Chemokine-receptor Mediated Dendritic Cell and T-cell Recirculation in Heart Failure and Transplantation

Chemokine-receptor gemedieerde dendritische cel en T-cel recirculatie tijdens hartfalen en na transplantatie

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Στους γονείς μου

"The human mind treats a new idea the way the body treats a strange protein.... it rejects it."

Peter Medawar

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Chapter 1

General Introduction

Introduction

Heart failure (HF) is a progressive disorder characterized by impaired cardiac function and often by circulatory congestion. Once considered a sole problem of left ventricular pump dysfunction, HF has now come to be perceived as a highly complex clinical syndrome that is manifested by many extracardiac features, including neuroendocrine activation and cytokine release [1]. Common clinical manifestations of HF such as dyspnoea and fatigue limit exercise tolerance, while fluid retention may lead to pulmonary and peripheral oedema. Ischemia through coronary artery disease and hypertension are the most common underlying causes of left ventricular systolic dysfunction. Other, less frequent, causes of dysfunction include primary myocardial disease (cardiomyopathy), congenital malformations and valvular disorders [2]. Virtually any form of heart disease can lead to HF while abnormalities of systolic and diastolic function frequently coexist. Whereas the presence of immune activation as well as the prognostic relevance of low-grade inflammation in chronic HF is now widely accepted [3], the site and the source of cytokine production remain the object of intense research [4]. In many cases the onset of the disease is completely unknown, clinically silent, and not fully understood. This is particularly true for patients with dilated cardiomyopathy, one of the types of myocardial disease that may result in HF, even at a young age [5].

The recent addition of beta-blockers and resynchronization therapy to the HF armamentarium have resulted in better clinical outcomes [6-8]. However, the prognosis of patients suffering from HF, remains very poor with estimated life expectancies far less than in many forms of cancer. In real end-stage disease, heart transplantation (HTx) is the only therapeutic option that can prolong life and improve quality of life considerably. Despite improvements in short-term heart allograft survival through the available immunosuppressive medication, late-graft loss due to rejection remains a clinical obstacle post-HTx [9]. In addition, complications of continual immunosuppressive therapy, which include markedly increased risks for opportunistic infections, nephrotoxicity, malignancies, diabetes, hypertension and additional cardiovascular disease, negatively affect the survival and quality of life of cardiac allograft recipients. For years, the transplantation community attempts to improve graft outcome while obviating the need for immunosuppression in selected patients. In this context, predicting rejection would be advantageous for the prevention of short- and long-term complications post-HTx. Identifying patients at high risk for rejection is important as appropriate adjustments in immunosuppressive

regimens may avoid over-immunosuppression of patients at a lower risk for rejection after clinical HTx.

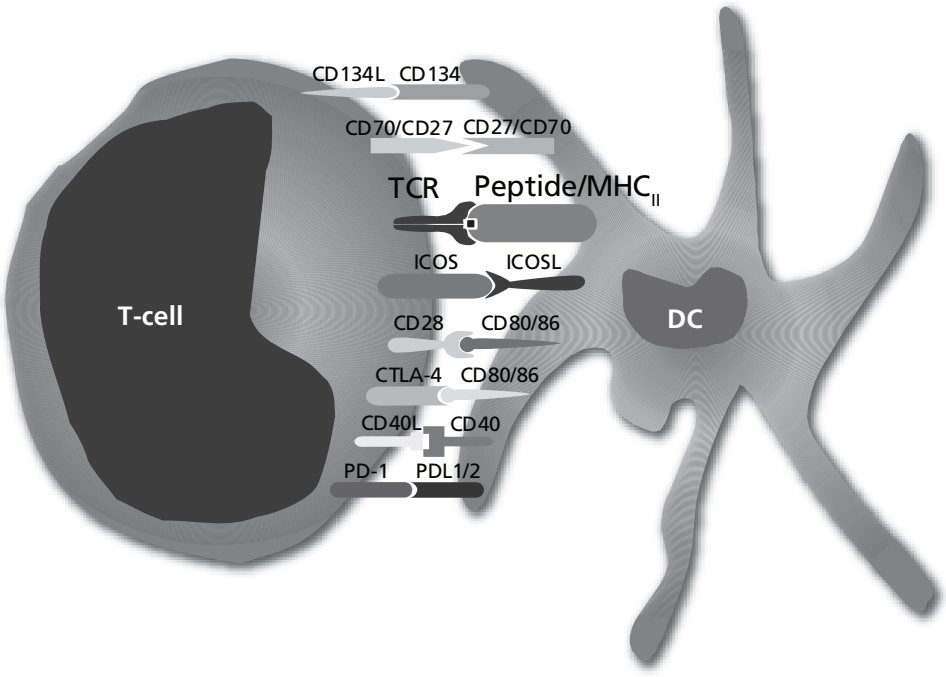

Figure 1: Co-stimulatory pathways. Signaling between dendritic cells (DCs) and T-cells is based primarily on ligation of the T-cell receptor with its antigen (peptide) bound to MHC molecules on the surface of DCs. Positive signals through CD80/86 – CD28, CD40L – CD40 and the TNF/TNF-R family of molecules (e.g. CD134 – CD134L, CD70/27 – CD27/70) induce T-cell proliferation, cytokine production and differentiation into effector T-cells. Negative signals like CTLA-4 – CD80/86 and PD-1 – PDL1/2 can result in anergy, loss of proliferative capacity and impaired cytokine production.

The primary basis for allograft rejection is the ability of T-lymphocytes to recognize, polymorphic versions of a variety of proteins referred to as alloantigens, through their antigen receptors. Alloantigens are encoded within the major histocompatibility complex (MHC) with hundreds of different alleles identified at the five major human MHC (HLA) loci (HLA-A, HLA-B, HLA-C, HLA-DR and HLA-DQ). Alloantigen specific T-cells can secrete cytokines – e.g. interferon-γ (IFN-γ) and interleukin-4 (IL-4) – that recruit and activate cells of the innate immune system (macrophages and eosinophils, respectively), which in turn can secrete tumor necrosis factor-α (TNF-α) and lymphotoxin which

cause direct tissue destruction [10]. Optimal activation of naïve CD4+ and CD8+ T-cells essential for rejection, is accomplished by both alloantigen recognition through the T-cell receptor (TCR) and the delivery of co-stimulatory signals through the binding of T-cell–surface receptors (e.g. CD28) to their ligands (e.g. CD80 or CD86), expressed on antigen-presenting cells, such as dendritic cells (DCs) [11] (Figure 1). Once activated and recruited by chemokines expressed in the graft, T-cells can directly kill donor target T-cells through perforin and granzyme B [12].

Chemokines and their receptors

Chemokines are a large family of 8 to 12 kDa glycoproteins, which primarily function as leukocyte chemoattractants [13,14]. According to the number and position of NH_2-terminal cysteine (C) residues, the majority of chemokines are subdivided into the CC (CCL1-28) and CXC (CXCL1-16) subfamilies, while the C family contains only 2 members (XCL1 and XCL2) and CX_3C only 1 member (CX_3CL1) [15,16]. Except for regulating leukocyte homeostasis, chemokines exert diverse biological effects on many cell types such as endothelial cells, fibroblasts and vascular smooth muscle cells. While contributing in physiological processes such as infection, angiogenesis [17], wound healing and hematopoiesis [18], excessive chemokine activation may result in inappropriate inflammation through immune cell activation [19], leading to cell and tissue damage.

Chemokines exert their effects through interactions with the chemokine receptors: a family of 7 transmembrane domain-containing G-protein coupled receptors (GPCRs). Signaling by chemokine receptors is mediated by heterotrimeric G-proteins containing $G\alpha_i$. These activate protein and lipid kinases such as mitogen-activated protein (MAP), Janus kinase-signal transducer and activator of transcription (JAK-STAT), and phosphatidyl inositol-3-kinase (PI3K), which mediate actin cytoskeleton rearrangement, changes in integrin affinity and avidity, leukocyte proliferation, differentiation, and apoptosis [20]. Within the chemokine system, there is a significant redundancy, as shown by the binding of multiple chemokines to a particular receptor and multiple receptors interacting with a particular chemokine. Currently, there are 10 identified CC chemokine receptors (CCR1-10), 6 CXC receptors (CXCR1-6), 1 C receptor (XCR1), and 1 CX_3C receptor (CX_3CR1) [15,16].

In total, three functional families of chemokines and their receptors have been defined [14]. The "homeostatic family" includes those molecules functioning specifically in the migration of leukocytes to and within lymphoid tissues, as well as those functioning in hematopoiesis, including chemokines such as CCL19

and CCL21 which bind to CCR7 [21-26], CXCL12/SDF-1 and its receptor CXCR4 [27-30] and CXCL13 and its receptor CXCR5 [31-33]. The "inflammatory family" includes a wide array of molecules involved in the migration of innate and adaptive immune effector cells to sites of inflammation. Chemokines in this group (CCL2, CCL4, CCL5, CXCL8, CXCL10) are expressed in response to a host of inflammatory stimuli, including pathogen-associated molecular products, and inflammatory cytokines such as TNFα and type I and type II interferons. These chemokines are expressed by tissue cells, fibroblasts, endothelial cells, DCs, monocytes, NK cells, and T-cells. The "dual function family" includes CCL1, CCL17, CCL25 (and receptors CCR8, CCR4, and CCR9, respectively), CXCL9 to CXCL11 (interacting with CXCR3), and CXCL16 (interacting with CXCR6), which function both in inflammation and in the migration of T-cells within the thymus [14]. Several chemokines within this group are involved in the recirculation of memory T-cells restricted to specific tissues such as the small intestine (CCL25/CCR9) [34,35] and skin (CCL17, CCL22/CCR4) [36,37].
Responsiveness to inflammatory and homeostatic chemokines is further regulated by desensitization of receptors after chemokine binding. Homologous desensitization occurs in a ligand-dependent manner and involves phosphorylation of the receptor by specific G-protein–coupled receptor kinases (GRKs), followed by β-arrestin-mediated targeting for endocytosis [38]. Ligand-independent desensitization also occurs, when stimulation of a heterologous GPCR leads to phosphorylation of others through second messenger-dependent kinases such as protein kinase C, and subsequent G-protein decoupling [39]. In this manner, cross talk between chemokine receptors has been demonstrated [40,41].

Dendritic- and T- cell trafficking as dictated by chemokines and their receptors

Dendritic cells (DCs) comprise a heterogeneous family of antigen presenting cells that possess the ability to prime naïve helper and cytotoxic T-lymphocytes [42]. Normally, phenotypically immature DCs migrate continuously into blood from the bone marrow. Under the influence of inflammatory chemokines, DCs reside in non-lymphoid tissues where they constantly process microbial or viral antigens [43]. Upon tissue damage, infection or inflammation, further pro-inflammatory cytokine and chemokine release induces DCs to phagocytose antigens and increase surface expression of the maturation marker CD83 as well as MHC class I & II molecules [44]. Antigen-loaded DCs begin 'homing' to lymphatic tissue through differential regulation of chemokine receptors

on their surface. Enhanced interaction of chemokine receptor CCR7 with its chemokine ligands CCL19 & CCL21, thereby enables mature DCs to migrate via afferent lymphatic vessels or blood to secondary lymphoid organs. Naïve and central memory T-cells also express CCR7 and migrate to secondary lymphoid tissues through specialized postcapillary venules that support high levels of lymphocyte extravasation from the blood, called high endothelial venules (HEVs). $CCR7^+$ T-cells stay in the T-cell area under influence of CCL19 and CCL21, whereas mature dendritic cells expressing CCR7 colocalize with T-cells for antigen presentation. Once in the T-cell area, T-cells are antigen primed by dendritic cells and undergo activation, multiplication, and functional maturation [45].

The initial DC- naïve T-helper (T_H) cell interaction is critical in that it defines the type of acquired immune response. Except for antigenic stimuli ('signal 1'), DCs are also equipped to deliver co-stimulatory information ('signal 2') and 'signal 3' for optimal differentiation of naïve T-cells into distinct T_H subsets [46]. Depending upon their progeny from a common myeloid or a common lymphoid precursor, the nature of antigen and cytokine/chemokine micro-environment, it has been shown that DC maturation influences subsequent T-cell responses with myeloid (m) and plasmacytoid (p) DCs preferentially polarizing to T-helper 1 (T_H1) or T-helper 2 (T_H2) responses *in vitro* [47].

Accordingly, the cellular makeup of infiltrates at inflammatory sites is determined both by the chemokines induced at the tissue site and the responsiveness of various cell types to those chemokines. After the priming process by DCs within secondary lymphoid tissues, multiple effector $CD4^+$ or $CD8^+$ T-cell subsets are generated which down-regulate CCR7 & CXCR4 and become less responsive to chemokines involved in trafficking of naïve cells to lymphoid tissues (CCL19, CCL21 & CXCL12). Additionally, effector T-cells up-regulate different sets of receptors for inflammatory chemokines, guiding further their migration to non-lymphoid sites of inflammation: T_H1/cytotoxic cells expressing CCR1, CCR2, CCR5, CXCR3, CX_3CR1 and CXCR6, whereas T_H2/cytotoxic cells express CCR4 and CCR3. Non-polarized memory T-cells are uncommitted T-cells and circulate in blood and lymphoid tissues in a manner similar to naïve T-cells [48] (Figure 2).

Chemokines and their receptors in activation of leukocytes during heart failure

Chronic low-grade inflammation with infiltrating leukocytes has been found in the failing human myocardium independent of the cause of failure [49-51]. By playing a crucial role in recruitment and activation of these cells, certain

chemokines (e.g. CCL2 & CXCL8) lead to damage and dysfunction of the cardiac muscle in patients with chronic HF through activation and production of reactive oxygen species (ROS), matrix metalloproteinases (MMPs) and inflammatory cytokines [52]. Representing a pathogenic loop in end-stage HF, oxidative stress may further activate the transcription factor NF-κB in various cell types, which again induces the synthesis of CXCL8 & CCL2 [53]. Enhanced MMP activity has been found in non-ischemic and ischemic forms of cardiomyopathy, possibly contributing to myocardial remodeling, and inflammatory chemokines are involved in this process [54]. Notably, increased cardiomyocyte gene expression of the CCL2 receptor (CCR2) has been demonstrated in the failing human myocardium, suggesting that CCL2 may play a pathogenic role in HF by directly acting on cardiomyocytes [55]. Chemokines may potentially modulate myocardial function both directly through effects on cardiomyocytes and indirectly through effects on infiltrating T-cells, monocytes, granulocytes and native fibroblasts or endothelial cells within the failing myocardium. Enhanced myocardial expression of chemokine receptors on both cardiomyocytes and infiltrating leukocytes in various forms of myocardial failure suggests a potential central role for chemokine related interactions in the pathogenesis of these disorders [56].

Besides chemotaxis and leukocyte activation, chemokines may also regulate several other biological processes of importance to the pathogenesis of HF, e.g. fibrosis, angiogenesis, apoptosis [57]. Furthermore, multiple DC-based mechanisms have been implicated in progression of HF after infectious autoimmune disease [58]. Chronic DC-driven myocardial inflammation has been demonstrated to result in functional impairment of the ventricles with hemodynamic characteristics of dilated cardiomyopathy. In such cases, tissue-resident DCs appear to be critical for the propagation and maintenance of HF after autoimmune myocarditis [59].

Chemokines and their receptors in leukocyte infiltration during heart allograft rejection

Acute rejection (AR) after HTx, is diagnosed by histological examination of routine endomyocardial biopsies (EMBs) from within the first weeks up to the 1st year after transplantation. Characteristically, AR is diagnosed histologically by allograft infiltration with CD4^{+} and CD8^{+} T-cells, cardiomyocyte lysis and necrosis [60]. Experimental data point to the chemokine receptors CXCR3 and CCR5, which are induced upon activation of T-cells, as key mediators of effector cell recruitment in response to the local elaboration of chemokines by

graft endothelial and parenchymal cells [61]. Data from clinical studies suggest that these pathways are also active in human recipients of cardiac allografts. Serial EMBs from clinical cardiac allograft recipients have demonstrated that expression of CXCR3 was temporally and spatially associated with $CD3^+$ T-cell infiltrates, and correlated with the histopathological diagnosis of AR [62]. The CXCR3 ligand, CXCL10, was rarely expressed in biopsies showing normal myocardium, but was markedly induced in endomyocardial biopsies from patients suffering from AR [63,64]. Furthermore, expression of CCL5 has been demonstrated by infiltrating neointimal lymphocytes and macrophages in arterioles and venules adjacent to the wall of coronary arteries showing graft vascular disease [65,66].

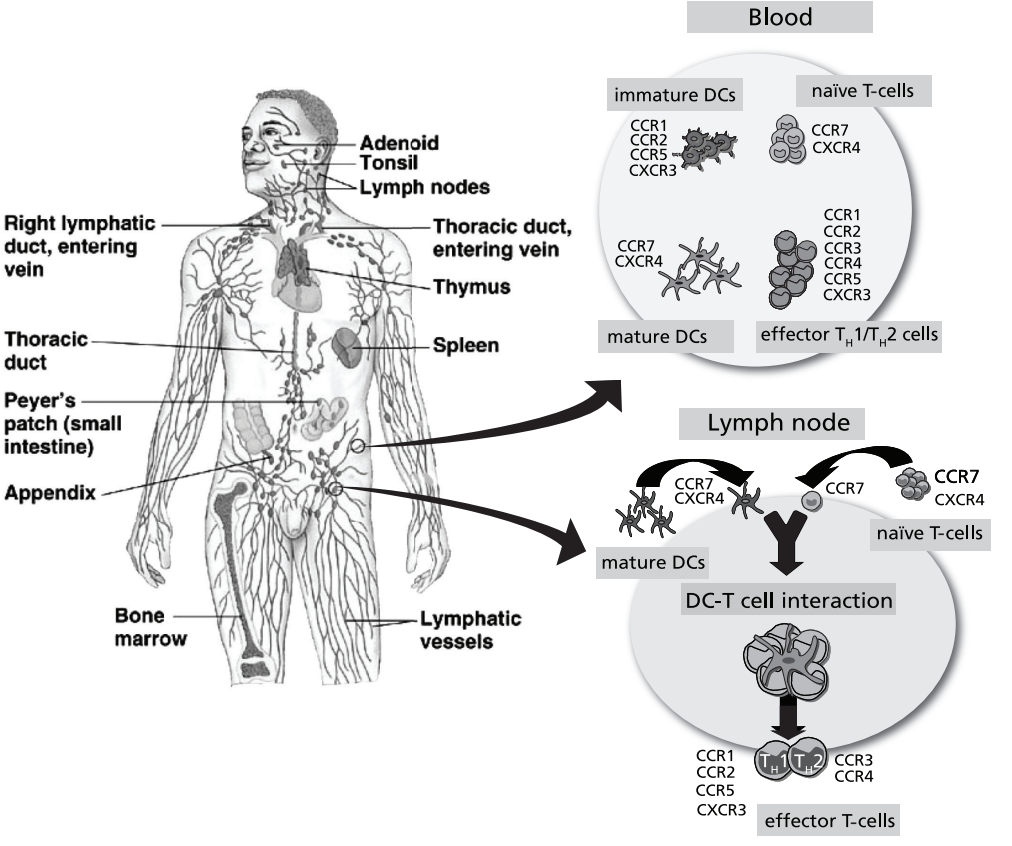

Figure 2: Chemokine receptor make-up of human dendritic cells (DCs) and T-cells. Peripheral blood contains both immature and mature DCs as well as naïve and effector T-cell populations. According to the immune response, polarized effector cells comprise of T_H1/cytotoxic or T_H2/cytotoxic cells, which arise after mature DC- naïve T-cell interaction in the lymph nodes.

As their immunostimulatory capacity also contributes to the pathogenesis of rejection, DCs have also been ascribed a role in heart allograft rejection [67,68]. Indirectly, evidence has been provided by experimental models of cardiac transplantation where it was demonstrated that DCs are critical for the induction of tolerance to the heart allograft [69-72]. However, the lack of accurate techniques for quantification of DC populations in humans has resulted in a limited number of studies questioning the role of mDCs and pDCs in AR after HTx in humans.

