

Up-Regulation of CD163 Expression in Subpopulations of Blood Monocytes After Kidney Allograft Transplantation

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Summary

M2 macrophages expressing CD163 are known to suppress immune responses but have been also found in biopsies of patients with chronic kidney allograft injury associated with interstitial fibrosis. The aim of our study was to evaluate the expression of CD163 in blood monocytes, precursors of tissue macrophages, in kidney allograft recipients with uncomplicated outcome (n=94) compared with those developing acute rejection (n=44). Blood samples were collected before the transplantation and at 1 week, 1 month and 1 year. The expression of CD163 increased during the first week after the transplantation not only in classical (CD14⁺CD16⁻) but also in intermediate (CD14⁺CD16⁺) and nonclassical (CD14^{low}CD16⁺) monocytes in all patients regardless of their rejection status. In patients developing acute rejection, higher pre-transplant expression of CD163 on blood monocytes was found. *In vitro* experiments confirmed strong induction of membrane CD163 on monocytes together with CD206 (an alternative marker of M2 macrophages) in response to IL-10. We assume from our data that dramatic upregulation of CD163 by peripheral blood monocytes may have a pathophysiological role in early phases after kidney allograft transplantation and high pre-transplant expression of CD163 on blood monocytes might be involved in events leading to acute rejection.

Key words

Blood monocytes • Subpopulations • Kidney transplantation • Acute rejection • CD14⁺CD16⁺ • CD163

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Introduction

Mononuclear phagocytes at different stages of differentiation from blood monocytes to tissue macrophages and dendritic cells play a key role in the initiation, effector mechanisms and regulation of immune responses (Moghaddam *et al.* 2018). In kidney transplantation, monocyte infiltration into the graft has been found to correlate with renal dysfunction (Girlanda *et al.* 2008). Monocytes producing IL-6 are in relationship with a decrease in glomerular filtration rate in patients with borderline changes suspicious for acute T cell-mediated rejection (Desy *et al.* 2018) but there is also an association between the number of peripheral monocytes and risk of chronic kidney disease in transplant recipients (Bowe *et al.* 2017). Specific subpopulations can be distinguished among monocytes by the expression level of membrane antigens CD14 (a receptor for bacterial LPS) and CD16 (FcγRIII) (Ziegler-Heitbrock *et al.* 2010). The “classical” monocytes (CD14⁺CD16⁻) are characterized by a strong CD14 expression in the absence of CD16 on their surface and two subpopulations monocytes have relatively low CD14 expression and detectable CD16 molecule on a membrane being classified as “intermediate” (CD14⁺CD16⁺) and “non-classical” (CD14^{low}CD16⁺)

(Wong *et al.* 2011) (Stansfield and Ingram 2015). These two functionally and phenotypically related subsets (Ong *et al.* 2019) showed higher pre-transplant numbers in patients developing acute rejection as compared with those with uncomplicated outcome (van den Bosch *et al.* 2017b).

In tissue macrophages originated from blood monocytes, CD163 expression is a characteristic marker of subpopulation suppressing immune responses known as M2 (alternatively activated) macrophages with a capability to release high amounts of IL-10 (Mayer *et al.*). CD163 positive macrophages are frequently found in malignant tumors with progressive growth (Shiraishi *et al.* 2018) and provide local immunosuppressive effects. This well conserved molecule serves as a scavenger molecule for hemoglobin-haptoglobin complexes (Fabrick *et al.* 2005) and binds cytokine TWEAK (Moreno *et al.* 2009) or microorganisms (Fabrick *et al.* 2009). The expression of CD163 in cultured human blood monocytes is upregulated by glucocorticoids, IL-10 and IL-6 (Maniecki *et al.* 2006) while pro-inflammatory factors including TNF alpha, IFN gamma and LPS downregulate the membrane form. In response to macrophage activation by Fc gamma cross-linking, LPS stimulation or by oxidative stress, CD163 is proteolytically cleaved by a metalloproteinase ADAM17 (Etzerodt *et al.* 2014) and undergoes shedding from the membrane of mononuclear phagocytes. This soluble form, sCD163, is detectable in serum and may be used as a biomarker in chronic inflammatory and metabolic diseases (Dige *et al.* 2014, Smiljanovic *et al.* 2018, Tanimura *et al.* 2015, Cinkajzlova *et al.* 2017).

Most of the studies in organ transplant patients are focused on detection of CD163 positive macrophages in allograft biopsies which represent an invasive option to obtain valuable data on molecular processes in kidney parenchyma (Trailin *et al.* 2020). In early kidney transplant biopsies, parenchymal infiltration with CD163⁺ macrophages correlated with interstitial inflammation, tubulitis, and peritubular capillaritis scores (Shin *et al.* 2015).

