Peripheral blood dendritic cells in human end-stage heart failure and the early post-transplant period: evidence for systemic Th1 immune responses

Petros Athanassopoulos*, Leonard M.B. Vaessen, Alex P.W.M. Maat, Aggie H.M.M. Balk, Willem Weimar, Ad J.J.C. Bogers

Departments of Cardiothoracic Surgery, Cardiology and Internal Medicine, Erasmus MC, Rotterdam, The Netherlands

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Abstract

Objectives: Dendritic cells (DCs) are antigen presenting cells that play a central role in inflammation, allograft rejection and immune tolerance. Myeloid (mDC) and plasmacytoid (pDC) subsets regulate immune reactions by polarising naïve T-helper cells into a Th1 or Th2 response, respectively. In this study we examined total peripheral blood DCs, mDC and pDC subsets in chronic heart failure (CHF) and clinical heart transplantation (HTx). Methods: We compared 16 heart transplant patients before and after HTx to 14 healthy controls. Whole blood was collected pre-HTx and 1-week post-HTx from patients and at corresponding time-points from controls. All patients received induction and maintenance immunosuppression post-HTx. mDCs and pDCs were measured by flow cytometry and were further characterised for maturation and homing potential to the secondary lymphoid organs with CD83 and CCR7, respectively. Data were expressed as absolute numbers/µl whole blood, percentage (%) mDC or pDC of total blood DCs and % positive DCs for CD83 and CCR7.

Results: CHF patients had more peripheral blood DCs compared to controls (P < 0.01) while only the mDC fraction was increased compared to controls (P = 0.01). Percentage CD83+ and CCR7+ mDCs was also higher than control levels (P < 0.05). One week post-HTx, total DCs, mDCs and pDCs decreased below controls (P < 0.001). At the same time % mDCs in peripheral blood increased markedly compared to CHF and control levels (P < 0.001). The % CD83+ mDC, % CD83+ pDC and % CCR7+ mDC also returned to control levels and only % CCR7+ pDC decreased below control levels (P = 0.005). Conclusions: Total peripheral blood DCs are elevated during CHF due to an increase in the mature fraction of the mDC subset suggesting a possible Th1 response in end-stage heart failure. The decrease in total DCs and mature mDCs and pDCs seen post-HTx, probably reflects immunological quiescence through adequate immunosuppression. Peripheral blood DC monitoring may provide a new insight into mechanisms of heart failure and allograft rejection by safe weaning from immunosuppression after clinical HTx.

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1. Introduction

Heart failure is a clinical syndrome with unknown pathophysiological parameters. Many molecular, genetic and biochemical pathways have been suggested as determinants for the development of overt myocardial dysfunction [1]. Progression of the disease to chronic heart failure (CHF) is based on intrinsic compensatory neurohormonal, cellular and mechanical signals, which lead to further cardiac deterioration and adverse remodelling. In the latter process, mononuclear phagocyte migration into the injured myocardial tissue is considered a pivotal step in potentiating cardiac inflammatory responses. The contribution of systemic immune activation to the pathogenesis of human CHF has been demonstrated by abnormal plasma profiles of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), IL-1β, IL-2, IL-6 [2], soluble cytokine receptors (sTNFR-I and sTNFR-II) [3],


* Corresponding author. Address: Department of Cardiothoracic Surgery, Erasmus MC, University Medical Center Rotterdam, Room Eu563a, Dr. Molewaterplein 50, P.O. Box 1738, 3015 GE Rotterdam, The Netherlands. Tel.: +31-10-463-5421; fax: +31-10-408-9443.

E-mail address: p.athanassopoulos@erasusmc.nl (P. Athanassopoulos).
and chemokines (CXCL-1, -5, -8 and CCL-2, -3, -5) [4,5] in several studies on ischaemic heart disease (IHD), dilated (DCM) or hypertrophic cardiomyopathy (HCM). Others have also been able to display the involvement of specific immune components which are capable of polarising the immune response towards a T-helper–1 type of reaction in experimental in vivo models of myocardial ischaemia-reperfusion injury [6], DCM [7] and myocarditis [8]. The factors causing this apparent Th1 deviation of immune responses in heart failure still remain to be elucidated.

