Immunosuppression and monocyte subsets

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ABSTRACT

Background. Monocytes are critical in innate immunity and transplantation. Three monocyte subsets exist, CD14++CD16-, CD14++CD16+ and CD14+CD16++ monocytes; cell counts of CD14++CD16+ and CD14+CD16++ monocytes are increased in pre-transplant chronic kidney disease. Interestingly, the effect of immunosuppressants on monocyte heterogeneity has not been well studied.

Methods. The impact of immunosuppressants on monocyte subsets was studied: (i) in 152 kidney transplant (KTx) recipients to characterize subset distribution in the steady state, (ii) in patients after autologous (n = 10) versus allogenic (n = 9) haematopoietic stem cell transplantation (HSCT) to analyse monocyte subset development and (iii) in an in vitro model to compare the effect of immunosuppressants on monocyte subset biology.

Results. In KTx, steroid intake was associated with higher total, CD14++CD16- and CD14++CD16+ monocyte counts, but fewer CD14+CD16++ monocytes, whereas intake of mycophenolate, calcineurin inhibitors (CNI) and mammalian target of rapamycin inhibitors (mTORI) did not affect monocyte (subset) counts. In linear regression analysis, only steroid intake was a significant determinant of monocyte (subset) counts: total monocytes (β = 0.331; P < 0.001), CD14++CD16- monocytes (β = 0.374; P < 0.001), CD14++CD16+ monocytes (β = 0.221; P = 0.010) and CD14+CD16++ monocytes (β = −0.169; P = 0.049). After HSCT, CD14++CD16- monocytes were the first to arise, followed by CD14++CD16+ and later by CD14+CD16++ monocytes. Monocyte subset distribution did not differ significantly in patients after autologous compared with allogenic transplantation. CNI, mycophenolate and methotrexate did not influence monocyte subset development, but modified surface receptor expression (CCR2, HLA-DR, ENG, TEK and TLR4) in allogenic HSCT.

Conclusion. Chronic low-dose steroids are associated with monocytosis and higher counts of CD14++CD16- and of proinflammatory CD14++CD16+ monocytes.

Keywords: CD14, CD16, immunosuppression, monocyte, transplantation

INTRODUCTION

Several lines of evidence suggest a prominent role of monocytes and monocyte-derived macrophages in transplant immunology [1]. In vitro monocytes were found to act as intermediaries between allogenic endothelial cells and T cells, thus mediating the induction of an adaptive immune response [2]. In patients with acute renal allograft rejection monocyte infiltration showed a strong association with acute allograft dysfunction [3] and was identified as an independent predictor of allograft survival [4]; in keeping, detection of monocyte markers by immunohistochemistry during protocol biopsies predicted renal allograft outcome [5].

Of note, monocytes are a heterogeneous leucocyte type with distinct phenotypic and functional characteristics. Three human monocyte subsets exist: classical CD14++CD16-, intermediate CD14+CD16+ and non-classical CD14-CD16++ monocytes, the latter have previously been summarized as CD16-positive cells [6]. Specifically, intermediate CD14+CD16+ monocytes are predisposed to potentially exert important
functions in transplantation due to their high endothelial affinity [7] and their ability to present antigen and to induce T cell proliferation [8], both of which are relevant for acute and chronic allograft injury.

The importance of monocyte subsets in the context of vascular injury in cardiovascular medicine has been confirmed in animal models [9] and clinical studies [10–13]. In contrast, only limited experimental [14, 15] and clinical [16, 17] data are available on monocyte heterogeneity in transplantation.

Of note, monocyte depletion inhibited experimental transplant vasculopathy [15]. Translation of these interesting results is hampered by knowledge gaps pertaining to the impact of immunosuppressants on monocyte subsets and the poorly researched developmental relationship of human monocyte subsets.

We therefore applied a three-pronged approach, first studying the effect of immunosuppressants on monocyte subset distribution in stable patients after kidney transplantation; second examining monocyte reconstitution after allogenic and autologous haematopoietic stem cell transplantation (HSCT) and third, analysing the course and the impact of immunosuppressants in an in vitro model of human monocyte subset generation.

**MATERIALS AND METHODS**

**Monocyte subset distribution in stable patients after kidney transplantation**

To characterize the impact of immunosuppressants on monocyte subsets in steady state, we performed flow cytometric analysis of monocyte subsets in 152 stable patients after kidney transplantation (KTx) recruited into the Heterogeneity of Monocytes and Echocardiography among allograft recipients in nephrology (HOMe ALONE) study. Patients referred for routine outpatient follow-up at the Department of Internal Medicine IV, Saarland University Medical Centre, were recruited. Inclusion criteria were stable clinical status and time since transplantation ≥ 12 months; patients were excluded in case of current infections, active malignant disease and acute kidney injury. Additionally, we excluded patients with steroid intake > 4 mg methylprednisolone from the present analyses.

To confirm the effects of steroid medication on monocyte subset distribution beyond the field of transplant medicine, we reanalysed data from our HOM SWEET HOMe study [12], which investigated monocyte subsets in patients at elevated cardiovascular risk referred for elective coronary angiography.

