the lack of biomarkers to predict rejection in kidney transplantation. In this study, we tested the hypothesis whether pretransplant numbers of proinflammatory CD16+ monocytes could predict acute rejection risk after kidney transplantation. We found that patients who developed acute rejection after transplantation possesed higher absolute numbers of CD16+ monocytes at the time of transplantation as compared to patients with no rejection. On the other hand, high numbers of classical monocytes pretransplant were significantly associated with lower rejection risk. In a pilot case-control study, we showed abundant accumulation of CD16+ monocytes at rejection in kidney graft tissue with a concomitant decrease of the absolute number of circulating CD16+ monocytes in peripheral blood. These findings indicate a migratory shift of CD16+ monocytes from the circulation towards the rejecting tissue.

Monocytes/macrophages have shown their potential value as biomarkers in several diseases [26, 31, 48-52]. Recently, we documented a skewed shift towards pro-inflammatory CD16+ monocytes in stable kidney transplant recipients which was already present at the time of transplantation and retained during the first 6 months posttransplant despite immunosuppressive therapy and a significant improvement of kidney function [47]. These monocytes were capable of IFNγ production [53]. Many studies have tried to identify the type and the function of injury-related monocytes in kidney transplant recipients and also in patientes with chronic renal failure undergoing dialysis. CD163 overexpression on circulating monocytes was shown to be useful in prediction of transplant function after kidney transplantation. CD163 over-expression seemed to significantly correlate with serum creatinine values 1 year after transplantation [54]. This subpopulation is involved in regulating the production of anti-inflammatory cytokines by macrophages. Recently, graft infiltration by CD16+ monocytes with smooth muscle cell like characteristics was documented during chronic transplant dysfunction, which suggests tissue remodeling including neointima formation in transplant arteries and formation of a pro-fibrotic matrix via TGF-β expression [55]. Patients with chronic renal failure undergoing dialysis have been shown to possess expanded CD14+CD16+ monocyte pools with production of proinflammatory cytokines like IL-1β, TNFα and IL-6 [56, 57]. Other studies showed that haemodialysis leads to temporary removal of monocytes in the pheripheral blood followed by the reappearance of activated CD14+CD16+ monocytes [58]. Although more detailed knowledge is now available with regard to phenotype and function of different subtypes of monocytes, no information regarding the molecular signature and functional roles of the monocyte subsets in relation to distinct acute and chronic rejection types is known as yet. Advances in genomics and proteomics will help to answer these open questions, to define the nature of monocyte subset in the context of disease, and to move the diagnostic, prognostic, and therapeutic biomarker discovery field forward.

This report describes a proof of principal study indicating the feasibility of CD16+ monocytes pretransplant number as a predictive marker for rejection. Here, we report on a novel concept of (CD16+) monocyte as a crucial immune cell type participating in the pathogenesis of rejection showing measurable subset differences before transplantation between patients who will develop rejection and patients who will remain free of rejection. Interestingly, at the time of rejection a measurable decrease in circulating CD16+ monocyte subset was paralleled by an increase in tissue infiltrating CD16+ monocytes implying a migration towards inflamed graft. On the other hand, these results should be confirmed in a larger prospective blinded validation fashion, as there are limitations and cautions. The studied population was selected based on available blood samples. Although almost equally distributed, a part of study population [4/5 (80%) of no rejection group and 8/11 (72%) of rejection group was re-transplanted, had a graft in situ and used immunosuppressive medication. All these parameters could have an effect on circulating monocyte subsets and numbers, which need to be taken into account in a future validation study. Next, we want to investigate the specificity of this finding by including more diagnostic groups in the future like patients with BK nephropathy, DGF, CMV infection, and urinary tract infections. Altogether, our data point out that higher pretransplant numbers of CD16+ monocytes are associated with significantly higher rejection risk, shorter rejection free survival, and may serve as an early biomarker to predict acute rejection after kidney transplantation. A calculated cut-off value of 23.5/µl CD16+ monocyte numbers pretransplant could have a sensitivity of 90% to detect the population at risk for rejection. Conversely, higher numbers of CD14+CD16- monocytes are significantly associated with no-rejection outcome. Interestingly, the absolute numbers of CD14+ monocytes can be measured using CD14+ beads offering a simpel, cheap and fast immunological monitoring tool for future validation studies.

Furthermore, next-step research is warranted to investigate the value of monocyte subset monitoring in risk stratification and personalized optimization of immunosuppressive treatment as one of the cornerstones of posttransplant clinical work-up of kidney transplant recipients.

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Author Contributions

TPP contributed in the process of design/experimenting/discussing/results/data analysis/ writing, LBH contributed in the process of sample collection and writing/reviewing, RK

contributed in the process of experimenting, NHRL contributed in the process of sample collection and writing/reviewing, FR contributed in design/discussing results and and writing/reviewing, DN contributed in the process of data analysis and and writing/reviewing, EWS contributed in the process of data analysis and writing/reviewing, JAG contributed in the process of sample collection and writing/reviewing, DLR contributed in the process of experimenting and writing/reviewing, MCG contributed in experimenting, writing and reviewing, CCB contributed in the process of sample collection and writing/reviewing and ATR contributed in the process of design/experimenting/discussing results/data analysis/writing.

Disclosure

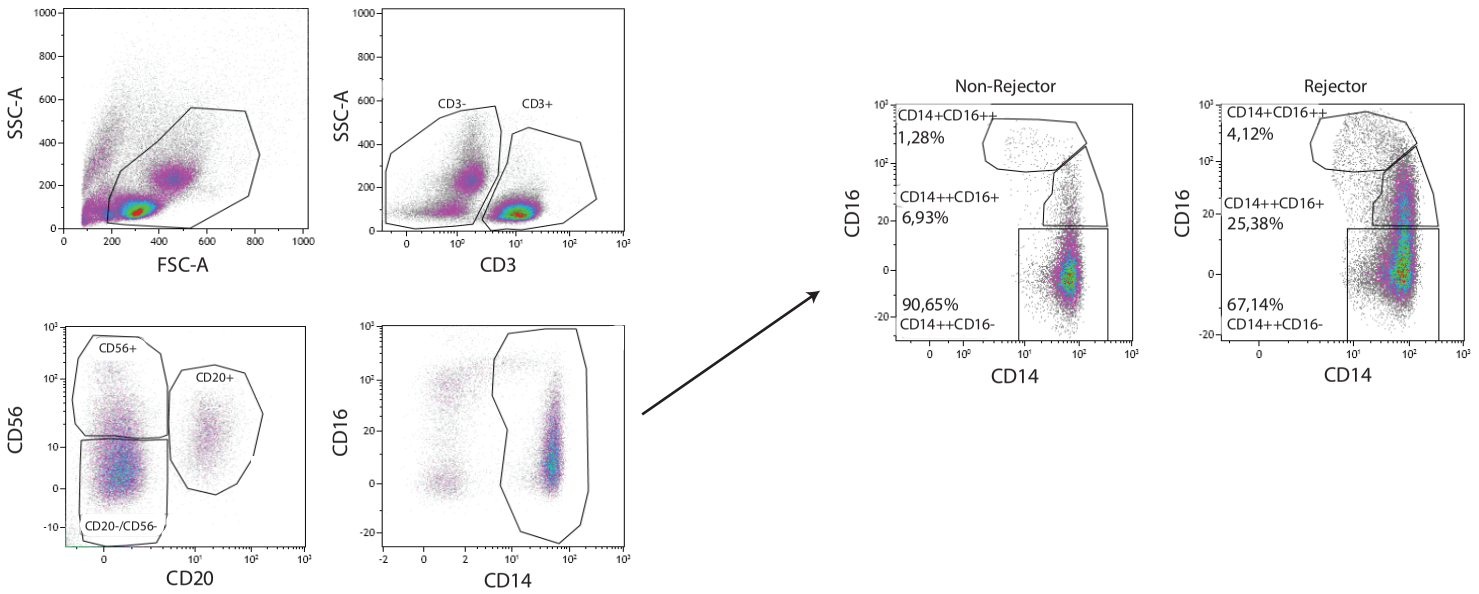
The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

Supplementary Materials

Supplementary Table 1. **Banff 2015 classification of biopsy proven rejections**

Rejection type	No. (%)
aABMR I	3/46 (6,52)
aABMR II	1/46 (2,17)
aABMR III	1/46 (2,17)
TCMR IA	12/46 (26,08)
TCMR IIA	14/46 (30,43)
TCMR IB	4/46 (8,69)
TCMR IIB	5/46 (10,86)
TCMR III	1/46 (2,17)
C4d+ Status	6/46 (13,08)
Other	5/46 (10,86)

Other: Bordeline TCMR (2/46), acute tubular necrosis (3/46)



Supplementary Figure 1. Gating strategy for monocytes

Representative FACS plots for the gating strategy at pretransplant time point to determine monocyte subset populations: CD3, CD20, and CD56 negative and CD14 and CD16 positive. Monocytes were characterised based on forward/sideward scatter, lack of expression of CD3, followed by lack of expression of CD20 and CD56 and then selected by positive expression of CD14 and CD16.

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Chapter 5

Kidney tissue resident microRNAs can discriminate between antibody mediated rejection and T-cell mediated rejection

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Abstract

MicroRNAs are important regulators of gene expression. The dysregulation of some microRNAs in kidney tissue has been associated with kidney transplant rejection. Here, we aimed to investigate whether kidney microRNA expression profiles could discriminate between the different histopathological rejection types. The microRNA expression levels were determined in total RNA isolated from 31 fresh frozen kidney biopsies derived from kidney transplant patients suffering from different forms of rejection. 8 patients displayed chronic active antibody-mediated rejection (c-aABMR), 9 presented with acute active antibody-mediated rejection (a-aABMR), 6 with T cell-mediated rejection type 1 (aTCMR I), and 8 with T cell-mediated rejection type 2 (aTCMR II). To investigate related pathways microRNA data was analyzed using Ingenuity software. Patients with a-aABMR could be distinguished from patients experiencing aTCMR by 55 differentially expressed microRNAs ($p=<0.01$), while patients with a-aABMR could be discriminated from c-aABMR by 5 differentially expressed microRNAs ($p=<0.01$). Pathway analyses indicated associations between these microRNAs and the inflammatory response network. Our exploratory study identifies microRNA signatures discriminating a-aABMR from aTCMR, and a-aABMR from c-aABMR. Further validation studies are needed to confirm these data in larger clinically and histopathologically well-defined patient cohorts.

Introduction

The use of microRNAs (miRNAs) as diagnostic biomarkers for rejection processes after kidney transplantation receives increasing attention. There is a clear unmet clinical need for the development of minimally invasive biomarkers that not only signal rejection but are also capable to distinguish between the different forms of rejection. The latter is particularly important as renal transplant recipients with different rejection types have a different prognosis and require different treatment modalities. Acute and chronic rejection are still one of the strongest negative prognostic factors associated with worse kidney transplant survival and function[1-3]. Currently, the diagnosis of rejection relies on a kidney transplant biopsy which comes with a potential risk of bleeding[4]. Molecular biology advances extending from genomics to proteomics and metabolomics have led to new possibilities to search for early surrogate markers for rejection in solid organ transplantation[5-8]. Urinary mRNA encoding cytotoxic proteins like granzymes, and other markers like FOXP3, OX-4, OX40L, PD1, PD-L1 or PD-L2 have been extensively investigated in clinical trials with promising results[9-18]. Unfortunately, none of these biomarkers has yet found its way into clinical practice. One of the reasons is the low biomarker specificity to discriminate rejection processes from infection which forms a major hurdle in their routine clinical use.

MiRNAs are small, noncoding RNAs with a length of 18 to 24 nucleotides. They fulfill an important role in gene expression regulation by binding in the context of the RNA induced silencing complex (RISC) to the 3'UTR of target mRNAs. MiRNA binding results in mRNA degradation and/or translation inhibition[19]. miRNAs display a tissue specific expression that can be severely deregulated in disease. This and the fact that they are stable present and easily detectable, not only in tissue but in body fluids like blood, urine and saliva make them highly suited as biomarker[20, 21]. MiRNAs affect most – if not all – cellular biochemical processes and play critical roles in the modulation of innate and adaptive immune responses. In the field of transplantation, Sui et al. were the first to identify 20 kidney tissue miRNAs which were differentially expressed during acute rejection after transplantation as compared to biopsies from transplanted kidneys that show no rejection[22]. Another study reported on 10 miRNAs to be underexpressed and 7 miRNAs overexpressed in acute rejection tissue samples compared to normal kidney transplant tissue. Their potential utility as rejection biomarkers was emphasized by miR-142-5p and miR-155 as ROC analyses indicated 100% sensitivity and 95% specificity for both of these miRNAs[23]. Urinary miR-210 downregulation and miR-10b upregulation were identified as specific urinary biomarkers for acute cellular kidney transplant rejection[24]. Scian et al. reported on a specific miRNA signature for chronic allograft dysfunction with interstitial fibrosis/tubular atrophy (IF/TA) compared to normal allograft tissue; five miRNAs were shown to be differentially expressed including miRNA-142-3p and miRNA-32 which were upregulated, and the downregulated miRNA-107, miRNA-211 and miRNA-204. A characteristic miRNA signature for IF/TA that correlates with

paired urine samples was identified[25]. miRNA profiles were studied in IF/TA biopsies of transplant patients and compared to normal surveillance biopsies showing that higher expression levels of miRNA-21, miRNA-142-3p/5p and miRNA-223 could be detected in IF/TA biopsies whereas miRNA-30 family members were expressed more abundantly in normal surveillance biopsies[26]. Of note, miRNA-21 is also found upregulated in a wide variety of oncological and cardiovascular disorders[27, 28]. MiRNA-21 promotes cell proliferation, migration, invasion and also apoptosis. Danger et al. showed miRNA-142-5p to be a specific biomarker for chronic antibody mediated rejection in PBMCs and biopsy samples of renal transplant patients as well as for the state of immunological tolerance[29],[30]. More recently, Wilflingseder et al. performed a large miRNA profiling study involving 65 renal allograft biopsies and identified seven significantly upregulated miRNAs in patients with delayed graft function including miRNA-21. In acute cellular rejection, 4 miRNAs (miRNA-150, miRNA-155, miRNA-663a and miRNA-638) where shown to be significantly upregulated. In antibody mediated rejection, six upregulated miRNAs were identified including miRNA-21 and miRNA-182[31]. Note that miR-150 is critical in regulation of B cell development and lymphopoiesis, whereas miRNA-155 is an important mediator of T-cell proliferation and CD8+ T cells cell responses[30].

In the present study, we aimed to investigate whether microRNAs can discriminate between different histopathological types of kidney allograft rejection. To this end we used a microarray profiling platform to determine miRNA expression profiles in 31 fresh frozen kidney biopsies (8 chronic active antibody mediated rejection, 9 acute active antibody mediated rejection, 6 acute T-cell mediated rejection type I, and 8 acute T-cell mediated rejection type II). Furthermore, using bioinformatic approaches we mapped biochemical pathways that are affected by selected miRNAs to identify potential biomarkers and new therapeutic targets.

Materials & Methods

Study population

Standard clinical and transplantation related characteristics were collected, as well as serum creatinine concentrations and corresponding eGFRs according to MDRD formula[32] at the time of biopsy (Table 1). Tissue samples are used according to the Dutch Code of Conduct 2011 and according to the declaration of Helsinki. For-cause kidney transplant fresh frozen biopsies with Banff 2015 categories of chronic, active antibody-mediated rejection (c-aABMR, n=8), acute, active ABMR (a-aABMR, n=9), acute T cell mediated rejection type I and II (aTCMR I: n=6, aTCMR II: n=8) were used for microarray experiments[33],[34].

Table 1. Clinical characteristics

	c-aABMR	a-aABMR	TCMR I	TCMR II
Number of patients	8	9	6	8
Age, mean±SEM, years	50 ± 6,6	39 ± 3,2	53 ± 8,0	50 ± 4,2
Ethnicity -- n (%)				
Caucasian	7 (87,5%)	6 (66,7%)	5 (83,3%)	6 (75%)
Black	0 (0%)	3 (33,3%)	0 (0%)	1 (12,5%)
Asian	1 (12,5%)	0 (0%)	1 (16,7%)	1 (12,5%)
Other	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Gender Recipient: Male, n (%)	6 (75%)	5 (55,55%)	3 (50%)	6 (75%)
Living kidney donation -- n (%)	7 (87,5%)	8 (88,8%)	5 (83,33%)	5 (62,5%)
Primairy kidney disease -- n (%)				
Hypertensive nephropathy	4 (50%)	6 (66,7%)	3 (50%)	3 (37,5%)
Diabetic nephropathy	0 (0%)	1 (11,1%)	0 (0%)	1 (12,5%)
Polycystic kidney disease	1 (12,5%)	0 (0%)	0 (0%)	0 (0%)
IgA nephropathy	0 (0%)	1 (11,1%)	0 (0%)	1 (12,5%)
Other	3 (37,5%)	1 (11,1%)	3 (50%)	3 (37,5%)
Type of dialysis prior to transplantation -- n (%)				
Pre-emptive	2 (25%)	0 (0%)	1 (16,7%)	0 (0%)
Hemodialysis	3 (37,5%)	7 (77,8%)	2 (33,3%)	3 (37,5%)
Peritoneal dialysis	3 (37,5%)	2 (22,2%)	4 (66,6%)	5 (62,5%)
Time on dialysis -- years, mean±SEM	0,87 ± 0,32	4,33 ± 0,81	2,00 ± 0,43	3,00 ± 0,19
Total no. HLA mismatches -- mean±SEM	2,75 ± 0,59	3,56 ± 0,29	3,83 ± 0,65	3,25 ± 0,49
Peak current PRA %, median (IQR)	0 (0-0)	0 (0-33)	0 (0-0)	0 (0-54)
Peak historical PRA %, median (IQR)	0 (0-13)	4 (0-96)	0 (0-10)	4 (0-98)
Maintenance Therapy -- n (%)				
prograft/prednison/cellcept	6 (75%)	6 (66,7%)	3 (50%)	7 (87,5%)
tacrolimus/prednison/cellcept	2 (25%)	3 (33,3%)	3 (50%)	1 (12,5%)
Basiliximab Induction therapy -- n (%)	4 (50%)	7 (77,8%)	2 (33,3%)	2 (25%)
Previous kidney transplantation -- n (%)	3 (37,5%)	6 (66,7%)	1 (16,7%)	3 (37,5%)
Delayed Graft function -- n (%)	2 (25%)	2 (22%)	1 (16%)	3 (38%)
Time point of biopsy postTX in days (mean±SEM)	1975 ± 505	284 ± 259	111 ± 62	357 ± 128
Creatinine level (µmol/l, mean±SEM				
Time of biopsy (protocol/indication)	212 ± 25	548 ± 131	297 ± 76	506 ± 137
eGFR (ml/min), mean±SEM				
Time of biopsy (protocol/indication)	30 ± 3	18 ± 6	17 ± 4	17 ± 4
Creatinine/Protein Ratio (mg/mmol), mean±SEM				
Time of biopsy (protocol/indication)	581 ± 362	2541 ± 1542	129 ± 86	114 ± 61
Return to dialysis after 1 year -- n (%)	2 (25%)	2 (22,2%)	3 (50%)	3 (37,5%)

Abbreviations: c-aABMR (chronic active antibody mediated rejection), a-aABMR (acute active antibody mediated rejection), aTCMR (acute T-cell mediated rejection type I or II), HLA (human leucocyte antigen), PRA (percent reactive antibody), SEM (standard error of the mean), IQR (interquartile range), postTX (post-transplantation), eGFR (estimated glomerular filtration rate).

MicroRNA profiling

RNA isolation from fresh frozen biopsy specimens was performed at Exiqon Services, Denmark. The quality of the total RNA was verified by an Agilent 2100 Bioanalyzer profile.

400 ng total RNA from both sample and reference (snoRNAs and snRNAs including U6) was labeled with Hy3™ and Hy5™ fluorescent tags, respectively, using the miRCURY LNA™ microRNA Hi-Power Labeling Kit, Hy3™/Hy5™ (Exiqon, Denmark) following the procedure described by the manufacturer. The Hy3™-labeled samples and a Hy5™-labeled reference RNA sample were mixed pair-wise and hybridized to the miRCURY LNA™ microRNA Array 7'th GEN (Exiqon, Denmark), which contains capture probes targeting all microRNAs for human registered in miRBASE version18.0. The hybridization was performed according to the miRCURY LNA™ microRNA Array Instruction manual using a Tecan HS4800™ hybridization station (Tecan, Austria). After hybridization the microarray slides were scanned and stored in an ozone free environment (ozone level below 2.0 ppb) in order to prevent potential bleaching of the fluorescent dyes. The miRCURY LNA™ microRNA Array slides were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc., USA) and the image analysis was carried out using the ImaGene 9.0 software (BioDiscovery, Inc., USA). The quantified signals were background corrected (Normexp with offset value 10, see Ritchie *et al*. 2007 [35]) and normalized using the quantile normalization method.