Aims of the thesis

Numerous studies have associated the state of immune activation with the presentation of HF as a clinical syndrome. As shown by transplantation studies, heart allograft rejection is characterized by the presence of infiltrating cells and a high local expression of inflammatory chemokines. The scope of this thesis is to acquire more insight into the circulating leukocyte chemokine receptor characteristics from patients with end-stage HF and of the same patients after implantation of a cardiac allograft. In the state of HF, except for expression patterns on circulating DCs, monocytes and granulocytes we investigated peripheral blood $CD4^+$ and $CD8^+$ T-cell chemokine receptor expression in patients, according to clinical severity of HF and pathophysiology of cardiac dysfunction. After HTx, we attempted to clarify the role of peripheral blood DCs, $CD4^+$ and $CD8^+$ T-cells in cardiac allograft rejection by examining their kinetics in relation to immune reactivity seen during AR.
Concerning HF, **chapter 2** describes alterations in the expression of CC- and CXC-chemokine receptors as well as the circulating frequency of peripheral blood $CD4^+$ and $CD8^+$ T-cell subsets, monocytes and granulocytes positive for the chemokine receptors questioned, from patients with end-stage HF. In **chapter 3**, changes in absolute numbers and frequency of phenotypically mature peripheral blood myeloid and plasmacytoid dendritic cells are presented in end-stage HF as well as early after HTx. **Chapter 4** is concerned with circulating dendritic cells according to the etiology of HF. Absolute numbers, frequency of phenotypically activated myeloid and plasmacytoid dendritic cells but also the surface expression levels of maturation marker CD83 and lymphoid homing chemokine receptor CCR7 on the two peripheral blood dendritic cell subsets, were measured according to pathophysiology of myocardial dysfunction in end-stage HF patients. Regarding HTx, **chapter 5** describes peripheral blood DC subset distribution, maturation and lymphoid homing properties in

relation to the histological diagnosis of acute cardiac allograft rejection early and late after HTx. In **chapter 6**, total peripheral blood DC incidence and DC subset reconstitution was investigated in time for both rejecting and non-rejecting heart allograft recipients considering administration of rejection therapy. Finally in **chapter 7**, we examined the differential contribution of inflammatory and homeostatic chemokines and their receptors on $CD4^+$ and $CD8^+$ T-cell recirculation in relation to corresponding intragraft chemokine and chemokine receptor expression during the process of rejection.

References

1. Mehra MR, Uber PA, Francis GS. Heart failure therapy at a crossroad: are there limits to the neurohormonal model? J Am Coll Cardiol 2003;41(9):1606-10.
2. Francis GS, Tang WH. Pathophysiology of congestive heart failure. Rev Cardiovasc Med 2003;4 Suppl 2:S14-20.
3. Conraads VM, Bosmans JM, Vrints CJ. Chronic heart failure: an example of a systemic chronic inflammatory disease resulting in cachexia. Int J Cardiol 2002;85(1):33-49.
4. Anker SD, von Haehling S. Inflammatory mediators in chronic heart failure: an overview. Heart 2004;90(4):464-70
5. Burkett EL, Hershberger RE. Clinical and genetic issues in familial dilated cardiomyopathy. J Am Coll Cardiol 2005;45(7):969-81.
6. Packer M, Bristow MR, Cohn JN, Colucci WS, Fowler MB, Gilbert EM, et al. The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. U.S. Carvedilol Heart Failure Study Group. N Engl J Med. 1996;334(21):1349-55.
7. Packer M, Coats AJ, Fowler MB, Katus HA, Krum H, Mohacsi P, et al; Carvedilol Prospective Randomized Cumulative Survival Study Group. Effect of carvedilol on survival in severe chronic heart failure. N Engl J Med. 2001;344(22):1651-8.
8. Cleland JG, Daubert JC, Erdmann E, Freemantle N, Gras D, Kappenberger L, et al. Cardiac Resynchronization-Heart Failure (CARE-HF) Study Investigators. The effect of cardiac resynchronization on morbidity and mortality in heart failure. N Engl J Med. 2005;352(15):1539-49.
9. Taylor DO, Edwards LB, Boucek MM, Trulock EP, Deng MC, Keck BM, et al. Registry of the International Society for Heart and Lung Transplantation: twenty-second official adult heart transplant report – 2005. J Heart Lung Transplant 2005;24(8):945-55.
10. Lechler RI, Sykes M, Thomson AW, Turka LA. Organ transplantation-how much of the promise has been realized? Nat Med 2005;11(6):605-13.
11. Hancock WW, Turka LA. Transplantation immunobiology. Immunol Rev 2003;196:5-6.
12. Shulzhenko N, Morgun A, Zheng XX, Diniz RV, Almeida DR, Ma N, et al. Intragraft activation of genes encoding cytotoxic T lymphocyte effector molecules precedes the histological evidence of rejection in human cardiac transplantation. Transplantation 2001;72(10):1705-8.
13. Rollins BJ, Rollins BJ. Chemokines. Blood 1997;90(3):909-928.

14. Moser B, Wolf M, Walz A, Loetscher P. Chemokines: multiple levels of leukocyte migration control. Trends Immunol 2004;25(2):75-84.
15. Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, et al. International union of pharmacology. XXII. Nomenclature for chemokine receptors. Pharmacol Rev 2000;52(1):145-76.
16. Murphy PM. International Union of Pharmacology. XXX. Update on chemokine receptor nomenclature. Pharmacol Rev 2002;54(2):227-9.
17. Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD, et al. CXC chemokines in angiogenesis. J Leukoc Biol 2000;68(1):1-8.
18. Youn BS, Mantel C, Broxmeyer HE. Chemokines, chemokine receptors and hematopoiesis. Immunol Rev 2000;177:150-74.
19. Luther SA, Cyster JG. Chemokines as regulators of T-cell differentiation. Nat Immunol 2001;2(2):102-7.
20. Mellado M, Rodriguez-Frade JM, Manes S, Martinez AC. Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation. Annu Rev Immunol 2001;19:397-421.
21. Gunn MD, Tangemann K, Tam C, Cyster JG, Rosen SD, Williams LT. A chemokine expressed in lymphoid high endothelial venules promotes the adhesion and chemotaxis of naïve T-lymphocytes. Proc Natl Acad Sci U S A 1998;95(1):258-63.
22. Gunn MD, Kyuwa S, Tam C, Kakiuchi T, Matsuzawa A, Williams LT, et al. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. J Exp Med 1999;189(3):451-60.
23. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell 1999;99(1):23-33.
24. Baekkevold ES, Yamanaka T, Palframan RT, Carlsen HS, Reinholt FP, von Andrian UH, et al. The CCR7 ligand elc (CCL19) is transcytosed in high endothelial venules and mediates T-cell recruitment. J Exp Med 2001;193(9):1105-12.
25. Kwan J, Killeen N. CCR7 directs the migration of thymocytes into the thymic medulla. J Immunol 2004;172(7):3999-4007.
26. Misslitz A, Pabst O, Hintzen G, Ohl L, Kremmer E, Petrie HT, et al. Thymic T-cell development and progenitor localization depend on CCR7. J Exp Med 2004;200(4):481-91.
27. Bleul CC, Fuhlbrigge RC, Casasnovas JM, Aiuti A, Springer TA. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). J Exp Med 1996;184 (3):1101-9.
28. Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. Nature 1998;393 (6685):595-9.
29. Ma Q, Jones D, Springer TA. The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. Immunity 1999;10(4):463-71.
30. Plotkin J, Prockop SE, Lepique A, Petrie HT. Critical role for CXCR4 signaling in

progenitor localization and T-cell differentiation in the postnatal thymus. J Immunol 2003;171 (9):4521-7.
31. Ansel KM, Ngo VN, Hyman PL, Luther SA, Forster R, Sedgwick JD, et al. A chemokine-driven positive feedback loop organizes lymphoid follicles. Nature 2000;406(6793):309-14.
32. Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T-cells with B cell helper function. J Exp Med 2000;192(11):1553-62.
33. Kunkel EJ, Campbell JJ, Haraldsen G, Pan J, Boisvert J, Roberts AI, et al. Lymphocyte CC chemokine receptor 9 and epithelial thymus-expressed chemokine (TECK) expression distinguish the small intestinal immune compartment: Epithelial expression of tissue-specific chemokines as an organizing principle in regional immunity. J Exp Med 2000;192(5):761-8.
34. Zabel BA, Agace WW, Campbell JJ, Heath HM, Parent D, Roberts AI, et al. Human G protein-coupled receptor GPR-9-6/CC chemokine receptor 9 is selectively expressed on intestinal homing T-lymphocytes, mucosal lymphocytes, and thymocytes and is required for thymus-expressed chemokine-mediated chemotaxis. J Exp Med 1999;190(9):1241-56.
35. Papadakis KA, Landers C, Prehn J, Kouroumalis EA, Moreno ST, Gutierrez-Ramos JC, et al. CC chemokine receptor 9 expression defines a subset of peripheral blood lymphocytes with mucosal T-cell phenotype and TH1 or T-regulatory 1 cytokine profile. J Immunol 2003;171(1):159-65.
36. Campbell JJ, Haraldsen G, Pan J, Rottman J, Qin S, Ponath P, et al. The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T-cells. Nature 1999;400(6746):776-80.
37. Reiss Y, Proudfoot AE, Power CA, Campbell JJ, Butcher EC. CC chemokine receptor (CCR)4 and the CCR10 ligand cutaneous T-cell-attracting chemokine (CTACK) in lymphocyte trafficking to inflamed skin. J Exp Med 2001;194(10):1541-7.
38. Luttrell LM, Lefkowitz RJ. The role of beta-arrestins in the termination and transduction of G-protein-coupled receptor signals. J Cell Sci 2002;115(Pt 3):455-65.
39. Ali H, Richardson RM, Haribabu B, Snyderman R. Chemoattractant receptor cross-desensitization. J Biol Chem 1999;274(10):6027-30.
40. Honczarenko M, Le Y, Glodek AM, Majka M, Campbell JJ, Ratajczak MZ, et al. CCR5-binding chemokines modulate CXCL12 (SDF-1)-induced responses of progenitor B cells in human bone marrow through heterologous desensitization of the CXCR4 chemokine receptor. Blood 2002;100(7):2321-9.
41. Hecht I, Cahalon L, Hershkoviz R, Lahat A, Franitza S, Lider O. Heterologous desensitization of T-cell functions by CCR5 and CXCR4 ligands: inhibition of cellular signaling, adhesion and chemotaxis. Int Immunol 2003;15(1):29-38.
42. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392(6673):245-52.
43. Sallusto F, Lanzavecchia A. Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. Immunol Rev 2000;177:134-40.
44. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of

dendritic cells. Annu Rev Immunol 2000;18:767-811.
45. von Andrian UH, Mempel TR. Homing and cellular traffic in lymph nodes. Nat Rev Immunol 2003;3(11):867-78.
46. Kalinski P, Hilkens CM, Wierenga EA, Kapsenberg ML. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol Today 1999;20(12):561-7.
47. Langenkamp A, Messi M, Lanzavecchia A, Sallusto F. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T-cells. Nat Immunol 2000;1(4):311-6.
48. Kim CH, Nagata K, Butcher EC. Dendritic cells support sequential reprogramming of chemoattractant receptor profiles during naïve to effector T-cell differentiation. J Immunol 2003;171(1):152-8.
49. Devaux B, Scholz D, Hirche A, Klovekorn WP, Schaper J. Upregulation of cell adhesion molecules and the presence of low grade inflammation in human chronic heart failure. Eur Heart J 1997;18(3):470-9.
50. Sharma R, Anker SD. Immune and neurohormonal pathways in chronic heart failure. Congest Heart Fail 2002;8(1):23-8, 48.
51. Yndestad A, Holm AM, Muller F, Simonsen S, Froland SS, Gullestad L, et al. Enhanced expression of inflammatory cytokines and activation markers in T-cells from patients with chronic heart failure. Cardiovasc Res 2003;60(1):141-6.
52. Aukrust P, Damas JK, Gullestad L, Froland SS. Chemokines in myocardial failure – pathogenic importance and potential therapeutic targets. Clin Exp Immunol 2001;124 (3):343-5.
53. Valen G, Yan ZQ, Hansson GK. Nuclear factor kappa-B and the heart. J Am Coll Cardiol 2001;38(2):307-14.
54. Spinale FG, Coker ML, Heung LJ, Bond BR, Gunasinghe HR, Etoh T, et al. A matrix metalloproteinase induction/activation system exists in the human left ventricular myocardium and is upregulated in heart failure. Circulation 2000;102(16):1944-9.
55. Damas JK, Gullestad L, Ueland T, Solum NO, Simonsen S, Froland SS, et al. CXC-chemokines, a new group of cytokines in congestive heart failure--possible role of platelets and monocytes. Cardiovasc Res 2000;45(2):428-36.
56. Sasayama S, Okada M, Matsumori A. Chemokines and cardiovascular diseases. Cardiovasc Res 2000;45(2):267-9.
57. Christopherson K, 2nd, Hromas R. Chemokine regulation of normal and pathologic immune responses. Stem Cells 2001;19(5):388-96.
58. Eriksson U, Kurrer MO, Sonderegger I, Iezzi G, Tafuri A, Hunziker L, et al. Activation of dendritic cells through the interleukin 1 receptor 1 is critical for the induction of autoimmune myocarditis. J Exp Med 2003;197(3):323-31.
59. Eriksson U, Ricci R, Hunziker L, Kurrer MO, Oudit GY, Watts TH, et al. Dendritic cell-induced autoimmune heart failure requires cooperation between adaptive and innate immunity. Nat Med 2003;9(12):1484-90.
60. Billingham ME, Cary NR, Hammond ME, Kemnitz J, Marboe C, McCallister HA, et al. A

working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group. The International Society for Heart Transplantation. J Heart Transplant 1990;9(6):587-93.

61. Hancock WW, Wang L, Ye Q, Han R, Lee I. Chemokines and their receptors as markers of allograft rejection and targets for immunosuppression. Curr Opin Immunol 2003;15 (5):479-86.
62. Melter M, Exeni A, Reinders ME, Fang JC, McMahon G, Ganz P, et al. Expression of the chemokine receptor CXCR3 and its ligand IP-10 during human cardiac allograft rejection. Circulation 2001;104(21):2558-64.
63. Zhao DX, Hu Y, Miller GG, Luster AD, Mitchell RN, Libby P. Differential expression of the IFN-gamma-inducible CXCR3-binding chemokines, IFN-inducible protein 10, monokine induced by IFN, and IFN-inducible T-cell alpha chemoattractant in human cardiac allografts: association with cardiac allograft vasculopathy and acute rejection. J Immunol 2002;169(3):1556-60.
64. Fahmy NM, Yamani MH, Starling RC, Ratliff NB, Young JB, McCarthy PM, et al. Chemokine and chemokine receptor gene expression indicates acute rejection of human cardiac transplants. Transplantation 2003;75(1):72-8.
65. von Hundelshausen P, Weber KS, Huo Y, Proudfoot AE, Nelson PJ, Ley K, et al. RANTES deposition by platelets triggers monocyte arrest on inflamed and atherosclerotic endothelium. Circulation 2001;103(13):1772-7.
66. Burns WR, Wang Y, Tang PC, Ranjbaran H, Iakimov A, Kim J, et al. Recruitment of CXCR3 and CCR5 T-cells and Production of Interferon-gamma-Inducible Chemokines in Rejecting Human Arteries. Am J Transplant 2005;5(6):1226-36.
67. Peche H, Heslan M, Usal C, Amigorena S, Cuturi MC. Presentation of donor major histocompatibility complex antigens by bone marrow dendritic cell-derived exosomes modulates allograft rejection. Transplantation 2003;76(10):1503-10.
68. Peche H, Trinite B, Martinet B, Cuturi MC. Prolongation of heart allograft survival by immature dendritic cells generated from recipient type bone marrow progenitors. Am J Transplant 2005;5(2):255-67.
69. Maksymowicz M, Olszewski WL, Cybulska E. Repopulation of donor heart by recipient bone marrow-derived dendritic cells prior to transplantation causes acute rejection by both the allogeneic and syngeneic recipient. Transplant Proc 2003;35(6):2374-5.
70. Tiao MM, Lu L, Tao R, Wang L, Fung JJ, Qian S. Prolongation of cardiac allograft survival by systemic administration of immature recipient dendritic cells deficient in NF-kappaB activity. Ann Surg 2005;241(3):497-505.
71. Liang X, Lu L, Chen Z, Vickers T, Zhang H, Fung JJ, et al. Administration of dendritic cells transduced with antisense oligodeoxyribonucleotides targeting CD80 or CD86 prolongs allograft survival. Transplantation 2003;76(4):721-9.
72. Garrovillo M, Ali A, Depaz HA, Gopinathan R, Oluwole OO, Hardy MA, et al. Induction of transplant tolerance with immunodominant allopeptide-pulsed host lymphoid and myeloid dendritic cells. Am J Transplant 2001;1(2):129-37.

Chapter 2

Altered Chemokine Receptor Profile on Circulating Leukocytes in Human Heart Failure

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Abstract

Chemokines and their receptors have been implicated in the pathogenesis of different forms of heart failure (HF). We examined CC- and CXC- chemokine receptor expression in fresh peripheral blood leukocyte populations from 24 end-stage HF patients consisting of coronary artery disease (CAD, n = 6) and hypertrophic cardiomyopathy (HCM, n = 7) or idiopathic dilated cardiomyopathy (IDCM, n = 8) or valvular disease (VD, n = 3) and compared the data with 18 healthy controls. Levels of CCR1, 2, 3, 4, 5 and 7, and CXCR1, 2, 3 and 4 were measured by flow cytometry, and the expression profile was assessed as molecules of equivalent soluble fluorochrome units as well as frequency (percentage) of $CD3^+$, $CD4^+$ & $CD8^+$ T-cells and monocytes or granulocytes. Frequency of $CD3^+$ $CXCR4^+$, $CD3^+$ $CXCR1^+$ and $CD3^+$ $CXCR3^+$ cells was significantly increased in HF patients whereas only CCR7 and CXCR4 expression levels were elevated on $CD3^+$ cells. Both $CD4^+$ $CXCR4^+$ and $CD8^+$ $CXCR4^+$ cell frequencies were significantly increased irrespective of cardiac disease etiology. Elevated CCR7 expression was less pronounced on $CD4^+$ than $CD8^+$ cells in patients with CAD and IDCM. Expression of CXCR4 on $CD8^+$ cells was upregulated substantially, independent of the cause of disease. $CD8^+$ $CXCR1^+$ and $CD8^+$ $CXCR3^+$ but not $CD4^+$ $CXCR1^+$ or $CD4^+$ $CXCR3^+$ cells were increased in the HF patients with IDCM and CAD, respectively. Expression of CXCR1 or CXCR3 on both $CD4^+$ and $CD8^+$ cells did not differ in all the groups. For monocytes, frequency of $CD14^+$ $CCR1^+$ and $CD14^+$ $CCR2^+$ cells were significantly decreased in CAD patients, whereas, increase in $CD14^+$ $CXCR4^+$ cell frequency was accompanied with elevated CXCR4 expression. On granulocytes, CXCR1 and CXCR2 receptors were downregulated in all patients, as compared to controls. Our results suggest that the altered expression profile of CC- and CXC- chemokine receptors on circulating leukocyte populations involve enhanced activation of the immune system perhaps as part of the pathogenic mechanisms in HF. Modulation of the chemokine network could offer interesting novel therapeutic modalities for end-stage HF.

Introduction

Heart failure (HF) is a clinical syndrome with a prevalence of 40-45 / 1000 people above the age of 55 years in the western world. HF is no longer considered a pure cardiac entity, but instead a syndrome because its etiology is diverse and involves several, initially adaptive and later detrimental, neurohumoral compensatory mechanisms [1]. Some aspects of HF are mediated by nonlethal alteration of cardiac function and structural changes of the ventricular myocardium induced by cytokines [2]. In murine models of myocarditis, inflammatory cytokines are induced rapidly in the myocardium and are continuously expressed during the chronic stage, even when the heart assumes the typical pattern of dilated cardiomyopathy in the absence of inflammatory processes [3,4]. In the pressure overloaded left ventricle, myocardium first develops adaptive hypertrophy, a pattern which in later stages undergoes transition to HF. Cytokines appear to play a significant role in the development of HF by accelerating myocardial hypertrophy, fibrosis and downmodulating cardiac function [5,6]. In the ischemic heart, the nonischemic myocardium develops hypertrophy associated with the progression of ischemic area scarring. This remodeling process initially exerts an important compensatory mechanism for ventricular function, but it later results in the development of HF [7].