**In vivo reconstitution of monocyte subsets after HSCT**

We prospectively analysed monocyte subpopulation reconstitution in 19 patients after HSCT, of whom 9 patients received allogenic HSCT after myeloablative conditioning regimens (AlloTx, n = 9), and another 10 patients underwent high-dose chemotherapy with subsequent autologous HSCT (AutoTx, n = 10). In the first 11 patients, flow cytometric analysis was done daily from d1 until full reconstitution/hospital discharge; in the remaining patients flow cytometric analysis was done on day 1, and daily from day 4 or day 5 until reconstitution/hospital discharge.

The local ethics committee approved the study and all patients gave their written consent. To analyse the effect of immunosuppressive drugs on monocyte subsets, we compared patients after autologous transplantation with patients undergoing allogenic stem cell transplantation. All patients received peripheral blood mononuclear cells (PBMCs). AlloTx patients received anti-thymocyte globulin (ATG genzyme; 4.5 mg/kg with related and 7.5 mg/kg with unrelated donors, respectively) on Days −4 through −2 for prophylaxis of graft versus host disease. During the study period, all AlloTx patients were on immunosuppressants comprising a calcineurin inhibitor (CNI); either cyclosporine A (target trough level 150–200 ng/mL) or tacrolimus (target trough level: 8–12 ng/mL) either as monotherapy or combined with either mycophenolate mofetil (2 × 1 g/day from Day +1 to Day +28) or methotrexate [15 mg/m² (Day +1), 10 mg/m² (Day +3; +6)].

All AutoTx and AlloTx patients received a single dose of 50 mg prednisolone prior to stem cell transfusion. Afterwards, corticosteroids were selectively administered for treatment of nausea, for prevention of allergic reaction to blood transfusions, or (in AlloTx) for treatment of graft versus host disease. Monocyte subset analysis was performed blinded to clinical characteristics of the respective patient.

**Flow cytometric analysis**

Monocyte subsets were identified via flow cytometry [fluorescence activated cell sorting (FACS) Canto II with FACS Diva Software; BD Biosciences, Heidelberg, Germany] in cell culture or in a whole-blood assay using 100 µL of ethylenediaminetetraacetic acid (EDTA)-anticoagulated blood according to our standardized staining and gating strategy, which has been validated against a proposed reference analysis strategy and virtually leads to identical results [8]. Briefly, monocytes were gated in a side scatter/CD86 dot plot, identifying monocytes as CD86-positive cells with monocyte scatter properties. CD14⁺ CD16⁻, CD14⁻ CD16⁺ and CD14⁻ CD16⁺⁺ monocyte subpopulations were then distinguished by their surface expression pattern of CD14 (LPS receptor) and CD16 (Fcγ/III receptor). Due to the dynamic process of early monocyte emergence after stem cell transplantation and in the in vitro model of monocyte generation, gates were set in order to capture this process best, thus slightly differing from gating when analysing monocyte subsets in individuals with normal peripheral blood counts.

Surface expression of different antigens was quantified as median fluorescence intensity (MFI) and standardized against coated fluorescent particles (SPHEROTM; BD Biosciences); background fluorescence (measured in negative controls) was subtracted. The following antibodies were used: anti-CD14 PerCP (Mc9), anti-CD16 PCy7 (3G8), anti-CD195 APC (2D7/CCR5), anti-CD282 Alexa Fluor 647 (11G7) and anti-CD192 Alexa Fluor 647 (48 607) from BD Biosciences; anti-CD74 FITC (5–329) and anti-CD105 APC (SN6) from ebioscience, Frankfurt, Germany; anti-HLA-DR FITC (L243) and anti-CD202b Alexa Fluor 647 (Ab33) from BioLegend, Fell, Germany; anti-CD143 FITC (9B9) and anti-CD192 FITC (2A9-1) from Biozol, Eching, Germany; anti-CD86 PE (HA5.2B7) from Beckman-
For isolation of CD34+ haematopoietic stem cells, EDTA-anticoagulated blood was drawn from healthy volunteers by venopuncture and PBMCs were immediately isolated by Ficoll-Paque (Lymphocyte Separation Medium; PAA, Colbe, Germany) gradient density centrifugation. CD34+ cells were isolated using the CD34 MicroBead Kit (Miltenyi Biotec, Germany) in a concentration of 105 cells/mL for 13 days in the Haematopoietic Progenitor Cell Expansion Medium DXF (PromoCell GmbH, Heidelberg, Germany) supplemented with the Cytokine Mix E (PromoCell GmbH), which contains the recombinant human growth factors TPO, SCF,flt3-ligand and IL-3. A 25-fold expansion rate of haematopoietic stem cells was observed within the 13 days. In the second step, expanded cells (2 × 106 cells/mL) were seeded in six-well plates in the Haematopoietic Progenitor Medium (PromoCell GmbH). Differentiation of haematopoietic stem cells into monocytes was flow cytometrically monitored after anti-CD86, anti-CD14 and anti-CD16 staining of cells daily from the same well, subdividing them into CD14−CD16−, CD14+CD16− and CD14+CD16+ cells.

