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Monocyte derived macrophages from lung transplantation patients have an increased M2 profile

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ABSTRACT

Introduction: Lung transplantation (LTx) is a last treatment option for patients with an end-stage pulmonary disease. Although the monocyte-macrophage lineage is accepted to be clinically important only little is known about the effect of immunosuppressive drugs in combination with chronic rejection. It is likely that local inflammatory conditions and immunosuppressive medication alter the activation state of macrophages. The goal of this study was to determine how monocyte derived macrophage subsets were affected in LTx patients.
Methods: PBMC's were obtained by ficoll density gradient centrifugation and cultured in RPMI with 10% FCS for 7 days. For identification and quantification of cultured monocyte derived macrophages fluorescence-activated cell sorting analysis was performed. Markers including; CD16, CD64, CD200r, CD163 and CD14 were used to determine M1, M2a and M2b macrophages.
Results: Transplantation patients showed an increased and frequency ($p = 0.0245$) for M2a macrophages compared to healthy controls. Also, median fluorescence intensity of CD163, CD64, HLA-DR and CD200r increased with transplantation.
Discussion: An increase in M2 phenotype macrophages in transplantation patients is in line with the latest findings in solid organ transplantation. M2 macrophages are associated with tissue-regeneration and diminished capacity of host defence, possibly leading to fibrosis development [1]. What this exactly means for the disease process and current clinical assessment requires further investigation.

1. Introduction

Long term survival following lung transplantation is still limited by acute and chronic lung allograft dysfunction (CLAD). CLAD eventually involves the long-term airflow obstruction and rejection of the allograft. Multiple triggers including infection and acid reflux are associated with innate and eventually acquired immunity against the allograft [2–4]. Alveolar macrophages provide a first line of defence against infections and irritants [1]. Already in 1976 it was observed that, in transplanted lungs, the macrophage population is maintained by the recruitment of blood monocytes [5]. Later research by Eguiluz–Gracia provided more results suggesting that blood monocytes can replenish the lung if alveolar macrophages are depleted in some way [6].

Macrophages are a heterogeneous population of cells that belong to the mononuclear phagocyte system and have been studied extensively over the years [5,7–10]. Macrophages are able to react to stimulation and regulation factors from the environment by changing phenotype and function. Their microenvironment is a critical determinant in their response to injury [1,11]. Mosser et al. introduced a classification based on function and with more room for phenotypical overlap [1]. This includes three main subsets namely; classically activated macrophages also known as M1 macrophages, wound-healing macrophages also known as M2a macrophages and immune regulatory macrophages also known as M2b macrophages [12].

Despite the clinical importance of the monocyte-macrophage lineage only little is known about the effect of immunosuppressive drugs in combination with chronic rejection. It is likely that local inflammatory conditions and immunosuppressive medication alter the activation state of macrophages. Shifting the balance between M1 and M2 macrophages could result in a dampened immune response against the graft or exasperate chronic graft rejection. Kannegieter et al. found *in vitro* that when cultured with tacrolimus and mycophenolic acid the

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polarization of macrophages shifts towards an M2 like phenotype [13]. While van den Bosch et al. found that skewed macrophage polarization towards an M2 type was a hallmark for acute heart allograft rejection [9].

To continue on these building blocks we investigate the differentiation of monocyte derived macrophages from lung transplantation patients *in vitro*. These patients have been taking immunosuppressive medication for a long period of time and a group of patients suffer from CLAD.

2. Materials and methods

2.1. Patients

EDTA blood samples were obtained from lung transplant recipients ($N = 38$), of which 8 were diagnosed with CLAD. Also, 10 healthy controls were included. Lung transplant recipients who received their lung transplantation at the Heart Lung Centre in Utrecht, the Netherlands and received follow up care at the St. Antonius Hospital and had Biobank informed consent were included. The diagnosis CLAD was defined as a decline in forced expiratory volume in one second (FEV1) of more than 20% from the baseline determined by an average of two measurements made at least three weeks apart in the absence of known causes [14].

2.2. Purification of blood monocytes and macrophage cell culture

PBMC's were obtained by Ficoll density gradient centrifugation and cultured in RPMI (Thermo Fisher Scientific, Waltham, MA USA) supplemented with 10% heat inactivated foetal calf serum (Thermo Fisher Scientific). After 2 h of incubation, culture plates were washed twice with PBS (Thermo Fisher Scientific) leaving only monocytes on the culture plate. Monocytes were incubated with RPMI and 10% heat inactivated foetal calf serum for 7 days. Trypsin (Thermo Fisher Scientific) was used to harvest macrophages following one week of incubation.