Systemic inflammatory responses triggered by hypoxia and ischemia are characterized by activation of various cell types and a massive release of endogenous mediators of inflammation which may produce severe clinical sequlae [8]. A series of experimental studies have demonstrated that the progressive nature of HF resides in a complex blend of structural, functional and biologic alterations leading to irreversible cardiac remodeling. A number of clinical studies have demonstrated that attraction of leukocytes into tissue is essential for inflammation, the host response to infection, and may also be involved in the pathogenesis of human end-stage HF [9]. The intrinsic systemic immune activation in human congestive HF has been demonstrated by abnormal plasma profiles of pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-2, IL-6, [10-13], soluble cytokine receptors (sTNFR-I and sTNFR-II),[14-16] and chemokines (CXCL1, 5 & 8 and CCL2, 3 & 5), [15-17] in patients with cogenital heart disease, coronary syndromes and cardiomyopathies of different origin.

Chemokines and chemokine receptors

Chemokines play an important role in the control and regulation of leukocyte homeostasis in normal and diseased tissue. They are small (8-11 kDa) heparin binding single polypeptide chemotactic cytokine (ranging from 70 to 100 aminoacids) that are involved in the trafficking of leukocytes. The chemokine superfamily is divided into four groups (CC-, CXC-, CX3C-, and XC-), according to the relative positioning of the first two closely paired and highly conserved cysteine residues in their protein sequence. Chemokines exert diverse biological effects also on other cell types such as endothelial cells, fibroblasts and vascular smooth muscle cells [18]. Although chemokine dependent functions are essential for the control of infection, wound healing and hematopoiesis, excessive chemokine activation may result in inappropriate inflammation leading to cell and tissue damage.

Chemokine actions are mediated by binding to a family of seven-transmembrane heterotrimeric G-protein-coupled receptors, named chemokine receptors [19,20]. Eighteen chemokine receptors (10 CC-, 6 CX-, 1 CX3C- and 1 XC-) have been cloned so far and are expressed on the surface of several cell types, including leukocytes and endothelial cells. Concerted action of chemokines and chemokine receptors has been suggested to play a role in the induction and maintenance of many immune-mediated disorders (e.g. rheumatoid arthritis and bronchial asthma) [21]. Biological functions for some of the chemokine receptors and the array of effects of their ligands on immune cells are summarized in Table 1.

Chemokines, chemokine receptors and cardiovascular diseases

There is growing evidence to suggest that chemokines and their receptors play an important pathogenic role in various cardiovascular disorders and different forms of myocardial failure.

Myocarditis

Association between chemokine expression and the influx of leukocytes has been reported in experimental models of infective and autoimmune myocarditits [22]. In these models, the cytokines interferon (IFN)-γ and TNF-α play a major role in modulating chemokine expression and consequently, the chemokine mediated leukocyte influx through chemokine receptors. In humans, high plasma levels of CCL-2 (monocyte chemoattractant protein-1), are associated with fatal outcomes in patients with acute myocarditis [23]. Furthermore, chemokines were shown to be produced by infected macrophages

Table 1: Ligands, biological function and chemotaxis of target T-cell types for CC- and CXC- chemokine receptors

Receptor	Ligands		Functional expression on immune cells	Roles in leukocyte trafficking
	selective	other		
CC subfamily				
CCR1	CCL-14	CCL-3, -5, -7, -15, -16, -23	Monocytes, Macrophages, B cells, Basophils, Eosinophils T-cells (T_H1), DCs	monocyte recruitment in most types of inflammation, DC migration to sites of inflammation
CCR2	CCL-2	CCL-1, -7, -12, -13	Monocytes, Macrophages, NK cells, Basophils, memory T-cells (T_H1), DCs	monocyte recruitment and effector T_H1 migration, memory T-cell & DC migration to sites of inflammation
CCR3	CCL-11, -24	CCL-5, -7, -8, -13, -15, -24, -26	Eosinophils, Basophils, MasT-cells, T-cells (T_H2)	eosinophil recruitment and effector T_H2 migration, to inflammatory sites, including lung and skin
CCR4	CCL-17, -22	–	T-cells (T_H2)	memory T-cell migration to the skin, effector T_H2 migration
CCR5	CCL-4, -5	CCL-3	Progenitors, Monocytes, Macrophages, T-cells (T_H1), DCs	monocyte recruitment and effector T_H1 migration, memory T-cell & DC migration to sites of inflammation
CCR7	CCL-19, -21	–	B-cells, T-cells (naïve, T_H1 & T_H2), DCs	naïve T-cell migration to lymph nodes and Peyer's patches, memory T-cell & DC migration to and within lymphoid tissues, B-cell migration

Table 1 (*Continued*)

Receptor	Ligands		Functional expression on immune cells	Roles in leukocyte trafficking
	selective	other		
CXC subfamily				
CXCR1	CXCL-8	CXCL-2, -3, -5, -6, -7	Monocytes, Macrophages, Neutrophils, MasT-cells, T-cells	monocyte, neutrophil recruitment and DC migration in most types of inflammation
CXCR2	CXCL-8	CXCL-1, -2, -3, -4, -5, -6, -7	Monocytes, Macrophages, Neutrophils, Mast Cells, T-cells	neutrophil recruitment to inflammatory sites including liver, lung and atherosclerotic lesions
CXCR3	CXCL-9, -10, -11	–	B-cells, NK cells, Eosinophils, Platelets, T-cells (T_H1)	recruitment of effector T_H1 cells to sites of inflammation
CXCR4	CXCL-12	–	Progenitors, B-cells, Monocytes, Macrophages, T- cells (naïve, T_H1 & T_H2), DCs	B-cell migration, progenitor and B cell development naïve T-cell migration to lymphoid tissues

CCL, CC ligand; CXCL, CXC ligand; NK, natural killer; DC, dendritic cell; T_H1, T-helper 1; T_H2, T-helper 2

or cardiomyocytes in *in vitro* and *in vivo* models of experimental *Trypanosoma cruzi* infection [24]. Recently, the expression of receptor CCR5 was found to be augmented on the surface of isolated leukocytes from chronic Chagasic cardiomyopathy patients and associated with the development of mild forms of the disease [25].

Cardiomyopathy

Increased CCL-2 levels have been demonstrated in animal models of cardiac decompensation secondary to pressure-overload or ischemia [26-28]. In transgenic mice, interstitial monocyte infiltration in the myocardium due to CCL-2 over-expression has been related to the development of a number of pathological changes characterizing HF, including cardiac hypertrophy, ventricular dilatation and depressed contractile function [29]. In patients, increased serum levels of CXCL-1 (growth-related oncogene α), CXCL-5 (epithelial-cell-derived neutrophil chemoattractant-78) and CXCL-8 (IL-8) have been found in chronic congestive HF resulting from both ischemic and nonischemic cardiomyopathy, with the highest levels present in those with the most advanced disease [16,30]. Also, enhanced expression of CCR2 and CXCR4 has been observed in the failing human myocardium [31].

Atherosclerosis

Enhanced expression of CCR2 and CXCR2 in macrophages has been reported in advanced murine and human atherogenesis [32]. Knock-out mice lacking these receptors or their corresponding ligands CCL-3 (macrophage inflammatory protein α) and CXCL-8, exhibit significantly reduced progression of atherosclerosis [33-35]. In humans, raised CCL-3 and CXCL-8 levels have been found both in plasma and in the atherosclerotic vessel itself with the levels being the highest in patients with acute coronary syndromes [36]. Furthermore, enhanced expression of CCL-2, CXCL-8 and CXCL-10 (interferon-inducible protein-10), has been found in human atherosclerotic lesions, possibly mediating chemoattractant and mitogenic effects on neutrophils, T-cells and vascular smooth muscle cells [37].

Heart failure

In congestive HF, both ischemia, cachexia as well as endotoxin translocation, are discussed as causes of inflammation. However, it is not clear whether whole body inflammation is involved in the pathogenesis of circulatory failure or it simply represents an epiphenomenon. In cardiogenic shock and severe HF, a

significant increase in plasma concentrations of TNF-α, IL-6 and the chemokine CXCL-8 as well as of soluble adhesion molecules has been observed, suggesting systemic inflammation similar to septic disease or polytrauma [8,13]. Chronic low-grade inflammation with infiltrating leukocytes has been described in the human failing myocardium [38], and chemokines, through their receptors, are thought to recruit and activate these cells. CCR2 mRNA has been demonstrated in the failing myocardium, suggesting that CCL-2 may modulate myocardial function by directly acting on cardiomyocytes [30]. Furthermore, chemokines are thought to lead indirectly to damage and dysfunction of the cardiac muscle through the release of other inflammatory cytokines (e.g. IL-1β and IL-6), activation and production of reactive oxygen species (ROS), matrix metalloproteinases (MMPs) and inflammatory cytokines [9]. CCL-2 has been described to stimulate the release of IL-1β and IL-6 in adult rat cardiomyocytes [39]. Moreover, chemokines may regulate fibrosis, angiogenesis and apoptosis [40,41]. CCL-2 and CXCL-8 may directly induce ROS generation in congestive HF patients. Oxidative stress activates NF-κB in various cell types, that could induce the synthesis of CXCL-8 and CCL-2, indicating a pathogenic loop in congestive HF [42, 43]. Enhanced MMP activity and selective MMP upregulation in non-ischemic and ischemic cardiomyopathy, contributes to myocardial remodeling [44], and CCL-2 may be involved in this process [45].
Seemingly chemokines modulate myocardial function directly through effects on cardiomyocytes and indirectly through effects on infiltrating leukocytes, fibroblasts or endothelial cells within the failing myocardium. Based on the potential pathogenic role of CC- and CXC- chemokines in the activation and possibly in the recruitment of peripheral blood neutrophils, T- cells and monocytes into the myocardium through chemokine receptors, we investigated CC- and CXC- chemokine receptor expression on peripheral blood leukocyte populations of patients with end-stage HF. To assess the significance of the inflammatory response through chemokine receptors with the etiology of HF, circulating leukocyte frequency and receptor expression were also analyzed in relation to underlying disease.

Materials and methods

Patient selection

Twenty-four patients with chronic symptomatic end-stage HF participated in this study. The patients were included if they: 1) were classified in New York Heart Association (NYHA) functional class III/IV, 2) had a Left Ventricular Ejection Fraction <40%, 3) were receiving optimal unchanged medical treatment during 1 month preceding the study and 4) were considered suitable for heart transplantation. Patients were excluded if they were re-transplanted or had evidence of significant concomitant pathology such as infections, pulmonary disorders, autoimmune or connective tissue disease. Their clinical situation was stable, with no change of medication during the last month before the investigation. All patients were screened for hypertension, hypercholesterolemia and atherosclerotic vascular disease. Diagnoses at transplantation were idiopathic dilated cardiomyopathy (IDCM), hypertrophic cardiomyopathy (HCM), coronary artery disease (CAD) and severe concomitant valvular disease (VD) (see Table 2). Control subjects were 18 laboratory co-workers matched for age and gender, who volunteered to donate blood for this investigation. This normal healthy control (NHC) population received no medication during follow-up and represented a baseline for all values obtained throughout our study. Demographic characteristics for groups, HF classification and hemodynamic parameters of the HF patients are depicted in Table 2. The research conformed with the principles outlined in the Declaration of Helsinki. All patients provided written informed consent in accordance with a protocol approved by the local medical ethical committee of the Erasmus University Medical Center, The Netherlands (MEC 215.732/2002/157).

Monoclonal antibodies

Allophycocyanin (APC)-conjugated CD3 (clone SK7), peridinin chlorophyll protein Cy5 (PerCPCy5)-conjugated CD8 (clone RPA-T8) and phycoerythrin (PE)-conjugated anti-CCR4 (clone 1G1), anti-CCR5 (CD195; clone 2D7/CCR5), anti-CXCR3 (CD183; clone 1C6/CXCR3) and anti-CXCR4 (clone 12G5) were purchased from BD Biosciences (San Jose, CA). Phycoerythrin (PE)-conjugated anti-CCR1 (clone 53504,111), anti-CCR2 (clone 48607,211), anti-CCR3 (clone 61828,11), anti-CCR7 (CD197w; clone 150503), anti-CXCR1 (clone 42705,111) and anti-CXCR2 (clone 48311,11) were obtained from R&D Systems Europe (Abingdon, UK). Fluorescein isothiocyanate (FITC)-conjugated CD14 (clone UCHM1) was provided by Serotec (Oxford, UK). Irrelevant PE-conjugated

control murine isotypic antibodies (IgG1, IgG2a & IgG2b) were purchased from BD Biosciences. Mouse anti-human CD45 FITC (clone F10-89-4) / CD14 PE (clone UHCM-1) reagent from Serotec was used to sample peripheral whole blood lymphocyte, monocyte and granulocyte presence. All antibodies were directly conjugated to FITC, PE, APC or PerCPCy5.

Table 2: Clinical parameters of study subjects

	HF patients	Controls
Age (years)	52.5 (37–65)	49.0 (27-60)
Gender, (males/females)	15/9	10/8
Duration of heart failure (years)	4.5 (2-15)	
NYHA functional class (III/IV)	8/16	
Cause of heart failure (NYHA III/IV)		
CAD	n = 6 (2/4)	
IDCM	n = 8 (2/6)	
HCM	n = 7 (3/4)	
VD	n = 3 (1/2)	
LVEF (%)	29.0 ± 3.5	
Medication (%)		
ACE inhibitor	n = 21 (88)	
HMG-CoA reductase inhibitors	n = 9 (38)	
β-blockers	n = 14 (58)	
Diuretics	n = 22 (92)	
Anti-coagulants	n = 11 (46)	
Anti-arrhythmics	n = 19 (79)	

NYHA = New York Heart Association classification, CAD = Coronary artery disease, IDCM = idiopathic dilated cardiomyopathy, HCM = hypertrophic cardiomyopathy, VD = valvular disease, LVEF = Left Ventricular Ejection Fraction, ACE = angiotensin converting enzyme, HMG-CoA = 3-hydroxy-3-methylglutaryl-CoA. Data on age and duration of heart failure are given as the median (range).

Immunophenotypic labeling of white blood cells

Whole blood cells from all patients were collected 4-6 h before transplantation and at 1- and 4- wk follow-up from the control group. All samples were obtained in 7- mL heparinized tubes during the morning, by using venous puncture and were processed within 3 h of blood collection. Blood was divided equally in 100-μL aliquots and all samples were placed directly into 5-mL polystyrene tubes containing 10 μL of CD3 (APC), 10 μL of CD14 (FITC), and 20 μL of CD8 (PerCPCy5). In separate tubes, 5 μL of anti- CCR1, 2, 3, 4, 5, 7 or anti- CXCR1, 2, 3, 4 were added to the aforementioned monoclonal antibodies (mAbs) to resolute peripheral blood leukocyte chemokine receptor expression, by

four-color flow cytometry. To minimize selective loss during the preparation procedure, cells were first stained with the mAbs for 20 minutes at room temperature, followed by lysis of the erythrocytes with fluorescence-activated cell sorter (FACS) lyzing solution (BD Biosciences). For each test, a sample of whole peripheral blood was incubated with 10 μL of the CD14PE/CD45FITC mAbs. After washing with FACSflow (BD Biosciences) 100,000 total leukocytes were acquired for chemokine receptor expression analysis on peripheral blood T-cells, monocytes, and granulocytes. White blood cell numbers in the samples were determined using an automated cell counter (Sysmex Microcellcounter F300, Goffin Meyvis, Etten Leur, The Netherlands).

Flow cytometric analysis

APC-stained $CD3^+$ cells were identified within lymphocytes (gate R2; ≥ 5000 events), based on the forward scatter (FSC) / sideward scatter (SSC) plot (Figure 1A). Within $CD3^+$ cells, PerCPCy5 $CD8^+$ and PerCPCy5 $CD8^-$ cells, accounted for $CD8^+$ and $CD4^+$ T-cells, respectively (Figure 1B). Definition of monocytes was based on CD14-FITC staining and SSC within all leukocytes (gate R6; ≥ 2000 events) (Figure 1C). Granulocytes were identified as $CD3^-CD14^-$ cells and were determined within gates R3 and R7 of the CD3-APC / CD14-FITC plot (Figure 1D). Surface PE markers for CCR1, 2, 3, 4, 5, 7 and CXCR1, 2, 3 and 4 were then analyzed with a FACSCalibur® four-color flow cytometer using the CellQuest Pro software program (BD Biosciences). To compare various measurements of chemokine receptor expression in time, the flow cytometer was calibrated using Calibration Beads Quantum 1000 (Bangs Lab Inc., Fishers, IN). Each bead contained a known amount of the PE fluorochrome. The fluorescence intensity was denoted as molecular equivalents of soluble fluorochrome (MESF) and was converted to a standard curve using Quick Cal software program (Bangs Lab) for Quantum Beads (Figure 2).

Statistical analysis

Expression levels of CCR1, 2, 3, 4, 5, 7 and CXCR1, 2, 3, and 4 on $CD3^+$, $CD4^+$, $CD8^+$ T-cells, monocytes and granulocytes are presented in MESF units. The percentage of positive peripheral white blood cells, represented the frequency of each leukocyte population positive for the receptor in question per subject at the time of sampling, as defined by flow cytometric analysis. All values are expressed in mean ± SEM unless stated otherwise. Continuous values for the NHC group were compared by the paired samples *t*-test or the Wilcoxon matched pairs test, as appropriate. Data sets were tested before comparisons in order to assess whether they passed normality. The unpaired Student's

t- test and the Mann-Whitney *U*-test were employed to compare differences between means of controls and patients, as appropriate. One-way ANOVA with Bonferroni's post-hoc analysis was applied for comparisons between more than two groups of patients. Two-sided tests were performed and a p-value < 0.05 was considered significant. SPSS 11.0.1 software (SPSS, Chicago, IL, USA) and the GraphPad statistical program (GraphPad, San Diego, CA, USA) were applied for analyses and graphics, respectively.

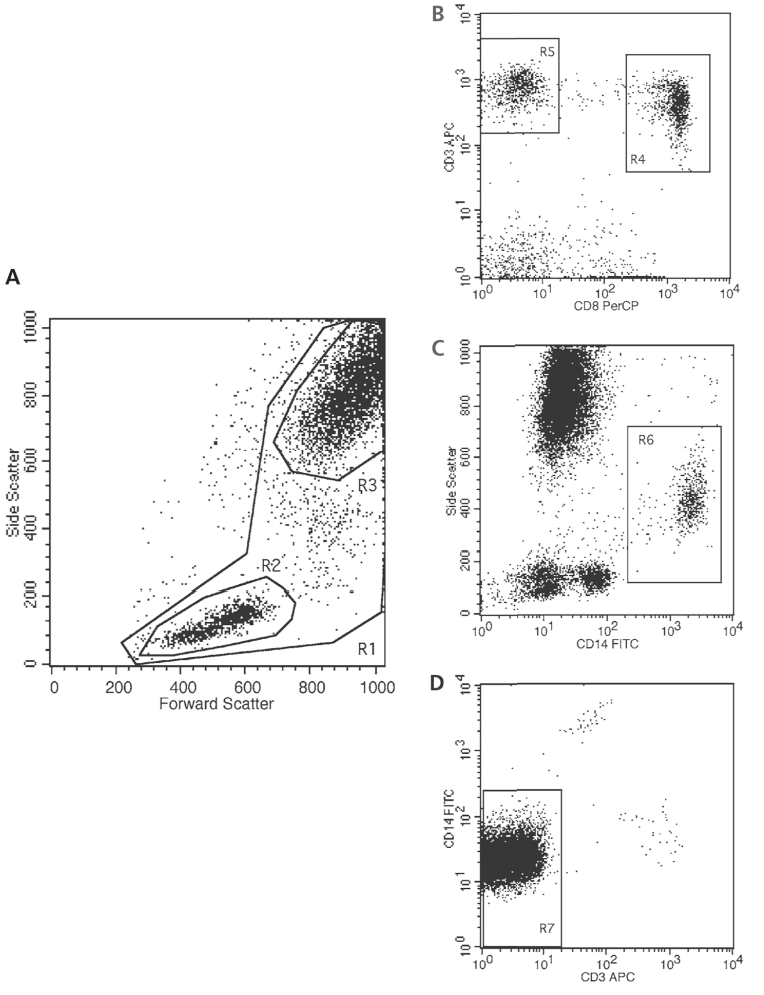

Figure 1: Selection of whole blood leukocyte subsets with four-color flow cytometry. (A) In total leukocytes gated within the lyzed cells (R1), lymphocytes (R2), and granulocytes (R3) were determined. (B) Within R1 & R2, CD3 cells positive or negative for CD8 ($CD8^+$and $CD4^+$cells, respectively) were characterized. (C) R1-gated $CD14^+$ cells were defined as monocytes (R6). (D) R1- and R3- gated cells negative for CD3 or CD14 were identified as granulocytes (R7).