For measurement of phagocytosis, Fluoresbrite yellow green carboxylate microspheres (0.75 μm; Polysciences, Eppelheim, Germany) were first opsonized for 30 min at 37°C with serum from healthy donors (diluted to 50% with Krebs Ringers PBS) and adjusted to 108 particles/mL for the phagocytosis assay. Then, 10 μL of opsonized particles were mixed with 1 × 10^6 cells (from Day 7 of differentiation) in 100 μL of culture medium and incubated with gentle shaking for 30 min at 37°C. Subsequently, the samples were washed with FACS buffer, stained at 4°C with anti-CD86, anti-CD14 and anti-CD16 and counts of FITC positive cells were determined flow cytometrically in each cell type (CD14−CD16−, CD14+CD16− and CD14+CD16+ cells).

For measurement of reactive oxygen species (ROS), 1 × 10^6 cells from Day 7 of differentiation were incubated with the cell-permanent carboxyl-H2DFDA (Life Technologies, Darmstadt, Germany) in a concentration of 10 μM for 15 min at 37°C and 5% CO2. Afterwards, cells were stained with anti-CD86, anti-CD14 and anti-CD16, and ROS levels were determined flow cytometrically as MFI within the three cell types (CD14−CD16−, CD14+CD16− and CD14+CD16+ cells).

The ability of distinct cell types (CD14−CD16− and CD14+CD16+) to induce CD4+ T cell proliferation was analysed by measuring the cytoplasmic dilution of CFDA-SE (Vybrant CFDA-SE Cell Tracer Kit; Life Technologies). For this experiment, cells at Day 7 of differentiation were separated into CD14−CD16− and CD14+CD16+ using CD14 Microbeads (Miltenyi Biotec) and cultivated overnight in 96-well plates at a density of 5 × 10^4 cells/well in the presence of 2.5 μg/mL staphylococcal enterotoxin B (SEB; Sigma–Aldrich, Munich, Germany). Within 24 h, CD4+ T cells were isolated from healthy donors using the CD4+ T Cell Isolation Kit II (Miltenyi Biotec) and labelled with 5 μM CFDA-SE for 10 min at 37°C. Then, 2 × 10^6 labelled CD4+ T cells were added to SEB-stimulated cells and counts of proliferating T cells were measured after 4 days as CFDA-SE dilution, identifying T cells after anti-CD3 (CD3 APC; SK7; BioLegend) staining.

For experiments with immune modulators, rapamycin, cyclosporin A and dexamethasone (all purchased from Biomol, Hamburg, Germany) were added to the Haematopoietic Progenitor Medium.

**Statistics**

Categorical variables are presented as percentages of patients and were compared using Fisher’s exact test. Continuous data are expressed as mean ± SD and compared using t-test for two independent samples. In the case of skewed distributions, the median (interquartile range) are given, the Mann–Whitney-U-test was used for two independent samples, and the Kruskal–Wallis test (followed by Dunn’s test as post hoc test) for more than two independent samples. A linear regression analysis was computed with time since transplantation, body mass index (BMI), high-density lipoprotein (HDL), total cholesterol and intake of steroids as independent variables and monocyte (subset) counts as dependent variables. The level of significance was set at P < 0.05.

**RESULTS**

**Monocyte subset distribution in stable kidney transplant recipients**

A total of 152 KTx patients were included into the study. The mean age was 56.1 ± 12.2 years, mean estimated glomerular filtration rate (eGFR) was 46.9 ± 17.0 mL/min/1.73 m2; mean count of CD14−CD16− monocytes was 586 ± 211 cells/μL, of CD14+CD16− monocytes was 35 ± 21 cells/μL and of CD14+CD16+ monocytes was 63 ± 34 cells/μL. Further characteristics are summarized in Table 1.

CNIs were taken by 115 (76%) patients, mycophenolate/mycophenolic acid was prescribed to 104 (68%) subjects, 27 (18%) patients took mammalian target of rapamycin inhibitors (mTORI) and methylprednisolone were part of the immunosuppressive regimen of 108 (71%) patients. The mean monocyte subset counts did not differ when stratifying patients by intake of mycophenolate/mycophenolic acid, CNI or mTORI, respectively (Table 2). In contrast, patients on steroid treatment had significantly higher total monocyte counts, CD14−CD16−, CD14+CD16− monocyte counts and lower CD14+CD16+ monocyte counts than patients without steroid intake (Table 2 and Figure 1A–D). Time since transplant in patients taking steroids was significantly shorter along with lower BMI, higher total and higher HDL cholesterol levels.
towards higher CD14++CD16− steroid therapy is associated with shifts of monocyte subsets.

In all patients, counts of monocyte subsets were analysed from Day +1 to hospital discharge. Indications for HSCT and conditioning regimens are presented in Table 4A and B. Figure 2 depicts representative examples from one patient after autologous HSCT and one patient after allogenic HSCT. CD14++CD16− cells were the first cells appearing in the peripheral blood (Days 5–6). Over the course of time, we observed a gradual increase of CD14++CD16− monocytes, seamlessly followed by the appearance of CD14++CD16+ and later of CD14+CD16++ monocyte counts.