There is growing evidence that the polarisation of immune responses resides on the progeny, development and phenotype of antigen presenting cells (APCs) [9]. Dendritic cells (DCs) are professional APCs that play a central role in establishing and controlling immune responses by priming naive helper and cytotoxic T lymphocytes [10]. Immature DCs are characterised by an increased potential for phagocytosis and a low co-stimulatory molecule (CD40, CD25) and maturation marker (CD80, CD83, CD86) expression. Ligation of inflammatory chemokine receptors like CCR1, CCR2 and CCR5 on their surface is thought to recruit immature DCs and their blood precursors to sites of inflammation and infection [11]. In this state, DCs reside in peripheral tissues, where they act as sentinels by capturing and processing microbial antigens and pro-inflammatory T cell-derived stimuli, constantly. Upon antigen encounter however, DCs become mature and exert effector functions such as antigen presentation and T-cell activation in the lymph nodes. In this maturation process, DCs undergo characteristic phenotypical and functional changes. They exhibit reduced phagocytic activity and expression of major histocompatibility complex (MHC) molecules, they upregulate co-stimulatory molecules and expression of maturation markers. They also secrete large amounts of immunostimulatory cytokines like IL-12 [12] and display a solely lymphoid chemokine receptor expression pattern (CCR7 and CXCR4) which enables them to migrate to the T-cell areas of draining secondary lymphoid organs, where they initiate adaptive immune responses [13].

In humans, two DC subsets, myeloid DCs (mDCs) and plasmacytoid DCs (pDCs), have been identified. The mDCs, derived from myeloid precursors, express cell markers CD11c, CD13 and CD33, and require exogenous granulocyte-macrophage colony-stimulating factor in their micro-environment for survival. mDCs, also called Type 1 DCs (DC1), produce high levels of IL-12 when stimulated with TNF-α or CD40 ligand and drive a potent Th1 polarised immune response. On the other hand, pDCs, which originate from lymphoid precursors, show a plasma cell-like morphology and express high amounts of IL-3 receptor-α chain (CD123), which is necessary for their survival and differentiation. pDCs, also called Type 2 DCs (DC2), can elicit an IL-4-independent Th2 polarisation of naive T cells [14].

The two distinct peripheral blood DC subsets follow different migratory patterns. Myeloid DCs capture foreign antigens and migrate into the regional lymph nodes through afferent lymphatics, where they present the antigens to T cells. Thus, mDCs are considered the primary Th1-inducing DCs responsible for the surveillance against pathogenic signals in the periphery. By contrast, pDCs enter the T-cell areas of the lymph nodes directly from the blood stream via high endothelial venules (HEVs). pDCs also express high levels of L-selectin, which mediates their extravasation by interaction with L-selectin ligand peripheral lymph node addressin, which in turn is exclusively expressed by HEVs [15].

On the basis of these distinct properties of the DC subsets, mDCs and pDCs are considered to be specialised APCs preferentially inducing a Th1 and Th2 response, respectively. In end-stage heart failure, it is suggested that Th1-biased immune responses may evolve from a skewing to a primarily myeloid blood DC population. To clarify whether the balance between mDCs and pDCs is altered in CHF, and to observe the effects of heart transplantation (HTx) on DC subsets we examined the numbers of mDCs and pDCs and the percentage mDCs and pDCs of total peripheral blood DCs. We further investigated the state of maturation and potential for homing to the lymphoid organs within each circulating DC subset from heart transplant candidates before and early after HTx.

2. Patients and methods

2.1. Study groups

The study protocol was approved by the local medical ethical committee on human research (MEC 215.732/2002/157). All patients were recruited from the Thoraxcenter, Erasmus Medical Center (Rotterdam, The Netherlands) and gave informed consent before entering the study. Patients with heart failure were meticulously defined by the New York Heart Association (NYHA) classification system at least 1 month prior to transplantation. The subjects included 16 heart transplant candidates, consisting of four patients with IHD, five patients with HCM and seven patients with DCM who underwent HTx.