MicroRNA target and pathway analysis:

Targeted genes of differentially regulated miRNAs were further analyzed using Ingenuity Pathway analyses (Ingenuity, USA), with respect to their molecular function, associated biological processes, and subcellular location.

Statistical analysis

Statistical analysis of microArray data was performed using R version 3.2.2 and BRB-ArrayTools Version 4.4.1. Data were analyzed by univariate- and Two-sample T-test. Nominal significance level of each univariate test was set at 0.01. Since the nature of this study is exploratory we did not run extensive multiple testing corrections.

Results

Baseline characteristics of rejecting kidney transplants

Standard clinical and transplantation related characteristics were collected as well as the serum creatinine concentrations and eGFR according to MDRD formula (Table 1)[36]. The median follow up time for a-aABMR, TCMR I and TCMR II was 9 months (IQR 0.2-13). All rejections were diagnosed within the first year after kidney transplantation with exemption of c-aABMR (median: 61 months, IQR: 44-91). MicroRNA expression profiles were studied in 31 for-cause kidney transplant biopsies with Banff 2015 categories[33] of either acute T cell mediated rejection type I or II (aTCMRI: n=6, aTCMRII: n=8), acute, active ABMR (a-aABMR, n=9), or chronic, active antibody-mediated rejection (c-aABMR, n=8). As we do not perform standard protocol biopsies for patients with stable kidney transplant function in our center, we were unable to investigate those microRNA profiles.

MicroRNA profiling of different Banff rejection categories

Figure 1 shows the unsupervised clustering of the top 50 most variable expressed microRNAs. Here, it is clear that the different subtypes already show some different expression patterns. Next, we analyzed the differences in microRNA expression profiles between a-aABMR vs. aTCMRI and aTCMRII, and between a-aABMR vs. c-aABMR.

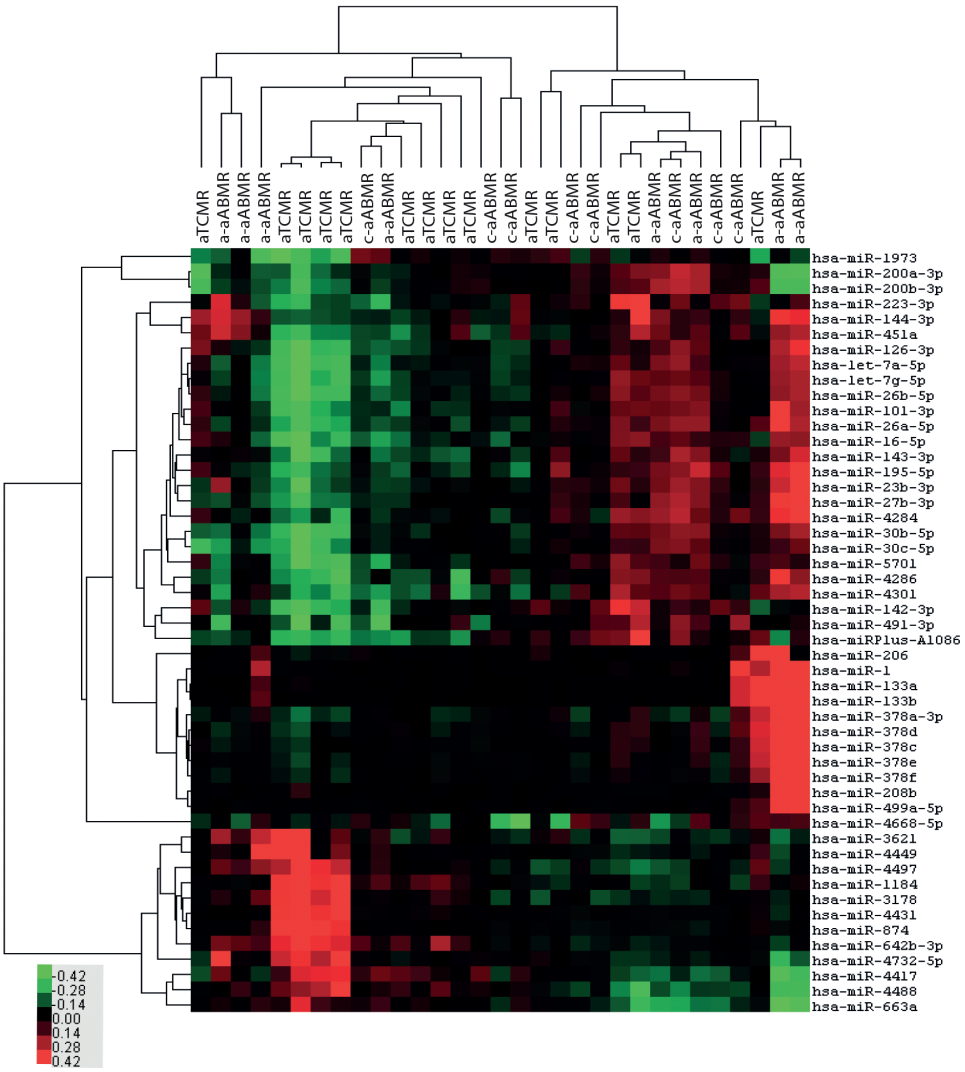

Figure 1. MicroRNA profiling of different histopathological subtypes of rejection

The heatmap was produced using the top 50 variably expressed microRNAs. The color scale illustrates the relative expression level of the indicated microRNA across all the samples, (red) expression <0.28, (green) expression >0.28.

Significantly differentially expressed microRNAs between a-aABMR and aTCMR I/II

Microarray analysis identified 55 significantly differentially expressed microRNAs between a-aABMR and aTCMRI/aTCMRII; p-values <0.01 (Figure 2A, B and Table 2). Among the top 5 differentially expressed microRNAs we observed an upregulation of hsa-miR-99a-5p (Fold Change (FC): 2.2, p=0.00094) and downregulation of hsa-miR-4756-3p (Fold Change: -2.1, p=0.0001944), hsa-miR-4290 (FC: -1.6, p=0.000524), hsa-miR-1284 (FC: -1.6, p=0.0011) and hsa-miR-4303 (FC: -1.3, p=0.0012) in a-aABMR compared to aTCMRI/aTCMRII. These 55 differentially expressed microRNAs between aTCMR and a-aABMR were analyzed using Ingenuity Pathway analysis, and were characterized as genomic mediators in pathways involved in inflammatory diseases, inflammatory responses, protein synthesis, cellular growth and proliferation processes. One network is displayed in Figure 3 and molecules identified in this network are linked to their microRNAs (Figure 3 and Supplementary Table 1).

Table 2. Significantly differentially expressed microRNAs between a-aABMR and aTCMR I/II

miRNA	Fold Change	adjusted P-value
hsa-miR-4756-3p	-2.165	0.0001944
hsa-miR-4290	-1.606	0.000524
hsa-miR-99a-5p	2.219	0.0009458
hsa-miR-1284	-1.606	0.0011456
hsa-miR-4303	-1.333	0.001228
hsa-miR-4778-3p	-1.29	0.0013518
hsa-miR-1972	-1.273	0.0013659
hsa-miR-3184-3p	-1.442	0.0014167
hsa-miR-877-3p	-1.659	0.0015449
hsa-miR-4300	-1.315	0.0018098
hsa-miR-4646-3p	-1.471	0.0019378
hsa-miR-320a	1.722	0.002107
hsa-miR-550b-3p	-1.327	0.0024474
hsa-miR-320b	1.581	0.0024878
hsa-miR-4538	-1.54	0.0026582
hsa-miR-4454	1.734	0.0026636
hsa-miR-22-3p	2.049	0.0026906
hsa-miR-3183	-1.621	0.0029419
hsa-miR-5002-5p	-1.406	0.0034821
hsa-miR-1976	-1.42	0.0037172
hsa-miR-3920	-1.248	0.0037711
hsa-miR-214-3p	1.814	0.0039018
hsa-miR-210	1.564	0.0039767
hsa-miR-4326	-1.193	0.004058

miRNA	Fold Change	adjusted P-value
hsa-miR-320c	1.599	0.0043812
hsa-miR-4728-3p	-1.607	0.0045045
hsa-miR-4286	3.004	0.0049026
hsa-miR-3124-3p	-1.548	0.0050548
hsa-miR-1285-5p	-1.633	0.0053395
hsa-miR-138-2-3p	1.847	0.0054998
hsa-miR-144-3p	4.03	0.0057715
hsa-miR-4743	-1.461	0.005934
hsa-miR-151a-3p	1.469	0.0060134
hsa-miR-5096	-1.46	0.0060656
hsa-miR-2114-5p	-1.294	0.0063726
hsa-miR-4328	2.153	0.006618
hsa-miR-145-5p	2.143	0.0073292
hsa-miR-100-5p	1.68	0.007891
hsa-miR-17-3p	1.311	0.0082762
hsa-miR-139-5p	1.489	0.0082949
hsa-miR-4451	-1.765	0.0083672
hsa-miR-654-5p	-1.402	0.008576
hsa-miR-4722-3p	-1.223	0.0087056
hsa-miR-23b-3p	2.512	0.0087352
hsa-miR-4797-5p	2.1	0.0088696
hsa-miR-1236	-1.824	0.0090253
hsa-miR-650	-1.3	0.0094688
hsa-miR-377-3p	1.408	0.0095252
hsa-miR-4804-3p	-1.459	0.0095933
hsa-miR-144-5p	1.231	0.0096551
hsa-miR-423-3p	1.533	0.0097147
hsa-miR-28-3p	1.224	0.0097947
hsa-miR-644b-3p	-1.313	0.0099017
hsa-miR-584-5p	-1.391	0.0099344
hsa-miR-191-5p	1.879	0.0099896

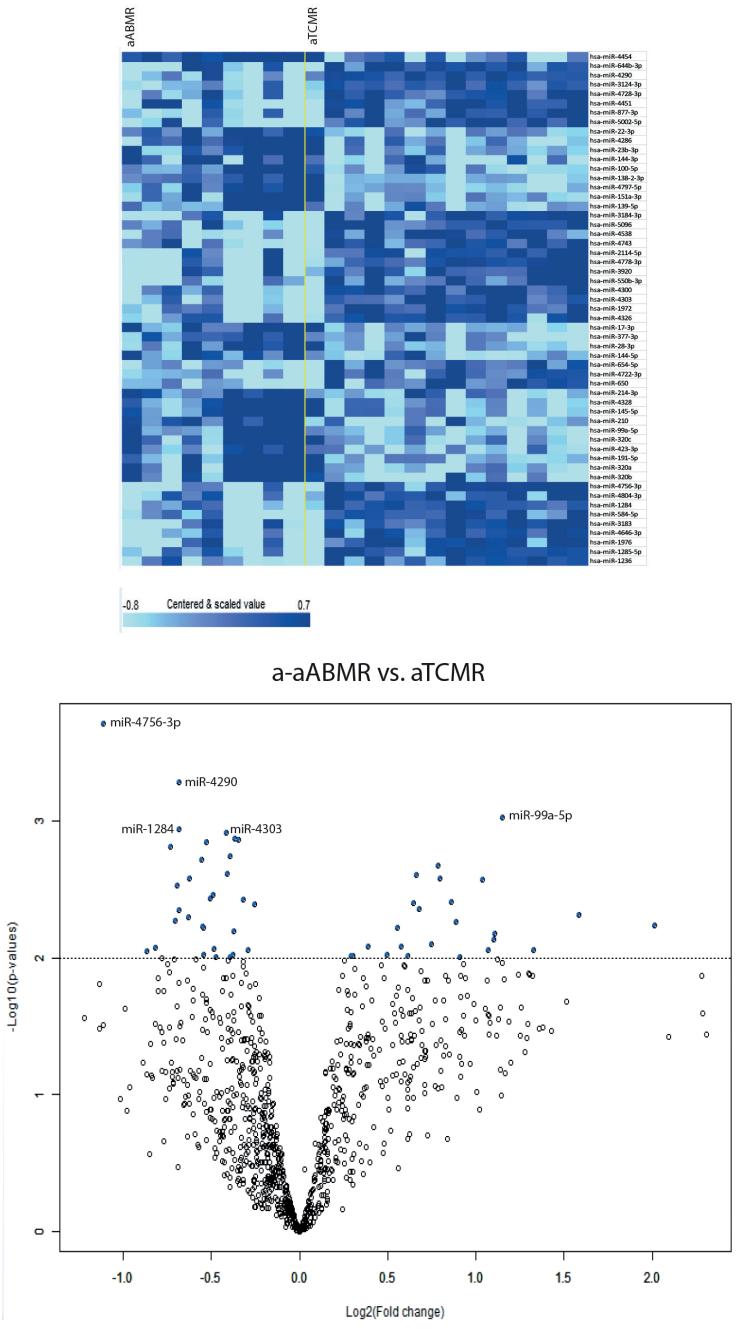

Figure 2. Differentially expressed miRNAs in acute active antibody mediated rejection versus acute T cell mediated rejection type I/II

(A) represents heatmap of expression values of significant miRNAs. Centered and scaled values are indicated as light blue expression <-0.8 and dark blue expression >0.7. Yellow line indicates different histomorphological group; a-aABMR (left) and aTCMR (right). (B) Volcano plot showing FC (log_2 values) and p-value (log_{10} values) for the 55 microRNAs of interest comparing a-aABMR and aTCMR I/II.

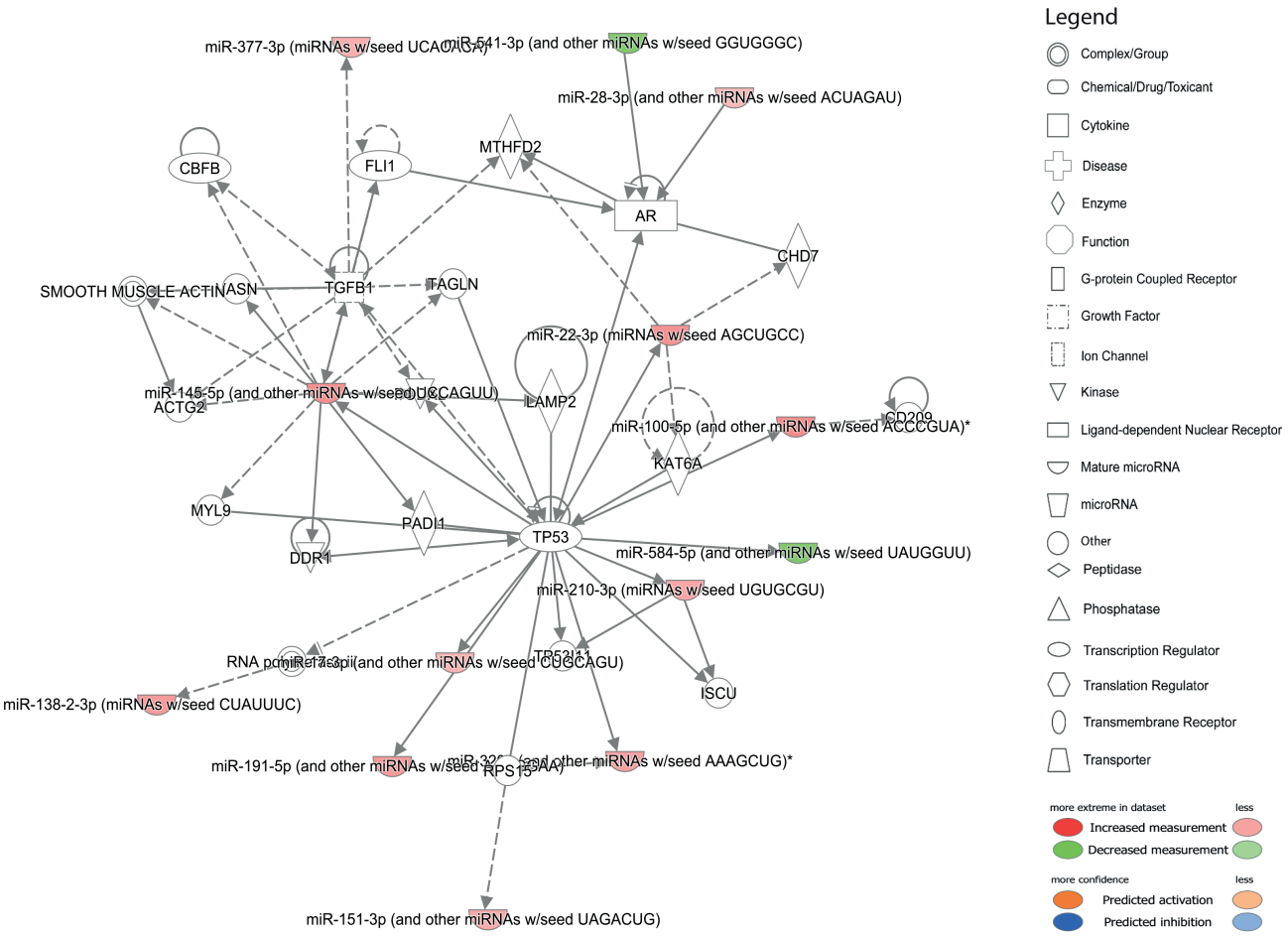

Figure 3. The comparison of a-aABMR and aTCMR I/II related pathways

Relevant genes and proteins associated with 55 differentially expressed microRNAs between a-aABMR and aTCMR I/II showing the indirect or direct involved protein interaction.

Significantly differentially expressed genes between a-aABMR and c-aABMR.
Microarray analysis identified 5 significantly differentially regulated microRNAs between a-aABMR and c-aABMR as tested by univariate testing and p-values <0.01 (Figure 4A, B and Table 3). Among the 5 differentially expressed microRNAs, we detected an upregulation of hsa-let-7f-1-3p (FC: 1.1, p=0.0038), hsa-miR-3679-3p (FC: 1.4, p=0.0099) and downregulation of hsa-miR-664-5p (FC: -1.2, p=0.0013), hsa-miR-3591-5p (FC: -1.5, p=0.0058) and hsa-miR-4268 (FC: -1.5, p=0.0072) in a-aABMR biopsies compared to c-aABMR biopsies.

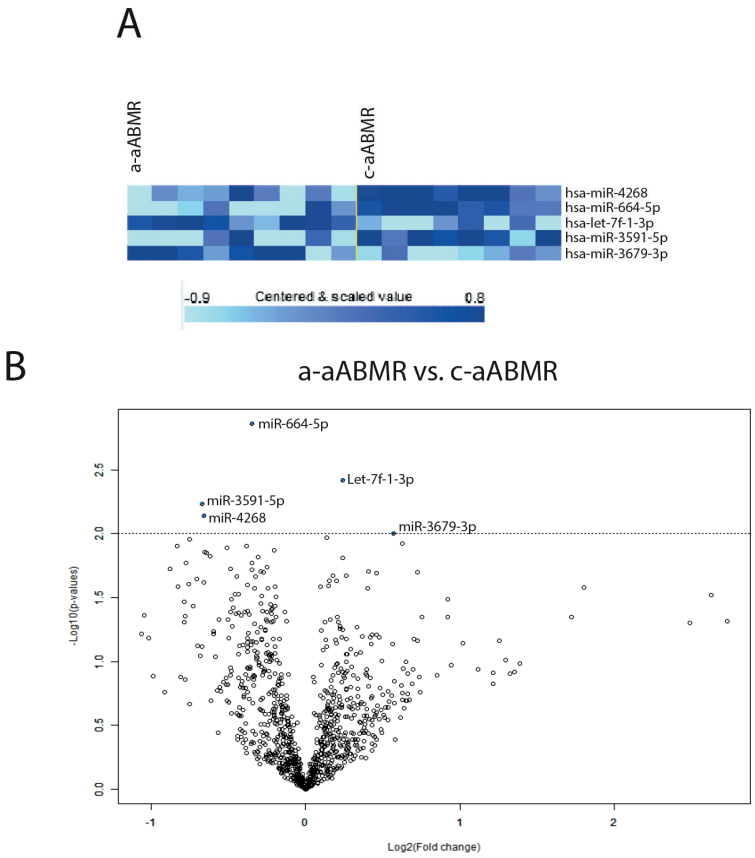

Figure 4. Differentially expressed miRNAs in acute active antibody mediated rejection versus chronic active antibody mediated rejection
(A) represents heatmap of expression values of significant miRNAs. . Centered and scaled values are indicated as light blue expression <-0.9 and dark blue expression >0.8. Yellow line indicates different histomorphological group a-aABMR (left) and aTCMR (right). (B) Volcano plot showing FC (log_2 values) and p-value (log_{10} values) for the 5 microRNAs comparing a-aABMR and aTCMR.