2.3. Flow cytometry

For identification and quantification of cultured monocyte derived macrophages fluorescence-activated cell sorting analysis was performed. Macrophages were stained for CD14 (63D3, Biolegend, San Diego, CA USA), CD16 (3G8, Biolegend), CD64 (10.1, Biolegend), CD163 (GHI/61, Biolegend), HLA-DR (L243 G466, BD Biosciences, San Jose, CA USA) and CD200r (OX-108, Biolegend). To prevent non-specific binding, cells were suspended in flow cytometry wash solution (BD Biosciences) with 10% human serum (Merck KGaA, Darmstadt, Germany).

3. Results

3.1. Overall surface markers expression

Culture of monocyte derived macrophages from lung transplantation patients and healthy controls was done free of antibiotics, growth factors and other macrophage stimulants known to influence macrophage differentiation. This way we aimed to better determine the effect of long term use of immunosuppressive medication on the monocyte-macrophage lineage. The median fluorescence intensity (MFI) of surface markers on monocyte-derived macrophages from transplantation patients is shown in Fig. 1.

3.2. A shift towards the M2a type macrophage in transplantation patients

Next, subsets of macrophages were determined based on specific surface marker expression. Classically activated macrophages or M1 were determined as CD16+ CD64+(CD200r-CD163-). Alternatively activated macrophages were determined as M2a when CD14+CD200r +(CD64-, CD16-, CD163-) and M2b when CD14+CD163+(CD64-, CD16-, CD200r-). Fig. 2 shows the frequency of total for all three macrophage subsets.

M1 macrophages showed an overall low and stable frequency in healthy controls and transplantation patients. M2b macrophages showed a higher but also a stable frequency. M2a macrophages showed a significant increase (*0.0245) in transplantation patients.

4. Discussion

In this study we cultured monocyte-derived macrophages from lung transplantation patients that have been using immunosuppressive medication for multiple years and healthy controls. Transplantation patients showed an increased frequency (* $p = 0.0245$) of M2a macrophages compared to healthy controls. CLAD diagnosed patients showed an increased MFI for CD14. Overall, expression of CD163, CD64, HLA-DR and CD200r MFI increased with transplantation.

This is a single centre study with a limited cohort. Even though this is small and modest study, these data can certainly be a contributing building block when bridging the gap between *in vitro* cell line studies and tissue staining studies during allograft rejection.

In vitro data on monocyte derived macrophages by Kannegieter et al. shows that direct stimulation with tacrolimus and mycophenolic acid (MPA) leads to macrophage polarization to an M2-like phenotype and increases expression of surface markers CD163 and CD200r [13]. These data are in line with our data. Kannegieter et al. used spiked macrophages from healthy volunteers while we cultured monocyte-derived macrophages from patients using immunosuppressive medication. Macrophage culture was executed without additional additives during incubation. This suggests that this *in vitro* effect also happens *in vivo*. The use of corticosteroids is accepted as a driver of polarization towards a more M2 phenotype [7,9,15–18].

The contribution macrophages have in organs transplantation has been a topic of interest and to date a number of studies indicate the accumulation of macrophages in both acute and chronic rejection injury models. Only recently van den Bosch et al. found that during acute heart transplant rejection significant more M2 macrophages are present in the tissue and almost no M1 macrophages [9]. Also, during chronic renal allograft rejection an increase of macrophages together with myofibroblasts is noted [19]. Others found similar results, indication that during chronic rejection an allograft is characterized by interstitial infiltration of macrophages [20,21].

Eardley et al. found that the infiltrating macrophages correlate with the degree and severity of damage and fibrosis found in the allograft [22]. In a healthy state M2 macrophages are intended to create an anti-inflammatory environment and response. Thereby promote healing and regeneration of wounds [23]. However, during chronic inflammation that persists for several weeks or months, macrophages are also capable of inducing scar formation or fibrosis [24]. By Toki et al. and Ikezumi et al. it was described that the accumulation of M2 macrophages correlates with the severity of fibrosis [25,26]. Nicod et al. demonstrated that major phenotypic changes occur in alveolar macrophages shortly after lung transplantation [27]. We also show a major shift towards an M2 profile in *in vitro* monocyte derived macrophages from lung transplantation patients.

It has become clear that macrophages are an important component during allograft rejection [24,28,29]. With this study we found that monocyte-derived macrophages from transplantation patients have an increased M2a phenotype in line with the latest finding in solid organ transplantation [1,8,11,15,24,30]. M2 macrophages are associated with a tissue-regenerated ability possibly leading to development of fibrosis and a diminished capacity of host defence [1]. However, what this exactly means for the disease process is not yet clear. The fact that respiratory tract infections increase the risk of developing chronic