The presence of CD163⁺ macrophages in kidney biopsies of antibody-mediated rejection was found to be associated with chronic glomerular injury and poor graft function, but did not significantly affect graft survival (Kim *et al.* 2018). In T cell mediated rejection of kidney allograft, CD163⁺ macrophages seem to be the main source of IL-18 (Stokman *et al.* 2016). In pediatric patients with chronic kidney allograft injury, CD163⁺ macrophages accumulate in areas of interstitial fibrosis and their

numbers correlate with the parameters of kidney function (Ikezumi *et al.* 2015). CD163⁺ macrophages are preferentially present in transplanted endomyocardial tissue and increase upon acute cellular rejection of heart allograft (van den Bosch *et al.* 2017a). On the other hand, high numbers of CD163⁺ macrophages predict favorable early graft outcome in living donor liver transplantation (Nigam *et al.* 2018).

There are only limited data regarding CD163 expression on peripheral blood monocytes of organ transplant patients. In living kidney recipients, CD163⁺ monocytes increased immediately after the transplantation having a correlation with serum creatinine at one week and with a decrease of sCD163 serum levels (Guillen-Gomez *et al.* 2014). Also in our previous study in cadaveric donors, numbers of CD163 positive monocytes were highly upregulated at one week after kidney allograft transplantation (Sekerikova *et al.* 2014). Whether these CD163 positive peripheral monocytes represent cells with immunosuppressive capacity similar to M2 macrophages is not clear, yet. In a recent study, numbers of peripheral blood CD163⁺CD206⁺ monocytes and their IL-10 production correlated positively with a progression of proteinuria in idiopathic membranous nephropathy (Hou *et al.* 2018) suggesting their potential profibrotic effect.

Here, we aimed to elucidate whether CD163 expression is differently regulated in subpopulations of peripheral blood monocytes of kidney transplant recipients and has an association with the outcome. Next, we wondered whether membrane expression of CD163 on blood monocytes correlates with CD206, an alternative marker of M2 macrophages, and has a relationship with its soluble form (sCD163) and an anti-inflammatory cytokine IL-10 in serum samples. Additionally, regulation of CD163 by cytokines was tested in cultures of peripheral blood monocytes.

Patients and Methods

Patients

The group of 138 patients who underwent renal transplantation from deceased donor in Transplant Center of the Institute for Clinical and Experimental Medicine in Prague was enrolled in the prospective study. The study protocol was approved by the Ethics Committee of the Institute for Clinical and Experimental Medicine (ID 118099).

Patient characteristics are shown in Table 1.

Table 1. Demographic characteristics.

	Normal outcome
Number of patients (n)	94
Type of acute rejection (n, %)	
Cellular	
Humoral/DSA+	
Humoral/DSA-	
Gender (male/female) (n)	61/33
Recipient age (median, range)	58 (22-80)
Donor age (median, range)	56.5 (1-81)
Cause of renal failure (n)	
Glomerulonephritis	11
Tubulointerstitial nephritis	7
Vascular diseases	6
Diabetes mellitus	9
Polycystic kidney	12
IgA nephropathy	15
Other causes	34
Dialysis (years) (median, range)	2.30 (0-11.9)
Cold ischemia time (h) (median, range)	14 (0-24)
First renal transplantation (n, %)	93 (98.9)
Deceased donor (n, %)	94 (100)
ABO incompatibilities (n)	0
PRA (%) (median, range)	10 (0-98)
HLA mismatches (median, range)	3 (1-6)
DSA positivity (n, %)	
Preformed DSA	8 (8.5)
De novo DSA	0
Induction therapy (n, %)	
Simulect	27 (28.7)
ATG	59 (62.8)
ATG, PE, IVIG	8 (8.5)
Maintenance IS (n, %)	
Tac, MMf, KS	86 (91.5)
Tac, KS	5 (5.3)
mTOR, MMf, KS	3 (3.2)

Induction treatment and maintenance immunosuppression

All patients received induction treatment according to regular Transplant Center protocol. Primary kidney transplant recipients with panel reactive antibodies (PRA) < 20 % and negative donor-specific antibodies (DSA) received basiliximab (N=43), patients with PRA > 20 % were on rabbit anti-thymocyte globulin (ATG) (N=77), those with known DSA positivity at the time of transplantation (N=18) received plasma exchange (PE)

prior to transplantation and intravenous immunoglobulin (IVIG) in addition to ATG. Majority of patients (N=125) was treated by a triple maintenance therapy consisting of tacrolimus, mycophenolate mofetil, and corticosteroids. Target through levels of tacrolimus were 10-15 µg/l in the first month after transplantation with continuous cessation to 4-6 µg/l after the third month. Ten patients were on dual therapy with tacrolimus and corticosteroids and remaining 3 were converted to everolimus in combination with

mycophenolate mofetil and corticosteroids early after transplantation.