Table 3. Significantly differentially expressed genes between a-aABMR and c-aABMR

miRNA	Fold Change	adjusted P-value
hsa-miR-664-5p	-1.272	0.0013678
hsa-let-7f-1-3p	1.182	0.0038045
hsa-miR-3591-5p	-1.597	0.0058369
hsa-miR-4268	-1.582	0.0072596
hsa-miR-3679-3p	1.484	0.0099494

Discussion

In this study, we compared miRNA expression profiles detected in biopsies from transplanted kidneys displaying different types of rejection (a-aABMR vs. aTCMR and between a-aABMR vs. c-aABMR). Furthermore, pathway analyses were performed to search for the target genes, and to elucidate the contributions of specific miRNAs in the rejection process. We identified 55 differentially expressed miRNAs between a-aABMR and aTCMR. The microRNAs found in this study are all part of immune and inflammation pathways which again shows the dominant role of these pathways in the anti-donor response. These pathways are particularly active in immune cells such as lymphocytes, monocytes – macrophages, NK cells and dendritic cells. These cells are all involved during rejection of the allograft. Clearly, larger cohorts including samples showing no signs of rejection are needed to validate these findings, and to determine the association of specific miRNAs with either rejection type, i.e. antibody mediated rejection or T cell mediated rejection.

The rapid technological advances enabling in depth investigations of the human genome and particularly microRNAs, also affects the field of transplantation immunology[22, 23, 25, 26, 37]. In order to better define differences in alloimmune inflammatory responses underlying different types of rejection, a molecular approach should be used. Here, we found large differences in miRNA expression profiles between a-aABMR and aTCMR related kidney biopsies, whereas less pronounced differences were found between a-aABMR and c-aABMR biopsies. Particularly the expression of microRNA-100-5p was different between a-aABMR and aTCMR. miRNA-100-5p reached one of the highest fold change expression levels abundantly present in kidney transplant tissue showing acute active antibody mediated rejection compared to acute cellular rejection. It is known that this miRNA is involved in the regulation of adhesion molecule *CD209* (DC-SIGN). The functional interaction between miRNAs-100-5p and *CD209* should be investigated in future studies[38, 39]. The majority of the infiltrated myeloid dendritic cells express *CD209* leading to activation of the T-cells[39]. Woltman et al. tested the reliability of DC-SIGN as a rejection marker performing double immunostaining experiments for DC-SIGN/BDCA-1 on fresh frozen kidney transplant samples. Although the effect of kidney DCs on allograft survival has not been defined completely, a prior study has shown that DCs density in rejecting allograft biopsies could

predict kidney transplant dysfunction[38]. DCs density was also able to predict poor allograft survival independent of clinical variables[40]. Given these findings, one can envision that manipulation of miRNA-100-5p expression and/or function can potentially be used to affect DC-SIGN function in rejecting kidney transplants.

Also miRNA-145-5p interacts with many cellular functions such as cell growth, cell proliferation, cell differentiation and apoptosis[41-43]. MiRNA-145-5p is expressed by different types of immune cells such as CD4+ and CD8+T-cells, and CD19+ B-cells[44]. MiRNA-145-5p has been directly related to the synthesis of TGF-B1 (transforming growth factor beta 1), VASN (vasorin), DDR1 (discoidin domain receptor tyrosine kinase 1), PADI1 (peptidyl arginine deiminase 1) and TP53 (tumor protein p53, and regulates the production of SMA (smooth muscle actin), TAGLN (transgelin) and ACTG2 (acta gamma 2). TGF-B1 has been demonstrated to affect kidney transplant survival in many ways. TGF-B1 is not only involved in tissue regeneration but can also act as an immunosuppressive factor repressing anti donor cellular immune responses[45, 46]. DDR1 is found to be one of the new mediators in chronic kidney disease as it modulates inflammatory cell recruitment, and is indirectly linked to smooth muscle actin, ECM deposition and fibrosis in renal disease in general[47, 48]. Vasorin together with lumican have been identified in plasma of patients with diabetic nephropathy. Soluble lumican protein core can bind LPS-mediated proinflammatory effects in macrophages[49].

As in many gene expression and in particular miRNA studies, the cell source of the detected discriminating miRNAs cannot be defined as yet unless in situ hybridization studies on formaline fixated parrafine imbedded corresponding kidney biopsies from the same patients are performed. Clearly, different kidney resident cell types contain a broad range of miRNAs, all with their unique targets and functions. Therefore, specificity and interpretation of kidney biopsy derived miRNAs is still a hurdle to be taken. Studies are ongoing at the moment to determine the cell source of the identified microRNAs of interest by *in situ* hybrydization. In order to understand the full biological impact of particular miRNAs, further identification of their mRNA targets in necessary as well as the cellular pathways in which they function. Without any doubt, this knowledge will be essential to point out the discriminating checkpoints during different rejection type processes. Clinically, this information is very useful as the treatment modalities and prognosis differ between various rejection types. Nevertheless, the clear differentially expressed microRNA profiles between different types of rejection, as demonstrated in this study, emphasize again the high potential of these small RNAs as biomarkers for post-transplant immunological injury and kidney transplant outcome.

Author contributions

Thierry P.P. van den Bosch contributed in collecting data, analyses and writing. Marcel Smid contributed in statistical analyses, writing and reviewing. Erik A. C. Wiemer contributed in interpretation, writing and and reviewing. Ajda T. Rowshani was responsible for the conceptualization and design, contributed in collecting data, experimenting, analyses, writing and reviewing.

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Chapter 6

Human monocytes produce interferon-gamma upon stimulation with LPS

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6

Abstract

Representing a crucial T-helper 1 cytokine, IFN-γ acts as an important bridge between innate and adaptive immunity and is involved in many acute and chronic pathologic states, such as autoimmune diseases and solid organ transplant rejection. At present, debate still prevails about the ability of human monocytes to produce IFN-γ. We aimed to investigate whether human monocytes possess the capacity to produce IFN-γ at mRNA and protein level. Using real time PCR, flow cytometric analysis and ELISA, we investigated the capacity of freshly isolated CD14+ monocytes of healthy individuals and kidney transplant recipients to produce IFN-γ after stimulation with IFN-γ and LPS or LPS alone. We observed increased IFN-γ mRNA levels in CD14+ monocytes after stimulation as compared to the unstimulated controls in both populations. In addition, stimulation with IFN-γ and LPS or LPS alone led to a significant increase in the percentage of CD14+ monocytes producing TNF-α and IFN-γ at protein level ($p<0.05$). A trend towards increased secreted IFN-γ production in supernatants was also observed after LPS stimulation using ELISA. We conclude that human monocytes from healthy individuals and kidney transplant recipients possess the capacity to produce IFN-γ.

Keywords
monocytes, IFN-γ, LPS, cytokines, macrophage, transplantation

Introduction

Interferon-gamma (IFN-γ) is a pleiotropic cytokine with pivotal roles in innate and adaptive immunity. It affects many biological functions primarily related to host defense and immune regulation, such as antiviral and antibacterial defense, cell cycle, apoptosis, and inflammation [1]. IFN-γ stimulation leads to up-regulation of MHC class I and class II molecules and increases antigen presentation by professional antigen-presenting cells (APCs) [2]. Activation of macrophages (Mph) by IFN-γ leads to cytokine secretion, upregulation of antigen processing and presenting pathways, and activation of antimicrobial and antitumor mechanisms [1]. In addition, IFN-γ attracts, matures and differentiates leukocytes [3]. It also enhances natural killer cell activity and regulates B-cell function [3-5]. In early host defense the production of IFN-γ by natural killer (NK) cells and possibly professional APCs is important, while T cells become the main producers of IFN-γ during the adaptive immune response [6]. Moreover, IFN-γ is critically important in the development of a type 1 T-helper cell response, by regulating differentiation, activation and homeostasis of T cells and inhibiting Th2 cell development.

Clinically, IFN-γ plays a major role in auto-immunity and solid organ transplant rejection. In multiple sclerosis (MS) increased IFN-γ and Interleukin-(IL)12 expression in the central nervous system and cerebrospinal fluid were correlated with increased disease activity [7]. In serum of MS patients the concentration of IFN-γ was significantly increased compared to healthy individuals [8]. In addition, administration of IFN-γ to patients with MS markedly exacerbated the disease [9]. In a mouse model, induced by transfer of myelin protein-specific CD8+ T cells neutralization of IFN-γ ameliorated the disease [10]. In a mouse model for rheumatoid arthritis, administration of IFN-γ exacerbated the severity of disease [11, 12]. Next to its role in auto-immunity, IFN-γ is also a key cytokine in transplant immunity. In mouse models for solid organ transplantation, high IFN-γ gene expression levels were associated with rejection [13]. Furthermore, IFN-γ-/- mice were unable to reject MHC class II-incompatible grafts whilst still rejecting MHC class I-incompatible grafts [14], probably due to the fact that IFN-γ up-regulates MHC class II expression by professional and non-professional APCs [15, 16]. In humans, early acute rejection episodes [17, 18] and poor long term kidney graft function [19] were predicted by high IFN-γ plasma levels and IFN-γ production during mixed lymphocyte reactions pre-transplantation. Finally, patients with pronounced clinical glomerulitis had significantly higher intrarenal IFN-γ mRNA compared to patients with subclinical glomerulitis and patients without any histological abnormalities [20]. In addition to the major role of IFN-γ in immune activation, it is also involved in down-regulation of adaptive immunity. This is particularly mediated via IFN-γ-induced expression of indoleamine 2,3-dioxygenase in APCs, which in turn stimulates differentiation of regulatory T cells [21].

In the field of solid organ transplantation, T-cells are the predominant cell types infiltrating acutely rejecting kidney transplants [22, 23]. T-cells are known to be required for acute rejection [24-26] Other immune cell types such as monocytes and NK cells compose up to now neglected immune cells, gaining more attention due to their nowadays recognized contribution to acute and chronic antibody mediated rejection [27-29]. Monocyte infiltration and specifically glomerular monocytes were associated with graft dysfunction and poor graft outcome [30, 31]. Furthermore, monocytic infiltrates seemed to drive the acute rejection in T-cell-depleted, alemtuzumab-treated kidney transplant recipients [32]. Moreover, one of the cardinal histopathological hallmarks of antibody mediated rejection is accumulation of monocytes and Mph in peritubular capillaries leading to acute, overt and often irreversible graft damage. IFN-γ is one of major pro-inflammatory cytokines driving rejection of the kidney graft [33, 34]. Whether monocytes contribute to rejection by IFN-γ production remains an important question for further investigations. At present, the production of IFN-γ by monocytes is debated in the literature. Most of the research has focused on the production of IFN-γ by Mph, but contrasting reports in this regard have been published in recent years. Schleicher et al. reported that RAG2-/- γ-chain-/- Mph did not produce IFN-γ after stimulation with IL-12, IL-18, lipopolysaccharide (LPS) alone or in combination with IFN-γ. They related the observed production of IFN-γ by Mph to the contamination of these Mph with other cell types [35]. However, Choudhry et al. found that RAG2-/- γ-chain-/- mice did retain control over *C. parvum* reproduction which was IFN-γ dependent [36]. In addition, they showed that the secretion of IFN-γ by Mph was stimulated by IL-18 and IL-12.

To further elucidate the possibility of IFN-γ production by monocytes, we aimed to investigate whether human monocytes obtained from healthy individuals and renal transplant recipients (Tx recipients) possess the capacity to produce IFN-γ at mRNA and protein level.

Materials & Methods

Patient characteristics

Whole blood was collected from kidney Tx recipients either at time of transplantation or 3 months after transplantation and from healthy individuals. All patients were treated with basiliximab (Simulect®) as induction therapy and the maintenance immunosuppressive regimen consisted of mycophenolate mofetil (cellcept®), tacrolimus (prograft®) and corticosteroids. Following the guidelines of the Ethical Committee of the ErasmusMC, all patients signed written informed consent.

Monocyte isolation and stimulation

Using a Ficoll-Hypaque density gradient (Lymphoprep™), PBMCs were isolated from anti-coagulated blood collected in sodium-heparin coated tubes. CD14+ beads (Miltenyi) were applied to purify the monocytes using magnetic activated cell sorting on an AutoMACS according to the manufacturers instruction. Cell purity was checked by flow cytometric analysis and was always higher than 95%.

Freshly isolated CD14+ monocytes were used to determine intracellular cytokine production. The cells were cultured in RPMI medium supplemented with 10% fetal calf serum and stimulated using either 100 ng/ml E. coli LPS (Sigma-Aldrich) and golgiplug (BD Biosciences) overnight or pre-treated for 2 hours with 20 ng/ml IFN-γ (U-Cytech) and then with a combination of 100 ng/ml LPS and golgiplug overnight. The optimal time for priming of monocytes with IFN-γ was determined by performing time-course experiments. Pre-stimulation with IFN-γ was done for 2, 4 or 6 hours before overnight stimulation with LPS. As no difference in the cytokine producing capacity of monocytes at different IFN-γ pre-stimulation time points could be observed, a 2 hour priming period with IFN-γ was used (data not shown).

Flow cytometric analysis of intracellular cytokine production

After overnight stimulation, cells were incubated with EDTA for 15 min and washed. Cells were exposed to CD14-Pacific Blue (Biolegend) in PBS containing 10% BSA for 30 minutes at 4°C. The cells were incubated with FACS lysing solution at room temperature for 10 min and with FACS permeabilizing solution 2 (BD Biosciences) for 15 min. Next, conjugated antibodies to TNF-α-Percp-Cy5.5 and IFN-γ-APC-Cy7 and their respective isotype controls (Supplementary Figures 1 and 2) (all Biolegend) were added to determine intracellular cytokine production. The cells were washed and analyzed using flow cytometry (FACSCanto II, BD Biosciences) and FACSDiva software. Gating was done on living cells and monocytes were identified based on CD14 expression. To study the shift of monocytes to Mph, cells were stained for CD68 using as a primary antibody mouse-anti-CD68 antibody (DakoCytomation) and a FITC-conjugated anti-mouse IgG1 secondary antibody (Biolegend). To study the amount of cell death after overnight incubation of the monocytes, cells were stained for 7-AAD (Biolegend).

ELISA

CD14+ monocytes were stimulated overnight with 100 ng/ml LPS. Supernatants were harvested the next day and frozen until use. The IFN-γ ELISA was performed according to manufacturers instructions (U-CyTech).

mRNA analysis

After stimulation of CD14+ monocytes with LPS alone for 2 hours or with IFN-γ for 2 hours followed by 2 hours with LPS cells were fixed in RNAlater (Ambion). RNA was isolated by RNA isolation kit according to manufacturer's protocol (Roche).

cDNA was generated using random primers and 275 ng RNA. To verify target gene mRNA expression the StepOnePlus™ Real-Time PCR system, taqman universal PCR master mix and the primer/probes for IFN-γ (Hs00174143.m1), TNF-α (Hs99999905.m1) and GAPDH (Hs9999043.m1) (all Applied Biosciences) were used to perform quantitative real-time PCR. Gene expression levels were normalized to GAPDH mRNA levels and presented as a ratio relative to the unstimulated controls.

Statistical analysis

Significant differences were determined using either the *t*-test, or one-way ANOVA. The results are presented as mean ± SEM. A p-value <0.05 was considered significant.

Results

Intracellular cytokine production by monocytes

Freshly isolated monocytes from healthy individuals and Tx recipients were used to stimulate with IFN-γ and LPS or LPS alone. Overnight incubation of the monocytes caused approximately 15% cell death as was observed by 7-AAD staining (Supplementary Figure 3). To determine the effectiveness of the stimulation protocols in activating monocytes, we investigated TNF-α production as a positive control in our system. No differences were observed between healthy individuals and Tx recipients for the unstimulated condition as well as for both stimulation protocols (Figure 1A). TNF-α was produced by 3% of the monocytes by both healthy individuals and Tx recipients in the unstimulated condition. Stimulation with both IFN-γ and LPS and LPS alone induced a significant increase in the percentage of TNF-α producing monocytes compared to unstimulated cells, both in healthy individuals and Tx recipients (mean ± SEM: 32.7% ± 5.6 and 26.7% ± 9.8 for healthy individuals and 20.6% ± 4.1 and 28.2% ± 5.7 for Tx recipients respectively) (Figure 1A,C).

Interestingly, both types of stimulation protocols increased the percentage of IFN-γ positive monocytes comparably (Figure 1B). The percentage of unstimulated monocytes producing IFN-γ was 1.1% and 1.3% for healthy individuals and Tx recipients. Stimulation with both IFN-γ and LPS or LPS alone significantly increased the percentage of IFN-γ-producing monocytes, with no difference between the two groups (mean ± SEM: 10.5% ± 2.2 and 11.6% ± 7.7 for healthy individuals and 7.3% ± 2.8 and 10.6% ± 4 for Tx recipients respectively) (Figure 1B,D). In addition, no significant difference was observed comparing IFN-γ and LPS and LPS alone for both healthy individuals and Tx recipients. Thus stimulation with LPS alone induces IFN-γ

production by monocytes and priming with IFN-γ prior to LPS exposure did not alter this response.

In addition, we investigated the monocyte-derived IFN-γ production by means of ELISA. We observed that unstimulated monocytes produced IFN-γ at background level (5 pg/ml), whereas increased IFN-γ concentration levels were detected in supernatants of LPS-stimulated monocytes confirming more enhanced production and secretion of IFN-γ by monocytes after LPS stimulation (mean ± SEM: 27.6 pg/ml ± 12.2) (data not shown).

IFN-γ production by CD68+ and CD68- monocytes

Cell surface expression of CD68 was determined to check whether a switch towards Mph phenotype had taken place during the procedure. Stimulation did not lead to a significant increase in cell surface expression of CD68, indicating that monocytes did not shift towards a Mph-like phenotype (Figure 2A-D). Importantly, production of IFN-γ did not reside specifically within monocytes with CD68 cell surface expression, since a large proportion of IFN-γ-positive monocytes did not express CD68 (Figure 2D). This indicates that IFN-γ production was not associated with a shift towards a more Mph-like phenotype.

IFN-γ mRNA expression by monocytes upon stimulation

A clear trend towards increased mRNA expression levels of IFN-γ was observed, indicating that stimulation with IFN-γ and LPS or LPS alone is potent in activating monocytes to produce IFN-γ (Figure 3A) underlying our findings at the protein level. Accordingly, we observed that the mRNA expression of TNF-α by monocytes was significantly increased with both types of stimulation compared to the unstimulated state (Figure 3B). The IFN-γ mRNA expression is more pronounced after 2 hours priming with IFN-γ itself followed by LPS stimulation, indicating that the priming and/or longer stimulation period may lead to more detectable production of IFN-γ mRNA probably due to differences in cytokine production kinetic. Although statistically not significant, the IFN-γ mRNA expression was more enhanced by monocytes obtained from healthy individuals than kidney transplant recipients which may be explained by the uremic condition of our patients (Supplementary Figure 4).