### Table 1. Patient characteristics of total cohort and stratified by intake of steroids

<table>
<thead>
<tr>
<th></th>
<th>Total cohort (n = 152)</th>
<th>Steroid (N) (n = 44)</th>
<th>Steroid (Y) (n = 108)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>56.1 ± 12.2</td>
<td>56.4 ± 12.1</td>
<td>56.0 ± 12.3</td>
<td>0.884</td>
</tr>
<tr>
<td>Sex (male)</td>
<td>97 (64%)</td>
<td>25 (57%)</td>
<td>72 (67%)</td>
<td>0.269</td>
</tr>
<tr>
<td>Diabetes mellitus (Y)</td>
<td>46 (30%)</td>
<td>13 (30%)</td>
<td>33 (31%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Smoking (Y)</td>
<td>16 (11%)</td>
<td>2 (4%)</td>
<td>14 (13%)</td>
<td>0.154</td>
</tr>
<tr>
<td>Time since Tx (years)</td>
<td>5.7 (2.3–9.5)</td>
<td>6.7 (5.3–8.6)</td>
<td>4.8 (1.8–9.8)</td>
<td>0.024</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.3 ± 5.8</td>
<td>29.1 ± 4.8</td>
<td>26.6 ± 6.0</td>
<td>0.015</td>
</tr>
<tr>
<td>BP systolic (mmHg)</td>
<td>146 ± 21</td>
<td>146 ± 21</td>
<td>146 ± 20</td>
<td>0.993</td>
</tr>
<tr>
<td>BP diastolic (mmHg)</td>
<td>85 ± 10</td>
<td>84 ± 9</td>
<td>86 ± 11</td>
<td>0.384</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m²)</td>
<td>46.9 ± 17.0</td>
<td>48.5 ± 14.0</td>
<td>46.3 ± 18.0</td>
<td>0.435</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>2.3 (1.0–5.8)</td>
<td>2.6 (1.3–6.6)</td>
<td>2.2 (0.9–5.8)</td>
<td>0.795</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>198 ± 38</td>
<td>187 ± 36</td>
<td>202 ± 39</td>
<td>0.023</td>
</tr>
<tr>
<td>LDL-C (mg/dL)</td>
<td>114 ± 32</td>
<td>109 ± 31</td>
<td>115 ± 32</td>
<td>0.261</td>
</tr>
<tr>
<td>HDL-C (mg/dL)</td>
<td>58 ± 18</td>
<td>52 ± 17</td>
<td>60 ± 18</td>
<td>0.017</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>139 (97–198)</td>
<td>135 (87–199)</td>
<td>142 (108–197)</td>
<td>0.601</td>
</tr>
</tbody>
</table>

N: no; Y: yes; Tx: transplantation; BMI: body mass index; BP: blood pressure; eGFR: estimated glomerular filtration rate; CRP: C-reactive protein; LDL-C: low-density lipoprotein cholesterol; HDL-C: high-density lipoprotein cholesterol. P-values below 0.05 are given in bold letters.

### Table 2. Monocyte (subset) counts according to intake of classes of immunosuppressants

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Total monocytes</th>
<th>Classical monocytes</th>
<th>Intermediate monocytes</th>
<th>Non-classical monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (n = 44)</td>
<td>565 ± 214</td>
<td>P &lt; 0.001</td>
<td>461 ± 181</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Y (n = 108)</td>
<td>733 ± 230</td>
<td></td>
<td>637 ± 202</td>
<td></td>
</tr>
<tr>
<td>CNI</td>
<td>N (n = 37)</td>
<td>714 ± 210</td>
<td>P = 0.345</td>
<td>612 ± 185</td>
</tr>
<tr>
<td></td>
<td>Y (n = 115)</td>
<td>675 ± 246</td>
<td></td>
<td>578 ± 219</td>
</tr>
<tr>
<td>MMF/MPA</td>
<td>N (n = 48)</td>
<td>703 ± 227</td>
<td>P = 0.501</td>
<td>614 ± 210</td>
</tr>
<tr>
<td></td>
<td>Y (n = 104)</td>
<td>675 ± 243</td>
<td></td>
<td>574 ± 212</td>
</tr>
<tr>
<td>mTORI</td>
<td>N (n = 125)</td>
<td>678 ± 245</td>
<td>P = 0.622</td>
<td>584 ± 219</td>
</tr>
<tr>
<td></td>
<td>Y (n = 27)</td>
<td>705 ± 200</td>
<td></td>
<td>600 ± 174</td>
</tr>
</tbody>
</table>

CNI: calcineurin inhibitor; MMF: mycophenolate mofetil; MPA: mycophenolic acid; mTORI: mammalian target of rapamycin inhibitor. N: no; Y: yes. P-values below 0.05 are given in bold letters.

Even after adjustment for those confounders, intake of steroids remained the only significant determinant of total monocyte and monocyte subset counts (Table 3).

Altogether, in stable kidney transplant recipients, chronic steroid therapy is associated with shifts of monocyte subsets towards higher CD14+CD16− and CD14+CD16+ monocyte counts and lower CD14−CD16++ monocyte counts.