Histopathology and rejection phenotypes

Kidney allograft biopsy samples were obtained using a percutaneous ultrasound-guided 16G biopsy needle for scheduled (performed routinely at 3 months post-transplant) and “for-cause” biopsies. All patients gave their informed consent and signed their agreement with each biopsy performed.

Biopsy-proven acute rejection was diagnosed histologically according to the revised Banff '2017 classification (Haas *et al.* 2018). From 44 patients with acute rejection, 16 were classified as T cell-mediated rejection characterized by tubulointerstitial inflammation or intimal arteritis in the absence of C4d and DSA. Twenty-eight patients had histologic signs of antibody-mediated rejection (glomerulitis, peritubular capillaritis and/or intimal arteritis in presence of C4d, in absence of C4d the sum of microvascular inflammation was at least 2). DSA were detectable in 13 patients, remaining 15 patients had undetectable DSA and the diagnosis of antibody-mediated rejection was thus based on histology.

The most frequent first-line treatment in patients with cellular rejection were methylprednisolone pulses in 10 patients, ATG in 2 patients and 3 patients were judged as steroid-resistant and ATG was used as a rescue therapy. Patients with DSA positive humoral rejection were treated with methylprednisolone, plasma exchange (PE) and intravenous immunoglobulins (IVIG) in 9 cases, less intensive treatment with methylprednisolone only or adjustment of maintenance immunosuppression was used in remaining patients mostly due to infectious complications. Eleven of 13 DSA negative patients with histologic antibody-mediated rejection were treated with corticosteroids. In four of them, PE and IVIG were added and in two patients, adjustment of maintenance immunosuppression was performed.

Tissue cultures of peripheral blood monocytes

Peripheral blood samples were collected at following time-points: before kidney transplantation and day 7, 1 month, and 1 year post-transplantation. PBMCs were purified from buffy coats from healthy donors by standard Ficoll-Paque gradient centrifugation. Briefly, 3 ml of Ficoll-Paque gradient was pipetted into a 15-ml centrifuge tube. The EDTA blood was diluted 1:1 in phosphate-buffered saline (PBS) and carefully layered over the Ficoll-Paque gradient (6 ml/tube). The tubes were

centrifuged for 30 min at 400× g. The cell interface layer was harvested carefully and the cells were washed once in PBS (for 10 min at 250× g) and were washed once again in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA), (for 10 min at 250× g).

Cells were cultured in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10 % heat inactivated fetal bovine serum (FBS), L-glutamine, penicillin and streptomycin (Sigma-Aldrich). Cells were then removed into 6-well tissue culture plates (Costar, Corning, NY, USA) at density of 2×10^6 cells per 2 ml per well and cultured under a 5 % CO₂ atmosphere at 37 °C. Monocytes seeded in 6-well tissue culture plates (Costar, Corning, NY, USA) at a density of 2×10^6 cells/ml were stimulated with IL-10 (R&D Systems, Minneapolis, MN, USA) at 10 ng/ml, unstimulated cells were used as controls.

Flow cytometry

Peripheral blood mononuclear cells (100 µl, approx. 1×10^6) were labeled with fluorochrome-conjugated monoclonal antibodies resuspended in PBS-BSA buffer for 20' at room temperature (RT) in the dark. Following monoclonal antibodies were used: anti-CD163 (clone GHI61, PE or clone RM3/1, PE), anti-CD206 (clone 15-2, APC) purchased from BioLegend, San Diego, CA, USA; anti CD14 (RM052 clone, APC-Alexa Fluor 750), anti-CD16 (clone clone 3G8, Pacific, Blue), from Beckman Coulter, Brea, CA, USA. Samples were measured on Navios flow cytometer (Beckman Coulter, Brea, CA, USA) and analyzed using CxP software and Kaluza software (Beckman Coulter, Brea, CA, USA), the percentage of positive cells and mean intensity fluorescence (MFI) were evaluated for each individual marker. The gating strategy of CD14⁺CD16⁺ subpopulations is shown in Fig. 1.

Statistics

Statistical analyses were performed by GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Based on the distribution of the data, parametric or nonparametric (Mann-Whitney, Kruskal-Wallis) testing was used. We performed logarithmical transformation and parametric testing by repeated measures ANOVA when comparing the flow cytometry data in different time-points. Clinical and flow cytometry data were correlated using Spearman rank correlation coefficient. Data were showed as median [min; max] according to the distribution and differences regarded statistically significant with $p < 0.05$.