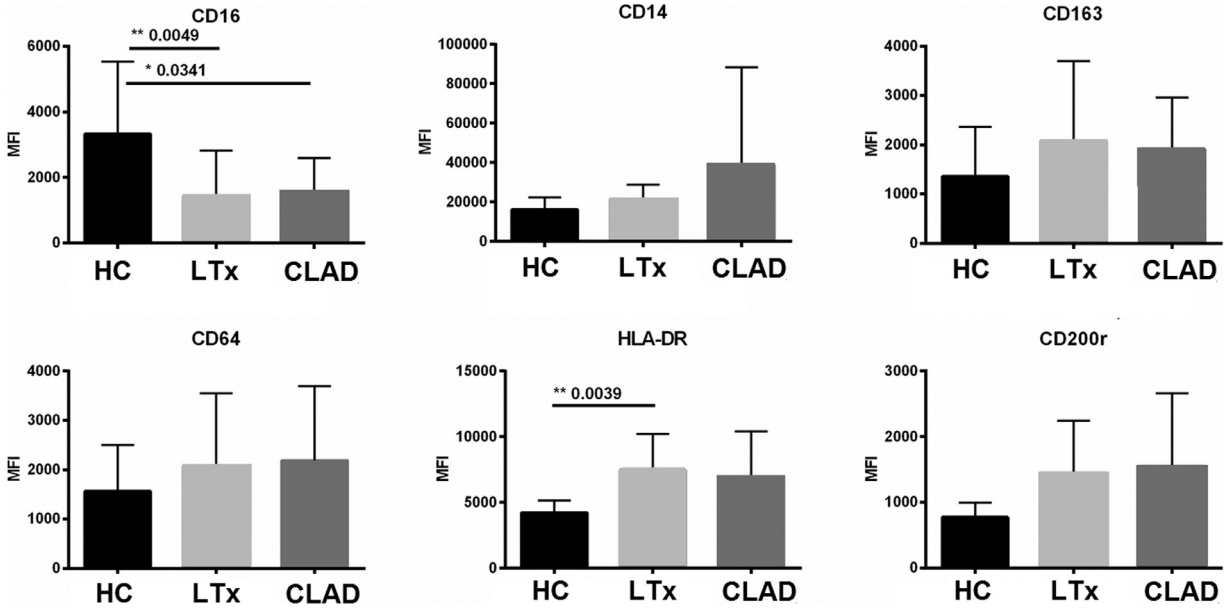

Fig. 1. Shows median fluorescent intensity for surface markers CD16, CD14, CD163, CD64, HLA-DR and CD200r. Each marker is plotted for healthy controls (HC, $N = 10$), for lung transplantation patients (LTX, $N = 30$) and for CLAD diagnosed transplantation patients (CLAD, $N = 8$). Healthy controls show higher MFI for CD16 while CLAD diagnosed patients show higher MFI for CD14. Overall, expression of CD163, CD64, HLA-DR and CD200r shown an increasing trend in transplantation.

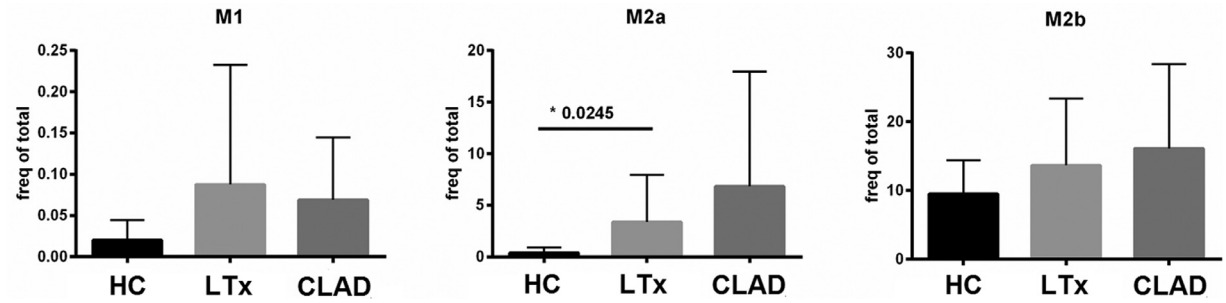

Fig. 2. Shows frequency of total for classically activated macrophages or M1, wound healing macrophages or M2a and immune regulatory macrophages also known as M2b. Each macrophage subset is plotted for healthy controls (HC, $N = 10$), for lung transplantation patients (LTX, $N = 30$) and for CLAD diagnosed transplantation patients (CLAD, $N = 8$).

rejection seems a logical association if the infiltrate of the monocyte-macrophage lineage is limited to a tissue regeneration profile. Also, current clinical assessment of cellular rejection following lung transplantation does not include macrophages. Therewithal, immunosuppressive regimen does not focus on macrophage functionality. Further studies are needed to further identify the functionality of macrophages in lung transplantation and their role in allograft rejection.

Declaration of Competing Interest

None of the authors of this manuscript have affiliations with organizations with direct or indirect financial interest in the subject matter discussed in the manuscript.

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