**Monocyte subset distribution in patients referred for elective coronary angiography**

To assess the association of steroid monotherapy with monocyte subset distribution beyond the field of transplant medicine, we compared 12 participants from the HOM SWEET HOMe study who received steroid monotherapy for the treatment of chronic obstructive pulmonary disease, asthma, arthritis, polymyalgia or giant cell arteritis, respectively, to 12 HOM SWEET HOMe participants without steroid intake. Patients were matched for gender, diabetic status, smoking status, age (range ± 5 years), eGFR (range ± 15 mL/min/1.73 m²) and BMI (± 5 kg/m²). These 12 patients on steroid therapy were excluded from the primary immunological analyses within the HOM SWEET HOMe study [12], which listed immunosuppressive medication as exclusion criteria.

The matching procedure was done blinded to monocyte subset distribution status. As demonstrated in Supplementary Table S1, the pairs were well matched. Consistent with the results in renal transplant recipients, we observed in patients on steroid medication significantly higher total (P = 0.035) and CD14+CD16− (P = 0.041) monocyte counts; in addition, they numerically had higher CD14+CD16+ (P = 0.157) and lower CD14−CD16++ (P = 0.285) monocyte counts, although statistical significance was not reached due to the small sample size (Supplementary Figures S1–S4).

**Reconstitution of human monocyte subsets in patients after HSCT**

In all patients, counts of monocyte subsets were analysed from Day +1 to hospital discharge. Indications for HSCT and conditioning regimens are presented in Table 4A and B. Figure 2 depicts representative examples from one patient after autologous HSCT and one patient after allogenic HSCT. CD14+CD16− cells were the first cells appearing in the peripheral blood (Days 5–6). Over the course of time, we observed a gradual increase of CD14+CD16− monocytes, seamlessly followed by the appearance of CD14+CD16+ and later of CD14−CD16++ monocytes. Monocyte subpopulation frequencies after HSCT plateaued at...
**FIGURE 1:** Association of steroids and monocyte subset distribution in kidney transplant recipients. (A) Total monocyte counts, (B) CD14$^+$CD16$^-$ monocyte counts, (C) CD14$^+$CD16$^+$ monocyte counts, (D) CD14$^+$CD16$^{++}$ monocyte counts in 44 patients on steroid-free versus 108 patients on steroid containing regimens; each dot represents one patient, the mean is depicted as the horizontal line; statistical analysis was done with the $t$-test.
Days 8–10 for CD14\(^{+}\)CD16\(^{-}\) monocytes, at Days 12–14 for CD14\(^{+}\)CD16\(^{+}\) monocytes and at Days 14–16 for CD14\(^{+}\)CD16\(^{+}\) monocytes.

Interestingly, the appearance of monocyte subsets in blood of patients after autologous transplantation was similar to the pattern observed in patients after allogeneic transplantation (Figure 2) during the continuous monitoring, potentially suggesting that the immunosuppressants given to patients after allogeneic transplantation had no impact on monocyte subset development. At the end of the study period, which was defined as the day of discharge with clinically stable status, with no need for steroid therapy in the preceding 96 h and with stable immunosuppressive therapy (in allogeneic HSCT patients), percentages of monocyte subsets did not differ between steroid-free patients after allogeneic HSCT (n = 5) and after autologous (n = 9) stem cell transplantation (CD14\(^{+}\)CD16\(^{-}\): 61.2 ± 16.3% versus 60.0 ± 6.7%; CD14\(^{+}\)CD16\(^{+}\): 18.8 ± 8.0% versus 15.4 ± 4.8%; CD14\(^{+}\)CD16\(^{+}\): 17.7 ± 9.2% versus 17.1 ± 4.1%; P > 0.05).

### Expression of surface markers on monocytes from patients after HSCT

In a previous report, we described distinctive surface markers of human monocyte subsets characterizing their respective role in immunity [8]. To investigate whether monocytes of patients after allogeneic HSCT taking immunosuppressants differ from monocytes of patients after autologous HSCT in their expression of functional monocyte markers, we analysed surface expression of these markers (CCR2, CD74, HLA-DR, ENG, TEK, CCR5, ACE, TLR2, TLR4 and CX3CR1) on three monocyte subsets in nine patients at Days 14–16 after autologous HSCT and in four patients at the same timeframe after allogeneic HSCT. These

### Table 3. Linear regression analysis with monocyte (subset) counts as dependent variable

<table>
<thead>
<tr>
<th>Time since Tx (years)</th>
<th>BMI (kg/m(^2))</th>
<th>HDL-C (mg/dL)</th>
<th>Total cholesterol (mg/dL)</th>
<th>Steroid (yes)</th>
</tr>
</thead>
</table>
| β standardized regression coefficient; classical monocytes: CD14\(^{+}\)CD16\(^{-}\) monocytes; intermediate monocytes: CD14\(^{+}\)CD16\(^{+}\) monocytes; non-classical monocytes: CD14\(^{+}\)CD16\(^{+}\) monocytes; Tx: transplantation; BMI: body mass index; HDL-C: high-density lipoprotein cholesterol. P-values below 0.05 are given in bold letters.