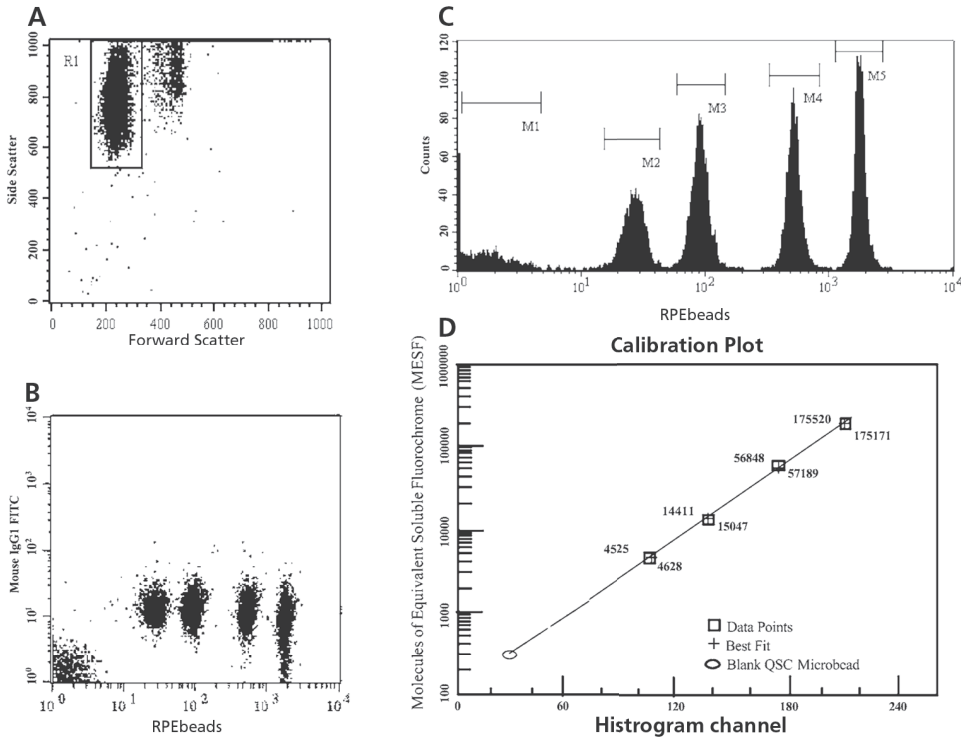

Figure 2: Calibration of the PE channel for surface expression analysis of CC- and CXC-chemokine receptors on peripheral blood leukocyte populations, in a four-color flow cytometer. (A) For each experiment, 10,000 events of Quantum 1000, Calibration Beads (R1) were identified within the forward scatter (FSC) / sideward scatter (SSC) plot, upon flow cytometric analysis. (B) R1-gated PE beads containing a specific amount of fluoresceïn were analyzed using the CellQuest Pro software program (BD Biosciences). (C) Fluorescence intensity was calculated through the geometric mean (Geomean = $10^{\Sigma \log(xi)/n}$) of the histogram signal acquired for each of the five gated PE beads. (D) Standard curve of fluoresence intensity constructed after using the QuickCal program for quantum beads software, whereby fluorescence intensity is depicted as MESF.

Results

Clinical and hematologic characteristics of HF patients

Number of T-lymphocytes, monocytes and granulocytes was comparable in all patient groups either with CAD, HCM, IDCM or VD (Table 3). None of the patients suffered from hypertension during the course of the study, and there were no significant differences in systolic ($p = 0.091$) or diastolic ($p = 0.236$)

pressure among patients with CAD, HCM, IDCM or VD (data not shown). Similarly, all patients had normal total cholesterol levels (< 6.5 mmol/L) with no marked differences between the groups (p = 0.707; data not shown). All CAD patients received statin therapy, whereas, only 14% of HCM, 13% of IDCM and 33% of VD patients were given statins. None of the patients presented with peripheral vascular atherosclerosis.

Chemokine receptor profile in controls and end-stage HF patients

Expression of CC- and CXC- chemokine receptors on peripheral blood $CD3^+$ T-cells and $CD4^+$ and $CD8^+$ T-cell subsets of healthy individuals did not reveal any significant differences in a period of 1 mo (p values ranged from 0.113 to 0.529). Similarly, they exhibited comparable expression of the same receptors on blood monocytes or granulocytes in time (p values ranged from 0.098 to 0.260) (data not shown).

$CD3^+$ T-cells

The majority of fresh peripheral blood $CD3^+$ T-cells from controls expressed CCR7 (75%) and CXCR4 (95%) on their surface (Figure 3A). As for controls, approx. 75% of peripheral blood $CD3^+$ cells from patients were found to be strongly positive for CCR7, but mean CCR7 expression was markedly increased on patient $CD3^+$ cells compared to controls (6047 MESF vs 3289 MESF; $p < 0.0001$) (Figure 3B). Both the percentage of $CXCR4^+$ cells (98%) and mean CXCR4 expression on patient $CD3^+$ cells (7971 MESF), were significantly increased against controls (95%, $p = 0.0008$ and 3546 MESF, $p < 0.0001$) (Figure 3A,B).

The frequency of $CD3^+$ T-cells that expressed CXCR1 and CXCR3 was increased in the patients compared with controls (17 vs 4%; $p = 0.0097$ and 55 vs 47%; $p = 0.045$, respectively) (Figure 3A) without overall changes in mean CXCR1 or CXCR3 expression levels (1243 MESF vs 1145 MESF; $p = 0.550$ and 3597 MESF vs 3889 MESF; $p = 0.478$) (Figure 3B). On $CD3^+$ cells from controls, there was low (11%) to hardly any (0.7%) expression for the rest of the inflammatory receptors (CCR1, CCR2, CCR3 and CXCR2) (Figure 3A), whereas frequencies of $CD3^+$ $CCR4^+$ and $CD3^+$ $CCR5^+$ cells were at 48 and 26%, respectively. Circulating frequency of $CD3^+$ cells and expression of these receptors on $CD3^+$ cells did not reveal any significant differences between controls and patients.

Table 3: Circulating leukocyte numbers and chemokine receptor profile in relation to HF etiology

	NYHA III-IV				
	CAD (n = 6)	**HCM (n = 7)**	**IDCM (n = 8)**	**VD (n = 3)**	**P value**
T-lymphocytes, cells/μL	766.1 ± 161.2	904.9 ± 172.2	1072 ± 303.8	478.6 ± 70.3	0.474
$CD3^+$ cells					
$CCR7^+$ %	74.0 ± 4.0	74.3 ± 8.4	76.3 ± 9.5	73.0 ± 5.0	0.996
MESF	6083 ± 228.1	5860 ± 343.9	6122 ± 847.1	4071 ± 494.2	0.062
$CXCR1^+$ %	24.5 ± 12.7	12.3 ± 1.0	21.0 ± 12.2	11.7 ± 5.9	0.817
MESF	1271 ± 77.3	1248 ± 158.9	1791 ± 716.2	813.0 ± 70.7	0.385
$CXCR3^+$ %	66.6 ± 7.9	58.8 ± 6.7	39.4 ± 7.7	48.0 ± 7.0	0.082
MESF	4787 ± 1059	3192 ± 184.6	4493 ± 903.7	2262 ± 400.2	0.152
$CXCR4^+$ %	98.6 ± 0.9	97.5 ± 0.8	99.1 ± 0.3	95.5 ± 2.5	0.111
MESF	7601 ± 1289	7036 ± 1033	8220 ± 540.4	7273 ± 2305	0.839
CD4+ cells					
$CCR7^+$ %	85.0 ± 5.1	91.0 ± 2.0	86.0 ± 1.0	77.5 ± 6.5	0.581
MESF	2979 ± 757.1	4678 ± 908.5	3600 ± 509.5	1573 ± 382.5	0.232
$CXCR1^+$ %	2.0 ± 0.6	3.0 ± 1.0	4.0 ± 3.0	9.0 ± 8.0	0.555
MESF	1357 ± 362.0	1113 ± 285.5	1033 ± 322.5	819.5 ± 109.5	0.789
$CXCR3^+$ %	41.0 ± 4.2	24.0 ± 1.0	42.5 ± 1.5	40.0 ± 2.0	0.101
MESF	5726 ± 1880	8598 ± 4460	3177 ± 154.2	2047 ± 301.0	0.376
$CXCR4^+$ %	95.2 ± 2.2	97.0 ± 2.0	99.0 ± 1.0	99.0 ± 1.0	0.594
MESF	4692 ± 1537	9038 ± 6119	3020 ± 1837	4937 ± 2485	0.344
CD8+ cells					
$CCR7^+$ %	45.5 ± 7.9	58.0 ± 9.0	39.5 ± 11.5	60.5 ± 39.5	0.508
MESF	4635 ± 274.5[a]	3049 ± 680.1	3680 ± 559.9[a]	2925 ± 876.5	0.037
$CXCR1^+$ %	13.0 ± 2.1	10.0 ± 6.0	19.0 ± 1.0	9.5 ± 3.5	0.315
MESF	1467 ± 212.5	1512 ± 527.5	5904 ± 3707	1009 ± 181.5	0.401
$CXCR3^+$ %	94.6 ± 2.0[a]	85.5 ± 1.5	87.0 ± 1.0	86.7 ± 2.7	0.042
MESF	4782 ± 939.6	2053 ± 235.6	4823 ± 1953	1862 ± 251.0	0.187
$CXCR4^+$ %	95.8 ± 1.2	96.5 ± 3.5	98.5 ± 1.5	98.0 ± 1.8	0.608
MESF	5486 ± 1519	8645 ± 1795	6277 ± 1079	5756 ± 2508	0.471

Table 3 *(Continued)*

	NYHA III-IV				
	CAD (n = 6)	**HCM (n = 7)**	**IDCM (n = 8)**	**VD (n = 3)**	**P value**
Monocytes, cells/μL	553.3 ± 157.0	398.5 ± 121.4	475.6 ± 121.8	905.0 ± 342.7	0.307
CCR1+ %	48.6 ± 7.9[a]	71.5 ± 7.2	72.3 ± 2.1	64.0 ± 2.1	0.041
MESF	1711 ± 335.6	2090 ± 574.5	1809 ± 265.2	1248 ± 293.5	0.678
CCR2+ %	68.4 ± 5.0[b]	86.2 ± 3.4	81.5 ± 8.9	79.0 ± 8.7	0.003
MESF	4543 ± 701.0	4925 ± 643.9	6266 ± 1310	3967 ± 1105	0.437
CXCR4+ %	93.7 ± 4.3	93.2 ± 3.8	93.3 ± 1.8	92.3 ± 6.2	0.997
MESF	7619 ± 1965	4971 ± 736.8	4853 ± 1004	5809 ± 3135	0.682
Granulocytes, cells/μL	6606 ± 715.9	6130 ± 1159	6560 ± 1062	9084 ± 3584	0.613
CXCR1+ %	99.2 ± 0.2	99.3 ± 0.2	99.8 ± 0.2	99.7 ± 0.3	0.236
MESF	25601 ± 4949	22284 ± 2990	32890 ± 1231	18849 ± 3857	0.085
CXCR2+ %	92.8 ± 5.5	97.7 ± 0.4	99.0 ± 0.5	98.7 ± 0.3	0.429
MESF	6611 ± 783.2	5099 ± 1173	8716 ± 2068	3728 ± 1534	0.173

[a]$p < 0.05$, [b]$p < 0.01$, from one-way ANOVA with Bonferroni's Multiple Comparison Test between patients with coronary artery disease (CAD), hypertrophic cardiomyopathy (HCM), idiopathic dilated cardiomyopathy (IDCM) and valvular disease (VD).

CD4+ and CD8+ T-cell subsets

In controls, a high proportion of CD4+ cells stained positive for CCR7 (80%) and CXCR4 (90%), whereas CD8+ cells were found to be positive for CCR7 (48%) and CXCR4 (88%) (Figures 4A and 5A). Elevated mean CCR7 expression on patient peripheral blood CD3+ cells versus controls was reflected less on the CD4+ subset (3314 MESF vs 2431 MESF; p = 0.02) (Figure 4B), but more on the CD8+ subset (3104 MESF vs 2033 MESF; p = 0.003) (Figure 5B). Compared with controls, percentage of CD4+ CXCR4+ (96 vs 89%) and percentage of CD8+ CXCR4+ (97 vs 89%) cells were both significantly increased (p = 0.001 and p = 0.009, respectively) (Figures 4A and 5A). Mean CXCR4 expression was upregulated substantially on both CD4+ and CD8+ subsets of patients compared to controls (CD4: 7231 MESF vs 3583 MESF; p < 0.0001 and CD8: 6201 MESF vs 3276 MESF; p = 0.006) (Figures 4B and 5B).

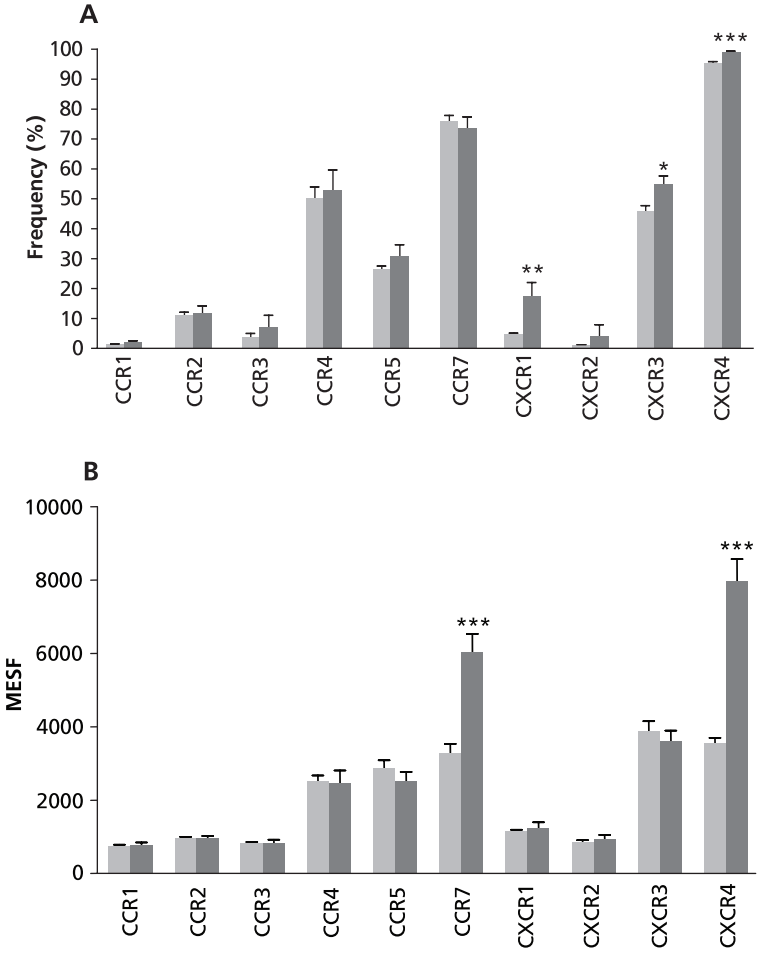

Figure 3: Chemokine receptor profile of peripheral blood CD3 T-cells from 18 healthy controls (light bars) and 24 patients with end-stage HF (dark bars). Percentage of positive cells (A) and expression levels in MESF (B) for all receptors studied are depicted accordingly. All values are expressed in mean ± SEM and are compared with normal healthy controls. *p < 0.05, **p < 0.01, ***p < 0.0005

Only the percentage of $CD8^+$ $CXCR1^+$ but not $CD4^+$ $CXCR1^+$ cells was increased in the patients (CD4: 5% vs 3%; p = 0.234 and CD8: 11% vs 5%; p < 0.0001). Similarly, the percentage of $CD8^+$ $CXCR3^+$ and not the percentage of $CD4^+$ $CXCR3^+$ cells was increased in patients compared with controls (CD4: 39 vs 43%; p = 0.437 and CD8: 91 vs 83%; p = 0.005) (Figure 5A). CXCR1 or CXCR3 expression on both $CD4^+$ and $CD8^+$ subsets from patients did not differ from the control levels of expression [CD4 (CXCR1: 1177 MESF vs 970.3 MESF; p = 0.186 and CXCR3: 4594 MESF vs 4924 MESF; *p* = 0.639)], [CD8 (CXCR1: 3241 MESF vs 2291.1

MESF; p = 0.113 and CXCR3: 3474 MESF vs 3911 MESF; p = 0.529)] (Figure 4B and 5B). In controls, only $CD4^+$ cell frequency corresponded with the $CD3^+$ cell frequency for CCR4 and CCR5 (49 and 17%), because $CD8^+$ cells expressed the same receptors at 43 and 47%, respectively (Figures 4A and 5A). As for $CD3^+$ cells, percentage and expression of CCR1, CCR2, CCR3 and CXCR2 on $CD4^+$ and $CD8^+$ T-cell subsets from controls were low. When studied between controls and patients, no obvious differences for these chemokine receptors could be detected on $CD4^+$ or $CD8^+$ T-cell subsets.