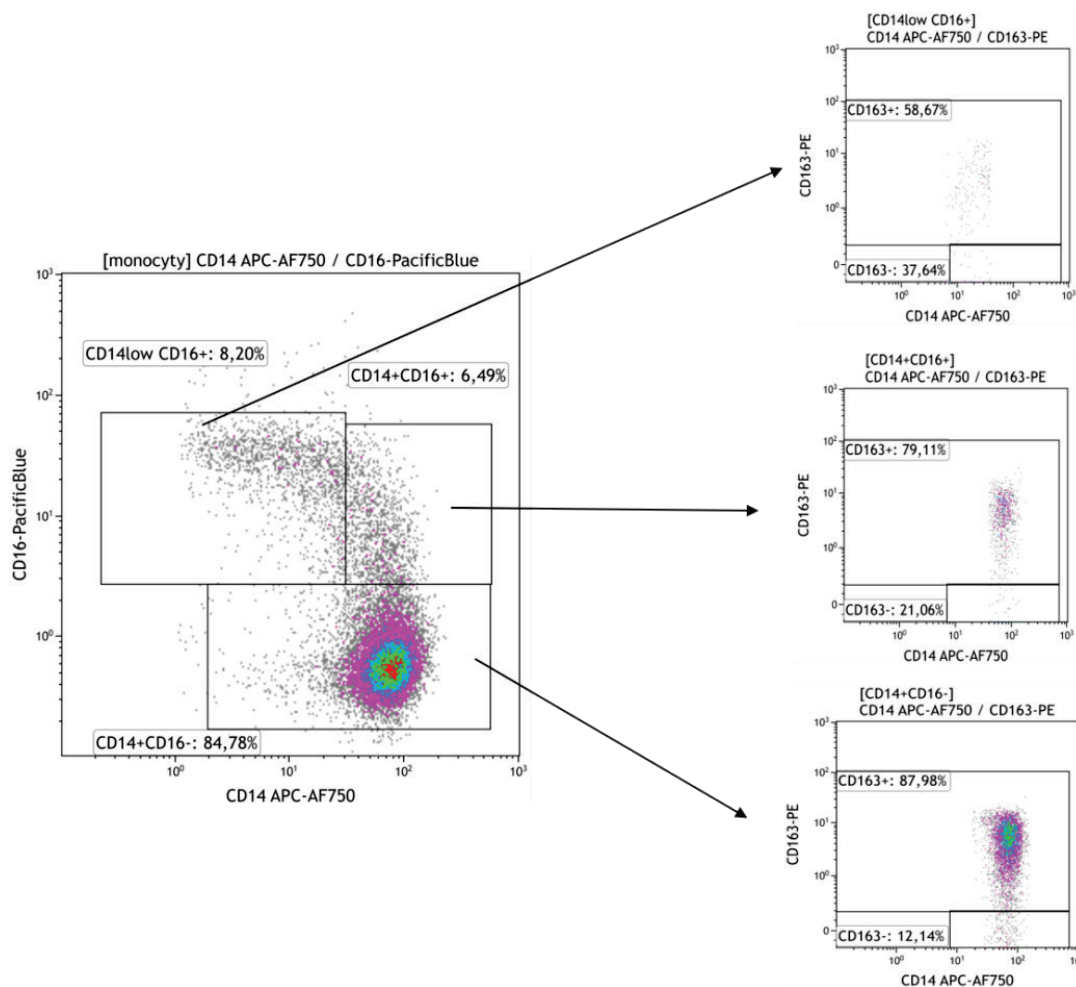


Fig. 1. The gating strategy to differentiate subpopulations of blood monocytes. Three subpopulations of peripheral blood monocytes were delineated by the expression level of CD14 and CD16 molecules into classical ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$) and nonclassical ($CD14^{low}CD16^+$) subsets (Fig. 1a). The expression of CD163 was then measured in the whole population of blood monocytes and selected three subpopulations.

Results

CD163 expression in different subpopulations of peripheral blood monocytes

The expression of CD163, a traditional marker of immunosuppressive M2 macrophages, was evaluated in peripheral blood monocytes subpopulations of patients before and one week, one month, and one year after kidney allograft transplantation. The subpopulations were consisted from the classical ($CD14^+CD16^-$) monocytes representing the predominant population and less frequently found intermediate ($CD14^+CD16^+$) and nonclassical ($CD14^{low}CD16^+$) monocytes with high pro-inflammatory potential. The membrane expression of CD163 was lower in nonclassical monocytes as compared to classical ($p < 0.001$) and intermediate ($p < 0.001$) ones and was markedly upregulated at one week after the transplantation in all three monocyte subpopulations ($p < 0.001$ for all comparisons) (Fig. 2). At one month,

percentage of $CD163^+$ cells decreased, particularly in non-classical monocytes ($p < 0.001$) but did not reach pre-transplant levels. No difference between one month and one year was found in the proportion of $CD163^+$ monocytes in any of subpopulations. Absolute numbers showed the same trend of CD163 induction after the transplantation but there was a decline of blood monocyte numbers after 1 month affecting proportionally all three subpopulations (data not shown).