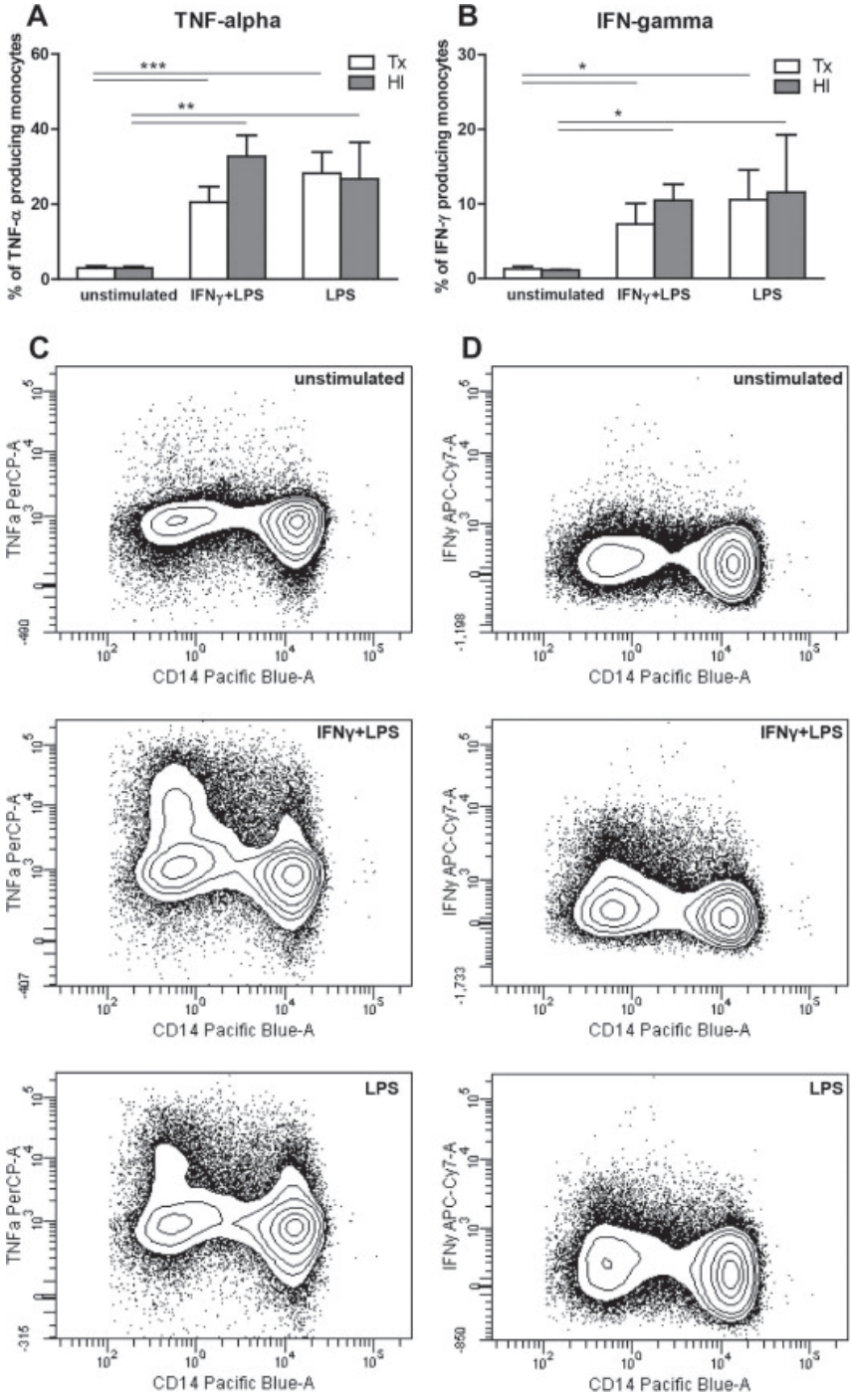
A
TNF-alpha
% of TNF-α producing monocytes
Tx
HI
***
**
unstimulated
IFNγ+LPS
LPS
B
IFN-gamma
% of IFN-γ producing monocytes
*
*
C
unstimulated
TNFa PerCP-A
CD14 Pacific Blue-A
D
IFNγ APC-Cy7-A
IFNγ+LPS
LPS

Figure 1. Human monocytes produce IFN-γ
Freshly isolated CD14+ monocytes from healthy individuals (HI) and renal transplant recipients (Tx) were used to test the production capacity of IFN-γ and TNF-α. Cells were stimulated overnight in the presence of golgiplug with either LPS alone or in combination with IFN-γ. (A) Both stimulation protocols led to a significant increase in the percentage of TNF-α-producing monocytes with equal efficacy (HI: mean ± SEM: 32.7% ± 5.6 for IFN-γ + LPS and 26.7% ± 9.8 for LPS alone, n=5; Tx: mean ± SEM 20.6% ± 4.1 for IFN-γ + LPS and 28.2% ± 5.7 for LPS alone, n=8). (B) Both stimulation protocols led to a significant increase in the percentage of IFN-γ-producing monocytes with equal efficacy (HI: mean ± SEM: 10.5% ± 2.2 for IFN-γ + LPS and 11.6% ± 7.7 for LPS alone, n=5; Tx: mean ± SEM 7.3% ± 2.8 for IFN-γ + LPS and 10.6% ± 4 for LPS alone, n=8). Representative flow cytometric plots of TNF-α (C) and IFN-γ (D) production are shown with unstimulated (upper panel), IFN-γ + LPS-stimulated (middle panel) or LPS-stimulated (lower panel) monocytes from a transplant recipient. *p<0.05, **p<0.01, ***p<0.001.

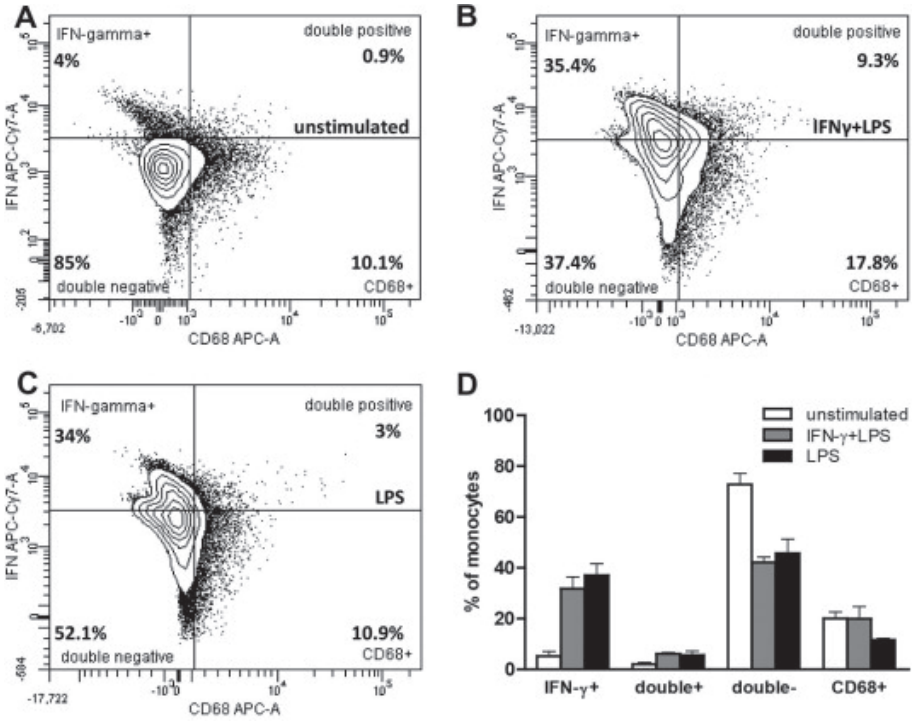

Figure 2. IFN-γ production not associated with a shift towards a Mph-like phenotype
Freshly isolated CD14+ monocytes from Tx recipients were used to test whether a shift towards Mph-like phenotype occurs. Cells were stimulated overnight in the presence of golgiplug with either LPS alone or in combination with IFN-γ and stained for IFN-γ and CD68 the next day. Representative flow cytometric plots showing CD68 versus IFN-γ are depicted in A-C. (D) Percentage of monocytes expressing CD68 versus IFN-γ (n=4).

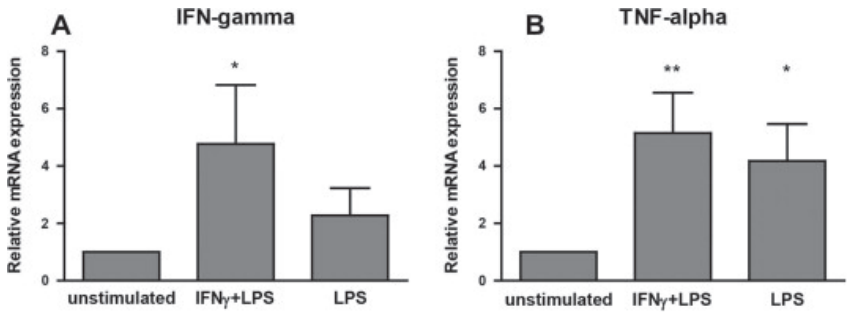

Figure 3. IFN-γ mRNA expression by monocytes upon stimulation

Freshly isolated CD14+ monocytes from Tx recipients were used to test the production capacity of TNF-α and IFN-γ mRNA. Cells were stimulated with either LPS for 2 hours or pre-stimulated with IFN-γ for 2 hours followed by 2 hours stimulation with LPS. Subsequently mRNA was isolated and qPCR performed. Target gene expression levels were normalized to GAPDH mRNA levels and presented as a ratio relative to the unstimulated control. Stimulation of monocytes with both stimulation protocols increased the mRNA expression of (A) IFN-γ and (B) TNF-α (n=5-8).

Discussion

Here, we show that human monocytes produce IFN-γ upon stimulation with LPS. We observed that stimulation of freshly isolated CD14+ human monocytes derived from healthy individuals and Tx recipients with LPS alone or combined stimulation with IFN-γ and LPS lead to a significant increase in the percentage of TNF-α- and IFN-γ-producing monocytes.

Although IFN-γ is mostly regarded as a T cell associated cytokine, it is also produced by other immune cell types like Mph and NK cells. No direct observations on human monocytes have been available so far. Several studies have shown that human monocyte-derived Mph and dendritic cells have the capacity to produce IFN-γ. Fenton et al. observed that infection of alveolar Mph with *M. tuberculosis in vitro* stimulated the release of IFN-γ protein and transiently induced an increase in IFN-γ mRNA levels using RT-in situ PCR [37]. Furthermore, human monocyte-derived dendritic cells are able to produce IFN-γ upon stimulation with either IL-12 or Salmonella infection [38, 39]. Recently, it was shown that monocytes produce high levels of IFN-γ in the presence of IL-2 and the bisphosphonate zoledronic acid [40]. These zoledronic acid-treated monocytes augmented, through the release of IFN-γ, TNF-related apoptosis-inducing ligand-mediated cytotoxicity of human NK cells. Although IFN-γ is well-known to activate a variety of signaling pathways in monocytes, we were interested in the potential of the monocytes to produce IFN-γ in response to the well-known stimuli such as LPS and/or IFN-γ/LPS. The effects of IFN-γ itself on monocytes are therefore not discussed here.

Recently, we documented the production of IFN-γ by monocyte subsets obtained from kidney transplant recipients after combined stimulation with IFN-γ and LPS or LPS alone

[41]. We observed that the percentage of both CD16+ monocyte subsets was significantly increased in transplant recipients compared to healthy individuals retaining for at least 6 months after transplantation, indicative of triggered innate immunity. Enhanced production capacity of TNF-α, IFN-γ and IL-1β by monocytes was observed at time of transplantation compared to healthy individuals. Remarkably, three months post-transplant, in presence of potent immunosuppressive drugs and despite improved kidney function, IFN-γ, TNF-α and IL-10 production capacity still remained significantly increased. We concluded that this shift could be one of the important drivers of early post-transplant cellular immunity. Uptake of IFN-γ from the extracellular medium after pre-stimulation with IFN-γ does not likely contribute significantly to the elevated percentage of IFN-γ+ monocytes, since results after stimulation with LPS alone were similar. These findings prompted us to investigate the IFN-γ production in both healthy individuals and Tx recipients more in detail using freshly isolated monocytes.

In this study, we demonstrate that monocytes express IFN-γ protein after stimulation by two different methods, namely flow cytometric evaluation representing the intracellular IFN-γ production and ELISA showing IFN-γ production and secretion by stimulated monocytes in supernatants. To be complete, IFN-γ mRNA production was also documented. Of note, the IFN-γ production by the monocytes was not associated with a shift towards CD68 positivity, indicating that these cells did not change in type towards a more Mph-like phenotype. Our data confirm that monocyte is a cell type capable of IFN-γ production. Whereas the IFN-γ production by macrophages and dendritic cells is believed to take place in tissue, T cells and monocytes could be designated as IFN-γ producing immune cells in circulation.

The immunosuppressive maintenance treatment received by the kidney transplant recipients had clearly no inhibitory effect on cytokine producing capacity of monocytes as there was no difference between the percentage of TNF-α and IFN-γ producing monocytes at pre-transplant and post-transplant time points. Of note, the release of TNF-α, IL-1β and IL-8, from monocytes isolated from cord blood was not inhibited by therapeutic levels of dexamethasone [42]. In addition, the inhibition of cytokine production was significantly lower in monocytes compared to T cells, indicating that monocytes are less sensitive to glucocorticoids as T-cells [43].

Taken together, our data show clearly that human monocytes possess the capacity to produce IFN-γ, shedding more light at their role in both linking innate and adaptive immunity, and the pathogenesis of kidney transplant related immunity.

Acknowledgements

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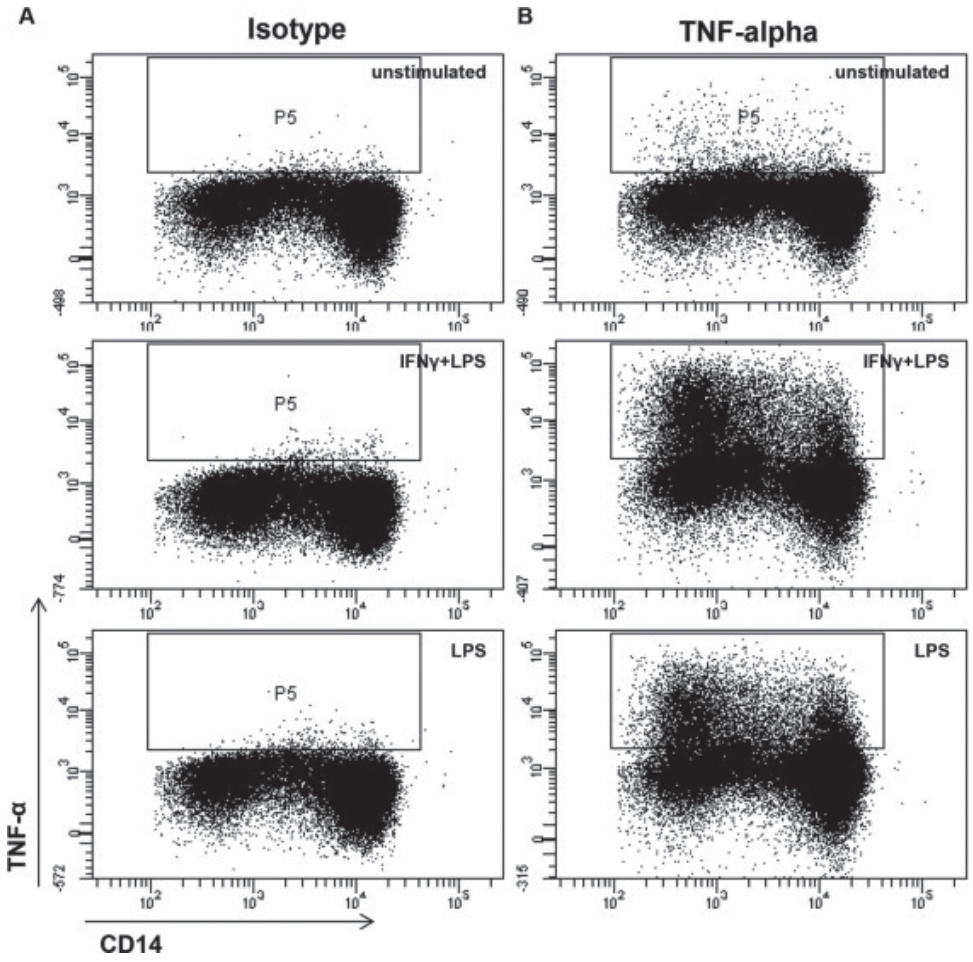
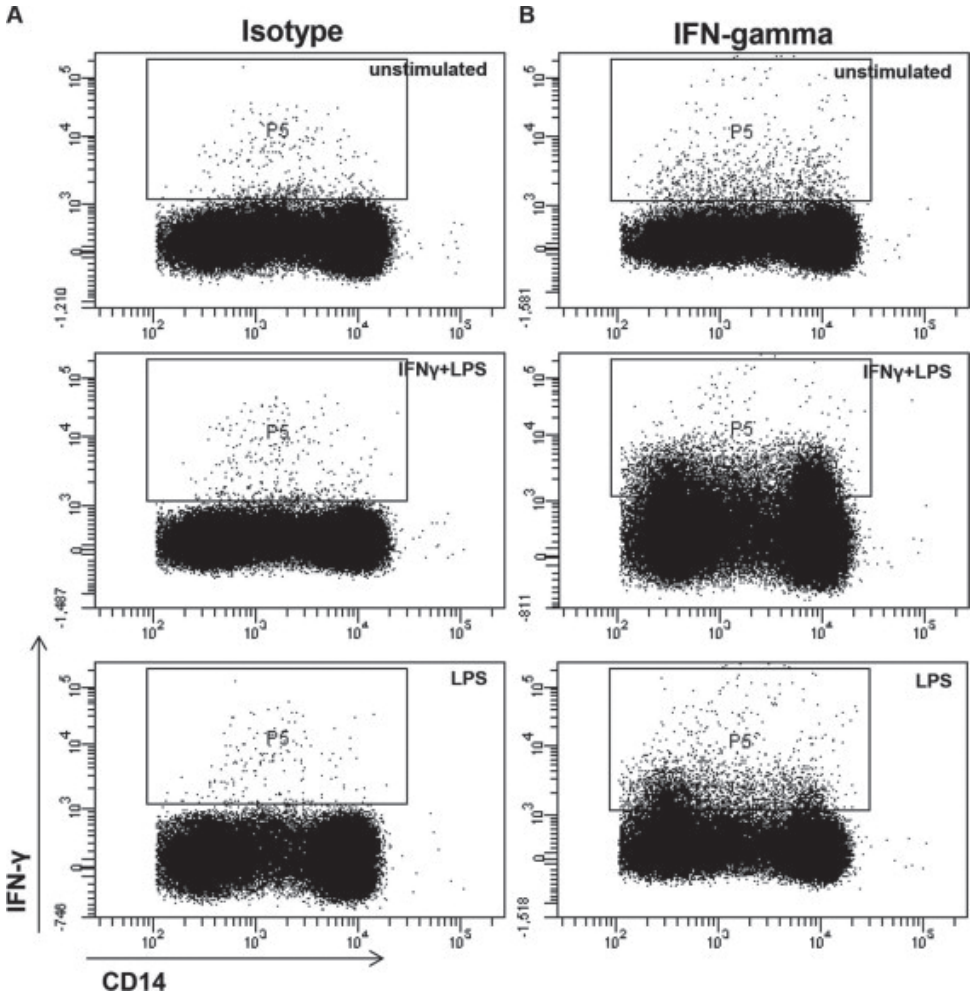
Supplementary Materials



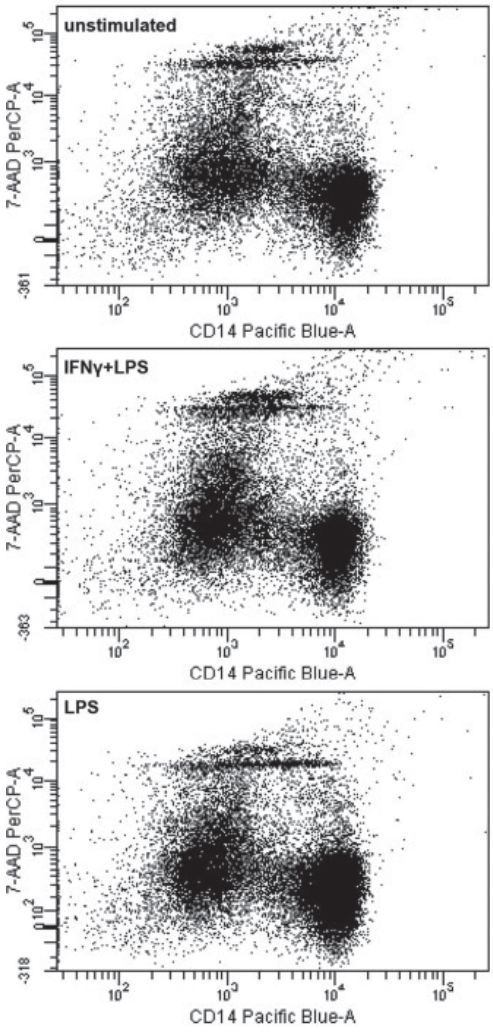
Supplementary Figure 1. Isotype control for TNF-α

Freshly isolated CD14+ monocytes were used to test the production capacity of TNF-α. Cells were stimulated overnight in the presence of golgiplug with either LPS alone or in combination with IFN-γ. Representative flow cytometric plots of (A) isotype control and (B) TNF-α are shown with unstimulated (upper panel), IFN-γ + LPS-stimulated (middle panel) or LPS-stimulated (lower panel) monocytes.

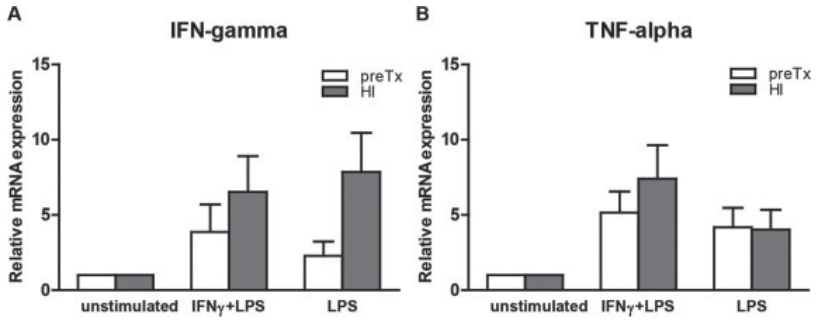


Supplementary Figure 2. Isotype control for IFN-γ

Freshly isolated CD14+ monocytes were used to test the production capacity of IFN-γ. Cells were stimulated overnight in the presence of golgiplug with either LPS alone or in combination with IFN-γ. Representative flow cytometric plots of (A) isotype control and (B) INF-γ are shown with unstimulated (upper panel), IFN-γ + LPS-stimulated (middle panel) or LPS-stimulated (lower panel) monocytes.



Supplementary Figure 3. 7-AAD staining of monocytes
Freshly isolated CD14+ monocytes from healthy individuals and Tx recipients were stimulated overnight in the presence of golgiplug with either LPS alone or in combination with IFN-γ and stained for 7-AAD the next day. A representative flow cytometric plot of 7-AAD staining is shown for unstimulated (upper panel), IFN-γ + LPS-stimulated (middle panel) or LPS-stimulated (lower panel) monocytes from a healthy individual.



Supplementary Figure 4.