All patients received typical medication against heart failure before transplantation (pre-HTx), namely a combination of an ACE inhibitor, a β-blocker, a diuretic, a statin, anti-arrhythmic and anti-coagulant agents. After transplantation, all patients received induction therapy in the form of horse-ATG (Imtix Sangstat BV, Lyon, France) at 3–8 i.v. dosages daily (1 dosage = 212.5 lymphocytyotoxic-units/kg per 24 h) until adequate Cyclosporin A (CsA) or Tacrolimus (FK506) trough levels were achieved (250–350 ng/ml and 12–16 ng/ml, respectively). The combination of Prednisolone, CsA or FK506 and Mycophenolate Mofetil (MMF) was used as maintenance immunosuppression. All patients received 75 mg prednisolone i.v. in the first post-transplant day and 50 mg/day for 5 consecutive days post-HTx.
At the time of venous puncture for DC enumeration (day 7–8 post-HTx) all patients received 40 mg prednisolone daily. CsA and FK506 were started at 8 and 0.3 mg/kg per 24 h oral dosages, respectively, divided in two doses daily, which were titrated further according to the corresponding trough levels. Two patients were already converted to FK506 within the first week post-HTx after experiencing CsA-induced nephrotoxicity. MMF was administered daily in dosages between 500 and 1500 mg, at the end of the induction therapy. Fourteen healthy volunteers, who received no medication, were also studied as control subjects.

2.2. Monoclonal antibodies

Allophycocyanin (APC)-conjugated CD11c, phycoerythrin-(PE)-conjugated anti-IL-3 receptor α chain (CD123), PE-conjugated CD83 and PE-conjugated anti-CCR7 (CD197w), peridinin chlorophyll protein (PerCP)-conjugated anti-HLA-DR and fluorescein isothiocyanate (FITC)-conjugated lineage cocktail 1 (lin 1) were purchased from Becton Dickinson (San Jose, CA, USA) and R&D Systems (Abingdon, UK). The lin 1 contains monoclonal antibodies (mAbs): CD3 (T cells), CD14 (monocytes/macrophages), CD16 (natural killer cells), CD19 (B cells), and CD56 (natural killer cells). PE- and PerCP-conjugated isotype control murine mAbs were obtained from Becton Dickinson.

2.3. Flow cytometric analysis

Peripheral blood cells, obtained from the subjects in a prospective manner, were analysed by four-colour flow cytometry. All blood samples from patients and controls were collected in the morning. To minimise selective loss during the preparation procedure, the cells were first stained with mAbs followed by lysing of the erythrocytes. Briefly, the blood cells were incubated with APC-, PE-, PerCP-, and FITC-conjugated mAbs for 20 min at room temperature. The erythrocytes were then lysed with FACS lysing solution (Becton Dickinson). After washing with FACSflow, the stained cells were analysed with a FACS Caliber flow cytometer and the CellQuest Pro software (Becton Dickinson). DCs were defined as the cells positive for PerCP-conjugated anti-HLA-DR mAb and negative for FITC-conjugated lin 1 mAbs. The number of total white blood cells in the samples was determined using an automated cell counter. CD11c conjugated with APC or CD123 conjugated with PE was used for identification of the mDC and pDC subsets. CD83 or anti-CCR7 was used for further characterisation of the maturation status and homing pattern of mDCs and pDCs, respectively. Absolute numbers of mDCs and pDCs were calculated from the white blood cell count multiplied by the proportion of each subset within the white blood cells. The percentage of mDCs and pDCs was derived from the total number of DCs as determined by flow cytometric analysis. Percentage positive mDCs or pDCs for CD83 or CCR7 was calculated from the total number of mDCs or pDCs, respectively.

2.4. Dendritic cell characterisation

For gating Lin− HLA-DR+ cells, whole peripheral blood cells were stained with anti-HLA-DR mAb and the Lineage Cocktail (Fig. 1a). In the gated cells we further defined the expression of CD11c and CD123 to determine the two distinct DC lineages, mDCs and pDCs were defined as Lin− HLA-DR+ CD11chigh CD123low and Lin− HLA-DR+ CD11clow CD123high, respectively. Representative profiles of CD11c and CD123 on peripheral blood DCs from a CHF patient are shown in Fig. 1b, which clearly indicates the two DC subsets. Acquired mDC and pDC subsets of controls and patients were analysed according to the maturation marker CD83 and the homing chemokine receptor CCR7 (Fig. 1c).