### Table 4. Characteristics of patients undergoing (A) allogeneic stem cell transplantation and (B) autologous stem cell transplantation

<table>
<thead>
<tr>
<th>Number</th>
<th>Age</th>
<th>Gender</th>
<th>Indication</th>
<th>Conditioning</th>
<th>Immunosuppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Allogeneic stem cell transplantation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>22.8</td>
<td>m</td>
<td>AML</td>
<td>FLAMSA</td>
<td>CSA/MMF</td>
</tr>
<tr>
<td>2</td>
<td>37.1</td>
<td>m</td>
<td>CML</td>
<td>BU/CY</td>
<td>CSA</td>
</tr>
<tr>
<td>3</td>
<td>54.6</td>
<td>m</td>
<td>AML</td>
<td>FLAMSA</td>
<td>CSA/MMF</td>
</tr>
<tr>
<td>4</td>
<td>58.8</td>
<td>f</td>
<td>AML</td>
<td>CLAMSA</td>
<td>CSA/MMF</td>
</tr>
<tr>
<td>5</td>
<td>63.9</td>
<td>m</td>
<td>DLBCL</td>
<td>FLU/BUCY</td>
<td>TAC/MMF</td>
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<tr>
<td>6</td>
<td>39.1</td>
<td>f</td>
<td>cALL</td>
<td>TBI/CY</td>
<td>CSA/MMF</td>
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<tr>
<td>7</td>
<td>63.8</td>
<td>f</td>
<td>AML</td>
<td>FLAMSA</td>
<td>CSA/MMF</td>
</tr>
<tr>
<td>8</td>
<td>52.7</td>
<td>m</td>
<td>DLBCL</td>
<td>FLU/BUCY</td>
<td>TAC/MMF</td>
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<tr>
<td>9</td>
<td>45.3</td>
<td>m</td>
<td>T-ALL</td>
<td>TBI/CLAMSA</td>
<td>CSA/MMF</td>
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<tr>
<td>(B) Autologous stem cell transplantation</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>1</td>
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<td>f</td>
<td>DLBCL</td>
<td>HD-TTBE</td>
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<tr>
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<td>MM</td>
<td>MEL</td>
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<td>MEL</td>
<td></td>
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<td>4</td>
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<td>m</td>
<td>FL</td>
<td>BEAM</td>
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TBI: total body irradiation; FLAMSA: fludarabine 30 mg/m\(^2\) (d –12 to –9), cytarabine 2000 mg/m\(^2\) (d –12 to –9), amsacrine 100 mg/m\(^2\) (d –12 to –9), TBI 2 x 2 Gy (d –5), cyclophosphamide 60 mg/kg (d –4 to –3), BU/CY: busulfan 3.2 mg/kg (d –7 to –4), cyclophosphamide 60 mg/kg (d –3 to –2), CLAMSA: clofarabine 30 mg/m\(^2\) (d –12 to –9), cytarabine 2000 mg/m\(^2\) (d –12 to –9), amsacrine 100 mg/m\(^2\) (d –12 to –9), TBI 2 x 2 Gy (d –5), cyclophosphamide 60 mg/kg (d –4 to –3), BU/CY: fludarabine 25 mg/m\(^2\) (d –8 to –4), cyclophosphamide 60 mg/kg (d –3 to –2), busulfan 3.2 mg/kg (d –6 to –4); TBI/CY: TBI 2 x 2 Gy (d –3 to –1), cyclophosphamide 60 mg/kg (d –5 to –4); TBI/CLAMSA: total body irradiation 2 x 2 Gy (d –12 to –11), cranial irradiation 24 Gy (d –10); clofarabine 30 mg/m\(^2\) (d –9 to –6), cytarabine 2000 mg/m\(^2\) (d –9 to –6), amsacrine 100 mg/m\(^2\) (d –9 to –6), cyclophosphamide 60 mg/kg (d –9 to –6); CSA: cyclosporine A 3 mg/kg (beginning d –1; subsequently blood target level 150–200 ng/mL); MMF: mycophenolate mofetil 2 g/d (d +1 to +28); TAC: tacrolimus 0.05 mg/kg (beginning d –1; subsequently blood target level 8–12 ng/mL); MTX: methotrexate 15 mg/m\(^2\) (d +1), 10 mg/m\(^2\) (d +3 +6); HD-TTBE: thiotepa 2 x 5 mg/kg (d –4 to –3), carmustine 400 mg/m\(^2\), etoposide 150 mg/m\(^2\) (d –5 to –3); MEL: melphalan 100 mg/m\(^2\) (d –3 to –2); BEAM: Carmustine 2 x 150 mg/m\(^2\) (d –6), cytarabine 2 x 200 mg/m\(^2\) (d –6 to –3), etoposide 2 x 100 mg/m\(^2\) (d –6 to –3), melphalan 140 mg/m\(^2\) (d –2); TBI/CY: total body irradiation 2 x 2 Gy (d –6 to –4), cyclophosphamide 60 mg/kg (d –3 to –2).
markers are centrally involved in distinct physiological and pathological processes such as inflammation and host defence (CD74, HLA-DR, TLR2 and TLR4), atherosclerosis (CCR2, CCR5, CX3CR1 and ACE) and angiogenesis (ENG and TEK). We previously described these markers as characteristic markers of the distinct monocyte subsets: CCR2 expression was a characteristic of CD14++CD16⁻ monocytes, whereas CD74, HLA-DR, ENG, TEK, CCR5, ACE, TLR2 and TLR4 expressions were characteristics of CD14⁺CD16⁺ monocytes and CX3CR1 expression was a characteristic of CD14⁺CD16⁺⁺ monocytes; the subset-specific expression pattern of the respective markers in patients after HSCT matched our previous description of the surface expression in healthy donors [8].