Freshly isolated CD14+ monocytes from healthy individuals (HI) and Tx recipients (preTx) were used to test the production capacity of (A) IFN-γ and (B) TNF-α mRNA. Cells were stimulated either with LPS for 2 hours or pre-stimulated with IFN-γ for 2 hours followed by 2 hours stimulation with LPS. Subsequently mRNA was isolated and qPCR performed. Target gene expression levels were normalized to GAPDH mRNA levels and presented as a ratio relative to the unstimulated control (n=3-8).

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Chapter 7

Targeting the monocyte-macrophage lineage in solid organ transplantation

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Abstract

There is an unmet clinical need for immunotherapeutic strategies which specifically target the active immune cells participating in the process of rejection after solid organ transplantation. The monocyte-macrophage cell lineage is increasingly recognized as a major player in acute and chronic allograft immunopathology. The dominant presence of cells of this lineage in rejecting allograft tissue is associated with worse graft function and survival. Monocytes and macrophages contribute to alloimmunity via diverse pathways: antigen processing and presentation, co-stimulation, pro-inflammatory cytokine production and tissue repair. Cross talk with other recipient immune competent cells and donor endothelial cells leads to amplification of inflammation and a cytolytic response in the graft.

Surprisingly little is known about therapeutic manipulation of the function of cells of the monocyte-macrophage lineage in transplantation by immunosuppressive agents. Although not primarily designed to target monocyte-macrophage lineage cells, multiple categories of currently prescribed immunosuppressive drugs, such as mycophenolate mofetil, mTOR inhibitors and calcineurin inhibitors, do have limited inhibitory effects. These effects include diminishing the degree of cytokine production, blocking co-stimulation and inhibiting the migration of monocytes to the site of rejection. Outside the field of transplantation, some clinical studies have shown that the monoclonal antibodies canakinumab, tocilizumab and infliximab are effective in inhibiting monocyte functions. Indirect effects have also been shown for simvastatin, a lipid lowering drug, and BET (Bromodomain and Extra-Terminal motif) inhibitors that reduce the cytokine production by monocytes-macrophages in patients with diabetes mellitus and rheumatoid arthritis.

To date, detailed knowledge concerning the origin, the developmental requirements and functions of diverse specialized monocyte-macrophage subsets justifies research for therapeutic manipulation. Here, we will discuss the effects of currently prescribed immunosuppressive drugs on monocytes/macrophages features and the future challenges.

Non-standardized abbreviation list

ATG	anti-thymocyte globulin
BET	bromodomain and extra terminal
CNI	calcineurin inhibitor
CTLA-4 Ig	cytotoxic T-lymphocyte-associated protein 4 immunoglobulin
ERK	extracellular regulated kinase
GRE	glucocorticoids response element
ICAM	intercellular adhesion molecule
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MHC	major histocompatibility complex
MMF	mycophenolate mofetil
MPA	mycophenolic acid
NFκB	nuclear factor kappa B
NFAT	nuclear factor of activated T-cells
NOD	nucleotide-binding oligomerization domain
RA	rheumatoid arthritis
SEB	staphylococcal enterotoxin B
TAT	tyrosine aminotransferase
TLR	toll like receptor

Introduction

Solid organ transplantation (SOT) is the preferred method to treat organ failure. Over the past decades, transplantation has become the preferred approach to treat solid organ failure. Striking improvement in short-term allograft survival, in particular of kidney allograft, has been achieved while long-term survival has lagged behind [1]. Intriguingly, this improvement is seen mainly in recipients who have never experienced a rejection episode, emphasizing the recipient's alloimmunity; in particular chronic antibody mediated rejection (cABMR) as a major determinant of overall transplant outcome [2, 3]. At present, there is an unmet clinical need to apply immunotherapeutic strategies to specifically target the active immune cells crucially participating in the process of rejection after SOT.

However, treatment with immunosuppressive drugs has exchanged the morbidity and mortality of organ failure for the risks of infection, cancer and increased mortality from cardiovascular disease. Although acute and chronic rejection, regardless of the type and the time of occurrence, are still major contributors leading to graft failure [1, 4, 5], cABMR is the main concern for the long term graft survival. Chronic antibody mediated rejection arises, at least in part, because immunosuppressive strategies do not completely inhibit rejection-related alloimmune responses specifically, resulting in slow progressive deterioration of graft function.

The monocyte-macrophage cell lineage is increasingly recognized as a major player in acute and chronic allograft immunopathology [6, 7]. The clinically used immunosuppressive drugs are not specifically directed against monocyte-macrophage lineage cells but still have some inhibitory effects. These cells contribute to alloimmunity via diverse pathways; antigen processing and antigen presentation, co-stimulation, pro-inflammatory cytokine production and tissue repair. Cross talk with other recipient immune competent cells and donor endothelial cells underlies amplification of inflammation at the graft site [8-10]. Interestingly, acute and chronic antibody mediated rejection are characterized amongst others by accumulation of monocyte-macrophage cells. Kidney graft infiltrating macrophages have been described to be a predictor of death-censored graft failure [11-21]. Macrophages are present in both acute antibody mediated rejection (ABMR) and acute cellular rejection (ACR) of solid organ transplants [19, 22]. In rejecting cardiac tissue, interstitial and intraluminal macrophage density correlates with effector alloantibodies and clinical antibody mediated rejection [22]. Even more, histopathological staining's for macrophages have been found to be positive prior to the onset of graft dysfunction indicating that macrophages can serve as potential diagnostic markers for transplant rejection [18]. Intravascular macrophages in the capillaries of endomyocardial tissue are shown to be a distinguishing feature of ABMR and are considered as one of the important histopathological diagnostic criteria in cardiac transplantation [22, 23].

A recent study showed that the severity of macrophage infiltration during ACR with arteritis is associated with impaired kidney function as measured by creatinine values up to 36 months post transplantation [19]. Importantly, Oberbarnscheidt et al. showed that monocyte recognition of allogeneic non-self persists over time, long after acute surgical inflammation has been subsided, indicating the important role of monocytes in the principle of long-term graft failure [24]. Recently, the presence of smooth muscle like-precursor cells within the non-classical monocyte subset has been described in kidney transplant patients. Characterization of non-classical monocytes in peripheral blood of kidney transplant patients undergoing chronic transplant dysfunction showed lower numbers compared to patients without chronic transplant dysfunction. Within the total living cell percentages of CD14+ monocytes there was no change observed, suggesting a shift within different subsets. Non-classical monocytes being reduced in transplant recipients with chronic transplant dysfunction may indicate a vital role in interstitial and vascular remodelling [25].

In stable kidney transplant recipients, a skewed balance towards pro-inflammatory CD16+ monocytes was shown at the time of kidney transplantation and during the first 6 months post-transplant. These monocytes were able to produce IFNγ, which acts as an important bridge between innate and adaptive immunity [26, 27].

In summary, the currently available knowledge concerning the immunobiology of specialized monocyte–macrophage subsets, their pathogenic role in rejection, and the still unmet clinical need to specifically prevent alloimmunity justify research on strategies for monocyte-macrophage directed therapeutics. In this review, we aim to discuss the relevant knowledge on monocyte-macrophage immunobiology. Briefly, to elaborate on the effects of currently available immunosuppressive drugs in relation to monocyte/macrophage lineage cells mainly focussed within, but also outside of the SOT field (**Table 1**), and eventually touch upon the future challenges and developments.

Table 1. Immunosuppressive drugs and the monocyte/macrophage lineage

Drug type	Effects on monocytes/macrophages	Key references
Basiliximab & ATG	• Basliximab targets the CD25 molecule (the IL-2 receptor) on activated T cells • ATG binds to multiple T-cell specific antigens and causes cell death via complement mediated cytotoxicity • Reduced number of monocytes *in vivo* • Upregulation of the anti-inflammatory M2 macrophage subset CD14+ CD163+ *in vivo*	Sekerkova et al, 2014
Alemtuzumab	• Targets CD52 on B cells, T cells, NK cells, dendritic cells and monocytes • Less effective in depleting monocytes than depleting T cells • Leads to a relative high expression of co-stimulatory molecules, IL-6 and NFκB	Hale et al, 1990 Kirk et al, 2003 Fabian et al, 1993 Rao et al, 2012

Drug type	Effects on monocytes/macrophages	Key references
Calcineurin inhibitors (tacrolimus & cyclosporin)	• No inhibitory effect on p38MAPK phosphorylation, but reduce cytokine production via ERK phosphorylation • Downregulate production of IL-6 and TNF-α after TLR stimulation *in vitro* • Impaired phagocytosis function and promotion of infection (CsA)	Escolano et al, 2014 Howell et al, 2013 Tourneur et al, 2013
Mycophenolate mofetil	• Diminished the production of IL-1β, IL-10 and TNF-α and decreased expression of TNF-receptor 1 on monocytes • Reduced monocyte migration through lower expression of adhesion molecules	Alisson et al, 2000 Weimer et al, 2003
Glucocorticoids	• Lower CD14+CD16++monocyte counts • Lower expression of B7 molecules leading to disturbed co-stimulation • Induction of anti-inflammatory response via increased IL-10 production • Impaired phagocytosis function	Rogacev et al, 2015 Girndt et al, 1998 Hodge et al, 2005 Blotta et al, 1997 Rinehart et al, 1974
mTor inhibitors	• Decreased chemokine and cytokine production • Combination therapy with steroids increased pro-inflammatory cytokine production	Lin et al, 2014 Oliveira et al, 2002 Weichhart et al, 2011
Belatacept/ abatacept	• Block CD80/86 molecules on antigen-presenting cells and inhibit co-stimulatory function • Lower migration and adhesion capacity • Decreased expression of the pro-inflammatory cytokines IL-12 and TNF-α	Latek et al, 2009 Bonelli et al, 2013 Wenink et al, 2011
Experimental drugs	• Canakinumab inhibits IL-1β production by monocytes • Sinomenine is associated with less monocyte migration, differentiation and maturation • 15-deoxyspergualin decreases monocyte proliferation, TNF-α production, phagocytosis and antigen presentation • Simvastatin and salsalate are associated with less monocyte activation and inhibition of IL-6 and IL-8 production in diabetes patients • Tocilizumab inhibits IL-6 production by monocytes • BET inhibitors are involved in epigenetic control of monocytes thereby preventing inflammation • Fish oils are associated with lower numbers of macrophages in obesitas patients and a reduced secretion of TNF-α *in vitro*	Hoffman et al, 1993 Ou Y et al, 2009 Wang et al, 2011 Perenyei et al, 2014 Donath et al, 2011 McCarty et al, 2010 Tono et al, 2015 Chan et al, 2015 Spencer et al, 2013 Zhao et al, 2013 Jialal et al. 2007

ATG anti-thymocyte globulin; **IL** interleukin; **NFκB** nuclear factor kappa-light-chain-enhancer of activated B cells; **MAPK**; mitogen-activated protein kinases; **ERK** extracellular signal–regulated kinase; **CsA** Cyclosporin A; **TNF** tumor necrosis factor; **BET** bromodomain and extra-terminal motif

Monocyte immunobiology

Monocytes and macrophages are mononuclear phagocytes with crucial and distinct roles in transplant immunity. Monocytes display a remarkable plasticity in response to signals from the microenvironment, enabling them to differentiate into various cell types. Several pro-inflammatory, metabolic and immune stimuli all increase the attraction of monocytes towards tissue [7]. Based on the expression of CD14 (LPS co-receptor) and CD16 (Fcγ receptor III), three phenotypically and functionally distinct human monocyte subsets: CD14++CD16- (classical), CD14++CD16+ (intermediate), and CD14+CD16++ (non-classical) monocytes can be defined [28-31]. Monocytes arise from myeloid precursor cells in primary and secondary lymphoid organs, such as liver and bone marrow. In humans, monocytes represent respectively 10% of the nucleated cells in peripheral blood, with 2 major reservoirs: the spleen and lungs that can mobilize monocytes on demand [32, 33]. Classical monocytes are able to start proliferating in the bone marrow in response to infection or tissue damage, and subsequently be released into the circulation in a CCR2 dependent manner (Figure 1) [34]. Intermediate and non-classical monocytes are thought to be descendants of classical monocytes that have been under control of transcription factor Nur77 (NR4A1) returned to the bone marrow [35]. Non-classical monocytes show a patrolling, distinct motility and crawling pattern [36]. Interestingly, intermediate monocytes show higher expression of major histocompatibility (MHC) class II molecules and thereby more related to non-classical monocytes [37, 38]. CD14+ monocytes can be recruited to the site of inflammation or areas of tissue injury where they can differentiate into macrophages and dendritic cells [39]. In steady state, circulating monocytes have minimal contribution to the maintenance of tissue resident macrophages [40, 41]. Depending on the microenvironment, activation stimuli and cross talk with other immunological effector cells, activation of macrophages alters their cytokine profile and co-stimulatory molecule expression. Monocyte differentiation to tissue macrophages is Colony Stimulating Factor 1 Receptor (CSF1R) dependent. Most tissue macrophages are seeded before birth in embryonic state, with varying contributions of primitive-derived and definitive-derived cells. Monocytic input to tissue macrophage compartments seems to be restricted to inflammatory settings, such as infection and acute graft rejection [39]. Monocyte chemotactic peptide-1 (MCP-1) is an important regulator of macrophage recruitment and was shown to be highly expressed in the kidney allograft, supporting the concept of recruitment of monocytes from the circulation [42].

Macrophages can be subdivided in ‘classically activated’ or ‘alternatively activated’. Classically activated macrophages are described as M1 macrophages, which are developed upon response to IFNγ, LPS or TNF-α. M1 macrophages express surface markers: MHCII, CD40, CD80, CD86 and CD11b. They can produce inflammatory cytokines such as: TNF-α, IL-1, IL-6, IL-8, IL-12, CCL2, CXCL9 and CXCL10. M1 macrophages are linked to the Th1 response and are mainly considered as pro-inflammatory macrophages whereas M2 are considered as mainly anti-inflammatory. M2 macrophages can be subdivided in M2a, M2b and M2c. M2a macrophages are generated on response to IL-4 and IL-13. Immune complexes and

TLR/IL-1R ligands activate M2b macrophages whereas M2c macrophages are activated by IL-10, TGF-β and glucocorticoids. M2 macrophages express surface markers: CD163, CD206 and CD209. M2 macrophages produce IL-10 and TGF-β mainly leading to tissue repair and scar formation. M2 macrophages are linked to Th2 response and show immune-modulatory functions [7, 39, 43]. Human regulatory macrophages (Mregs) are in a specific state of differentiation with a robust phenotype and potent T-cell suppressor function. These Mregs arise from CD14+ peripheral blood monocytes during 7-day culture exposed to M-CSF and activation by IFNγ [44]. Mregs express several molecules such as MHCII, FCγR, IFNγR, TLR-4 and PD-L1 as shown in Figure 1 [45]. Shifting the balance between regulatory macrophages and/or monocytes on the one hand, and the effector macrophages and proinflammatory monocytes on the other hand could theoretically result in dampening the immune response against the graft and the immunological tolerance, or to aggravation of graft rejection. To date, two clinical trials investigated the feasibility of regulatory macrophages in promoting allograft acceptance with promising results [46, 47]. Moreover, recently, a new homogeneous monocyte subpopulation of human G-CSF induced CD34+ monocytes with powerful immunosuppressive properties upon human allogeneic T-cell activation was described. Such tolerogenic monocytes could be used for novel immune-regulatory or cellular therapy development [48].

Recently, an adaptive feature of innate immunity has been described as “trained immunity”. Trained immunity is defined as a nonspecific immunological memory resulting from rewiring the epigenetic program and the functional state of the innate immunity [49]. Twenty naïve patients were vaccinated for bacille Calmette-Guérin (BCG) to investigate mechanisms of the enhanced immune function. Interestingly, these authors identified trained monocytes in the circulation of *BCG*-vaccinated individuals for at least 3 months suggesting that reprogramming takes place at the level of progenitor cells in the bone marrow [50]. Recent evidence emerged to indicate that innate immune memory could be transferred via hematopoietic stem and progenitor cells. In vitro studies showed effects lasting for days [51, 52], whereas other reports showed memory effects for weeks [53]. These interesting observations might be explained by alterations in epigenetic (de)methylation profiles after antigenic stimulation. Altering the epigenetic program by pharmacological means leading to behavioral changes of monocytes could be a promising method to restore or modify the healthy gene/protein expression in the pro-inflammatory microenvironment. The phenomenon of trained immunity in alloreactivity and transplantation may be a very interesting area of future research: i.e. innate memory towards donor antigens resulting from cross-reactivity with other microbial and/or viral agents.

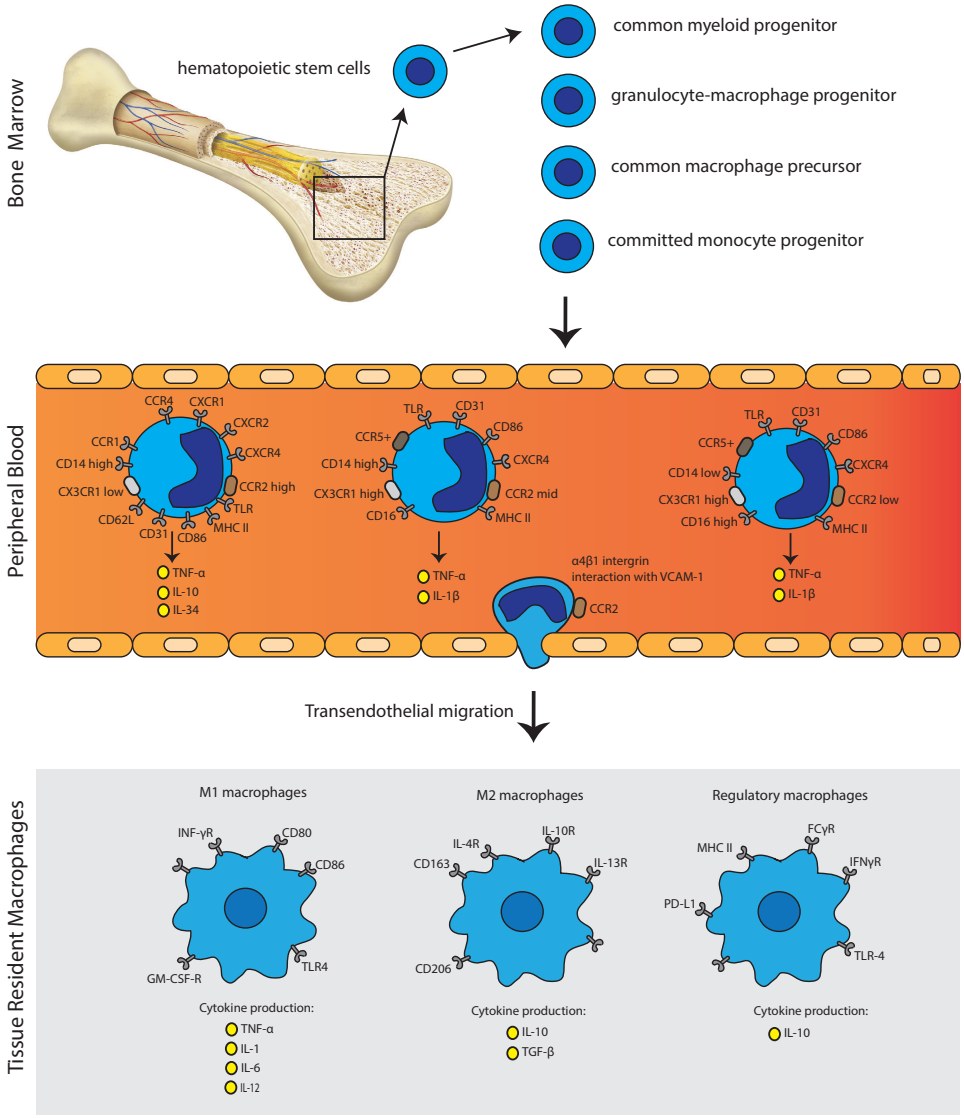

Figure 1. Monocyte immunobiology

Monocytes arise from myeloid precursor cells in primary lymphoid organs, including liver and bone marrow. In the peripheral blood, monocytes can be subdivided in three distinct subsets according to their CD14 and CD16 expression profile. Monocytes can undergo transendothelial migration through α4β1 integrin interaction with VCAM-1. Activation of monocytes is followed by the polarization of macrophages to acquire proinflammatory phenotype (M1), anti-inflammatory phenotype (M2) or the regulatory phenotype (Mreg). The secretion of distinct pro- or anti-inflammatory cytokines, next to expression patterns of surface molecules characterizes each phenotype.

rATG and basiliximab and monocyte/macrophage cell lineage

Rabbit Anti-thymocyte globulin (rATG) is a polyclonal antibody with mainly T cell depleting capacities. rATG can also induce B cell apoptosis, and stimulates Treg and NKT cell generation [54]. After rATG treatment, cytokine dependent homeostatic proliferation of T cells is initiated [55]. Basiliximab (anti CD25 monoclonal antibody) blocks the CD25 receptor on the surface of activated T cells. Studies on the effects of basiliximab or rATG on monocytes/macrophages are scarce. However, one report showed a reduction in the percentage of CD14+CD16+ monocytes when PBMC were cultured *in vitro* in the presence of rATG [56]. In contrast, this cell type was not affected by basiliximab, although low expression levels of CD25 on stimulated monocytes and macrophages are described [57, 58]. These authors also reported a reduction of circulating CD14+CD16+ monocytes in kidney transplant patients treated with rATG during the first week after transplantation, while this was not seen for basiliximab induction therapy. Another part of the same study showed an upregulation of the percentage of CD14+CD163+ monocytes in either basiliximab or rATG -treated kidney transplant recipients, which could be detected for a longer time period in the circulation than in patients without induction therapy. CD14+CD163+ monocytes are precursors for M2 macrophages and these cells are well known for their anti-inflammatory effect, suggesting that the upregulation of CD14+CD163+ cells may contribute to a better outcome after transplantation. However, this study only described the changes in the CD14+CD16+ monocyte subset after rATG or basiliximab therapy, while the effect on other subsets such as the classical CD14++CD16- monocytes remains unknown. Therefore, it is unclear whether the pro-inflammatory immune response by monocytes is changed in the presence of rATG or basiliximab.