The expression of CD163 in patients with acute rejection of kidney allograft

When comparing CD163 expression in relationship to clinical outcome, the upregulation of CD163 by peripheral blood monocytes after the transplantation was found in both patients with uncomplicated outcome ($p < 0.001$) and those with acute rejection ($p < 0.001$). Subjects with kidney allograft rejection had higher percentage of $CD163^+$ monocytes before the trans-

plantation ($p < 0.05$) (Fig. 3). The data were not affected by the type of rejection (antibody mediated rejection, cell mediated rejection). In most kidney transplant recipients,

initial upregulation of CD163 was followed by a decrease at one month after the transplantation ($p < 0.001$) and did not change at one year (Fig. 3).

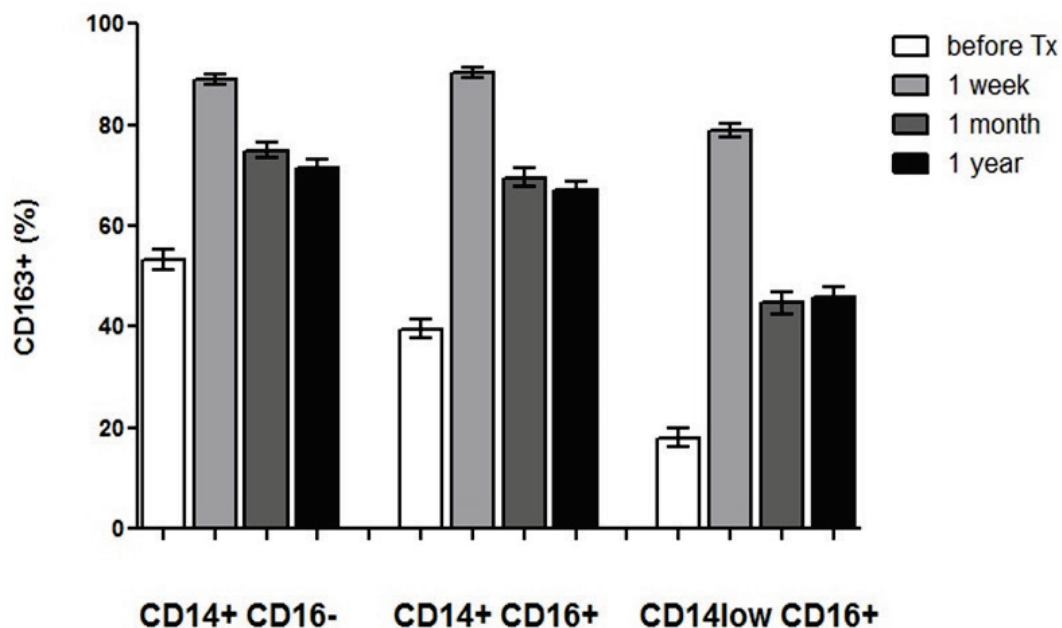


Fig. 2. CD163 expression in subpopulations of blood monocytes from kidney allograft recipients. The expression of CD163 was evaluated in classical (CD14⁺CD16⁻), intermediate (CD14⁺CD16⁺) and nonclassical (CD14^{low}CD16⁺) peripheral blood monocytes obtained from patients ($n=138$) before the kidney transplantation and then one week, one month, and one year after the surgery. The percentage of CD163 positive cells was lower in nonclassical monocytes as compared to classical and intermediate ones ($p < 0.001$) but the expression of the molecule was upregulated at one week after the kidney transplantation in all three subsets ($p < 0.001$) and then decreased after one month ($p < 0.001$). The expression of CD163 after one year did not change as compared to values at one month and remained higher as compared to pre-transplant percentage of positive cells ($p < 0.001$).