When comparing patients after allogenic with patients after autologous HSCT, subset-specific expression of all surface markers, except for CD74, was lower at Days 14–16 after allogenic HSCT (Figure 3); specifically, CCR5, ACE, TLR2 and CX3CR1 tended to be down-regulated, whereas expression of CCR2, HLA-DR, ENG, TEK and TLR4 was significantly lower (P < 0.05). Of note, whether these observations are solely attributable to the intake of immunosuppressants or are partly explained in the context of an allogenic or chimeric immune system in patients after allogenic HCT cannot be discerned within our study.

In vitro generation of monocytes from CD34⁺ haematopoietic stem cells and impact of immunosuppressants on in vitro generation of human monocyte subsets

Finally, we analysed in vitro monocyte subset generation and the impact of routinely applied immunosuppressants. In this model, CD34⁺ haematopoietic stem cells first differentiated into CD14⁺CD16⁻ monocytes (Days 2–3; with maximal cell counts at Days 7–9); subsequently cultured cells acquired CD16, thus resembling CD14⁺CD16⁺ monocytes (Figure 4A). This course potentially reflects the phenotype changes of monocytes observed in vivo after HSCT. However, no CD14⁺CD16⁺⁺ monocytes were detected in vivo.

To validate monocyte subset identity between in vitro generated monocytes and in vivo developing monocytes, we performed comparative phenotypic and functional analyses. We first measured expression of surface antigens (CD74, HLA-DR, ENG, TEK, CCR5, ACE, TLR2 and TLR4), which are in vivo characteristic markers of circulating CD14⁺CD16⁺ monocytes, on monocyte subsets on Days 4–18 of in vitro culture. Throughout the in vitro culture period, CD14⁺CD16⁺ monocytes showed highest expression of all those characteristic surface markers (Supplementary Figure S5).

Furthermore, we verified functional characteristics of in vitro generated monocytes subsets: CD14⁺CD16⁺ monocytes had a high capacity to phagocyte (Supplementary Figure S6) and to induce CD4⁺ T cell proliferation after SEB stimulation (Supplementary Figure S7); moreover, compared with CD14⁺CD16⁻ and CD14⁺CD16⁻ cells, CD14⁺CD16⁺ monocytes produced highest levels of ROS (Supplementary Figure S8), resembling functional characteristics of their circulating CD14⁺CD16⁺ monocyic counterparts in vivo after HSCT.

The impact of rapamycin (100 nM), cyclosporine A (250 ng/mL) or dexamethasone (250 nM) was tested on the generation of CD34⁺ haematopoietic stem cells to CD14⁺CD16⁺ monocytes (Figure 4B). The proliferation inhibitor rapamycin reduced the development of CD14⁺CD16⁺ monocytes more potently (3.7-fold reduction in CD14⁺CD16⁺ monocyte percentage at Day 8) than dexamethasone (2.2-fold reduction,
Figure 3: Expression of characteristic surface proteins on monocyte subsets in patients after HSCT. Expression of surface proteins was determined as MFI in nine patients after autologous HSCT and in four patients after allogenic HSCT. Statistical analysis was performed using the Mann–Whitney–U-test. *P < 0.05 and **P < 0.01.
respectively), while development of CD14<sup>++</sup>CD16<sup>+</sup> monocytes was not significantly inhibited by cyclosporine A (Figure 4B).

### DISCUSSION

Modern immunosuppressive regimens, which predominantly target T cells, have achieved remarkable improvements in allograft survival, especially in 1 year graft function [19]. However, long-term allograft survival has not improved proportionally [20]. A contemporary understanding of the underlying mechanisms of allograft loss points to an important role of monocytes and monocyte-derived macrophages in these processes.

First, glomerular monocyte accumulation as evidenced during episodes of acute rejection has been identified as an independent predictor of long-term allograft failure after initially successful treatment of rejection episodes [4]. Along the same lines, macrophage infiltration detected by protocol biopsies in low-risk living kidney donation recipients predicted later graft failure [21].

Second, monocytes have been found to interact as intermediaries between endothelial cells and the adaptive immune system thus facilitating rejection processes in vitro [2]. Furthermore, in the absence of T cells after alemtuzumab treatment, monocytes have been identified as key players driving kidney allograft rejection after living donation [22] and finally, selective macrophage depletion in mice prevented the development of cardiac allograft vasculopathy [23]. Altogether, there is a wealth of pre-clinical and clinical data supporting an important role of monocytes and monocyte-derived macrophages in transplantation.