Alemtuzumab and monocyte/macrophage cell lineage

The humanized monoclonal antibody alemtuzumab targets the CD52 molecule which is expressed at different levels on B cells, T cells, NK cells, dendritic cells and monocytes. The CD52 molecule, also known as CAMPATH-1 antigen, is a glycoprotein of which the precise function is unclear, although it might be involved in T-cell migration and co-stimulation [59]. However, monocytes are known to be less sensitive for the depleting effects of alemtuzumab than lymphocytes, despite their high CD52 expression [60-63]. For example, in acute cellular rejection dominated by monocytes, alemtuzumab treatment did not show depletion of monocytes in tissue, confirming the low sensitivity of monocytes to alemtuzumab treatment [64]. An explanation for this low susceptibility could be the high expression levels of complement inhibitory proteins, which protect monocytes from complement mediated lysis [63]. Another study showed repopulation of monocytes within 3 months after alemtuzumab therapy, while the recovery of T and B cells takes usually more than 1 year. Consequently, the low susceptibility of monocytes for alemtuzumab is thought to be one of the reasons for renal graft dysfunction after induction therapy with alemtuzumab, such as

reperfusion and rejection [65]. So far, this low susceptibility of monocytes to alemtuzumab therapy could be partially explained by the high expression of complementary inhibitory proteins that protect monocytes from getting lysed after alemtuzumab treatment [63]. After alemtuzumab treatment, tissue monocytes in the rejecting graft showed an increased expression of the co-stimulatory molecules CD80 and CD86, a higher intracellular expression of NFκB and stronger production of IL-6 compared to patients without alemtuzumab therapy [61]. Moreover, this pro-inflammatory cytokine production could facilitate kidney allograft rejection after alemtuzumab therapy, although other cell types, such as NK cells, could also contribute to rejection processes after alemtuzumab therapy [66].

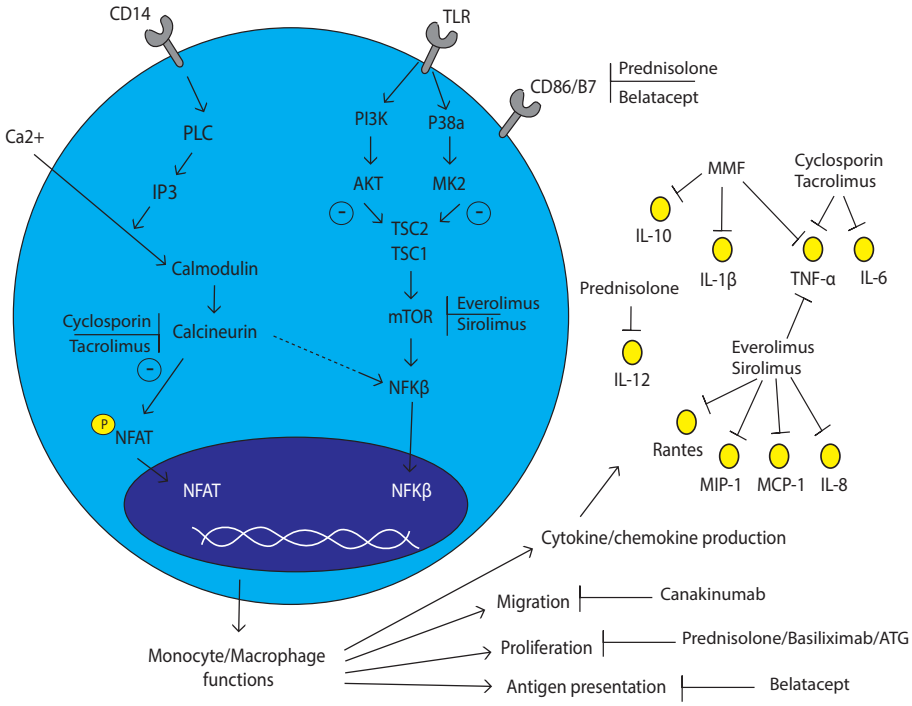

Figure 2. Monocyte and macrophage lineage cell and the effect of immunosuppressive drugs.
The effect of currently prescribed immunosuppressive drugs with several inhibition spots on and in monocyte/macrophage lineage cells.

Calcineurin inhibitors and monocyte/macrophage cell lineage

Tacrolimus and cyclosporine A inhibit the calcineurin pathway in T cells, which is also present in other cell types. As a consequence, the activation of the Nuclear Factor of Activated T cells (NFAT) is blocked, leading to a reduced production of IL-2 and IFN-γ by T cells [67, 68]. Calcineurin inhibitors (CNI) also have an effect on the MAPK signalling pathway via the inhibition of p38MAPK phosphorylation and consequently, reduced production of cytokines,

such as IL-2, IL-10, TNF-α and IFN-γ [69]. The calcineurin and MAPK pathway are also present in macrophages, although the inhibitory effects of CNIs on T cells and macrophages are different [70]. In more detail, tacrolimus was found to have no inhibitory effect on p38MAPK phosphorylation at low (5 ng/ml) and high (50 ng/ml) concentrations in LPS-activated monocytic THP-1 leukaemia cells [71]. However, another member of the MAPK pathway, ERK, did show less phosphorylation in the presence of a high concentration (50 ng/ml) of tacrolimus in monocytes as measured by western blotting, leading to a lower production of TNF-α. Kang et al. reported that monocyte signalling pathways were activated instead of inhibited by CNI via the inhibition of the calcineurin pathway and, as a consequence, the activation of the NFκB signalling pathway [70]. However, the concentrations of CNIs used in this study were supratherapeutic. Therefore, the observed induction in cytokine production, shown in this study, could also be explained by toxic lysis of the monocytes [72]. Overall, these studies suggest that CNIs cannot supress the activation of monocytes to the same degree as in T-cells.

Recognition of damage-associated molecular patterns (DAMP's) by toll like receptors (TLR) on the surface of monocytes leads to the activation of these cells and plays an important pathogenic role during transplant rejection [73-75]. Both tacrolimus and cyclosporine can inhibit TLR signaling of PBMC in liver transplant patients, as shown by decreased production of IL-6 and TNF-α after TLR stimulation [72]. CNIs act differently in suppressing the cytokine production upon TLR activation. For example, cyclosporine inhibits the production of TNF-α mediated by TLR7/8 and the production of IL-6 mediated by TLR2 and TLR7/8 signalling significantly more than tacrolimus [72]. Moreover, monocytes from renal transplant recipients treated with tacrolimus showed an increased production of IL-1β, TNF-α, IL-6, IL-10 after stimulation with LPS, in comparison to cyclosporine treated patients [76]. Thus, the effect of CNIs on monocytes differs between tacrolimus and cyclosporine.

The different outcomes of tacrolimus and cyclosporine on cytokine production concerns only one of the monocyte/macrophage functions. Bacterial infections can have a significant impact on the graft after transplantation. Cyclosporine inhibits the phagocytosis of bacteria by macrophages via the alteration of NOD-1 expression. The NOD-1 expression depends on the activation of the transcription factor NFAT, which is the main target of CNI [77]. Thus, cyclosporine can promote bacterial infections after transplantation by altering phagocytic capacity of macrophages more rigorously.

Mycophenolate mofetil and monocyte/macrophage cell lineage

Mycophenolate mofetil (MMF) has led to significantly reduced rejection rate as compared to its counterpart azathioprine [78-80]. The active metabolite mycophenolic acid (MPA) reduces the synthesis of guanosine nucleotides via the inhibition of inosine monophosphate dehydrogenase, which is a more specific metabolic pathway for T and B cells than for other cell types [81, 82] .

Circulating monocytes of kidney transplant recipients suffering from chronic rejection who were treated with MMF showed a decreased capacity to produce IL-1β, IL-10 and TNF-α as compared to circulating monocytes of chronic rejection patients who were not treated with MMF. Cytokine production capacity was measured by flow cytometry and confirmed by PCR on gene expression level [83]. Moreover, the expression of the TNF-receptor 1 was decreased in the MMF treated group, suggesting a favourable effect in patients with chronic rejection [83]. Furthermore, MMF reduced the expression of the adhesion molecules; intercellular adhesion molecule (ICAM)-1 and MHC II on isolated human monocytes [84].

Glucocorticoids and monocyte/macrophage cell lineage

The immunosuppressive and anti-inflammatory effects of glucocorticoids are redundant and cover different stages of alloreactivity triggered by activation of donor-specific T cells after transplantation. Steroids can bind via passive diffusion to the intracellular glucocorticoid receptor. After translocation to the nucleus, steroids bind to the glucocorticoid response elements (GRE's) that have a connection with promotors of different genes. The anti-inflammatory effect of glucocorticoids is based on the transrepression of inflammatory gene transcription, such as the inhibition of the transcription factors AP-1 and NFκB, and the transactivation of anti-inflammatory genes, including tyrosine aminotransferase (TAT) and the induction of IκB [85-88]. In this way, glucocorticoids control antigen presentation, cytokine production and proliferation of lymphocytes.

In monocytes, glucocorticoids specially affect the heterogeneity of monocyte subsets [89-91]. Flow cytometric analysis revealed that steroid treatment of stable kidney transplant patients for more than 12 months is associated with an increased absolute number of CD14++CD16- and CD14++CD16+ monocyte subsets compared to patients without steroid intake. As a consequence, the counts for the non-classical CD14+CD16++ monocyte subset, were significantly lower [89]. Furthermore, glucocorticoids inhibit the upregulation of B7 molecules on the surface of human monocytes, which can negatively affect the antigen presenting function of the cell [92, 93]. The B7 family consists of many peripheral membrane proteins, including CD80 and CD86, which are all involved in the co-stimulatory signal needed for T cell activation. This suggests that glucocorticoid therapy in combination with belatacept therapy (blocking CD80/CD86) could theoretically block the immune response by T cells induced via antigen presenting monocytes after transplantation.

The production of the anti-inflammatory cytokine IL-10 by monocytes is increased under treatment with methylprednisolone while the production of the pro-inflammatory cytokines IL-12, IL-1 and TNF-α are down-regulated in the presence of glucocorticoids [94, 95]. Addition of 16 μg/ml of glucocorticoids *in vitro* leads to a decreased uptake of bacteria by monocytes, indicating that the phagocytosis of bacteria by monocytes is downregulated [96]. Glucocorticoids are also known to drive the polarization of macrophages to a M2 phenotype [43, 97]. This indicates that glucocorticoids drive the cytokine production by monocyte to

a more anti-inflammatory phenotype and inhibits the phagocytic function of monocytes. Glucocorticoids enhance the uptake of apoptotic cells by macrophages trough the induction of Mer-Tk (MER proto-oncogene tyrosine kinase), thereby inducing macrophage reprogramming toward a regulatory phenotype, also called Meff, for macrophages performing efferocytosis [98-100]. This approach has been evaluated in the treatment of collagen-induced arthritis (Bonnefoy F et al., Arthritis Res Ther. 2016 Aug 11;18(1):184), as well as acute graft rejection (Wang Z et al., Am J Transplant. 2006 Jun;6(6):1297-311.) justifying further exploration in the field of transplantation.

Inhibitors of the mammalian target of rapamycin (mTOR) and monocyte/macrophage cell lineage

The mammalian target of rapamycin (mTOR) signalling pathway is involved in the activation, proliferation, differentiation and translocation of T cells. Inhibitors of mTOR, such as everolimus and sirolimus, are therefore very useful after transplantation [101]. The same mTOR inhibitors do also have an inhibitory effect on human monocytes by suppressing the production of the chemokines MCP-1, RANTES, IL-8, MIP-1α and MIP-1β [102]. Furthermore, the downstream effects of rapamycin therapy are characterized by a decreased production of the monocyte-derived cytokine IL-6 and an increase of TGF-beta production in comparison to MMF, as it was shown by fine-needle biopsy cultures from kidney transplant patients treated with either a cyclosporine-rapamycin-prednisone or a cyclosporine-MMF-prednisone therapy one week after transplantation [103]. This resulted in a more tolerogenic effect of the monocytes and less graft rejection during the first 6 months after transplantation in comparison to a MMF based drug therapy. Moreover, combined therapy of mTOR inhibitors and glucocorticoid therapy increased the production of the pro-inflammatory cytokines IL-12, TNF-α and IL-1β [104]. Altogether, mTOR inhibitors can inhibit cytokine production by monocytes shortly after transplantation, although a combination therapy with prednisone should be regarded with caution.

Belatacept and monocyte/macrophage cell lineage

Belatacept, a fusion-protein consisting of the extracellular domain of the human cytotoxic T-lymphocyte antigen (CTLA)-4 antigen linked to a Fc-fragment of immunoglobulin G1 (IgG1), inhibits the co-stimulatory signal between the CD80/CD86 molecules on antigen presenting cells and the CD28 molecule on T cells, thereby preventing T cell activation [105]. Monocytes express CD80/CD86 molecules and, as a consequence, the antigen presenting function of monocytes is blocked by belatacept [106-108]. This suggests that belatacept inhibits the antigen presenting function of monocytes/macrophages. In one case of acute rejection within 3 months after transplantation, the blockade of CD80/CD86 was incomplete under belatacept treatment, suggesting the importance of higher belatacept tissue concentrations needed to completely block monocyte antigen presentation function [106].

Thus belatacept, in controlled dosages, blocks the expression of CD80/CD86 on monocytes, thereby inhibiting their antigen presenting function and activation of T cells.

The older variant of belatacept, abatacept (CTLA-4Ig), is frequently used in the treatment of patients with rheumatoid arthritis (RA) [109]. After treatment with abatacept, the number of circulating monocytes was increased, and the phenotype of these cells was significantly changed, due to down regulation of actin fibers. For example, the capability of monocyte migration was negatively changed even as the number of adhesion molecules *in vitro*. Data were verified with monocytes from healthy controls. The reduced number of adhesion molecules and migration capacity could be a reason for the increased number of monocytes in the peripheral blood that cannot pass endothelial barriers, whereby it is no longer possible for the monocyte to contribute in inflammation.

Binding of abatacept to the CD80/CD86 receptor on macrophages from healthy blood donors is associated with decreased production of the pro-inflammatory cytokines IL-12 and TNF-α, suggesting again a role for abatacept/belatacept in changing the pro-inflammatory environment via macrophages after transplantation [110].

Other experimental drugs and monocyte/macrophage cell lineage

Although no monocyte specific drugs as such exist now, multiple experimental and less known drugs do influence monocyte functions. Looking outside the box of currently used immunosuppressive drugs in solid organ transplantation, there are a few compounds with immune-inhibitory effects, which theoretically could be interesting in combating alloimmunity. For example, the human monoclonal antibody canakinumab, originally designed as an interleukin-beta (IL-1β) inhibitor for the repression of inflammation in autoimmune diseases, can also inhibit the IL-1β production by monocytes [111]. A high expression of IL-1β is noticed in the most severe liver transplant rejection episodes and at the time of kidney transplantation, suggesting the importance of blocking its production by monocytes [112, 113]. However, treatment of kidney transplant recipients with canakinumab can inhibit IL-1β secretion in many other cell types, leading to undesirable side effects [114].

Infliximab, originally used in the treatment of autoimmune diseases, is another monoclonal antibody targeting monocyte TNF-α production. Monocytes and macrophages are main producers of TNF-α, suggesting the importance of infliximab for targeting monocytes [115]. Beside the effect on TNF-α production, monocytes from Crohn's disease patients treated with therapeutic concentrations of infliximab showed also increased apoptosis via the activation of caspase-3, 8 and 9 [116].

Furthermore, the herbal medicine sinomenine was found to reduce migration of activated human monocyte cells and inhibits human monocytes-derived DC differentiation and maturation [71, 117]. In addition, peripheral blood monocytes from healthy donors cultured for 60 hours in the presence of different concentrations of sinomenine showed an enhanced

production of IL-6 and a decreased expression of IL-8, which is important for cell migration [118]. This would suggest a positive effect of sinomenine on monocyte infiltration and migration, although there is still an increased production of pro-inflammatory cytokines. However, this research was performed using monocytic THP-1 cell-line, and isolated peripheral blood monocytes from healthy donors, so that possible effects with regard to transplantation are still unknown.

15-deoxyspergualin or gusperimus is a relatively long known immunosuppressive drug with an inhibitory effect on monocyte proliferation, TNF-α production and phagocytotic functions of monocytes. More recently, it was been suggested that gusperimus can also be effective in suppressing the antigen presentation function of monocytes in transplantation [119]. Another member of the spergualin family is LF15-0195. This drug is known for its inhibitory effect on monocyte accumulation in the tubulo-interstitial compartment of rat kidneys and was shown to have beneficial effects in the treatment of glomerulonephritis [120].

In diabetes mellitus, macrophage accumulation and activation play a central role in disease progression. Research on simvastatin, a drug to lower elevated lipid levels, has been shown to effectively lower IL-6, IL-8, TNF cytokine and superoxide anion production by monocytes isolated from human blood samples of patients with diabetes mellitus type 1 [121]. In addition, simvastatin reduces the NFκB activity in monocytes with approximately 60%, which causes the inhibition of IL-6 and IL-8 production. Treatment of IgA nephropathy with the drug atorvastatin showed a reduction of monocyte proliferation [122]. In diabetes mellitus type 2 patients this drug lowers the TNF-alpha production by monocytes [123]. Other studies in diabetes mellitus patients have shown potential effects of salsalate on macrophages activation. Salsalate, a prodrug of salicylic acid, is also known for the inhibition of the NFκB pathway in macrophages [124, 125]. This suggests a working mechanism for salsalate that is similar to simvastatin. Both drugs can be promising compounds to inhibit monocyte and macrophage activation.

In RA, research on therapeutic drugs to target monocytes and macrophages is more common because of the important role of monocytes in developing this disease. In addition, TNF-α is a key player known to cause inflammation in RA and is mainly produced by monocytes [126]. Some of the drugs used to suppress inflammation in RA could also have a potential in transplantation. For example, a decreased number of CD14+ CD16+ monocytes was found after treatment of RA patients with tocilizumab, an IL-6 receptor blocker [127]. In addition, production of IL-6 by monocytes from healthy donors was reduced when tocilizumab was added *in vitro*. The drug also induces the apoptosis of SEB (staphylococcal enterotoxin B)-activated monocytes [128]. These results suggest that tocilizumab could theoretically impair the monocyte responses after transplantation. Furthermore, bromodomain and extra terminal (BET) inhibitors are developed to control the intracellular chromatin regulation responsible for the activation of monocytes, thereby inhibiting inflammation processes

induced by monocytes. In more detail, CD14+ monocytes were isolated from blood samples of healthy volunteers and cultured in the presence of BET inhibitors and IFN-β, IFN-γ, IL-4 and IL-10 stimuli, where after the intracellular activation cytokine response were suppressed [129]. In RA patients this epigenetic control by BET inhibitors could suppress the production of pro-inflammatory cytokines and chemokines such as CXCL10. This would indicate that BET inhibitors could also inhibit monocyte activation after transplantation, although this is very speculative and require more research.

Fish oil based drugs, such as lovaza, are used to lower triglyceride levels in obesity. These fish oil compounds demonstrated a reduction in the number of macrophages and reduced MCP-1 blood levels [130]. Eicosapentaenoic acid, one of the major fatty acids in fish oil, reduces the secretion of TNF-α by human monocytic THP-1 cells, via the inhibition of the intracellular NFκB activation [131]. This suggests also a suppressing role for fatty acids in monocyte activation that could have a potential effect in transplantation as well.

Future challenges and developments

Therapies targeting monocytes and macrophages in (SOT) could intervene at different points with monocyte actions and their subsequent functions (Figure 3). First, the activation and function of the cells can be inhibited at multiple stages: Signaling pathway activation, antigen presentation and cytokine production. Blockade of the intracellular signaling pathways inhibits the activation of monocytes and macrophages. For example, the use of specific MAPK inhibitors, such as SB203580, blocks the activation of monocytes [132]. However, these drugs will also block the activation of many other cell types. Targeting antigen presentation is even more difficult than targeting signaling pathways. It is known that the Fcγ-receptor on monocytes is involved in the recognition and processing of donor antigen specific antibodies [133, 134]. Blocking this receptor with specific antibodies could inhibit the antigen presentation function of monocytes. Furthermore, already existing drugs that reduce the cytokine production by monocytes and macrophages, for example canakinumab, infliximab and tocilizumab, mainly target the inhibition of one single cytokine. To be more effective, monocyte specific drugs should be developed to inhibit the production or the effects of multiple cytokines at once, thereby reducing side effects.