2.5. Statistical analysis

The unpaired (Mann–Whitney) and the paired (Wilcoxon) t-tests were used for statistical analysis with the GraphPad statistical program (GraphPad Software Inc., San Diego, CA, USA). A P-value of <0.05 was considered significant. Values are expressed as the mean ± SEM of absolute DC numbers or percentage (%) positive myeloid and plasmacytoid dendritic cells.

3. Results

3.1. Clinical characteristics

Between healthy controls and patients with CHF, there was no significant difference in age or sex proportion (Table 1). The heart transplant candidates from all three categories (IHD, HCM, DCM) were classified clinically with end-stage heart failure (NYHA III–IV). The blood total leucocyte counts were not significantly higher in heart failure than in controls (P = 0.24), although a relative lymphopenia (P < 0.01) and a relative granulocytosis (P < 0.0001) were apparent in the CHF group against controls (Table 1). However, at 1 week after transplantation total leucocyte count was significantly higher than in controls (P < 0.0001) or patients during CHF (P < 0.02). This was attributed to a relative increase of granulocyte proportions (P < 0.001) and a concomitant decrease in lymphocytes (P < 0.02) when compared to the CHF condition, probably due to the rebound effect of horse-ATG (Table 1). No age or sex proportion difference was found between patients with HCM or DCM and patients with IHD or between patients classified with NYHA III and IV heart failure.
3.2. Numbers of blood mDCs and pDCs in heart transplant recipients and normal subjects

The % of DCs in peripheral blood of controls was stable at 0.32 ± 0.02 of total white blood cells (Fig. 2) and remained constant during a 1-month follow-up (data not shown). The % DCs in CHF patients (0.44 ± 0.05) was higher than controls (P = 0.001). At 1 week post-HTx we saw a marked decrease in the % of DCs (0.04 ± 0.01; P < 0.001) (Fig. 2a).

By analysing the distribution of absolute numbers of DCs in peripheral blood of individual subjects we observed that controls had a total DC number at 16.2 ± 1.8 cells/µl. For patients during CHF, total DC numbers at 31.3 ± 4.7 cells/µl were higher than controls (P = 0.008) (Fig. 2b). The mDC numbers in CHF patients were also higher than in the healthy controls (20.2 ± 3.8 vs 8.2 ± 1.0 cells/µl; P = 0.01) (Fig. 3a). However, the numbers of blood pDCs in CHF patients were no different to the corresponding pDC numbers in normal subjects (7.5 ± 0.9 vs 6.5 ± 1.0 cells/µl; P = 0.45) (Fig. 3b). One week post-transplant, however, total DC numbers were lower than the pre-transplant and control condition at 3.1 ± 0.6 cells/µl (P < 0.0001) (Fig. 2b). The mDC and pDC numbers were 2.5 ± 0.6 and 0.5 ± 0.1 cells/µl, respectively, both clearly lower than the pre-transplant (P ≤ 0.0005) and control condition (P < 0.0001) (Fig. 3a and b).

When compared to normal subjects, heart failure patients pre-HTx, exhibited a significant increment of 8.4% in blood mDCs (mDC: from 56.3 ± 1.7 to 64.7 ± 2.9; P < 0.01) and an equal decrement in pDCs (pDC: from 43.7 ± 1.5 to 35.3 ± 3.2; P < 0.01) (Fig. 3c). We noted that amongst CHF patients, the highest % mDCs were attributed to subjects with manifested DCM and HCM (88.3 ± 3.1 and 66.0 ± 2.8, respectively) compared to subjects with IHD (52.9 ± 7.8) (data not shown). In the first week post-HTx...
an increase in % blood mDCs to 81.8 ± 3.2 accompanied by a concomitant decrease in % blood pDCs to 18.2 ± 3.2, was dramatic for all transplanted patients when compared to the pre-HTx condition (P < 0.001) (Fig. 3c).