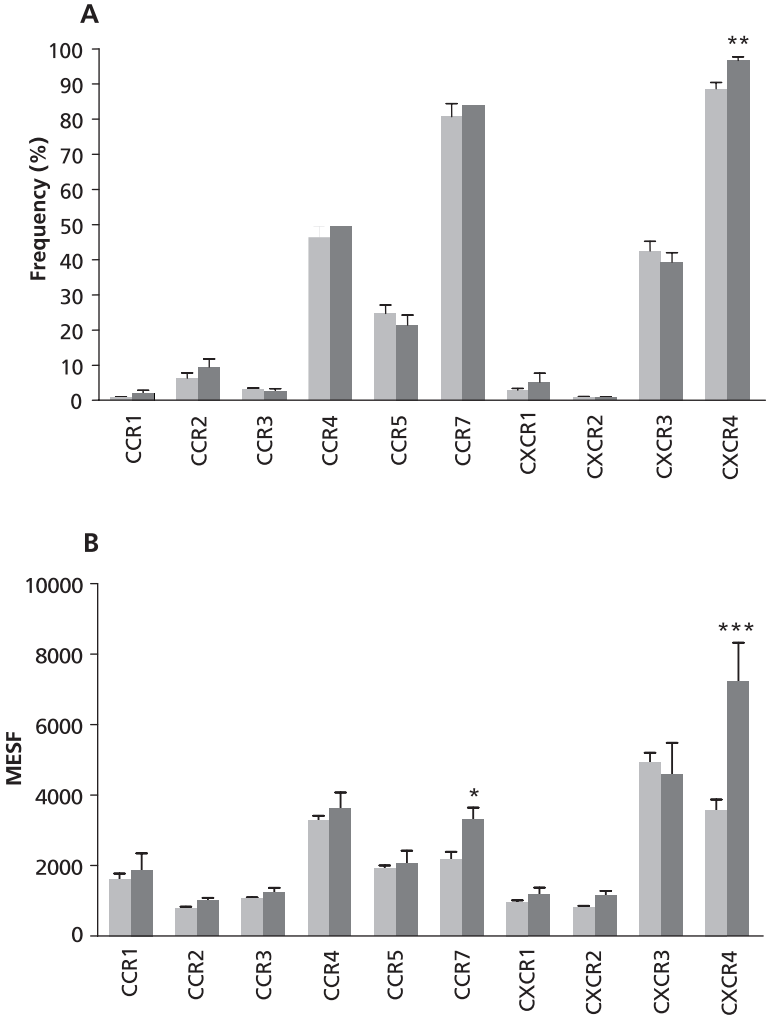

Figure 4: Chemokine receptor profile of peripheral blood CD4 T-cells from 18 healthy controls (light bars) and 24 patients with end-stage HF (dark bars). Percentage of positive cells (A) and expression levels in MESF (B) for all receptors studied are depicted accordingly. All values are expressed in mean ± SEM and were compared with normal healthy controls. *p < 0.05, **p < 0.01, ***p < 0.0005

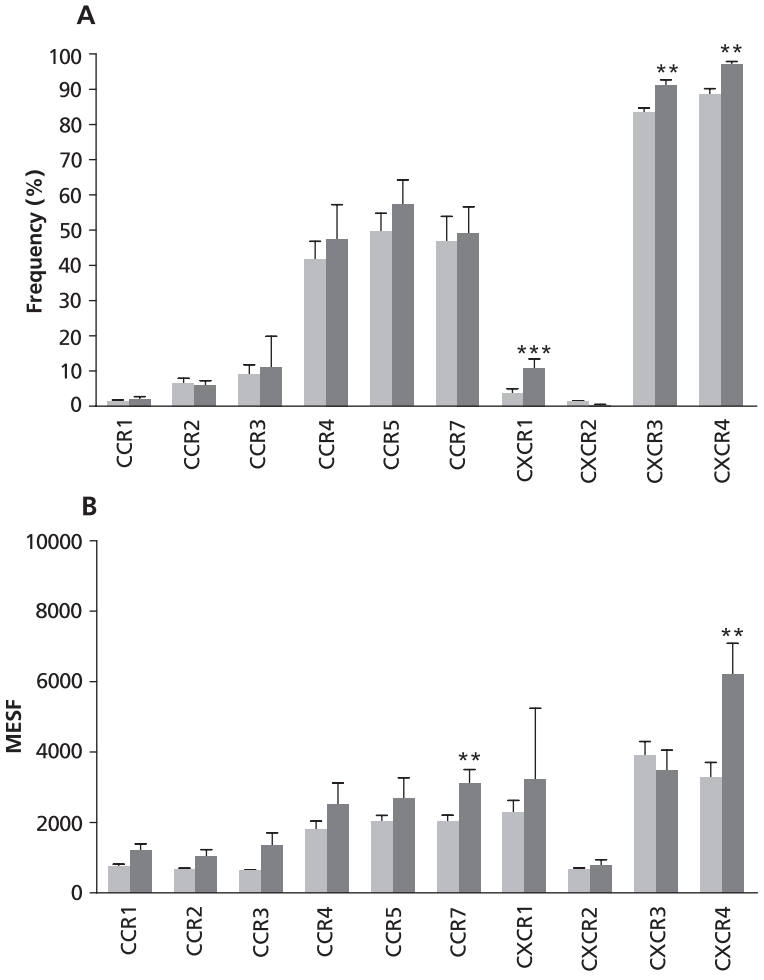

Figure 5: Chemokine receptor profile of peripheral blood CD8 T-cells from 18 healthy controls (light bars) and 24 patients with end-stage HF (dark bars). Percentage of positive cells (A) and expression levels in MESF (B) for all receptors studied are depicted accordingly. All values are expressed in mean ± SEM and were compared with normal healthy controls. **p < 0.01, ***p < 0.0005

Monocytes

Approximately 40 and 90% of circulating monocytes in controls expressed CCR7 and CXCR4 on their surface, respectively. A proportion of monocytes also expressed CCR1 (80%) and CCR2 (87%) (Figure 6A). Patient peripheral blood monocytes revealed a significant decrease in the frequency of $CCR1^+$ cells (from 77% in controls to 60% in patients; p = 0.032), and $CCR2^+$ cells (from 88% in controls to 77% in patients; p = 0.006). Additionally, a concomitant

increase in CD14+ CXCR4+ cells (from 89 to 95%; p = 0.041) in comparison with controls was observed (Fig. 6A). CXCR4 expression was upregulated from 3579 MESF to 5395 MESF in patients (p = 0.039) but not expression levels of CCR1 (1748 MESF) or CCR2 (4906 MESF), which remained at the same levels as the expression in controls (1946 MESF and 4388 MESF, respectively) (Figure 6B). No substantial percentage of monocytes in controls (4-9%) was observed to express CCR3, CCR5, or CXCR3 whereas CCR4+, CXCR1+ and CXCR2+ monocytes reached a frequency of 43, 58, and 56%, respectively. The frequencies and expression levels for these receptors did not differ significantly between controls and patients.

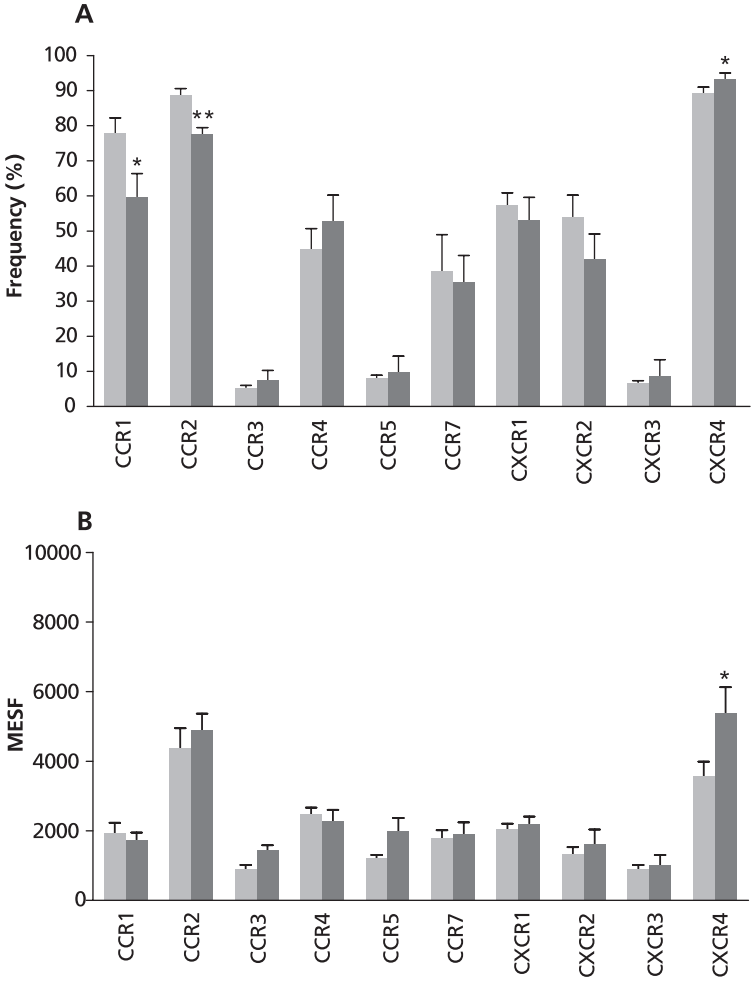

Figure 6: Chemokine receptor profile of peripheral blood monocytes from 18 healthy controls (light bars) and 24 patients with end-stage HF (dark bars). Percentage of positive cells (A) and expression levels in MESF (B) for all receptors studied are depicted accordingly. All values are expressed in mean ± SEM and were compared with normal healthy controls. *p < 0.05, **p < 0.01

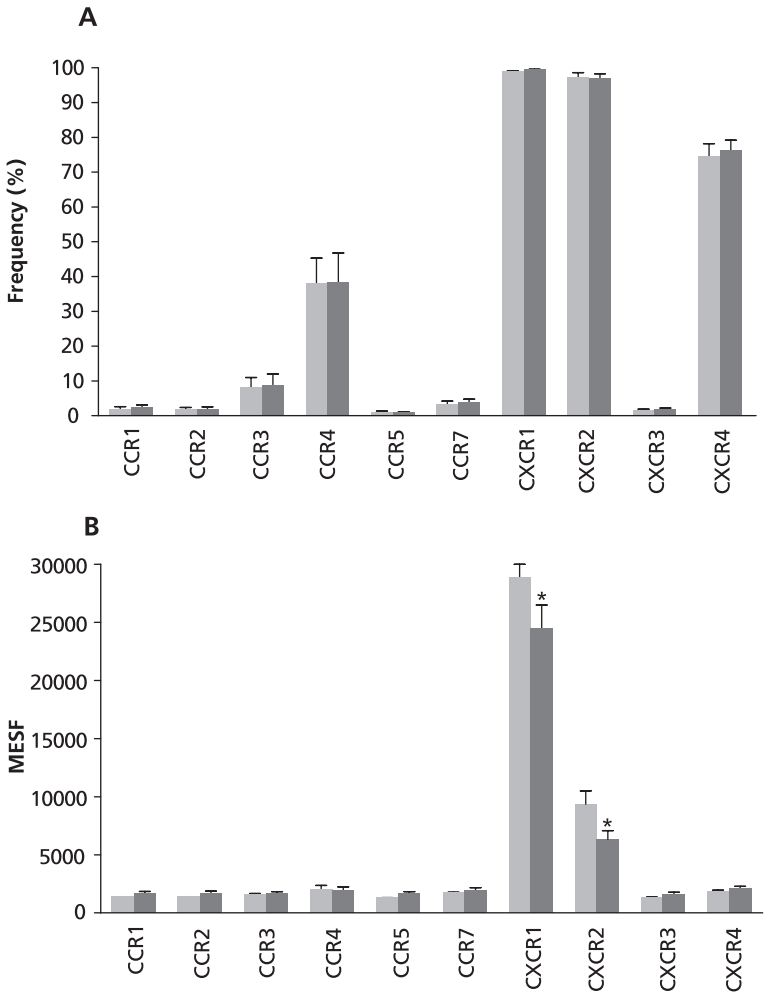

Figure 7: Chemokine receptor profile of peripheral blood granulocytes from 18 healthy controls (light bars) and 24 patients with end-stage heart failure (dark bars). Percentage of positive cells (A) and expression levels in MESF (B) for all receptors studied are depicted accordingly. All values are expressed in mean ± SEM and were compared with normal healthy controls. *$p < 0.05$

Granulocytes

The bulk of blood granulocytes in controls expressed CXCR1 (99%) and CXCR2 (97%), whereas notable proportion of these cells also expressed CCR4 (39%) and CXCR4 (75%) (Figure 7A). CCR1, CCR2, CCR3, CCR5, CCR7, and CXCR3 were expressed only at a fraction of the total population of granulocytes (1-10%) in patients and controls. Both $CXCR1^+$ and $CXCR2^+$ granulocyte frequencies were

equally high between patients and controls ($CXCR1^+$: 99 vs 98%; p = 0.112 and $CXCR2^+$: 97 vs 98%; p = 0.488) (Figure 7A). Only a marginal, albeit significant downregulation of the CXCR1 and CXCR2 receptor expression was observed in patients, compared with controls. CXCR1 levels decreased from 29671 MESF in controls to 24480 MESF in the patients (p = 0.034). The downregulation of CXCR2 levels from 9342 MESF to 6318 MESF was of analogous proportions (p = 0.032) (Figure 7B).

Chemokine receptor profile in HF patients according to disease etiology

We further analyzed the frequency of $CD3^+$, $CD4^+$ and $CD8^+$ T-cells, monocytes, and granulocytes as well as the expression profile of chemokine receptors in relation to the underlying disease etiology. Both frequency of $CD3^+$ $CXCR4^+$ cells and the expression of CXCR4 on $CD3^+$ cells were substantially increased in all patient groups, irrespective of disease etiology (p = 0.002 and p < 0.0001, respectively). Expression of CXCR4 was higher only on $CD8^+$ but not $CD4^+$ cells for all patients than controls (p = 0.030 and 0.128, respectively; data not shown). As seen in Table 3, although percentage of $CD4^+$ $CXCR4^+$ and percentage of $CD8^+$ $CXCR4^+$ cells were not significantly different with respect to the cause of disease (p = 0.594 and 0.608), both $CD4^+$ $CXCR4^+$ and $CD8^+$ $CXCR4^+$ cell frequencies from all patients revealed a tendency to be higher than the corresponding frequencies from controls (p = 0.067 and 0.117, respectively; data not shown). Within HF patients, the CAD and IDCM groups tended towards higher mean expression levels of CCR7 on $CD3^+$ cells, compared to HCM and VD patients (p = 0.062). This was reflected on higher CCR7 expression on $CD8^+$ cells (p = 0.037) but not $CD4^+$ cells (p = 0.232) of the CAD and IDCM patients (Table 3).

In patients with CAD, there was a tendency for higher percentage of circulating $CD3^+$ $CXCR3^+$ cells than in IDCM, HCM or VD patients (p = 0.082), but a significantly higher percentage of $CD8^+$ $CXCR3^+$ cells in the CAD patients (p = 0.042) were observed. Although differences were not significant, patients with IDCM showed the highest percentage of $CD8^+$ $CXCR1^+$ cells and expression of CXCR1 on $CD8^+$ cells compared to other etiologies of cardiac disease (Table 3). For monocytes, CAD group accounted for the decrease in frequencies of both $CCR1^+$ $CD14^+$ and $CCR2^+$ $CD14^+$ cells as compared to IDCM, HCM or VD (p = 0.041 and 0.003, respectively). With the exception of IDCM patients, the CAD, HCM and VD groups exhibited lower expression of CXCR1 and CXCR2 on granulocytes compared to controls (Table 3; Figure 7B). However, granulocyte CXCR1 and CXCR2 expression levels were not significantly different according to cause of cardiac disease (p = 0.085 and p = 0.173, respectively; Table 3).

Discussion

In the present study we assessed blood from heart transplant recipients before transplantation and healthy individuals to clarify whether circulating leukocyte chemokine receptor expression profile is modified during NYHA III-IV HF. We found that conditions of systemic hypoxia and low-grade inflammation as seen in end-stage HF, impose alterations both in the leukocyte surface expression as well as the frequency of peripheral blood T-cell, monocyte and granulocyte populations bearing CC- or CXC- chemokine receptors.

Leukocyte populations from fresh peripheral blood of healthy individuals express a wide variety of CC- and CXC- chemokine receptors, selectively. For example, T-cells exhibit high expression of the homeostatic CCR7 and CXCR4 on both $CD4^+$ and $CD8^+$ subpopulations, with preferential expression of CCR4 at intermediate levels. Interestingly, within T-cells the majority of $CD8^+$ but not $CD4^+$ cells, were positive for the inflammatory CXCR3 and CCR5 at intermediate levels. In contrast, monocytes were demonstrated to express high levels of CCR2 and CXCR4, whereas the majority of these cells were dimly positive for CCR1, CCR4, CXCR1 and CXCR3 at lower frequencies. Alternatively, granulocytes strongly expressed CXCR1, were highly positive for CXCR2 and intermediate for CXCR4.

Our results show that peripheral blood T-cells from HF patients exhibited upregulated expression of the chemokine receptor CXCR4, which was reflected more on $CD8^+$ than $CD4^+$ cells, independent of etiology of the disease. Given that the CXCR4 ligand, CXCL-12 (stromal derived factor-1), is increased in patients with chronic ischemic heart disease [46], and in various stages of human congestive HF [47], it is plausible that elevated CXCR4 expression on both circulating $CD4^+$ and $CD8^+$ cells in our patient cohort, reflects enhanced migration of both T-cell subsets with a naïve (CXCR4) phenotype towards lymphoid organs in end-stage HF. These results concur with the upregulation of an additional lymphoid tissue homing chemokine receptor -CCR7- expression on the surface of both circulating $CD4^+$ and $CD8^+$ cells in our patient cohort. Interestingly, CAD and IDCM patients had a higher CCR7 expression on $CD8^+$ cells than patients with HCM and VD. Seemingly, increased expression of homeostatic chemokine receptors (CCR7 and CXCR4) on naïve peripheral blood T-cell subsets could result in accelerated homing of $CD4^+$ or $CD8^+$ cells to lymphoid tissues for T-helper (T_H)1 or T_H2 priming during end-stage HF (Figure 8). In this state, lymph nodes could play a central role in T-cell and dendritic cell (DC) homing, because lymphatic endothelial cells have been

shown to increase CCL21 (secondary lymphoid tissue chemokine) expression upon pre-conditioning by increased concentrations of TNFα [48].

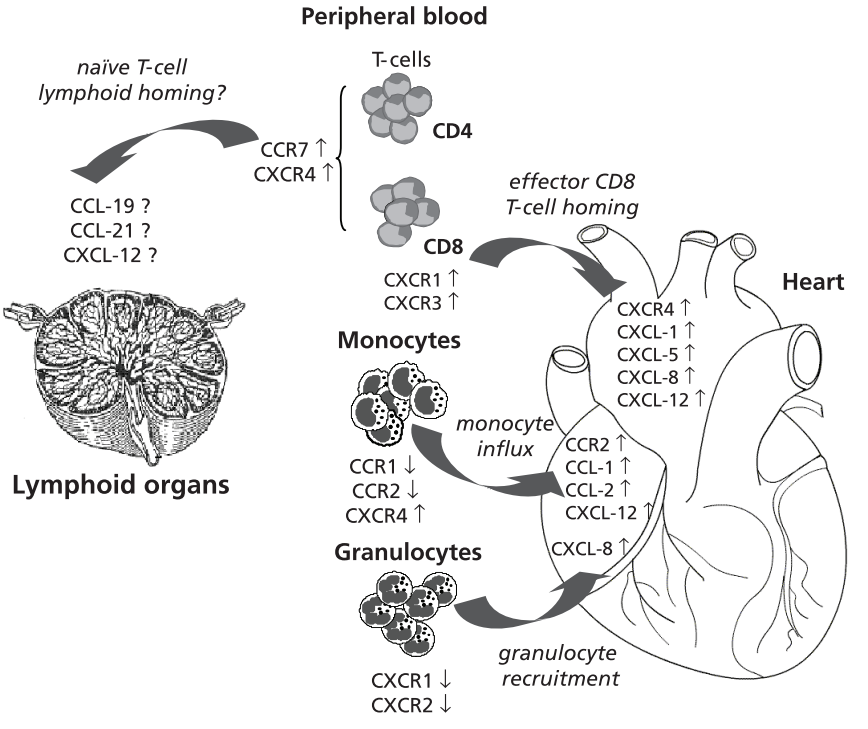

Figure 8: Schematic diagram depicting possible cellular interactions through chemokines and their receptors both in circulation and at the tissue sites in human heart failure. Altered chemokine receptor expression could be implicated in: CD4 and CD8 T-cell lymphoid homing through CCR7 and CXCR4, effector CD8 myocardial T-cell infiltration through CXCR1 and CXCR3, myocardial monocyte influx through CCR1, CCR2 and CXCR4, and granulocyte recruitment through CXCR1 and CXCR2. Some of these mechanisms may be associated with the cause of cardiac disease.

We have previously shown that myeloid DCs, which are able to prime T-cells towards T_H1 responses [49], with a mature lymphoid-homing $CCR7^+$ phenotype are present at higher numbers in the circulation of HF patients compared with controls [50]. However, in peripheral blood, we did not see any clear polarization of $CD4^+$ cells towards a T_H1 (CCR1, CCR2, CCR5, CXCR3) or T_H2 (CCR3, CCR4) phenotype. On the contrary, $CD8^+$ $CXCR3^+$ and $CD8^+$ $CXCR1^+$ T -cell frequency in the venous blood of our patients was increased. As CXCR3 is known to be induced on effector-memory T-cells [51], and CXCR1 was recently

shown to be expressed on human effector $CD8^+$ T-cells [52], our results provide the evidence for a small fraction of $CD8^+$ cells with a T_H1-type inflammatory inducing capacity in the peripheral blood of end-stage HF patients. Enhanced CXCR3 and CXCR1 expression on CD8 cells might be specific for patients with CAD and IDCM, respectively. Conceptually, these cells should exhibit affinity for homing into inflammatory sites through CXCL-1, CXCL-5 or CXCL-8, and in the case of our patients this should be the myocardium (Figure 8). Indeed, several studies have demonstrated an activated state of myocardial T-lymphocytes [53], and cytotoxic T-cells after myocardial infarction, which are able to recognize and kill even healthy cardiomyocytes *in vitro* [54]. Furthermore, patients with ischemic heart disease exhibited increased frequencies of circulating cytotoxic T-lymphocytes, irrespective of their clinical presentation [12]. Moreover, in another study, a peripheral blood effector-memory $CD8^+$ pool with an increased lymph-node homing potential through CD62L (Leu-8), was found to influx the myocardium of patients with dilated cardiomyopathy, continuously [55].