Second, delivering any potential new drug to the target cell, in this case, monocytes and macrophages, is a major point of intervention, which could lower the side effects. One can envision a delivery system using the phagocytosis function of the monocyte/macrophage, whereby macrophages can ingest immunosuppressive drug loaded-inactivated bacteria or liposomes carrying the potential new drug [135]. However, the monocyte is not the only cell type with a phagocytic system. Therefore the surface of these bacteria or liposomes should be modified to facilitate the specific recognition by the monocyte/macrophage in order to

overcome side effects. Another approach to target monocytes and macrophages via their phagocytotic function is to use apoptotic cells through a process that is known as efferocytosis [99, 100]. Phagocytosis of these apoptotic cells by monocytes and macrophages will induce an anti-inflammatory response at the tissue level and may induce immunological tolerance. Furthermore, *ex vivo* experiments showed a decrease in CD11b expression on macrophages [136], suggesting that treatment with apoptotic cells induces the generation of Mregs. As mentioned above (paragraph "Glucocorticoids and monocyte/macrophage cell lineage"), the uptake of apoptotic cells can be enhanced by treatment with glucocorticoids [100].

The third point of therapeutic efficacy would be the manipulation of the nature of these cells. The future of *in vivo* manipulation of macrophages is intriguing; phenotypes could be changed by transfection with adenovirus, modulation of nuclear transcription factor NR4A1 (Nur77) or by modulation of local microenvironment with cytokines to polarize macrophages to reparative phenotype [35]. Targeting all monocytes and macrophages indiscriminately could also be a disadvantage as regulatory and effector macrophages also have beneficial effects including the control of infections and the induction of regulatory cells [137].

Moreover, inhibition of all macrophages will also affect the number of Mregs, which are important for inducing tolerance after transplantation [138]. Too much inhibition of effector macrophages or Mregs could lead to graft rejection or complications, such as atherosclerosis and cardiovascular diseases. Furthermore, currently pre-scribed immunosuppressive drugs might miss the power to upregulate Mregs efficiently. In experimental mouse models, Mregs have demonstrated anti-inflammatory and T-cell suppressing effects (Other beneficial effects of Mregs are described in the paragraph "Monocyte immunobiology" [139, 140]. A more specific upregulation of these cells could be an approach to beneficially shift the balance towards macrophages controlling immune responses including those in organ transplant patients. Ideally, after SOT, the balance of macrophage subsets should be in favour of macrophages that control the anti-donor response, while the accumulation of macrophages with pro-inflammatory and antigen presentation characteristics should be decreased [141, 142]. For example reduced function of the detrimental functions of macrophages involved in alloreactivity might be a useful therapy, although more research is needed to find a specific approach. Another way to differentiate between effector and controlling functions of macrophages could be by polarizing cells into M1 and M2 subsets.

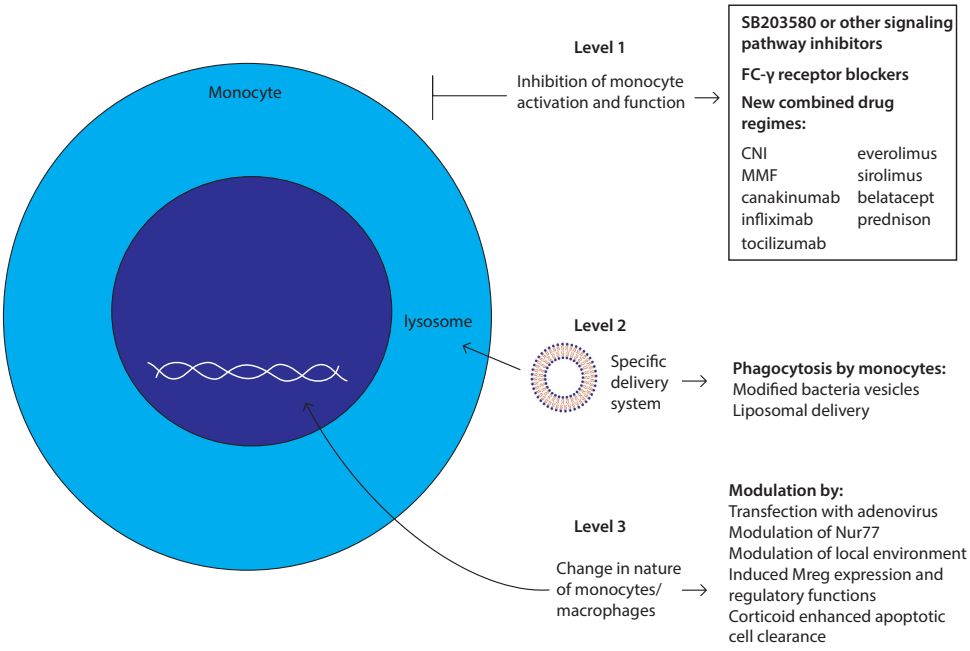

Figure 3. Future challenges and developments: Strategies to target monocytes/macrophages.
New therapies targeting monocytes and macrophages could intervene at three levels with monocyte actions and their subsequent functions as depicted, and described in manuscript body.

Targeting specific signaling pathways involved in this polarization process like the Notch signaling pathway could change the nature of these cells to an more anti-inflammatory phenotype [143]. NFκB signaling, controlled by the Notch pathway, is associated with pro-inflammatory macrophage responses, while a more anti-inflammatory phenotype is induced via the ERK pathway [143, 144]. Targeting these pathways with specific stimuli may change the phenotype of macrophages. Stimuli that induce macrophage polarization towards a M1 phenotype are GM-CSF, IFN-γ and LPS, while IL-4, IL-13 and IL-10 enhance a M2 macrophage phenotype [145]. Future insight and research are necessary to investigate the effect of these manipulated macrophages on healthy and diseased tissue.

Ideally, a potential new drug inhibiting monocytes-macrophages at these three levels would change the spectrum of not only rejection treatment or prevention after (SOT) but also the course of many autoimmune mediated diseases. Either alone or in combination with other existing immunosuppressive drugs, this field constitutes a challenging area of future therapeutic research.

Author Contributions

NMK and TPP contributed in the process of writing/design and discussing, DAH contributed in the process of discussion and reviewing, CCB contributed in the process of writing/design/discussing and reviewing, ATR contributed in the process of writing/design/discussing and reviewing.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose as described by Frontiers in Immunology.

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Chapter 8

Summary and Discussion

Summary

In the last decade it became apparent that apart from cells of the adaptive immune system such as the well-known T and B lymphocytes also cells of the innate immune system i.e., NK cells, DC and monocytes actively contribute to the rejection response after organ transplantation. In this response DC and monocytes present antigen, secrete pro- and anti-inflammatory cytokines, proliferate, differentiate while NK cells secrete cytokines and are cytolytic. The central aim of this thesis is to examine the role of monocytes and macrophages in various types of rejection and in fibrotic processes in immunosuppressed kidney and heart transplant recipients.

In chapter 2, we aimed to identify and correlate monocyte – macrophage profiles in tissue (endomyocardial biopsies) and corresponding blood samples both prior and during an acute cellular rejection episode in heart transplant recipients. At tissue level, striking CD16+ monocyte infiltration was observed during rejection. An interesting observation was done for the infiltrating macrophages. During acute rejection significantly more CD68+CD163+ M2 macrophages were present in the allograft than in specimens without histological signs of rejection. Moreover, acute rejection was associated with severe fibrosis in 1-year biopsies ($p < 0.001$). In the peripheral blood lower frequencies of CD16+ monocytes were found in patients compared to healthy individuals. At the time of rejection, significantly increased CD54 (adhesion molecule) and HLA-DR (activation marker) expression levels were found on peripheral CD16+ monocytes with retained cytokine production potential. The association of acute rejection with development of fibrosis at later stages after transplantation links the consequences of tissue injury with development of more chronic, fibrotic processes which might lead to failure of the allograft at later stages.

The elucidation of the molecular mechanisms underlying the observed cellular shifts in both peripheral blood and allograft may enable the timely adjustment of immunosuppressive drugs aiming to improve outcome after transplantation. In addition, it may lead to the discovery of molecular biomarkers that are also helpful in finding molecular targets for specific immunosuppressive therapy.

In chapter 3, we hypothesized that there are significant compartmental differences in monocyte – macrophage infiltration between different types of rejection that affect the outcome of kidney transplants. To address this hypothesis, we studied 48 kidney transplant rejection biopsies diagnosed according to the latest Banff 2015 by staining for monocyte and macrophage subtypes. Next, we correlated the histopathological findings with other rejection-associated variables in time up to 12 months after the biopsy procedure using multivariate analysis. We found that CD163+CD68+ M2 type macrophages were associated with clinical diagnoses of rejection, regardless of Banff category. Importantly, we found that CD163+CD68+ macrophages were associated with worse kidney graft function in the long-

term. Compartmental infiltration analysis showed that glomerular and perivascular infiltration of monocyte subtypes and tubulointerstitial infiltration of mainly CD16+ monocytes were associated with rejection. Furthermore, we learnt that there are significant compartmental differences in the distribution of different macrophage and monocyte subtypes between c-aABMR vs. a-aABMR, and a-aABMR vs. TCMR II/ I. Compartmental infiltration showed potential diagnostic patterns as shown by glomerular monocyte infiltration during c-aABMR, prominent perivascular presence of CD68+ CD163+ in a-aABMR and TCMRII, and high perivascular CD16+ monocyte infiltration during TCMR II. Comparing a-aABMR to TCMR I/II; glomerular CD16+ monocyte infiltration hallmarked TCMR I, while perivascular infiltration of mainly CD16+ monocytes and CD68+CD163 + macrophages signified TCMR II. Glomerular CD68+ CD163+ macrophage infiltration was significantly higher in a/a ABMR. Comparing a-aABMR with c-aABMR; we found that a prominent glomerular infiltration by all monocyte subsets in c-aABMR, also significantly more CD14+ perivascular monocyte distribution was observed in c-aABMR. In this study, perivascular infiltration by CD68+CD163+ macrophages signified a-aABMR. Further research to the underlying compartmental mechanisms of specific patterns together with larger validation studies will set a benchmark about the role of monocytes-macrophages in rejection processes.

In chapter 4, we questioned if differences in monocyte subset composition of the pretransplantation blood samples associated with rejection after kidney transplantation. Recipients who developed an acute rejection response had higher absolute numbers of CD16+ monocytes at the time of transplantation than patients who remained free from this complication. Higher absolute numbers of classical CD14+ monocytes were associated with lower rejection risk. In a pilot-case-control study, we studied 6 kidney transplant rejection biopsies and timely matched kidney transplant control biopsies without rejection showing normal transplant tissue. We found higher numbers of tissue resident CD16+ monocytes in rejection biopsies. In parallel, we observed a clear decrease in circulating CD16+ monocytes indicating the migration of these proinflammatory monocytes from the circulations towards rejecting kidney transplant tissue. Altogether, our data show that higher pretransplant numbers of CD16+ monocytes are associated with a significantly higher rejection risk, shorter rejection free survival, and may serve as an early biomarker to predict acute rejection after kidney transplantation.

In chapter 5, we focused on tissue expression of micro(mi)RNAs in 32 kidney rejection biopsies and if these profiles discriminate between the different Banff rejection categories. We compared microRNAs expression profiles between a-aABMR vs. aTCMR and a-aABMR vs. c-aABMR, and performed pathway analyses to search for the target proteins and the contributions of specific miRNAs in the rejection process. We identified 55 differentially expressed microRNAs between a-aABMR and aTCMR. MicroRNA-100-5p was identified as one of the most differentially expressed miRNA's. From the literature it is known that this microRNA-100-5p controls the expression of CD209 (DC-SIGN) which is expressed by both

macrophages and dendritic cells and contribute to the rejection process by subverting the alloimmune response [1]. The microRNAs found in this study are all part of immune and inflammation pathways which again shows the dominant role of these pathways in the anti-donor response. This pathways are enriched with immune cells such as lymphocytes, monocytes – macrophages, NK cells and dendritic cells. These cells are all involved during rejection of the allograft. Nevertheless, it is clear that more studies are warranted in larger cohorts also including samples showing no signs of rejection i. to validate our findings, and ii. to determine the association of specific miRNAs with either a-aABMR or aTCMR.

Because IFN-γ plays a pivotal role in both innate and adaptive immunity we studied in **chapter 6**, the ability of monocytes to produce this cytokine. For these studies we used peripheral blood samples of healthy individuals and immunosuppressed kidney transplant recipients. Upon LPS stimulation increased IFN-γ mRNA and protein levels were produced by CD14+ monocytes from both controls and patients. Interestingly, we noticed that stimulation of freshly isolated CD14+ monocytes of healthy individuals and transplant patients by LPS alone or in combination with IFN-γ, lead to a significant increase in the percentage of TNF-α and IFN-γ producing monocytes. Thus, monocytes do have the capacity to produce IFN-γ, an important mediator in the rejection process.

In chapter 7, we discussed the effects of currently given immunosuppressive drugs on the monocyte – macrophage functions as well as future opportunities for the development of novel immunosuppressive drugs for transplantation. After reviewing the literature, it was clear that surprisingly little is known about the impact of immunosuppression on monocyte-macrophages functions. There is some first evidence that immunosuppressant's such as MMF and mTOR inhibitors have limited inhibitory effects. Also CNIs do suppress the activation of monocytes but not to the same degree as in T-cells, described effects of immunosuppressive drugs on monocytes-macrophage functions include: inhibition of cytokine transcription and subsequent production, and inhibition of migration of these cells to the site of rejection. In addition to the literature focusing on these immunosuppressive drugs, we speculate that future studies targeting the monocyte-macrophages should be designed aiming to i: inhibit monocyte activation and function, ii: develop specific delivery systems via lipid vesicles and iii. manipulate the nature of monocytes and macrophages by modulation of Nur77 or modulation of the microenvironment and polarize them towards reparative and anti-inflammatory phenotypes. Ideally, new immunosuppressive drugs should inhibit the monocyte/macrophage at all these levels, which might prevent and also can be used to treat rejection.

Discussion

The overall aim of this thesis is to improve our understanding about the role of monocytes – macrophages in rejection responses after transplantation required to achieve improved graft outcomes. Presence of high numbers of monocyte-macrophages has been associated with poor allograft function and survival [2-4]. Here, we analyzed whether specific monocyte-macrophages profiles in grafted tissue and circulation are associated with biopsy proven rejection in heart transplant recipients [2-4]. The decreased CD16+ monocytes and increased CD16- monocytes frequencies in the peripheral blood suggest transendothelial migration of pro-inflammatory CD16+ monocytes to the allograft tissue at the time of rejection [5, 6].

In addition, presence of CD16+ monocytes in the allograft was associated with advanced vascular dysfunction in patients who develop early coronary artery disease [7]. In line with these findings we also found a shift in the balance between CD14+ CD16+ monocyte subsets and M1-M2 macrophages that were associated with the presence of fibrosis after rejection in heart patients. CD16+ monocytes and M2 macrophages secrete cytokines and growth factors known for their involvement in fibrotic processes such as TGF-β and Galactin-3 a protein associated with cardiac fibrosis and atrial fibrillation [8]. Also after heart transplantation these factors secreted by the infiltrated monocytes – macrophages might contribute to fibrosis of the damaged heart transplant [9, 10].

Also in kidney transplantation the role of monocytes-macrophages in allogeneic responses has been recognized. Our observation that higher pretransplant numbers of CD16+ monocytes were associated with a significantly higher rejection risk and shorter rejection free survival shows importance of this cell population in anti-donor reactivity. This potential role of CD16+ monocytes makes this cell population an interesting marker to further develop as a biomarker for rejection. However, high numbers of circulating monocytes might be influenced by the underlying kidney disease and/or renal replacement therapy. For example, studies reporting data of patients on dialysis showed increased levels of circulating CD16+ monocytes that produced high levels of the pro-inflammatory cytokines IL-1β, TNF-α and IL-6 [11]. Another study reported that patients on hemodialysis have a temporary absence of their circulating monocytes that recover after the dialysis procedure and reappear in the circulation as highly activated CD16+ monocytes [12]. Our studies show promising results but need validation in other cohorts of kidney transplant patients. This will confirm that absolute number of CD16+ monocytes can be used as to identify patients at risk for rejection.

Diagnosis of rejection is made by histological examination of the kidney biopsy in combination with the clinical parameters of rejection. Histopathological lesions are non-specific and the diagnosis depends on the pathologist's evaluation with limited reproducibility between pathologists [13-16]. As described in the introduction of this thesis, the BANFF criteria have

led to improvement of diagnostic accuracy of different types of rejection in kidney transplant recipients [17-19]. We here report that M2 type macrophages are significantly associated with clinical rejection regardless of Banff category. The large number of M2 macrophages and their active state could lead to the continuous production of TGF-β and several other growth factors that promote proliferation of myofibroblasts, activation of epithelial-mesenchymal transition/endothelial-mesenchymal transition and extra cellular matrix deposition [20]. Interestingly, the compartmental differences between subtypes of rejection indicate that these cells could accumulate in specific compartments of the kidney. Hoffmann et al. reported that high numbers of activated CX3CR1+CD68+ macrophages are present in acute tubulointerstitial and acute vascular rejection [21]. A recent study by Bergler *et al*. showed that glomerular CD68+ macrophage infiltration with active expression of Ki67 (proliferation marker) could be a surrogate for ABMR, indicating that infiltrating macrophages act as active triggers of alloimmune inflammation and severe graft injury [22]. Moreover, cases with severe glomerular endothelial injury associated with monocyte-macrophage infiltration in patients on alemtuzumab anti-rejection therapy have been described [23-26].

In view of rapid advances in our knowledge of human genome and microRNAs, also the field of transplantation immunology will take advantage of these new insights [27-31]. In order to better define differences in distinct alloimmune inflammatory responses, which give rise to different types of rejection, a molecular approach could be used. In our study, we found significant differences between a-aABMR and TCMR, whereas less differences were found between a-aABMR and c-aABMR. The latter might be due to a small sample size. Particularly the expression of microRNA-100-5p was different between a-aABMR and aTCMR. It is known that this microRNA is indirectly involved with adhesion molecule CD209 (DC-SIGN) and functional studies should demonstrate the functional interaction between microRNAs-100-5p and CD209 [32, 33]. The majority of the infiltrated myeloid dendritic cells express CD209. This molecule stimulates T-cells [33]. Also microRNA-145-5p, controlled by TGF-β1, interacts with many cellular functions such as cell growth, cell proliferation, cell differentiation and apoptosis [34-36]. MicroRNA-145-5 is expressed by CD4+ and CD8+T-cells, and CD19+ B-cells [37]. Our data provide interesting new data but also clearly shows the limitations of miRNA studies which requires further studies before conclusions can be drawn that impact diagnostic decisions. Specificity and interpretation of kidney biopsy derived microRNAs is a hurdle to be taken. For example many different cell types of the kidney contain a broad range of miRNAs, all with their unique targets and functions. Therefore, a limitation of biopsy studies is that the cellular source expressing a particular miRNA is unknown. Also miRNAs can interact with multiple genes. To determine the biological impact of a particular miRNA also mRNA expression should be studied. This will lead to a better understanding of the cells and pathways involved in rejection processes. Another limitation is that the present microRNA library is being constantly updated as more validated targets are incomplete or due to ongoing experiments [31].

Looking outside the box of currently used immunosuppressive drugs in solid organ transplantation; there are other compounds with immune-inhibitory effects that are of interest for the organ transplantation field. First, the human monoclonal antibody canakinumab, originally designed as an IL-1β inhibitor for the repression of inflammation in autoimmune diseases, might also inhibit the IL-1β production by monocytes involved in allogeneic reactions after transplantation [38]. A high expression of IL-1β is noticed in the most severe liver transplant rejection episodes and at the time of kidney transplantation, suggesting the importance of blocking its production by monocytes [39, 40]. Second, infliximab, originally used in the treatment of autoimmune diseases, is a monoclonal antibody targeting TNF-α production, a cytokine produced in high amounts by activated monocytes and macrophages. Therefore, infliximab could also target monocyte functions during rejection [41]. Besides the effect on TNF-α production, monocytes from Crohn's disease patients treated with therapeutic concentrations of infliximab also showed increased apoptosis via the activation of caspase-3, 8 and 9 [42]. A first study in kidney transplantation by the Berlin group reported excellent 5-year outcome [43]. Third, production of IL-6 by activated monocytes obtained from healthy donors was reduced when tocilizumab was added *in vitro*. The drug also induced apoptotic cell death of SEB (staphylococcal enterotoxin B)-activated monocytes [44]. These results support the hypothesis that tocilizumab inhibits monocyte responses after transplantation [45-47]. Recently, it was demonstrated that tocilizumab can suppress TNF-α production in monocytes during in vitro antibody-dependent cellular cytotoxicity assay suggesting a role of IL-6 pathway in TNF- α monocyte activation [48].