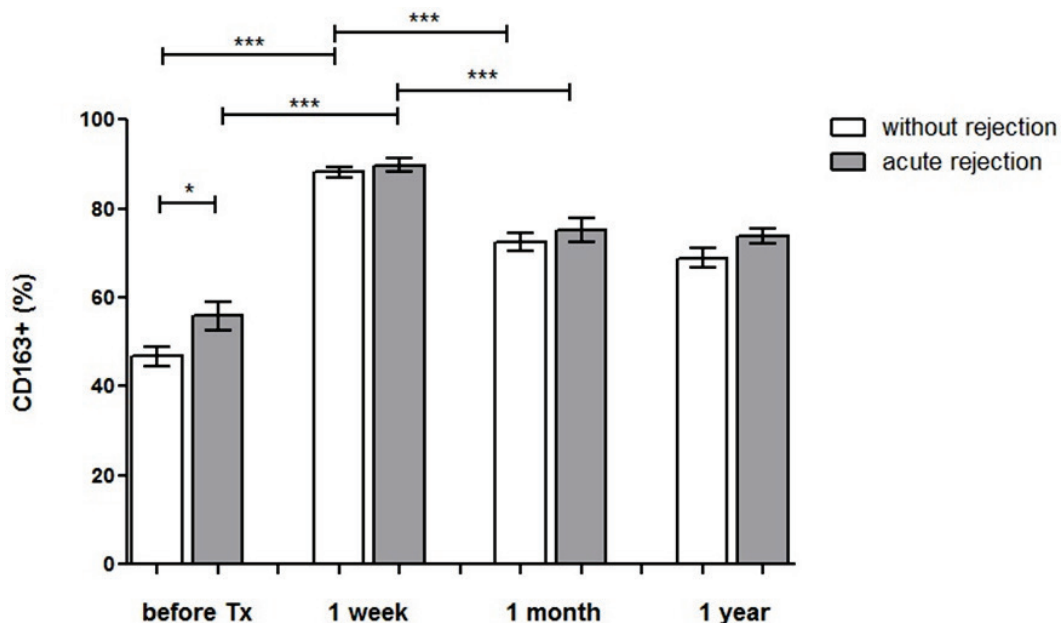


Fig. 3. CD163 expression after the transplantation in patients with acute rejection. The expression of CD163 was evaluated on blood monocytes obtained from patients before the kidney transplantation and one week after the surgery. We compared two groups of patients, those with uncomplicated outcome ($n=94$) and those developing acute rejection ($n=44$). Post-transplant CD163 expression was upregulated in both groups of patients but those with acute rejection had higher pre-transplant percentage of CD163 positive monocytes. The initial upregulation of CD163 was followed by a subsequent decrease at one month after the transplantation and did not change at 1 year. * $p < 0.05$; *** $p < 0.001$.

CD206 expression in peripheral blood monocytes of kidney allograft recipients

The expression of CD206, an alternative marker of M2 macrophages, on peripheral blood monocytes was much lower as compared to CD163 and these two markers of M2 macrophages did not show any mutual association. The expression of CD206 on peripheral blood monocytes (Fig. 4) was upregulated during the first week after kidney allograft transplantation only in acute rejection group ($p < 0.05$).

In vitro modulation of CD163 and CD206 in blood monocytes

The expression of CD163 on monocytes derived from peripheral blood of healthy donors stimulated with IL-10 at 10 ng/ml increased in a culture until day 3 (Fig. 5ab). The expression of CD206 was also upregulated by IL-10 (Fig. 5cd) but, similarly to *in vivo* data, no correlation was found between the expression of CD163 and CD206 (data not shown). In our *in vitro* experiments, two anti-CD163 antibodies were used directed against different epitopes. When the analysis of CD163 expression by monocytes was performed from whole blood samples, RM3/1 clone of monoclonal antibody did not show any detectable expression but the staining pattern was observed

after the gradient separation of mononuclear cells or in buffy coats (6a). In a culture of separated mononuclear cells, both antibodies showed the same trend of CD163 induction by IL-10 (Fig. 6b).

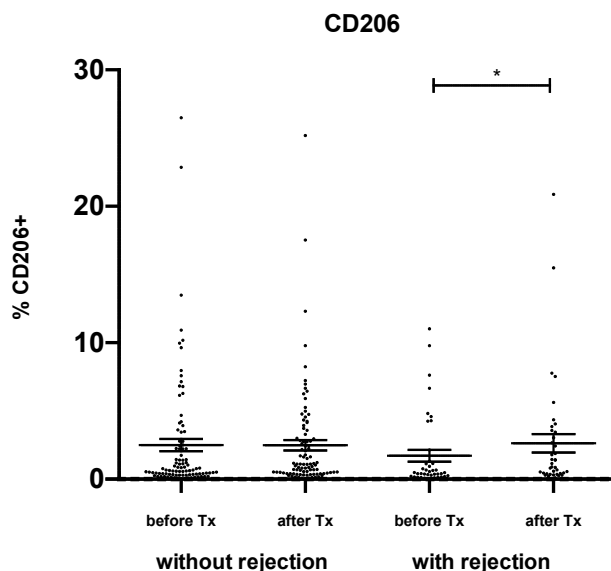


Fig. 4. CD206 expression in peripheral blood monocytes. In kidney transplant recipients, the expression of CD206 was determined on peripheral blood monocytes. The expression of CD206 was relatively low as compared to CD163 and increased during the first week after the transplantation only in patients with acute rejection. Data are expressed as mean \pm SEM; * $p < 0.05$.