In monocytes, we saw an increased frequency of $CD14^+$ $CXCR4^+$ cells and an elevated expression of CXCR4, except for a decreased frequency of $CD14^+$ $CCR1^+$ and $CD14^+$ $CCR2^+$ cells, compared with healthy volunteers. These results can be explained by elevated CCL-2 expression, found in animal models of cardiac decompensation secondary to pressure-overload or ischemia [43-45]. Furthermore, both CCL-2 and CCL-1 (macrophage inflammatory protein 1α) have been correlated to interstitial monocyte infiltration of the myocardium with development of a number of pathological changes including cardiac hypertrophy, ventricular dilatation, and depressed contractile function, and their levels are the highest in those with the most advanced disease [16]. The lower $CD14^+$ $CCR1^+$ and $CD14^+$ $CCR2^+$ cell frequency in patients with CAD, was responsible for the overall decrease of $CCR1^+$ and $CCR2^+$ monocyte frequencies in chronic HF patients compared with controls. We suggest that in such conditions, $CCR1^+$ and $CCR2^+$ monocyte frequencies are decreased in peripheral blood of HF patients because these monocyte subsets infiltrate myocardium. This view can be supported by the particularly enhanced CCR2 expression in the human failing myocardium, together with the expression of the constitutive chemokine receptor CXCR4 [30]. Alternatively, the effects of statin therapy may be superimposed on the effects of the disease in monocytes, particularly in CAD patients, because hydroxymethylglutaryl coenzyme A reductase inhibitors (statins) are known to reduce CCR1 and CCR2 gene expression of peripheral blood mononuclear cells [56]. However, low oxygen concentrations induce high expression of CXCR4 in monocytes, monocyte-derived macrophages and

endothelial cells [57]. Hence, it is conceivable that local and systemic hypoxic conditions encountered in end-stage HF, account for the elevated frequency of $CD14^+$ $CXCR4^+$ cells and the enhanced CXCR4 expression on peripheral blood monocytes. Given the increased chemotactic responsiveness of CXCR4 to its specific ligand CXCL-12 under hypoxia [58], we believe that CXCR4, in a concerted action with CCR1 and CCR2 on monocytes, could play an important role in the continuous colonization of failing myocardium with monocytes and macrophages from blood in patients with CAD during end-stage HF (Figure 8).

Virtually, all circulating granulocytes were positive for both IL-8 receptors (CXCR1 and CXCR2), implying their constitutive expression in the peripheral blood of both patients and healthy individuals. However, with the exception of the IDCM group, expression of both receptors was shown to be downregulated in the patients compared with controls. This could be the result of systemic presence of TNF-α which has been demonstrated to cause proteolytic degradation of both receptors for CXCL-8 (IL-8) [59]. Alternatively, the reduced surface CXCR1 and CXCR2 expression levels could be explained by the internalization of the receptor-ligand complex. This allows destruction of the ligand, receptor recycling, and a continuous cellular response to the chemokine, suggesting that both receptors have been engaged during neutrophil recruitment (Figure 8). Indeed, both CXCL-8 mRNA levels in mononuclear blood cells and serum CXCL-8 protein concentration, are elevated in patients with congestive HF [17,30], and such high concentrations have been demonstrated to down-regulate both CXCR1 and CXCR2 receptors [60].

Some of our results contrast the findings of other studies that have demonstrated enhanced overall chemokine receptor mRNA levels as assessed by RNAse protection assays in PBMCs of patients with end-stage HF. The reasons for this discrepancy could be sought in the different techniques used for assessing chemokine receptor expression. Although Damas et al. used mRNA-based strategies [17,31], our study evaluated the expression of chemokine receptor protein using flow-cytometry. Even though protein evaluation is a better estimate of function than the expression of mRNA, studies that assess the migration of leukocytes from these patients must be performed in the future, to firmly establish a role for any given chemokine receptor.

Nevertheless, we believe that the strength of our study resides in the technique used to measure chemokine receptor expression on T-cells, monocytes and granulocytes. As chemokine receptors undergo internalization and endocytic recycling, the detection sensitivity of receptor expression may also depend

on the manipulations used to isolate the cells being studied. A small signal induced by subtle changes such as temperature shifts, media changes (e.g. Ficoll Paque), or even extended duration of sample storage might induce internalization of the receptor, rendering the cell apparently negative [61]. Recently, it was demonstrated that the staining of whole blood and performing flow cytometric analysis without a lyzing procedure is the least interfering protocol with chemokine receptor expression [62]. Hence, by using whole blood and processing cells within 3h after collection, we used optimal conditions for isolating the cells in order to best identify cell subset expression of chemokine receptors.

The enhanced myocardial expression of chemokines and their corresponding receptors on both cardiomyocytes and infiltrating leukocytes in various forms of myocardial failure suggests a central role for chemokine-related interactions in the pathogenesis of HF. Our study further supports the notion that in end-stage HF, alterations of chemokine receptor expression may not only be a new parameter of enhanced immune activation, but may also reflect an important mechanism contributing to the progression of HF with underlying etiology. The capacity to control activation and migration of inflammatory cells suggests chemokines and their receptors as novel targets for therapeutic intervention in a number of diseases characterized by chronic inflammation. In acute and chronic myocardial failure, modulation of the chemokine network may represent interesting novel therapeutic modalities.

References

1. Haddy FJ (2003): Heart failure - incidence and survival. N Engl J Med 348:660.
2. Conraads VM, Bosmans JM, Vrints CJ (2002): Chronic heart failure: an example of a systemic chronic inflammatory disease resulting in cachexia. Int J Cardiol 85:33-49.
3. Matsumori A, Matoba Y, Sasayama S (1995): Dilated cardiomyopathy associated with hepatitis C virus infection. Circulation 92:2519-25.
4. Shioi T, Matsumori A, Sasayama S (1996): Persistent expression of cytokine in the chronic stage of viral myocarditis in mice. Circulation 94:2930-7.
5. Bozkurt B, Kribbs SB, Clubb FJ, Jr., Michael LH, Didenko VV, Hornsby PJ, Seta Y, Oral H, Spinale FG, Mann DL (1998): Pathophysiologically relevant concentrations of tumor necrosis factor-alpha promote progressive left ventricular dysfunction and remodeling in rats. Circulation 97:1382-91.
6. MacGowan GA, Mann DL, Kormos RL, Feldman AM, Murali S (1997): Circulating interleukin-6 in severe heart failure. Am J Cardiol 79:1128-31.

7. Bristow MR (1998): Why does the myocardium fail? Insights from basic science. Lancet 352 Suppl 1:SI8-14.
8. Hasper D, Hummel M, Kleber FX, Reindl I, Volk HD (1998): Systemic inflammation in patients with heart failure. Eur Heart J 19:761-5.
9. Aukrust P, Damas JK, Gullestad L, Froland SS (2001): Chemokines in myocardial failure - pathogenic importance and potential therapeutic targets. Clin Exp Immunol 124:343-5.
10. Levine B, Kalman J, Mayer L, Fillit HM, Packer M (1990): Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. N Engl J Med 323:236-41.
11. Matsumori A, Yamada T, Suzuki H, Matoba Y, Sasayama S (1994): Increased circulating cytokines in patients with myocarditis and cardiomyopathy. Br Heart J 72:561-6.
12. Mazzone A, De Servi S, Vezzoli M, Fossati G, Mazzucchelli I, Gritti D, Ottini E, Mussini A, Specchia G (1999): Plasma levels of interleukin 2, 6, 10 and phenotypic characterization of circulating T-lymphocytes in ischemic heart disease. Atherosclerosis 145:369-74.
13. Sharma R, Bolger AP, Li W, Davlouros PA, Volk HD, Poole-Wilson PA, Coats AJ, Gatzoulis MA, Anker SD (2003): Elevated circulating levels of inflammatory cytokines and bacterial endotoxin in adults with congenital heart disease. Am J Cardiol 92:188-93.
14. Ferrari R, Bachetti T, Confortini R, Opasich C, Febo O, Corti A, Cassani G, Visioli O (1995): Tumor necrosis factor soluble receptors in patients with various degrees of congestive heart failure. Circulation 92:1479-86.
15. Aukrust P, Ueland T, Lien E, Bendtzen K, Muller F, Andreassen AK, Nordoy I, Aass H, Espevik T, Simonsen S, Froland SS, Gullestad L (1999): Cytokine network in congestive heart failure secondary to ischemic or idiopathic dilated cardiomyopathy. Am J Cardiol 83:376-82.
16. Aukrust P, Ueland T, Muller F, Andreassen AK, Nordoy I, Aas H, Kjekshus J, Simonsen S, Froland SS, Gullestad L (1998): Elevated circulating levels of C-C chemokines in patients with congestive heart failure. Circulation 97:1136-43.
17. Damas JK, Gullestad L, Aass H, Simonsen S, Fjeld JG, Wikeby L, Ueland T, Eiken HG, Froland SS, Aukrust P (2001): Enhanced gene expression of chemokines and their corresponding receptors in mononuclear blood cells in chronic heart failure--modulatory effect of intravenous immunoglobulin. J Am Coll Cardiol 38:187-93.
18. Luster AD (1998): Chemokines--chemotactic cytokines that mediate inflammation. N Engl J Med 338:436-45.
19. Horuk R (2001): Chemokine receptors. Cytokine Growth Factor Rev 12:313-35.
20. Olson TS, Ley K (2002): Chemokines and chemokine receptors in leukocyte trafficking. Am J Physiol Regul Integr Comp Physiol 283:R7-28.
21. Murdoch C, Finn A (2000): Chemokine receptors and their role in inflammation and infectious diseases. Blood 95:3032-43.
22. Cook DN, Beck MA, Coffman TM, Kirby SL, Sheridan JF, Pragnell IB, Smithies O (1995): Requirement of MIP-1 alpha for an inflammatory response to viral infection. Science 269:1583-5.

23. Fuse K, Kodama M, Hanawa H, Okura Y, Ito M, Shiono T, Maruyama S, Hirono S, Kato K, Watanabe K, Aizawa Y (2001): Enhanced expression and production of monocyte chemoattractant protein-1 in myocarditis. Clin Exp Immunol 124:346-52.
24. Machado FS, Martins GA, Aliberti JC, Mestriner FL, Cunha FQ, Silva JS (2000): Trypanosoma cruzi-infected cardiomyocytes produce chemokines and cytokines that trigger potent nitric oxide-dependent trypanocidal activity. Circulation 102:3003-8.
25. Talvani A, Rocha MO, Ribeiro AL, Correa-Oliveira R, Teixeira MM (2004): Chemokine receptor expression on the surface of peripheral blood mononuclear cells in Chagas disease. J Infect Dis 189:214-20.
26. Shioi T, Matsumori A, Kihara Y, Inoko M, Ono K, Iwanaga Y, Yamada T, Iwasaki A, Matsushima K, Sasayama S (1997): Increased expression of interleukin-1 beta and monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 in the hypertrophied and failing heart with pressure overload. Circ Res 81:664-71.
27. Behr TM, Wang X, Aiyar N, Coatney RW, Li X, Koster P, Angermann CE, Ohlstein E, Feuerstein GZ, Winaver J (2000): Monocyte chemoattractant protein-1 is upregulated in rats with volume-overload congestive heart failure. Circulation 102:1315-22.
28. Kakio T, Matsumori A, Ono K, Ito H, Matsushima K, Sasayama S (2000): Roles and relationship of macrophages and monocyte chemotactic and activating factor/monocyte chemoattractant protein-1 in the ischemic and reperfused rat heart. Lab Invest 80:1127-36.
29. Kolattukudy PE, Quach T, Bergese S, Breckenridge S, Hensley J, Altschuld R, Gordillo G, Klenotic S, Orosz C, Parker-Thornburg J (1998): Myocarditis induced by targeted expression of the MCP-1 gene in murine cardiac muscle. Am J Pathol 152:101-11.
30. Damas JK, Gullestad L, Ueland T, Solum NO, Simonsen S, Froland SS, Aukrust P (2000): CXC-chemokines, a new group of cytokines in congestive heart failure--possible role of platelets and monocytes. Cardiovasc Res 45:428-36.
31. Damas JK, Eiken HG, Oie E, Bjerkeli V, Yndestad A, Ueland T, Tonnessen T, Geiran OR, Aass H, Simonsen S, Christensen G, Froland SS, Attramadal H, Gullestad L, Aukrust P (2000): Myocardial expression of CC- and CXC-chemokines and their receptors in human end-stage heart failure. Cardiovasc Res 47:778-87.
32. Gerszten RE, Mach F, Sauty A, Rosenzweig A, Luster AD (2000): Chemokines, leukocytes, and atherosclerosis. J Lab Clin Med 136:87-92.
33. Boring L, Gosling J, Cleary M, Charo IF (1998): Decreased lesion formation in CCR2-/- mice reveals a role for chemokines in the initiation of atherosclerosis. Nature 394:894-7.
34. Boisvert WA, Santiago R, Curtiss LK, Terkeltaub RA (1998): A leukocyte homologue of the IL-8 receptor CXCR-2 mediates the accumulation of macrophages in atherosclerotic lesions of LDL receptor-deficient mice. J Clin Invest 101:353-63.
35. Gu L, Okada Y, Clinton SK, Gerard C, Sukhova GK, Libby P, Rollins BJ (1998): Absence of monocyte chemoattractant protein-1 reduces atherosclerosis in low density lipoprotein receptor-deficient mice. Mol Cell 2:275-81.

36. Aukrust P, Berge RK, Ueland T, Aaser E, Damas JK, Wikeby L, Brunsvig A, Muller F, Forfang K, Froland SS, Gullestad L (2001): Interaction between chemokines and oxidative stress: possible pathogenic role in acute coronary syndromes. J Am Coll Cardiol 37:485-91.
37. Mach F, Sauty A, Iarossi AS, Sukhova GK, Neote K, Libby P, Luster AD (1999): Differential expression of three T lymphocyte-activating CXC chemokines by human atheroma-associated cells. J Clin Invest 104:1041-50.
38. Devaux B, Scholz D, Hirche A, Klovekorn WP, Schaper J (1997): Upregulation of cell adhesion molecules and the presence of low grade inflammation in human chronic heart failure. Eur Heart J 18:470-9.
39. Damas JK, Aukrust P, Ueland T, Odegaard A, Eiken HG, Gullestad L, Sejersted OM, Christensen G (2001): Monocyte chemoattractant protein-1 enhances and interleukin-10 suppresses the production of inflammatory cytokines in adult rat cardiomyocytes. Basic Res Cardiol 96:345-52.
40. Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM, Elner SG, Strieter RM (1992): Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science 258:1798-801.
41. Hesselgesser J, Taub D, Baskar P, Greenberg M, Hoxie J, Kolson DL, Horuk R (1998): Neuronal apoptosis induced by HIV-1 gp120 and the chemokine SDF-1 alpha is mediated by the chemokine receptor CXCR4. Curr Biol 8:595-8.
42. Li N, Karin M (1999): Is NF-kappaB the sensor of oxidative stress? Faseb J 13:1137-43.
43. Valen G, Yan ZQ, Hansson GK (2001): Nuclear factor kappa-B and the heart. J Am Coll Cardiol 38:307-14.
44. Spinale FG, Coker ML, Heung LJ, Bond BR, Gunasinghe HR, Etoh T, Goldberg AT, Zellner JL, Crumbley AJ (2000): A matrix metalloproteinase induction/activation system exists in the human left ventricular myocardium and is upregulated in heart failure. Circulation 102:1944-9.
45. Yamamoto T, Eckes B, Mauch C, Hartmann K, Krieg T (2000): Monocyte chemoattractant protein-1 enhances gene expression and synthesis of matrix metalloproteinase-1 in human fibroblasts by an autocrine IL-1 alpha loop. J Immunol 164:6174-9.
46. Heeschen C, Lehmann R, Honold J, Assmus B, Aicher A, Walter DH, Martin H, Zeiher AM, Dimmeler S (2004): Profoundly reduced neovascularization capacity of bone marrow mononuclear cells derived from patients with chronic ischemic heart disease. Circulation 109:1615-22.
47. Valgimigli M, Rigolin GM, Fucili A, Porta MD, Soukhomovskaia O, Malagutti P, Bugli AM, Bragotti LZ, Francolini G, Mauro E, Castoldi G, Ferrari R (2004): CD34+ and endothelial progenitor cells in patients with various degrees of congestive heart failure. Circulation 110:1209-12.
48. MartIn-Fontecha A, Sebastiani S, Hopken UE, Uguccioni M, Lipp M, Lanzavecchia A, Sallusto F (2003): Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. J Exp Med 198:615-21.
49. Vieira PL, de Jong EC, Wierenga EA, Kapsenberg ML, Kalinski P (2000): Development of T_H1-inducing capacity in myeloid dendritic cells requires environmental instruction. J Immunol 164:4507-12.

50. Athanassopoulos P, Vaessen LM, Maat AP, Balk AH, Weimar W, Bogers AJ (2004): Peripheral blood dendritic cells in human end-stage heart failure and the early post-transplant period: evidence for systemic T_H1 immune responses. Eur J Cardiothorac Surg 25:619-26.
51. Ko S, Deiwick A, Jager MD, Dinkel A, Rohde F, Fischer R, Tsui TY, Rittmann KL, Wonigeit K, Schlitt HJ (1999): The functional relevance of passenger leukocytes and microchimerism for heart allograft acceptance in the rat. Nat Med 5:1292-7.
52. Takata H, Tomiyama H, Fujiwara M, Kobayashi N, Takiguchi M (2004): Cutting edge: expression of chemokine receptor CXCR1 on human effector CD8+ T-cells. J Immunol 173:2231-5.
53. Noutsias M, Pauschinger M, Schultheiss H, U Kh (2002): Phenotypic characterization of infiltrates in dilated cardiomyopathy - diagnostic significance of T-lymphocytes and macrophages in inflammatory cardiomyopathy. Med Sci Monit 8:CR478-87.
54. Varda-Bloom N, Leor J, Ohad DG, Hasin Y, Amar M, Fixler R, Battler A, Eldar M, Hasin D (2000): Cytotoxic T-lymphocytes are activated following myocardial infarction and can recognize and kill healthy myocytes in vitro. J Mol Cell Cardiol 32:2141-9.
55. Holzinger C, Schollhammer A, Imhof M, Reinwald C, Kramer G, Zuckermann A, Wolner E, Steiner G (1995): Phenotypic patterns of mononuclear cells in dilated cardiomyopathy. Circulation 92:2876-85.
56. Waehre T, Damas JK, Gullestad L, Holm AM, Pedersen TR, Arnesen KE, Torsvik H, Froland SS, Semb AG, Aukrust P (2003): Hydroxymethylglutaryl coenzyme a reductase inhibitors down-regulate chemokines and chemokine receptors in patients with coronary artery disease. J Am Coll Cardiol 41:1460-7.
57. Schioppa T, Uranchimeg B, Saccani A, Biswas SK, Doni A, Rapisarda A, Bernasconi S, Saccani S, Nebuloni M, Vago L, Mantovani A, Melillo G, Sica A (2003): Regulation of the chemokine receptor CXCR4 by hypoxia. J Exp Med 198:1391-402.
58. Ceradini DJ, Kulkarni AR, Callaghan MJ, Tepper OM, Bastidas N, Kleinman ME, Capla JM, Galiano RD, Levine JP, Gurtner GC (2004): Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat Med 10:858-64.
59. Khandaker MH, Mitchell G, Xu L, Andrews JD, Singh R, Leung H, Madrenas J, Ferguson SS, Feldman RD, Kelvin DJ (1999): Metalloproteinases are involved in lipopolysaccharide- and tumor necrosis factor-alpha-mediated regulation of CXCR1 and CXCR2 chemokine receptor expression. Blood 93:2173-85.
60. Richardson RM, Pridgen BC, Haribabu B, Ali H, Snyderman R (1998): Differential cross-regulation of the human chemokine receptors CXCR1 and CXCR2. Evidence for time-dependent signal generation. J Biol Chem 273:23830-6.
61. Shalekoff S, Tiemessen CT (2001): Duration of sample storage dramatically alters expression of the human immunodeficiency virus coreceptors CXCR4 and CCR5. Clin Diagn Lab Immunol 8:432-6.
62. Berhanu D, Mortari F, De Rosa SC, Roederer M (2003): Optimized lymphocyte isolation methods for analysis of chemokine receptor expression. J Immunol Methods 279:199-207.

Chapter 3

Peripheral Blood Dendritic Cells in Human End-Stage Heart Failure and the Early Post-Transplant Period: Evidence for Systemic Th1 Immune Responses

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Abstract

Objective: 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 polarizing naïve T-helper cells into a T_H1 or T_H2 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 ($p = 0.01$). % $CD83^+$ 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 T_H1 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 heart transplantation.