3.3. Maturation status and homing potential of mDCs and pDCs in heart transplant recipients and normal subjects

Before transplantation, CD83 and CCR7 positive mDCs were significantly higher than control (%CD83 + mDC: 22.2 ± 4.6 vs 12.4 ± 1.8; P = 0.02) (%CCR7 + mDC: 28.2 ± 6.7 vs 13.9 ± 2.1; P = 0.01). While the numbers of mDCs remained low post-transplant, %CD83 + mDCs and %CCR7 + mDCs decreased to control levels (10.1 ± 2.3 and 14.8 ± 3.7, respectively) (Fig. 4a). At 1-week post-HTx, the numbers of pDCs were also lower than the pre-HTx or the control condition. Pre-HTx, CD83 + pDCs but not CCR7 + pDCs were significantly higher than controls (%CD83 + pDC: 16.9 ± 4.2 vs 3.0 ± 0.5; P < 0.0001) (%CCR7 + pDC: 73.2 ± 5.9 vs 68.1 ± 1.9; P = 0.29). At 1-week post-HTx, however, %CD83 + pDCs and %CCR7 + pDCs decreased significantly (5.7 ± 1.7 and 40.9 ± 7.6, respectively), but the decrease in CCR7 + pDCs with 33% was more prompt than the decrease in CD83 + pDCs (Fig. 4b).

4. Discussion

In the present study, we directly enumerated two distinct DC subsets, mDCs and pDCs, in the peripheral blood of patients with end-stage heart failure (NYHA III–IV) before and shortly after transplantation, using four-colour flow cytometry. We found significantly elevated total DC numbers and a marked increase of circulating mDCs with
a concomitant decrease of pDCs in patients with end-stage CHF, leading to an apparent alteration of the mDC:pDC balance toward mDCs. These data suggest that a Th1 predisposition of immune responses in heart failure may be associated with a polarised mDC:pDC balance towards mDCs.

Two hypotheses on the origin of immune activation in patients with CHF have been proposed, based on experimental and clinical data. The first suggests that bowel wall oedema may lead to bacterial translocation with subsequent endotoxin release and immune activation [16]. The second indicates that in CHF, the heart might also contribute to inflammatory cytokine and chemokine production. This has been demonstrated by the fact that TNF-α and CCL2 (MCP-1) are produced by the failing human myocardium but not by a normal heart [17]. However, no single source of cytokine production seems sufficient to fully explain the multiple organ involvement and the systemic low-grade inflammation seen in CHF. Although chronic physiological shear stress as seen in extensive atherosclerosis and CHF, are the most potent stimuli inducing immune responses with peripheral or myocardial pro-inflammatory cytokine and chemokine production [19]. In this context, DCs may play a central role, acting as scavengers for peripheral stress stimuli when they are immature and as the amplifiers of systemic activation through the involvement of lymphoid organs at a mature state.

During a systemic immune response, DCs are considered critical determinants for the polarisation of naive T cells into Th1 or Th2 cells. Rissoan and co-workers [13], demonstrated that the distinct subsets of human DCs, mDC and pDC, induce the different profiles of T-cell responses during immune activation. Consistent with this, several reports have shown that mDCs produce a large amount of the pro-inflammatory IL-12 and preferentially induce Th1 development which augments inflammatory responses, whereas pDCs secrete lower amounts of IL-12 and primarily elicit Th2 development [20]. Contrary to these observations, other studies have described also a plasticity of the DC subsets in directing T-cell responses. Certain anti-inflammatory molecules such as IL-10, transforming growth factor-β, and prostaglandin E2, are capable of stimulating immature mDCs to induce Th2 differentiation [21,22]. Therefore, functional differences between DCs in guiding T-cell responses might depend not only on their lineage but also on the micro-environment of cytokines and/or inflammatory mediators produced in immune responses. It is generally agreed though, that a reciprocal equilibrium of mDCs and pDCs with IL-12 regulates T-helper reactions, proposing the mDCs as the principal APCs inducing a Th1 response in humans.

We demonstrated that in a pathological state, such as end-stage heart failure, DC subsets may exhibit altered migratory properties. The profound shift in mDC:pDC balance towards mDCs in patients with CHF must reflect systemic Th1 polarisation. Furthermore, the mDCs and pDCs encountered in the blood of the CHF patients included in our study were also mature, according to their upregulation for CD83. The concurrent CCR7 increase on the mDCs must account not only for maturation of this subset but also for increased potential of the mDCs encountered in CHF to migrate towards secondary lymphoid organs. An observation which suggests that mDCs are indeed more efficient in eliciting systemic inflammatory responses through the lymph nodes in end-stage heart failure.