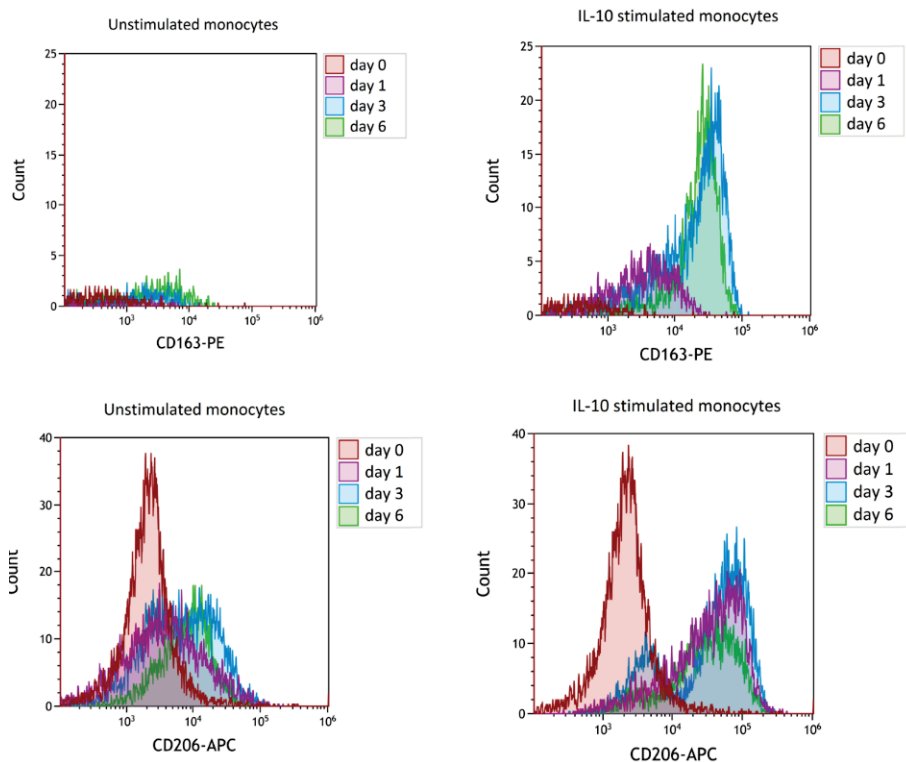


Fig. 5. Flow cytometry plots showing induction of CD163 and CD206 on cultured monocytes by IL-10. Peripheral blood mononuclear cells were cultured in the presence or absence of IL-10 (10 ng/ml) and the expression of CD163 and CD206 was analyzed on day 0, day 1, day 3, and day 6. The expression of CD163 on IL-10 stimulated monocytes increased dramatically on day 3, CD206 expression was upregulated by IL-10 from day 1.

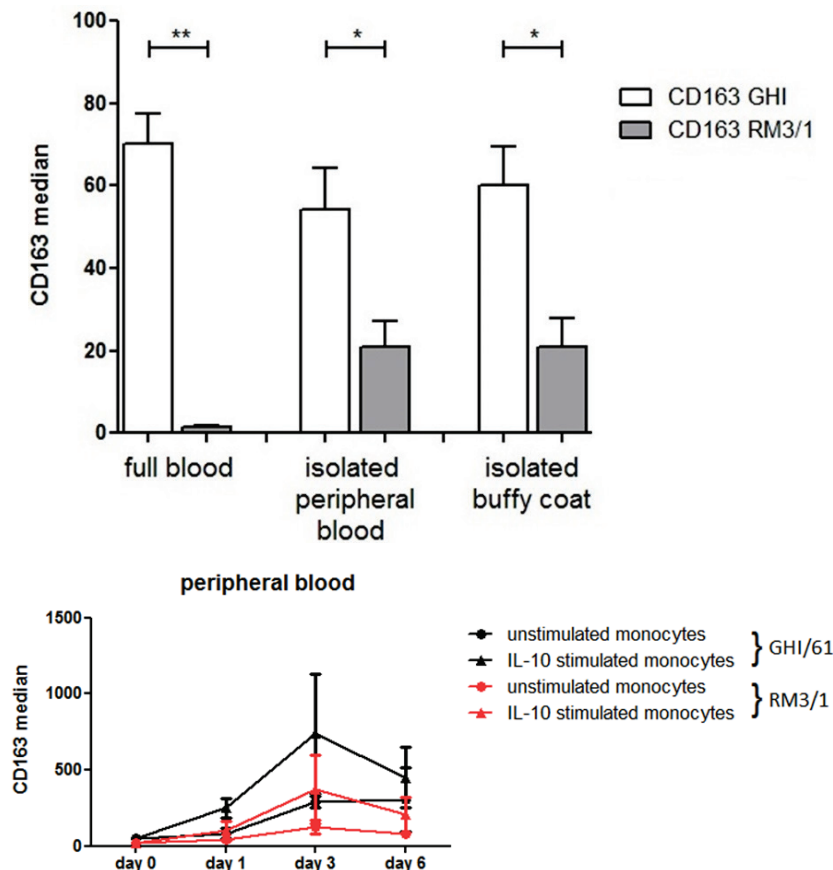


Fig. 6. Modulation of two different epitopes of CD163 by IL-10. Peripheral blood mononuclear cells were cultured for 6 days in the presence or absence of IL-10 (10 ng/ml) and the expression of CD163 was measured by two monoclonal antibodies (RM3/1 and GHI/61) recognizing different epitopes. In whole blood samples, only the GHI/61 clone showed clear membrane expression of CD163 in blood monocytes but staining by RM3/1 emerged after gradient centrifugation of mononuclear cells or in buffy coats (6a). In tissue cultures of mononuclear cells, both epitopes of CD163 on monocytes were markedly upregulated by IL-10 on day 3 (6b).