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], and chemokines (CXCL1, -5, -8 and CCL2, -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 polarizing 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 T_H1 deviation of immune responses in heart failure still remain to be elucidated.

There is growing evidence that the polarization 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 naïve 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 T_H1 polarized 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 T_H2 polarization of naïve 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 T_H1-inducing DCs responsible for the surveillance against pathogenic signals in the periphery. By contrast, plasmacytoid DCs 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 specialized APCs preferentially inducing a T_H1 and T_H2 response, respectively. In end-stage heart failure, it is suggested that T_H1-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.

Patients and Methods

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 one 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 to 8 i.v. dosages daily (1 dosage = 212,5 lymphocytotoxic-units/kg/24hrs) until adequate Cyclosporin A (CsA) or Tacrolimus (FK506) trough levels were achieved (250-350 ng/mL and 12-16ng/mL, respectively). The combination of Prednisolone, CsA or FK506 and Mycophenolate Mofetil (MMF) was used as maintenance immunosuppression. All patients received 75mg prednisolone i.v. in the first post-transplant day and 50mg/day for 5 consecutive days post-HTx. At the time of venous puncture for DC enumeration (day 7-8 post-HTx) all patients received 40mg prednisolone daily. CsA and FK506 were started at 8mg/kg/24hrs and 0.3mg/kg/24hrs oral dosages respectively, divided in 2 doses daily which were titrated futher 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 1500mg, at the end of the induction therapy. Fourteen healthy volunteers, which received no medication, were also studied as control subjects.

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.

Flow cytometric analysis

Peripheral blood cells, obtained from the subjects in a prospective manner, were analyzed by four-colour flow cytometry. All blood samples from patients and controls were collected in the morning. To minimize selective loss during the preparation procedure, the cells were first stained with mAbs followed by lyzing of the erythrocytes. Briefly, the blood cells were incubated with APC-, PE-, PerCP-, and FITC-conjugated mAbs for 20 minutes at room temperature. The erythrocytes were then lysed with FACS lyzing solution (Becton Dickinson). After washing with FACSflow, the stained cells were analyzed with a FACSCaliber 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 lin1 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 were used for further characterization 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.

Dendritic cell characterization

For gating Lin^- $HLA\text{-}DR^+$ cells, whole peripheral blood cells were stained with anti-HLA-DR mAb and the Lineage Cocktail (Figure 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^- $HLA\text{-}DR^+$ $CD11c^{high}$ $CD123^{low}$ and Lin^- $HLA\text{-}DR^+$ $CD11c^{low}$ $CD123^{high}$, respectively. Representative profiles of CD11c and CD123 on peripheral blood dendritic cells from a CHF patient are shown in Figure 1B, which clearly indicates the two DC subsets. Acquired mDC and pDC subsets of controls and patients were analyzed according to the maturation marker CD83 and the homing chemokine receptor CCR7 (Figure 1C).

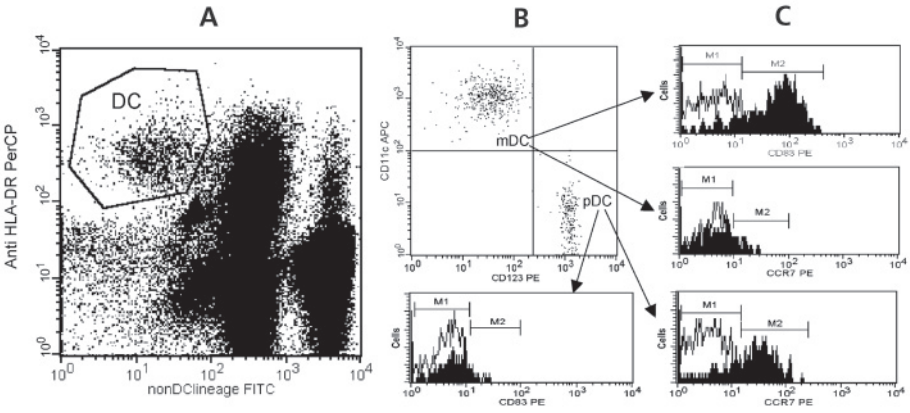

Figure 1: Typical example of peripheral blood DC phenotype in a CHF patient. Dendritic cells (DC) were identified by positive HLA-DR and negative lineage markers (A). DCs were further analyzed for the expression of CD11c and CD123. mDCs and pDCs were defined as Lin^{-} $HLA\text{-}DR^{+}$ $CD11c^{high}$ $CD123^{low}$ and Lin^{-} $HLA\text{-}DR^{+}$ $CD11c^{low}$ $CD123^{high}$, respectively (B). Characterization of the maturation status was achieved by analysis of the acquired mDCs and pDCs for CD83 and CCR7 positivity (M2) when compared to negative (M1) isotype controls, respectively (C).

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 $\pm$ SEM of absolute dendritic cell numbers or percentage (%) positive myeloid and plasmacytoid dendritic cells.

Results

Clinical characteristics

Between healthy controls and patients with CHF, there was no significant difference in age or sex proportion (see table 1). The heart transplant candidates from all 3 categories (IHD, HCM, DCM) were classified clinically with end-stage heart failure (NYHA III-IV). The blood total leukocyte 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 leukocyte 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 or NYHA IV heart failure.

Table 1: Clinical characteristics of study subjects

		Heart Failure (NYHA III-IV)				
	Control (n = 14)	**Total (n = 16)**	**IHD (n = 4)**	**HCM (n = 5)**	**DCM (n = 7)**	**Post-HTx (n = 16)**
Age, years	48.4 ± 3.6	52.6 ± 2.1	51.7 ± 4.9	53.0 ± 4.6	53.2 ± 3.8	52.6 ± 2.1
Gender, m:f	8:6	9:7	2:2	3:2	4:3	9:7
WBC, 10^3cells/μL	5.8 ± 0.6	7.1 ± 0.9	8.8 ± 0.8	8.3 ± 2.1	6.7 ± 0.9	11.6 ± 1.3***†
% lymphocytes	28.6 ± 1.4	18.8 ± 2.7**	17.3 ± 4.2*	18.4 ± 4.8*	19.9 ± 2.9*	7.9 ± 2.8***†
% monocytes	5.9 ± 0.5	5.6 ± 0.7	6.0 ± 2.0	7.0 ± 1.0	4.4 ± 0.9	5.2 ± 0.5
% granulocytes	65.5 ± 1.5	75.6 ± 2.6***	76.7 ± 5.6*	74.6 ± 4.0*	75.7 ± 2.0**	86.9 ± 1.9***§

IHD = Ischaemic Heart Disease; HCM = Hypertrophic Cardiomyopathy; DCM = Dilated Cardiomyopathy. Post-HTx, patients used induction and maintenance immunosuppression as described in Section 2.1. Age in years, white blood cell (WBC) counts and % values are expressed as the mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$; all compared to normal healthy controls. † $p < 0.02$, § $p < 0.001$; compared to total (NYHA III-IV).

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 (Figure 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 dendritic cells (0.04 ± 0.01; $p < 0.001$) (Figure 2A).

By analyzing the distribution of absolute numbers of dendritic cells 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) (Figure 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; $p = 0.01$) (Figure 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; $p = 0.45$) (Figure 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 ($p < 0.0001$) (Figure 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 ($p \leq 0.0005$) and control condition ($p < 0.0001$) (Figure 3A and 3B).

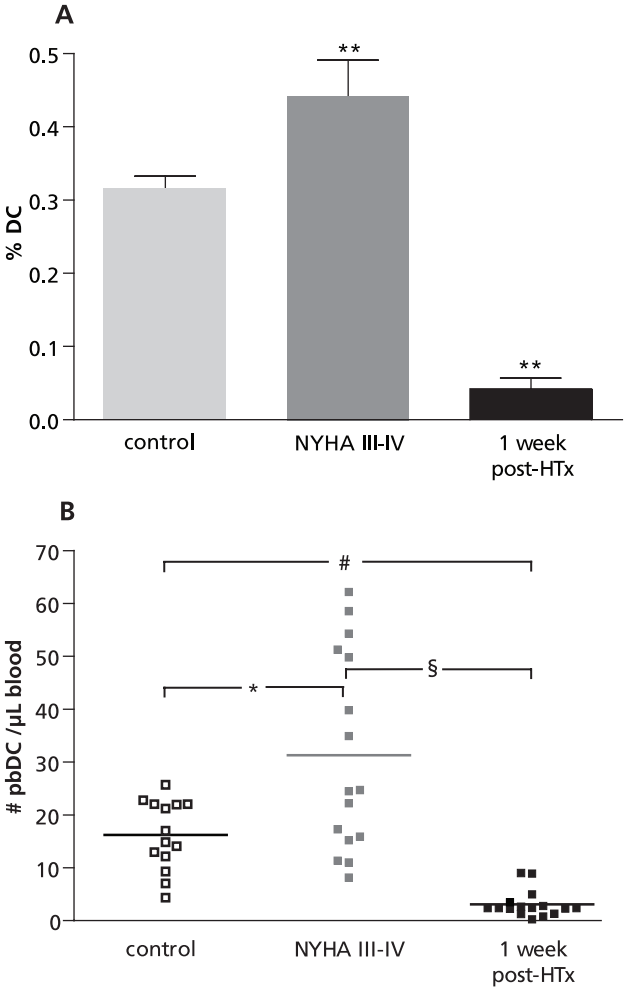

Figure 2: Percentage DCs of total white blood cells (A) and absolute numbers of total peripheral blood DCs (pbDC) (B) in controls (n = 14) and patients with end stage heart failure, before (NYHA III-IV) and 1 week after heart transplantation (post-HTx), (n = 16). ** $p < 0.01$, *** $p < 0.0001$; both compared to normal healthy controls.

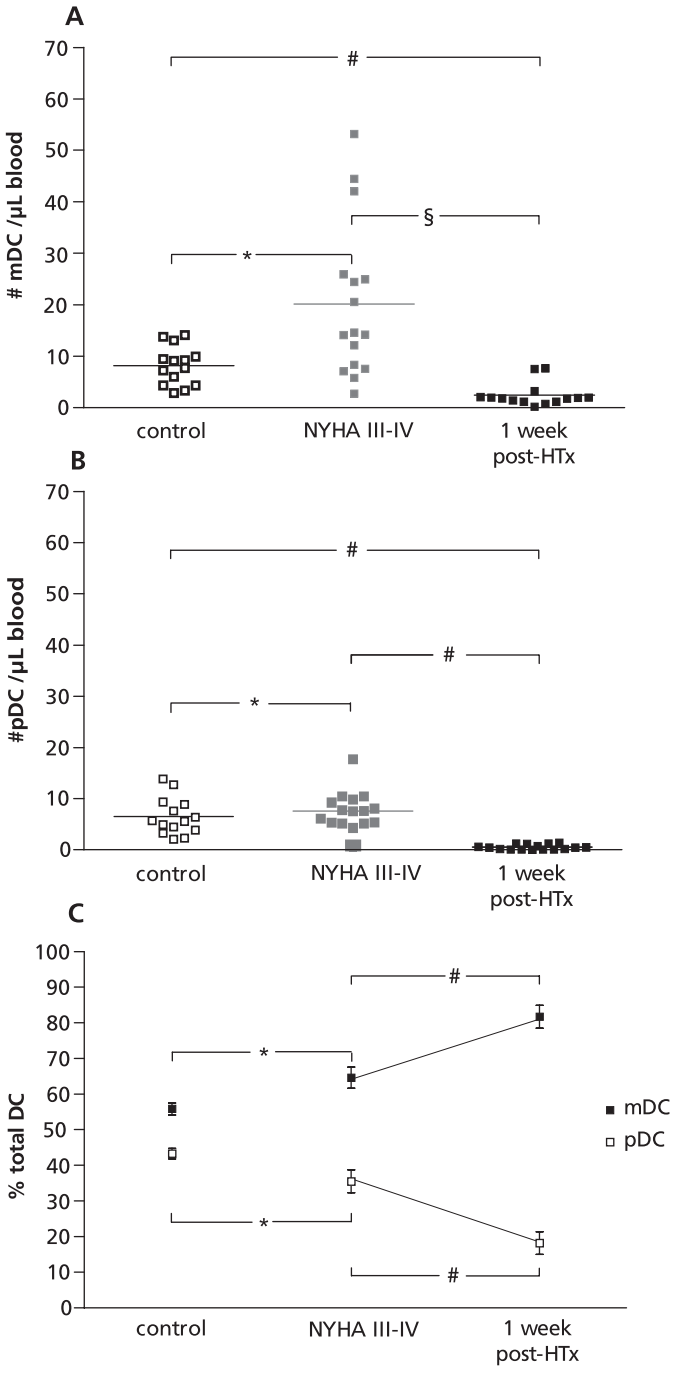

Figure 3: Absolute numbers of (A) mDCs, (B) pDCs and (C) percentage of mDCs and pDCs in the peripheral blood of all subjects. mDCs and pDCs were defined as shown in Figure 1. Absolute numbers of mDCs and pDCs, and the % mDCs or pDCs of total DCs, were determined as described in the Flow cytometric analysis section. Values represent means ± SEM from 16 patients with end-stage heart failure before (NYHA III-IV) and 1 week after heart transplantation (post-HTx) and 14 normal subjects.

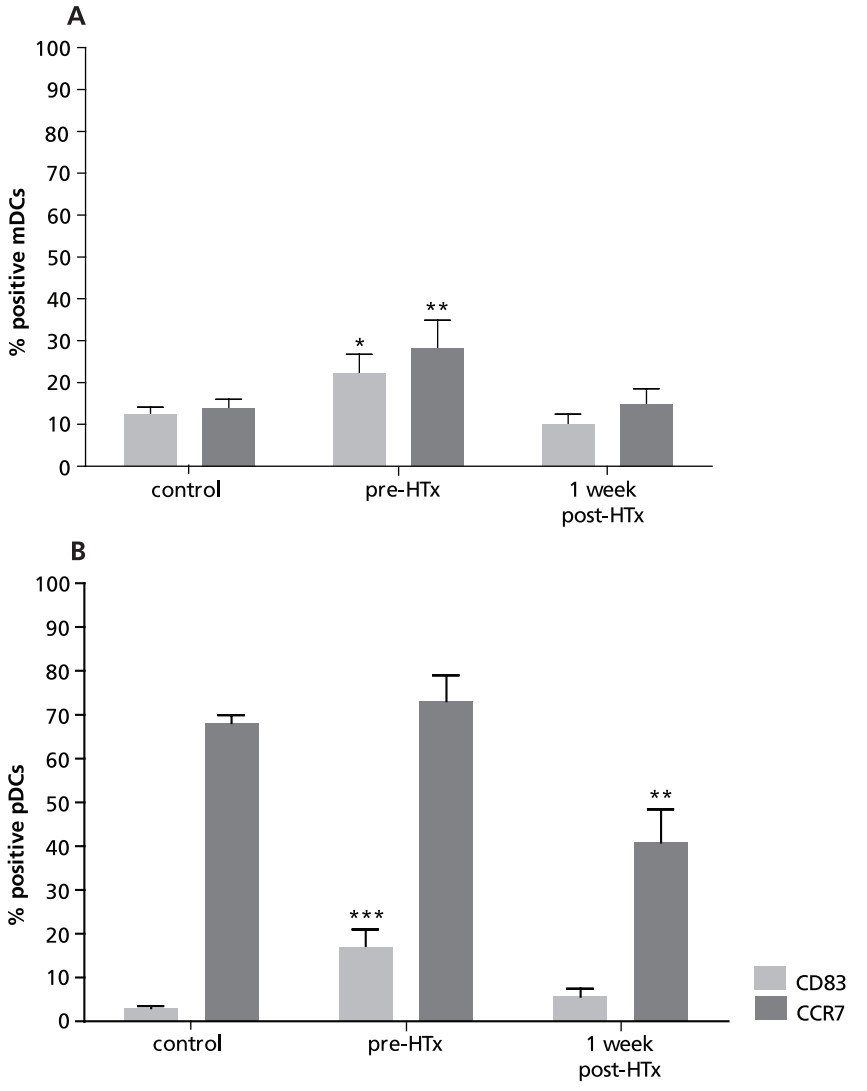

Figure 4: Percentage of positive (A) mDCs and (B) pDCs for CD83 and CCR7 in the peripheral blood of 16 patients with end-stage heart failure before (pre-HTx) and 1 week after heart transplantation (post-HTx) and 14 normal subjects. * $p < 0.05$, **$p < 0.01$, ***$p < 0.0001$; all compared to normal healthy controls.

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; $p < 0.01$) (Figure 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 ($p < 0.001$) (Figure 3C).

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) (Figure 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 (Figure 4B).

Discussion

In the present study, we directly enumerated two distinct dendritic cell 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-color 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 T_H1 predisposition of immune responses in heart failure may be associated with a polarized mDC: pDC balance towards mDCs.

Two hypotheses on the origin of immune activation in patients with chronic heart failure 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 may exert anti-inflammatory effects on intact vessels [18], tissue hypoperfusion, hypoxia, cell death and haemodynamic overload in combination with pathological 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, dendritic cells 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, dendritic cells are considered critical determinants for the polarization of naïve T-cells into T_H1 or T_H2 cells. Rissoan and coworkers [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 T_H1 development which augments inflammatory responses, whereas pDCs secrete lower amounts of IL-12 and primarily elicit T_H2 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 to stimulate immature mDCs to induce T_H2 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 T_H1 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 T_H1 polarization. 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 ischemia/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 posttansplant 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 T_H0 to T_H1 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 may 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].

Dendritic cell 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 antigen presenting cells, 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 dendritic cells 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 characterize 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 heart transplantation accounted for higher percentages of circulating mature myeloid DCs needs to be investigated in the future.

In summary, the present data clearly indicate that in patients with heart failure, the mDC:pDC balance polarizes towards mature mDCs in the peripheral blood, which may be associated with T_H1 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.

Acknowledgements

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References

1. Jessup M, Brozena S. Heart failure. N Engl J Med 2003;348:2007-18.
2. Parissis J, Filippatos G, Nikolaou V, Adamopoulos S. Cytokines and anti-cytokine therapeutic approaches to chronic heart failure. Eur J Intern Med 2002;13:356-64
3. Ferrari R, Bachetti T, Confortini R, Opasich C, Febo O, Corti A, Cassani G, Visioli O. Tumor necrosis factor soluble receptors in patients with various degrees of congestive heart failure. Circulation 1995;92:1479-86.
4. Aukrust P, Ueland T, Muller F, Andreassen AK, Nordoy I, Aas H, Kjekshus J, Simonsen S, Froland SS, Gullestad L. Elevated circulating levels of C-C chemokines in patients with congestive heart failure. Circulation 1998;97:1136-43.
5. Damas JK, Gullestad L, Ueland T, Solum NO, Simonsen S, Froland SS, Aukrust P. CXC-chemokines, a new group of cytokines in congestive heart failure--possible role of platelets and monocytes. Cardiovasc Res 2000;45:428-36.
6. Yang Z, Szabo C. Production of interleukin-12 is under the control of endogenous interleukin-10 in myocardial ischemia-reperfusion. Shock 2001;15:77-9.
7. Marriott JB, Goldman JH, Keeling PJ, Baig MK, Dalgleish AG, McKenna WJ. Abnormal cytokine profiles in patients with idiopathic dilated cardiomyopathy and their asymptomatic relatives. Heart 1996;75:287-90.
8. Eriksson U, Kurrer MO, Sonderegger I, Iezzi G, Tafuri A, Hunziker L, Suzuki S, Bachmaier K, Bingisser RM, Penninger JM, Kopf M. Activation of dendritic cells through the interleukin 1 receptor 1 is critical for the induction of autoimmune myocarditis. J Exp Med 2003;197:323-31.
9. Kalinski P, Hilkens CM, Wierenga EA, Kapsenberg ML. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol Today 1999;20:561-7.
10. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998; 392:245-52.
11. Sallusto F, Lanzavecchia A. Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. Immunol Rev 2000;177:134-40.
12. Cella M, Scheidegger D, Palmer-Lehmann K, Lane P, Lanzavecchia A, Alber G. Ligation of CD40 on dendritic cells triggers production of high levels of interleukin-12 and enhances T-cell stimulatory capacity: T-T help via APC activation. J Exp Med 1996;184:747-52.
13. Rissoan MC, Soumelis V, Kadowaki N, Grouard G, Briere F, de Waal Malefyt R, Liu YJ. Reciprocal control of T helper cell and dendritic cell differentiation. Science 1999;283: 1183-6.
14. Liu YJ. Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. Cell 2001;106:259-62.
15. Penna G, Vulcano M, Sozzani S, Adorini L. Differential migration behavior and chemokine production by myeloid and plasmacytoid dendritic cells. Human Immunology 2002; 63:1164-71.