Beyond the field of transplantation, CD16-expressing monocyte subsets have been found to be implicated in autoimmune disease such as Kawasaki disease and inflammatory bowel disease (IBD). Interestingly, in the case of IBD, extracorporeal apheresis of CD16-positive monocytes has been found to reduce disease severity [24] thus raising the question whether targeting monocyte subsets in autoimmune inflammatory conditions and transplantation might be beneficial.

Of special interest in transplantation and clinical immunology may be the intermediate CD14<sup>++</sup>CD16<sup>+</sup> monocyte subset, because circumstantial evidence suggests high endothelial affinity coupled with proinflammatory virtues of CD14<sup>++</sup>CD16<sup>+</sup> monocytes.

**FIGURE 4**: Generation of monocyte subsets in vitro and impact of immunosuppressants on in vitro monocyte generation. (A) After initial expansion of CD34<sup>+</sup> cells, differentiation was initiated and the process was flow cytometrically monitored for 18 days. One representative example of three independent experiments is shown. (B) Acquisition of monocyte surface markers was flow cytometrically monitored for 8 days after the addition of rapamycin (100 nM), cyclosporine A (250 ng/mL) or dexamethasone (250 nM) to the differentiation medium after initial expansion of CD34<sup>+</sup> cells. Data are presented as means ± SEM of three independent experiments. Representative dot plots for Day 8 are shown. Statistical analysis was performed using Kruskal–Wallis followed by Dunn’s test as post hoc test to compare treated samples versus control. *P < 0.05 and **P < 0.01.
monocytes; among those are their combined expression of the chemokine receptor triad, CCR5, CCR2 and CX3CR1 [25], their potential to home to activated endothelial cells and to attract further monocytes and T cells [26] and their highest inflammatory capacity of all monocyte subsets [8, 27], coupled with their ability to facilitate adaptive immune responses by inducing T cell proliferation after antigen presentation [8].

Taken together, the importance of monocytes in clinical immunology, be it transplantation or autoimmune disease, mandates a better understanding of the effect of immunosuppressants on monocyte subsets.

HSCT provides a unique tool for the in vivo evaluation of the temporal sequences of human monocyte subset differentiation and the effect of immunosuppressants thereupon, with the limitation that in allogeneic transplantation a chimeric or even full allogeneic immune system is in operation. Interestingly, only one report looked at monocyte reconstitution of two subsets (CD14++CD16− and CD16-positive cells) early after autologous stem cell transplantation [28]; however, the developmental course of the three subsets has not been rigorously followed. Moreover, the effect of immunomodulating drugs on monocyte heterogeneity has been largely overlooked so far. We report here that classical CD14+CD16− monocytes are the first monocyte subset to emerge after HSCT, followed by intermediate CD14++CD16− monocytes and finally non-classical CD14+CD16++ monocytes.

We further probed monocyte biology in an in vitro model. Although monocytes and macrophages have been previously generated from CD34+ progenitor cells [29], no study has so far looked in vitro on the impact of various immunosuppressants on monocyte heterogeneity. In vitro, only rapamycin and steroids significantly inhibited generation of CD14+CD16− monocytes.

Following HSCT immunosuppressive drugs such as the CNIs cyclosporine A and tacrolimus, mycophenolate mofetil/mycophenolic acid and methotrexate do not substantially alter monocyte subset distribution but merely modulate cell surface receptor expression of CCR2, HLA-DR, ENG, TEK, CCR5, ACE, TLR2, TLR4 and CX3CR1. Functional characteristics of monocyte subsets could thus be altered since these receptors are implicated in various physiologic and pathophysiologic processes, ranging from inflammation and host defence (CD74, HLA-DR, TLR2 and TLR4) to atherosclerosis (CCR2, CCR5, CX3CR1 and ACE) and angiogenesis (ENG and TEK).

The clinical relevance of our findings was demonstrated in a large cohort of stable kidney transplant recipients. In these patients, we could confirm that maintenance low-dose steroid-containing regimens are associated with higher CD14++CD16− and CD14++CD16+ monocyte counts, and lower CD14+CD16++ monocyte counts, whereas neither the intake of CNI, nor the intake of MMF/MPA or mTORI, affected cell counts of monocyte subsets. Even after correction for confounders, steroids remained the only significant determinant of monocyte subset distribution. Furthermore, in a small cohort of patients with preserved renal function, receiving steroid monotherapy, we could confirm the association of steroid intake and monocyte subset distribution observed in KTx patients.

Given the association of CD14++CD16+ monocytes with atherosclerotic vascular disease [30], their elevated counts observed under maintenance steroid therapy might partly explain higher cardiovascular risk of transplant recipients on steroid-containing immunosuppressive regimens [31].

Taken together, routinely applied immunosuppressants in KTx, apart from steroids, have no significant impact on monocyte heterogeneity. For monocyte-targeted therapy, alternative immunosuppressive strategies have to be considered, e.g. via immune-modifying microparticles as currently tested in preclinical and early clinical development [32].

SUPPLEMENTARY DATA

Supplementary data are available online at http://ndt.oxfordjournals.org.

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CONFLICT OF INTEREST STATEMENT

The authors have nothing to disclose.

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