Discussion

Our data demonstrated in kidney allograft recipients, that CD163 expression was dramatically upregulated in all three subpopulations of peripheral blood monocytes including highly pro-inflammatory non-classical ($CD14^{low}CD16^+$) subset. This observation is in contrast with traditional concept of CD163 as a marker of mononuclear phagocytes downregulating immune responses, specifically M2 macrophages (Hu *et al.* 2017). Also in our previous pilot study by Sekerkova *et al.* (2014), we presumed that $CD14^+CD16^+$ monocytes (both intermediate and non-classical) are pro-inflammatory while $CD14^+CD163^+$ are anti-inflammatory monocytes but our recent data demonstrated that this simplified classification is not valid. We can only speculate whether $CD206^+$, $CD163^+$, or double positive $CD163^+CD206^+$ monocytes represent a M2-like subset of monocytes with immunosuppressive properties but functional data are necessarily needed in this respect.

Pre-transplant distribution of classical, intermediate, and non-classical monocytes did not differ with respect to the clinical outcome as suggested by others (van den Bosch *et al.* 2017b). An alternative marker of M2 macrophages, CD206, did not correlate

with the expression of CD163 on peripheral blood monocytes and showed post-transplant upregulation only in acute rejection group.

The expression of CD163 on blood monocytes before the transplantation was higher in patients in a risk of acute rejection in comparison with kidney allograft recipients with uncomplicated outcome. Furthermore, we observed comparable results in patients with T cell-mediated rejection and those with antibody-mediated rejection, irrespective of their DSA status. The role of CD163 upregulation kidney transplantation is not clear but may reflect either higher need to eliminate hemoglobin/haptoglobin complexes in conditions of chronic tissue injury or a mechanism to increase TWEAK signaling important in post-ischemic repair (Akahori *et al.* 2015). In this respect, higher pre-transplant proportions of CD163 in patients at risk of acute rejection might be associated with more pronounced chronic inflammatory changes in renal parenchyma. There are multiple preexisting donor-derived factors affecting transplant outcome (Van Loon *et al.* 2020). This finding indirectly supports the view that $CD163^+$ monocytes are functionally different from $CD163^+$ M2 tissue macrophages and probably do not exclusively downregulate immune responses. We suppose that the induction of

CD163 on blood monocytes reflects rather the effect of immunosuppression than immune response against the kidney allograft. Recently, corticotherapy was found to upregulate CD163 expression by lung macrophages in patients with COPD (Higham *et al.* 2020).

The upregulation of CD163 by IL-10 has been already described by others (Ritter *et al.* 1999) but our *in vitro* experiments with cytokine stimulation of blood monocytes showed that also CD206 expression in cultured peripheral monocytes was induced by IL-10. In our previous study, upregulation of CD163 expression by monocytes has been shown in a culture with immunosuppressive drugs thymoglobulin or corticosteroids (Sekerikova *et al.* 2014) while calcineurin inhibitors targeting exclusively T lymphocytes (Hoskova *et al.* 2017) probably do not have any effect in this respect. We performed a couple of experiments with THP-1 monocyte/macrophage cell line where the percentage of CD163⁺ cells was much lower than in blood monocytes but the cytokine regulation showed the same trend (data not shown) with high variability between the single experiments. The reason to compare two monoclonal antibodies directed against different epitopes of CD163 emerged from our observation that RM3/1 antibody, excellent for staining of tissue M2 macrophages or a subset of alveolar macrophages (Striz *et al.* 1993), did not provide sufficient binding to peripheral blood monocytes and was thus replaced by

GHI/61 in our panel of antibodies. Under tissue culture conditions, both monoclonal antibodies showed the same profile of CD163 induction in response to IL-10. We can just speculate that CD163 three-dimensional conformation in fresh peripheral blood monocytes differs from those in tissue macrophages or cultured mononuclear phagocytes but different extracellular calcium dependence of different monoclonal antibodies (Maniecki *et al.* 2011) may play a role, too.

Mononuclear phagocytes represent an important part of immune system and some of their properties including memory (Saeed *et al.* 2014) or allorecognition (Zecher *et al.* 2009) lead us to revise the traditional view of innate and adaptive immunity (Cerny and Striz 2019). We believe that better understanding of mechanisms regulating phenotypic pattern of blood monocytes in kidney allograft transplantation might bring new potential biomarkers or therapeutic targets.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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