After reviewing the literature about the effects of immunosuppressive drugs on monocyte and macrophage functions it is obvious that the current clinically used immunosuppressive drugs do not specifically target this cell population. Looking into the future, several recently developed immunosuppressive drugs that are used in autoimmunity are of interest for the clinical transplantation field. The necessity of implementation of these agents and translation of developments in preclinical models targeting monocytic cells to the clinic is clear. These agents might also inhibit acute and chronic alloimmune responses, which will lead to improved outcomes.

Conclusions

The described research in this thesis aimed to investigate the role of monocytes and macrophages in rejection processes after kidney and heart transplantation. It was concluded that there are clear shifts of monocyte subset composition in both peripheral blood and transplanted organ during rejection after heart transplantation. A predominant presence of CD16+ monocytes and M2 type macrophages in the allograft was found which

was associated with an increased risk for the development of fibrosis detectable in 1-year post-transplant biopsies. In rejectors after kidney transplantation, high absolute numbers of CD16+ monocytes were measured in their subsequent pretransplantation blood sample. These first findings should be further explored for validation as well as the mechanisms involved. Only then this could be further developed as a predictive marker for rejection. Tissue infiltration with CD68+CD163+ macrophages was significantly associated with worse transplant function after rejection. The presence of glomerular and perivascular monocytes and CD68+CD163+ macrophages in the rejecting graft tissue can serve as a hallmark of clinical rejection independent of histopathological Banff assignment. Compartemental infiltration showed potential specific diagnostic patterns with possible clinical implementation in typing and grading of rejection in relation to graft outcome.

Overall, this thesis provides novel insights about the role of monocytes - macrophages in the allograft rejection process. Our data show that these cells play pivotal roles in both TCMR and ABMR. Given our findings, we postulate that based on monocyte features i. diagnostic tests should be developed to better identify patients at risk for rejection and ii. that immunosuppression therapy should be applied also targeting monocytes – macrophages actions.

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Chapter 9

Nederlandse Samenvatting

Samenvatting

Bij patiënten met eindstadium nier- of hartfalen is transplantatie de enige mogelijkheid voor een betere kwaliteit van leven en levensverwachting. Deze kwaliteit van leven kan nooit helemaal gelijk zijn aan die van gezonde personen omdat transplantatie patiënten hun leven lang medicijnen zullen moeten blijven slikken die ervoor zorgen dat het getransplanteerde orgaan niet kan worden afgestoten.

De nieren zijn belangrijke organen in het menselijk lichaam en hebben verschillende functies zoals het verwijderen van afvalstoffen, regulatie van vocht en zouten, regulatie van de bloeddruk en de productie van hormonen die aanmaak van rode bloedcellen stimuleren. De nieren kunnen beschadigd raken door verschillende oorzaken zoals hoge bloedruk, diabetes, medicijn gebruik en auto-immuunziektes. Patiënten bij wie de nieren steeds slechter functioneren zijn uiteindelijk aangewezen op dialyse totdat er een donornier beschikbaar komt. Transplantatie van een nier is de beste oplossing om de nierfunctie voor de patiënt te vervangen.

Een klein deel van patiënten met eindstadium hartfalen als gevolg van uitgebreide coronair ziekte, hartspierziekte zoals cardiomyopathie of een aangeboren hartafwijking ondergaan een harttransplantatie wanneer andere medische of chirurgische behandelingen niet meer mogelijk of afdoende zijn. Het probleem na transplantatie is dat het lichaamsvreemde orgaan wordt aangevallen door het afweersysteem van de patiënt. Om dit te voorkomen dan wel te onderdrukken krijgen nier- en harttransplantatiepatiënten afweerremmende geneesmiddelen genaamd immunosuppressiva voorgeschreven. Er zijn verschillende afweer onderdrukkende medicijnen maar desalniettemin kan er een acute afstotingsreactie bij nier- en harttransplantatiepatiënten plaats vinden waarvoor zij dan extra geneesmiddelen moeten krijgen. In het eerste jaar na niertransplantatie gebeurd dit bij ongeveer 15% van de patiënten en na harttransplantatie bij 25% van de patiënten.

Er zijn verschillende soorten van afstoting, meest voorkomend in de eerste 6 maanden na transplantatie is de acute afstoting (TCMR) waarbij de afweercellen genaamd T- cellen van de patiënt worden geactiveerd tegen de eiwitten (antigenen) van de donornier. Ook is er een chronische vorm (ABMR) van afstoting. Bij deze vorm van afstoting spelen antilichamen en complement factoren een belangrijke rol. Bij deze afstotingsreacties zijn verschillende typen cellen van het aangeboren en verworven afweersysteem betrokken. Onderzoek heeft laten zien dat naast cellen van het verworven immuunsysteem zoals de T- en B-cellen ook cellen van het aangeboren immuunsysteem zoals “natural killer cells” (NK cellen), “dendritic cells” (DC cellen) en monocyten/macrofagen, aan de afstotingsreactie bijdragen. Echter, er is nog maar weinig bekend over wat precies de rol en bijdrage van deze monocyten en macrofagen in de diverse typen van afstoting is.

Monocyten en macrofagen kun je onderverdelen in verschillende subsets. Monocyten bestaan uit 3 subtypes: klassieke monocyten (CD14++CD16-), intermediaire monocyten (CD14++CD16+) en niet-klassieke monocyten (CD14+CD16++). De CD16+ monocyten staan er om bekend dat ze na stimulatie met antigeen hoge hoeveelheden van pro-inflammatoire cytokinen kunnen produceren. Macrofagen kun je ook onderverdelen in verschillende subtypes: M1 macrofagen (CD68+CD80+) die bekend staan als pro-inflammatoire cellen die hoge hoeveelheid cytokines maken, en M2 macrofagen (CD68+CD163+) die bekend staan als anti-inflammatoire cellen die voornamelijk betrokken zijn bij de opruimreactie (bindweefselvorming) na een ontsteking.

In dit proefschrift onderzoeken wij de rol alsmede de bijdrage van monocyten-macrofagen in afstotingsreacties maar ook in productie van ongewenste bindweefselvorming in getransplanteerde nieren en harten.

In hoofdstuk 2 onderzochten wij de bijdrage van verschillende typen monocyten en macrofagen in het afstotingsproces in hartweefsels en bloedsamples van dezelfde patiënten zowel voor, als tijdens en na een acute cellulaire afstoting na harttransplantatie. We vonden veel geïnfiltreerde CD16+ monocyten in het getransplanteerde hart tijdens de acute afstotingsreactie. Opvallend daarbij was dat er tijdens de acute afstoting een groot aantal CD68+CD163+ M2 type macrofagen werd gevonden in het weefsel in vergelijking tot het hartweefsel zonder histologische afstoting. Acute afstoting was tevens geassocieerd met fibrosevorming tot 1 jaar na de afstotingsreactie. In het bloed van deze patiënten werden er lagere frequenties van CD16+ monocyten gemeten in vergelijking met gezonde individuen. Tijdens een afstotingsreactie vonden we ook een verhoogde concentratie van allerlei moleculen zoals CD54 en HLA-DR op de CD16+ monocyten die uiteindelijk cytokine productie stimuleren. De belangrijkste bevinding was dat schade opgedaan tijdens de afstoting, 1 jaar na afstoting nog meer chronische schade zal veroorzaken en daarbij een belangrijke rol meespeelt in het uiteindelijk falen van het harttransplantaat.

In hoofdstuk 3 toetsten we de hypothese of er in de verschillende compartimenten van getransplanteerde nieren er verschillen zijn in aantal en type infiltrerende monocyten en macrofagen. Daarnaast hebben we de relatie tussen aanwezigheid van deze cellen met de histopathologische diagnoses onderzocht. Om de hypothese te testen hebben wij 48 niertransplantatie biopten die gediagnosticeerd zijn met de laatste Banff 2015 classificatie immunohistochemisch gekleurd voor markers die voorkomen op monocyten en macrofagen. Daarnaast werden de histopathologische bevindingen gecorreleerd met afstoting gerelateerde variabelen in de tijd tot 12 maanden na biopsie, daarbij gebruikmakend van multivariate analyse. Wij vonden dat M2-type macrofagen geassocieerd waren met de klinische diagnose van afstoting, onafhankelijk van de Banff categorie. Bovendien was een belangrijke bevinding dat M2 macrofagen geassocieerd waren met een slechtere nierfunctie op de langere termijn. We zagen dat veel infiltrerende CD16+ monocyten in de glomerulaire

ruimtes, langs en in de bloedvaten en in de tubulointerstitiële ruimte infiltreerden. Er bleken significante verschillen te bestaan tussen c-aABMR versus a-aABMR, en a-aABMR versus TCMR I/II in deze compartimenten. De patronen van compartiment infiltratie correleert met diagnostische categorieën. Zo zagen wij glomerulaire infiltratie tijdens c-aABMR, prominente aanwezigheid van M2-macrofagen bij de vaten in a-aABMR en hoge infiltratie van CD16+ monocyten in de vaten tijdens TCMR II. Glomerulaire infiltratie van CD16+ monocyten in TCMR I terwijl er hoge infiltratie van CD16+ monocyten en M2 macrofagen bij de vaten tijdens TCMR II werd gevonden behoren tot de belangrijke bevindingen. Vergelijking van a-aABMR met c-aABMR leerde ons dat een hoog aantal infiltrerende monocyten van alle subtypes in de glomeruli tijdens c-aABMR met daarbij ook significant meer CD14+ monocyten bij de vaten werden gevonden. In deze studie vonden wij dat perivasculaire infiltratie van M2 macrofagen op a-aABMR duidt. Verder onderzoek naar de onderliggende mechanismen van deze specifieke compartiment verdeling in een grotere cohort zal een duidelijker beeld verschaffen over de rol van monocyten en macrofagen tijdens het afstotingsproces.

In hoofdstuk 4 onderzochten wij of voorafgaand aan de transplantatie procedure er al verschil aantoonbaar was in bepaalde subpopulaties van monocyten in het bloed van niertransplantatiepatiënten en of deze geassocieerd zijn met afstoting. Wij vonden een hoger aantal CD16+ monocyten op het pretranplantatie tijdspunt in patiënten die een acute afstoting ontwikkelden in vergelijking tot niertransplantatiepatiënten bij wie geen afstoting optrad. Een hoog aantal van CD14+ monocyten in het bloed was geassocieerd met een lager risico op het optreden van afstoting. In een pilot-case-control studie hebben wij 6 nierbiopten met afstoting vergeleken met controle biopten zonder afstoting. In de afstotingsbiopten waren veel infiltrerende CD16+ monocyten aanwezig. Deze bevindingen duiden erop dat circulerende CD16+ monocyten naar de afstotingsreactie van de getransplanteerde nier migreren. Onze data laat zien dat een hoog aantal absoluut CD16+ monocyten in de circulatie van de transplantatie patiënt significant geassocieerd is met hoger risico op afstoting, kortere afstotingsvrije overleving en mogelijk kan dienen als een vroege biomarker om afstoting te voorspellen na transplantatie.

In hoofdstuk 5 toetsten we of de expressie van bepaalde microRNAs onderscheid kan maken tussen de verschillende vormen van afstoting. Hiertoe zijn microRNA profielen van 31 nierbiopten vergeleken met de a-aABMR versus aTCMR status en de a-aABMR versus c-aABMR status. Tevens is er een pathway analyse uitgevoerd om inzicht te krijgen in de betrokken eiwitten die gereguleerd worden door specifieke microRNAs welke een rol kunnen spelen in het afstotingsproces. Tussen a-aABMR en aTCMR werden er 55 significant verschillende microRNAs gevonden. Hierbij werd microRNA-100-5p geïdentificeerd als de meest significante en dus als belangrijkste. Vanuit de literatuur is bekend dat microRNA-100-5p de expressie van CD209 (DC-SIGN) reguleert; dit eiwit komt tot expressie in zowel macrofagen als dendritische cellen en welke beide bijdragen aan het afstotingsproces. De microRNAs die wij in deze studie hebben gevonden zijn allen betrokken bij immuun en/

of ontstekingsprocessen, en dus compatibel met hun betrokkenheid bij de anti-donor response. De microRNAs hebben uiteindelijk via regulatie van productie en functie van eiwitten allen een rol in de communicatie tussen verschillende typen immuun cellen zoals lymfocyten, macrofagen, NK cellen en dendritische cellen. Er zijn zeker nog meer studies nodig om in andere cohorten en met name ook in biopten zonder tekenen van afstoting onze eerste bevindingen te valideren. Daarnaast natuurlijk ook om te bestuderen of er specifieke microRNAs geassocieerd zijn met het optreden van a-aABMR of aTCMR welke een toegevoegde waarde kan hebben bij de afstotingsdiagnostiek.

Omdat interferon-gamma een belangrijke rol speelt in de aangeboren en verworven immuniteit hebben wij **in hoofdstuk 6** onderzocht of monocyten deze cytokine kunnen produceren. Voor deze studie hebben wij bloed van gezonde personen en niertransplantatiepatiënten die immunosuppressiva gebruiken, bestudeerd. Wanneer de CD14+ monocyten werden gestimuleerd met LPS (lipopolysachariden) werden de mRNA niveaus van IFNγ eiwit maar ook het eiwit zelf verhoogd tot expressie gebracht in monocyten van gezonde personen en van de transplantatiepatiënten. Deze resultaten laten zien dat monocyten de capaciteit hebben om IFNγ te produceren.

In hoofdstuk 7, bediscussiëren wij de effecten van de op dit moment voorgeschreven afweerremmende geneesmiddelen op monocyten en macrofagen alsmede de ontwikkeling van nieuwe immunosuppressiva voor de transplantatiegeneeskunde. Na het lezen van de literatuur werd duidelijk dat er weinig bekend is over het effect van immunosuppressiva op monocyten – macrofagen functies. Er is bewijs dat de afweerremmende geneesmiddelen zoals mycofeno-laat-mofetil (MMF) en mechanistic target of rapamycin (mTOR) remmers in beperkte mate een remmend effect hebben op monocyten functies. Ook calcineurine remmers (CNIs) remmen de activiteit van monocyten, maar minder sterk dan die van T cellen. De effecten van immunosuppressiva op monocyten – macrofagen functies zijn onder andere remming van cytokine transcriptie en productie en remming van de migratie van deze cellen naar de plek van afstoting/ontsteking. Voorstellen voor nieuwe studies worden besproken die betrekking hebben op het gebruik van geneesmiddelen die monocyten en macrofagen remmen. Idealiter, deze nieuwe geneesmiddelen moeten de functie van monocyten en macrofagen beter onderdrukken dan de huidige medicatie. Ook het ontwikkelen van een specifiek afleveringssysteem met behulp van lipide vesicles en het manipuleren van het micromilieu en polarisatie van monocyten tot een anti-ontsteking fenotype zijn bediscussieerd. Het meest ideale scenario zou de ontwikkeling van een nieuw immunosuppressivum zijn die op meerdere punten in de activatie cascade de voor afstotingsreactie verantwoordelijke afweercellen kan remmen en daarmee afstoting kan voorkomen dan wel genezen.

Conclusies

De beschreven onderzoeken in deze dissertatie tonen aan dat monocyten en macrofagen een belangrijke rol spelen in zowel TCMR als ABMR. Zo hebben wij aangetoond dat M2 macrofagen en CD16+ monocyten betrokken zijn bij acute afstoting van het harttransplantaat, en dat dit mogelijk leidt tot blijvende schade welke 1-jaar na afstoting terug werd gevonden. Tevens vonden wij in de niertransplantatie patiënten dat een hoog aantal circulerende CD16+ monocyten een grotere kans op afstoting kan voorspellen. Zo ook de infiltratie van deze CD16+ monocyten naar de afstotende nier tezamen met de aanwezigheid van M2 macrofagen in specifieke compartimenten een indicatie kunnen geven van het soort afstoting. Tevens toonden wij aan dat de huidige afweerremmende middelen niet goed de monocyten – macrofagen functies kunnen onderdrukken.

Onze belangrijkste bevinding uit al deze studies is dat CD16+ monocyten en hun verder gedifferentieerde vorm M2 macrofagen een belangrijke rol (als zowel pro-inflammatoir als mede anti-inflammatoir) spelen in het afstotingsmechanisme. Gezien deze bevindingen, moeten wij: testen ontwikkelen gebaseerd op de functies van monocyten – macrofagen welke de afstotingsdiagnostiek kunnen ondersteunen. Dit moet leiden tot het eerder traceren van patiënten met een hoog risico op afstoting. Deze patiënten moeten afstotingsremmende geneesmiddelen voorgeschreven krijgen die juist de functies monocyten en macrofagen beter kunnen onderdrukken waarmee de afstotingsreactie kan worden voorkomen.

Appendices

Curriculum vitae auctoris

List of publications

PhD portfolio

Acknowledgements (Dankwoord)

Curriculum vitae auctoris

Thierry Paulus Pierre van den Bosch werd geboren op 7 augustus 1990 te Rotterdam. Na het afronden van de middelbare school op het Melanchton college te Bleiswijk, ging hij middelbaar laboratorium onderwijs studeren in Leiden. Na het voltooien vervolgde hij zijn studie met het hoger laboratorium onderwijs. In 2012 begon hij met zijn afstudeerstage bij de afdeling Pathologie aan het Erasmus MC onder leiding van dr. Rob Verdijk. In augustus 2013 begon hij aan zijn promotieonderzoek op de nefrologie en transplantatie sectie van de afdeling Inwendige Geneeskunde in het Erasmus MC – Universitair Medische Centrum Rotterdam onder begeleiding van prof. dr. Carla Baan en dr. A.T. Rowshani. Op 1 augustus 2017 is Thierry in dienst bij de afdeling Pathologie waar hij werkt aan het opzetten immunohistochemische kleuringen, multiplex IHC, microRNA ISH en beeldanalyses.

List of publications

This thesis

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Meeting Abstracts

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PhD portfolio

Summary of PhD training and teaching activities
Name PhD student: Thierry P.P. van den Bosch
Erasmus MC Department: Internal Medicine
Promotor: Prof. Dr. C.C. Baan
Copromotor: Dr. A.T. Rowshani
PhD period: 1 august 2013 – 1 august 2017
Research School: Molecular Medicine

PhD training

Research and General Academic skills	**Year**	**ECTS**
The Basic Human Genetics Course	2012	0.5 ECTS
The Course Biomedical Research Techniques XI	2012	1.5 ECTS
The Introductory Course on Statistics & Survival Analysis	2012	0.5 ECTS
The Course on Molecular Medicine	2013	0.7 ECTS
The Workshop on Photoshop and Illustrator CS6 for PhD students	2013	0.3 ECTS
The Course on Advances in Comparative Pathology	2013	0.3 ECTS
The Course on Basic introduction in SPSS	2013	1.0 ECTS
The Course on R	2015	1.4 ECTS
The Biomedical English Writing Course	2015	2.0 ECTS
The Course on Advanced Immunology	2016	2.0 ECTS
Research Integrity	2016	0.3 ECTS
(Inter)national conferences and presentations		
Bootcongres Leiden – Nederlandse Transplantatie Vereniging 26-28 March (2 orals)	2014	1.7 ECTS
Bootcongres Bournemouth - Nederlandse Transplantatie Vereniging 11-13 March (2 posters)	2015	1.7 ECTS
International society of Heart and Lung Transplantation Conference 15-18 April (1 poster) (Nice, France)	2015	1.7 ECTS
Bootcongres Zeist – Nederlandse Transplantatie Vereniging 8-9 March (3 orals + 1 poster)	2017	2.0 ECTS
American Transplant Congress (ATC) – Chicago USA 29 April – 3 May (1 oral + 2 posters)	2017	2.0 ECTS
Seminars and Workshops		
Klinisch review symposium - Nederlandse Transplantatie Vereniging	2014	0.3 ECTS
Klinisch review symposium - Nederlandse Transplantatie Vereniging	2015	0.3 ECTS
Internal Medicine – Science days Antwerp (1 poster)	2015	1.7 ECTS

Teaching Activity

Supervision of two medical students (Minor Transplantation)	2015	4.0 ECTS
Lectures during Minor Transplantation Erasmus MC	2015/2016	0.6 ECTS
Lectures for HLO students (Leiden)	2016/2017	1.0 ECTS
Supervision vaardigheidsonderwijs pathologie	2016/2017	4.0 ETCS
International society of Heart and Lung Transplantation Conference	2015	1.7 ECTS

Received grants

Astellas Pharma Travel Grant	2015
Erasmus MC Trusfunds Travel Grant	2015
NTV bootbeurs	2017
NTV Scholingsbeurs	2017
International society of Heart and Lung Transplantation Conference	2015

Received awards

Best abstract prize (Bootcongres)	2017
Poster of Distinction (American Transplant Congress)	2017
Nomination for the what's hot what's new presentation (ATC)	2017

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Thierry