In our patient cohort, the balance shifted towards mDCs also shortly after HTx. An observation, which suggests a mechanism of immune deviation by acute heart injury, probably due to trauma or during the transplantation procedure through ischaemia/reperfusion injury. Cell damage and death have been demonstrated to present danger signals that program DCs to mature and secrete IL-12 [23]. In murine heart allografts, chemokines CCL2 (MCP-1), CXCL1 (Gro-α) and CXCL10 (IP-10) have been detected by the fifth post-transplant day. Thereby, intragraft
released pro-inflammatory cytokines increase expression of MHC molecules and co-stimulatory molecules on graft derived and recipient infiltrating DCs [24]. Our results suggest that both donor and recipient DCs are considered to be programmed to drive the differentiation of Th0 to Th1 cells and to initiate acute rejection.

However, after transplantation, the numbers of circulating mDCs and pDCs were decreased significantly, suggesting that the components of the immunosuppressive treatment administered already in the first week after transplantation (e.g. h-ATG, corticosteroids, calcineurin inhibitors or MMF) are able to induce immunological quiescence. Indeed the impact on pDC numbers was more striking, suggesting that pDCs might be differentially affected by this specific immunosuppressive drug regimen. One can only speculate that pDCs, (a) become prone to die earlier, (b) migrate quicker into the lymphoid organs or (c) are selectively more expeditiously inhibited in their egress from bone marrow, when compared to the mDCs post-HTx. Both circulating DC subsets were also rendered into an immature state. This was confirmed by the lack of the maturation markers CD83 and CCR7 on mDCs and pDCs, suggesting that immunosuppression might exert a potent effect not only on the maturation status but also on the migration characteristics of the mDCs and pDCs. Nevertheless, the differential homing pattern of pDCs due to downregulation of CCR7 below control levels can be explained, since pDCs in contrast to mDCs, have a defective responsiveness to the CCR7 ligands (CCL19 and CCL21). Moreover, pDCs seem to be able to enter the T-cell areas of the lymph nodes from the blood stream directly via HEVs only through chemokine receptor CXCR4 and its ligand SDF-1 (CXCL12) interaction [15].

DC recirculation appears to be a dynamic process but the principles governing DC subset recruitment, migration and thus blood mDC:pDC balance remain unknown. In heart failure, it is also uncertain whether the alteration in blood mDC:pDC balance reflects an intrinsic DC aberrancy or it follows from commitment imposed by extrinsic factors inducing ventricular remodelling or cardiac decompensation. Because IL-12 has a potent capacity to recruit granulocytes and CD34+ progenitor cells from the bone marrow into the circulation, it might also selectively mobilise mDCs or their precursor into the peripheral blood [25]. It is accepted that IL-12, as the principal pro-inflammatory cytokine produced by APCs, could be regulated by mediators with opposing functions produced in inflammation, such as TNF-α and IL-10 [26]. Therefore it is also likely that the interaction of DCs with differential systemic or lymphoid levels of TNF-α and IL-10 may contribute to a selective perseverance of mDCs in heart failure patients and the early post-HTx period. Further studies will be required to clarify the precise mechanisms causing this presence of mDCs in abundance.

We are aware of the limitations of our study. Our patient group in which we measured and characterised circulating DCs is possibly too small in order to conclude that mDCs, their state of maturation or homing potential could be correlated precisely to the clinical classification of NYHA III or IV CHF. Moreover, the clinical data used to characterise the subjects were available from their last pre-transplant screening in the out-patient department of our clinic and did not match precisely the time-point of blood sampling for DC enumeration. However, the fact that CHF patients had generally higher total peripheral blood DC numbers than healthy individuals is intriguing. In particular, the observation that subjects with DCM or HCM prior to HTx accounted for higher percentages of circulating mature mDCs needs to be investigated in the future.

In summary, the present data clearly indicate that in patients with heart failure, the mDC:pDC balance polarises towards mature mDCs in the peripheral blood, which may be associated with Th1 biased immune responses in later stages of heart failure. We suggest that DC stimulation may possibly be a biological mechanism involved in the pathogenesis of end-stage heart failure. The decrease in total DC, mature mDCs and pDCs after transplantation probably reflects immunological quiescence through adequate immunosuppression.

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References