16. Niebauer J, Volk HD, Kemp M, Dominguez M, Schumann RR, Rauchhaus M, Poole-Wilson PA, Coats AJ, Anker SD. Endotoxin and immune activation in chronic heart failure: a prospective cohort study. Lancet 1999;353:1838-42.
17. Damas JK, Eiken HG, Oie E, Bjerkeli V, Yndestad A, Ueland T, Tonnessen T, Geiran OR, Aass H, Simonsen S, Christensen G, Froland SS, Attramadal H, Gullestad L, Aukrust P. Myocardial expression of CC- and CXC-chemokines and their receptors in human end-stage heart failure. Cardiovasc Res 2000;47:778-87.
18. Yamawaki H, Lehoux S, Berk BC. Chronic physiological shear stress inhibits tumor necrosis factor-induced proinflammatory responses in rabbit aorta perfused ex vivo. Circulation 2003; 108:1619-25.
19. Paulus WJ. Cytokines and heart failure. Heart Fail Monit 2000;1:50-6.
20. Smits HH, van Rietschoten JG, Hilkens CM, Sayilir R, Stiekema F, Kapsenberg ML, Wierenga EA. IL-12-induced reversal of human T_H2 cells is accompanied by full restoration of IL-12 responsiveness and loss of GATA-3 expression. Eur J Immunol 2001;31:1055-65.
21. Kalinski P, Vieira PL, Schuitemaker JH, de Jong EC, Kapsenberg ML. Prostaglandin E(2) is a selective inducer of interleukin-12 p40 (IL-12p40) production and an inhibitor of bioactive IL-12p70 heterodimer. Blood 2001;97:3466-9.
22. King C, Davies J, Mueller R, Lee MS, Krahl T, Yeung B, O'Connor E, Sarvetnick N. TGF-beta1 alters APC preference, polarizing islet antigen responses toward a T_H2 phenotype. Immunity 1998;8:601-13.
23. Gallucci S, Lolkema M, Matzinger P. Natural adjuvants: endogenous activators of dendritic cells. Nature Medicine 1999;5: 1249-55.
24. Fairchild RL, Kobayashi H, Miura M. Chemokines and the recruitment of inflammatory infiltrates into allografts. Graft 2000;3 Suppl 1; S24.
25. Bliss SK, Butcher BA, Denkers EY. Rapid recruitment of neutrophils containing prestored IL-12 during microbial infection. J Immunol 2000;165:4515-21.
26. Elenkov IJ, Chrousos GP, Wilder RL. Neuroendocrine regulation of IL-12 and TNF-alpha/IL-10 balance. Clinical implications. Ann N Y Acad Sci 2000;917:94-105.

Chapter 4

Blood Dendritic Cell Levels and Phenotypic Characteristics in Relation to Etiology of Human Heart Failure: Implications for Dilated Cardiomyopathy

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Abstract

Background: Dysregulation of dendritic cell (DC) mediated immune responses towards auto-antigens, is considered an important feature in the maintenance of experimentally induced heart failure (HF). In order to evaluate the role of blood DCs in cardiomyopathies of different origins, we examined myeloid (mDC) and plasmacytoid (pDC) subset levels and maturation characteristics, according to HF severity and etiology in humans.

Methods and Results: Absolute numbers of mDCs and pDCs in 12 New York Heart Association (NYHA) class-II, 28 NYHA class III-IV HF patients and 18 healthy controls, were studied by four-color whole blood flow cytometry. End-stage (NYHA III-IV) HF patients had comparable circulating DC subset levels to NYHA-II patients and controls. However, within the NYHA III-IV group total DC levels in patients with non-ischemic dilated cardiomyopathy (DCM) were higher ($p < 0.001$) than in patients with coronary artery disease (CAD), hypertrophic cardiomyopathy (HCM) or other HF etiology. This was due to a significant increase of primarily mDCs ($p < 0.0001$) and to a lesser extent of pDCs ($p < 0.05$) in idiopathic DCM patients, independent of systolic or diastolic cardiac dysfunction. Maturation marker CD83 and lymphoid homing chemokine receptor CCR7 surface expression was enhanced only on mDCs, but not pDCs from DCM patients ($p < 0.05$), compared to patients with CAD, HCM or other underlying cardiac pathophysiology.

Conclusions: Total blood DC levels in end-stage HF are elevated in patients with DCM. Whole blood DC characterization may lead to new insights into the pathophysiology of idiopathic DCM in humans.

Introduction

Growing evidence suggests that immunologic and inflammatory processes may play a pathogenic role in the development of different forms of myocardial dysfunction resulting in heart failure (HF) [1]. As such, non-ischemic dilated cardiomyopathy (DCM) encompasses diseases of the heart characterized by dilatation and impaired systolic function of one or both ventricles [2]. Traditionally, contributing factors like cardiotoxic agents, positive familiy history, alcohol abuse, nutritional deficiency, neuromuscular disorders and a post-partal state have been implicated in the development of the disease [3]. Other possible underlying pathophysiological mechanisms have included genetic predisposition (e.g. HLA-DR4 [4]), cardiomyocyte metabolic, energetic and contractile defects (PKC-α [5], cardiac C-protein [6], SERCA2a & phospholamban [7]) and abnormal modulation of cellular immune responses as represented by activated blood T-lymphocytes [8] and infiltrating memory T-cells and macrophages [9].

New insight into the etiology of DCM has provided evidence for initial viral infection prior to manifestation of HF symptoms [10]. Molecular mimicry between viral and cardiotropic endogenous molecules is hypothesized to trigger pathogenic heart-specific autoreactivity through autoantibody production against intracellular cardiac epitopes, like α-myosin heavy chain [11]. Alternatively, myocardial persistence of enteroviruses, is considered crucial for the development of DCM through adaptive $CD4^+$ and cytolytic $CD8^+$ T-cell driven autoimmune myocarditis [12,13]. As part of innate immunity, dendritic cells (DCs) are thought to become undesirable participants in the pathologic condition of DCM, by continuously presenting self-antigens to autoreactive T- cells [14].

Under physiological conditions, DC progenitors emerge from bone marrow and home to virtually all tissues where they take up and process foreign material as well as apoptotic and necrotic self-tissue [15]. Tissue-resident DCs appear to be critical for the propagation and maintenance of HF with autoimmune origins. Studies in murine models of post-inflammatory HF, have demonstrated that non-specific activation of α-myosin loaded DCs via Toll-like receptors, is sufficient to induce ongoing autoimmune myocarditis [16]. Recently, chronic DC-driven myocardial inflammation was shown to result in ventricular functional impairment with hemodynamic characteristics resembling DCM [17].

In humans, two major DC subsets have been identified, differing in their phenotype and influence on T-cell priming [18]. Myeloid DCs (mDCs) expressing

CD11c, are involved in cellular autoreactivity [19], while plasmacytoid DCs (pDCs) express CD123 and are regarded as crucial effectors in antiviral innate immunity [20]. Both DC subsets are able to promote polarization of naïve T-cells into T_H1 or T_H2 immune responses [21]. However, as pDC primed T-lymphocytes also differentiate into T-regulatory cells [22], it is postulated that pDCs may promote peripheral tolerance to self-antigens. Following tissue damage, infection, or inflammation, DC subsets phagocytose antigens, increase maturation marker CD83 as well as MHC class I & II molecule expression and migrate via afferent lymphatic vessels or blood to secondary lymphoid organs in order to present antigens and co-stimulatory signals to naïve T-cells [23]. Homing of immature DCs to areas of inflammation and of antigen-loaded DCs to lymphatic tissue is mediated through differential regulation of chemokine receptors on their surface, whereby expression of CCR7 enables mature DCs to migrate towards regional lymph nodes [24].

In this study, we hypothesized that the contribution of peripheral blood DCs in progression or etiology of cardiac dysfunction, would be reflected on the absolute levels of circulating DC subsets in patients with heterogeneous severity or pathogenesis of HF. After identifying peripheral blood mDCs and pDCs, we examined their levels according to the New York Heart Association (NYHA) classification, in HF patients with cardiomyopathies of different origins. Based on the distinct phenotypic properties between mature and immature DC subsets, surface maturation marker and lymphoid chemokine receptor expression were analyzed in relation to underlying cause of cardiac disease.

Methods

Patients and control subjects

We performed a single-center cross-sectional assessment of blood DC parameters in 28 consecutive adults with end-stage HF before transplantation (NYHA class III-IV) and 12 HF patients with coronary artery disease (CAD) before elective coronary artery bypass grafting (CABG) (NYHA-II). Written informed consent was obtained from each patient before entering the study. End-stage HF patients were subgrouped according to etiology of cardiac dysfunction in CAD (n = 9), hypertrophic cardiomyopathy (HCM, n = 7) and non-ischaemic DCM (n = 8) categories. Additionally, 3 patients presented with severe valvular disease (VD: 1 congenital, 2 as a complication of rheumatic disease) and 1 patient developed severe acute heart failure due to giant-

cell myocarditis (Table 1). After transplantation, diagnosis was confirmed by macroscopical examination of the explanted heart and microscopical evaluation of cardiac tissue sections for various histopathologic features, as described previously [25]. Haemodynamically, all patients were stable, with no change of medication during the last month before the investigation. No subjects with recent infections, pulmonary disorders, renal failure, collagen vascular disease or cancer were included in the study. Eighteen healthy laboratory co-workers and staff members from the Erasmus MC (age 48.8 ± 11.5, 10 men and 8 women) without any history of cardiac disease, functioned as controls. All subjects were of Caucasian descent. None of the study participants were taking antibiotics or oral contraceptive therapy at the time of assessment. The investigation conformed with the principles outlined in the Declaration of Helsinki and was approved by the Committee for Scientific Research with Humans of the University Medical Center Rotterdam (MEC 215.732/2002/157).

Table 1: Clinical and hemodynamic characteristics

	NYHA II	NYHA III-IV
Age, years	63 ± 13	52 ± 9
Sex, male/female	7/5	16/12
Duration of heart failure, years	3 ± 1	5 ± 3
Cause of HF (class II or III/IV)		
CAD	12	9 (5/4)
HCM	–	7 (3/4)
DCM	–	8 (2/6)
Other*	–-	4 (2/2)
LVEF, %	43 ± 11	29 ± 9
Medication, n (%)		
Nitrates	5 (42)	9 (32)
Diuretics	7 (58)	22 (79)
β-blockers	7 (58)	14 (50)
Digoxin	2 (17)	11 (39)
ACE-I or ARB	5 (42)	24 (86)
Coumarins	6 (50)	12 (43)
Statins	6 (50)	9 (32)

* Three patients with valvular disease and one with giant-cell myocarditis. NYHA = New York Heart Association functional class, CAD = Coronary artery disease, HCM = hypertrophic cardiomyopathy, DCM = non-ischaemic dilated cardiomyopathy, LVEF = Left Ventricular Ejection Fraction, ACE-I = angiotensin converting enzyme inhibitor, ARB = angiotensin II receptor blocker. Data are given as mean ± SD.

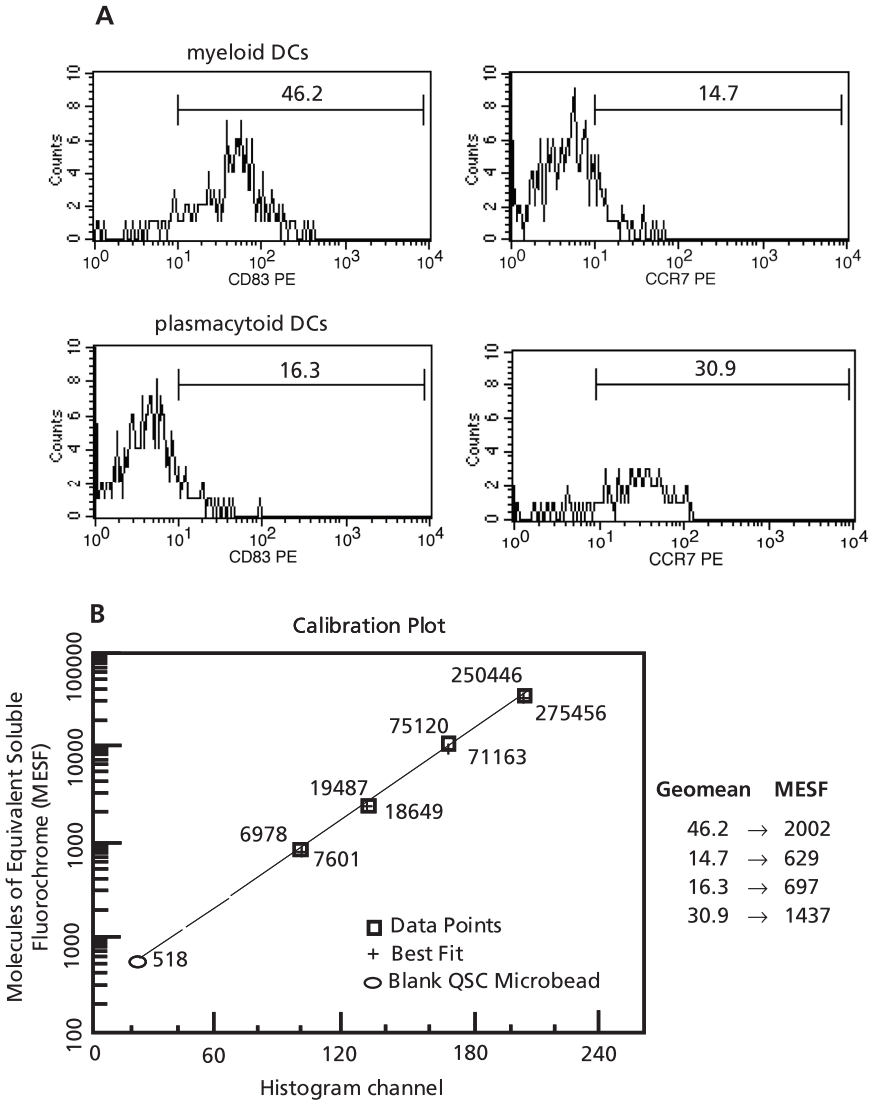

Figure 1: (A) Surface expression of maturation marker (CD83) and lymphoid chemokine receptor (CCR7) on circulating myeloid and plasmacytoid dendritic cells. Fluorescence intensity was calculated through the geomean of the histogram signal for each of the two molecules on mDCs or pDCs. (B) Standard curve of fluoresence intensity constructed after using the QuickCal program for quantum beads software, whereby fluorescence intensity is depicted as molecular equivalents of soluble fluorochrome (MESF).

Clinical and hemodynamic parameters

Typing for DQB1 and DRB1 human leukocyte antigens (HLA) was performed on PCR amplified DNA, using sequence specific oligonucleotides, [26], and results are shown as HLA-DQ and -DR antigens according to the latest HLA Dictionary [27]. Clinical condition was evaluated on the basis of the NYHA classification through the Modified Framingham Criteria for diagnosis of heart failure [28]. Trans-thoracic echocardiography was performed using a Hewlett Packard Sonos 5500 ultrasonograph with a 3.75 MHz transducer (Hewlett Packard, Andover, MA, USA) and interpreted by 2 blinded, independent observers. The primary parameter for evaluation of systolic heart function was the left ventricular ejection fraction (LVEF). In 23 patients this parameter was assessed quantitatively by echocardiography through 2- and 4-chamber views. For 17 patients LVEF was measured by radionuclide angiography using a 20mCi/740Mbq Tc^{99m} tracer on the Orbiter® system (Siemens, Erlangen, Germany). To evaluate diastolic heart function, 2-dimensional Doppler echocardiography was performed to measure peak early (E) / peak atrial (A) mitral inflow (E/A ratio), deceleration time of E (DT) and pulmonary vein velocity, within the valve orifices near the leaflet tips through an apical 4-chamber view. Diastolic function was defined as being normal, impaired during relaxation, pseudonormal or restrictive [29].

Circulating dendritic cell immunophenotypic characterization

Fresh whole blood cells from the transplant patients were collected 4-6 hours before transplantation and at 1 day pre-operatively from the CABG group. Heparinized pyrogen-free tubes were used to divide all specimens in 300 μL aliquots for DC immunophenotypic characterization by four-color flow cytometric analysis. Within non-DC $lineage^{-}$ $HLA\text{-}DR^{+}$ cells, peripheral blood mDC ($CD11c^{high}$ $CD123^{low}$) and pDC ($CD11c^{low}$ $CD123^{high}$) subsets were delineated as described previously [30]. All samples were processed within 3 h after venous puncture. Accuracy and reproducibility of this assay for whole blood DC subset assessment have been confirmed previously [31].

Dendritic cell subset maturation marker and lymphoid chemokine receptor expression

The mDC and pDC subsets of controls and patients were analyzed for maturation marker (CD83) and homing chemokine receptor (CCR7) as described previously [31]. In order to compare various measurements of CD83 and CCR7 expression in time, the flow cytometer was calibrated before each experiment [32] using 5 different Quantum Medium Level Calibration Beads with a known amount of the PE fluorochrome (Bangs Lab, Fishers, IN, USA).

Fluorescence intensity for CD83 and CCR7 was calculated through the geometric mean of the histogram signal acquired for each of the molecules on mDCs or pDCs (Figure 1A). Cell surface expression was denoted as molecular equivalents of soluble fluorochrome (MESF) after conversion of fluorescence intensity to a standard curve using Quick Cal software program (Verity Software House, Topsham, ME, USA) for Quantum Beads (Figure 1B).

Statistical analysis

Data are given as mean ± SD unless stated otherwise. For comparison of two groups of individuals the Mann-Whitney *U*-test (two-tailed) was used. When more than two groups were compared, one-way ANOVA or the Kruskal-Wallis test were applied as appropriate. Post-hoc analysis was performed using Bonferroni's or Dunn's multiple comparisons procedures, accordingly. Coefficients of correlation (r) were calculated by the Spearman rank test. All tests were performed with the GraphPad statistical program (GraphPad Software, San Diego, CA, USA). Probability values are two-sided and considered significant when $\alpha < 0.05$.

Results

Clinical characteristics and immune parameters of study subjects

For demographic characteristics, groups differed only in age, as NYHA-II patients were on average 11 years older than NYHA III-IV patients. Clinically, mean duration of HF was longer in NYHA III-IV than NYHA-II patients. LVEF was more depressed in NYHA III-IV patients, compared to NYHA-II patients. The use of diuretics, digoxin and ACE inhibitors was more common among NYHA III-IV patients. Statin use was more pronounced in NYHA-II patients, as could be expected in CAD patients (Table 1). Within the NYHA III-IV group, proportionally more CAD patients received statin therapy compared to HCM, DCM and patients of other HF pathophysiology. Blood total leukocyte counts of NYHA-II patients tended towards higher levels ($p = 0.063$), while a relative lymphopenia ($p < 0.0001$) and granulocytosis ($P < 0.01$) were apparent in both NYHA-II and NYHA III-IV groups of HF patients against controls (Table 2).

Circulating DC levels and severity of heart failure

Within the CAD group, blood mean mDC numbers did not differ significantly between NYHA-II or NYHA III-IV patients and controls [CAD, NYHA III-IV: 11 ± 7; CAD, NYHA-II: 11 ± 3; controls: 9 ± 5 cells/μL, $p = 0.397$] (Figure 2A). Also, blood mean pDC levels were comparable between patients and controls [CAD, NYHA

III-IV: 8 ± 5; CAD, NYHA-II: 6 ± 4; controls: 7 ± 5 cells/μL, p = 0.535] (Figure 2B). When circulating DC levels were examined within all end-stage HF patients (NYHA III-IV), mean mDC numbers were not different between the NYHA-III and NYHA-IV groups [NYHA-III: 18 ± 12 vs NYHA-IV: 15 ± 13 cells/μL, p = 0.557]. Similarly, although pDC levels tended to be higher in the NYHA-III patients, mean pDC numbers were not significantly different between the two groups [NYHA-III: 11 ± 6 vs NYHA-IV: 7 ± 3 cells/μL, p = 0.083] (data not shown).

Circulating DC levels and etiology of end-stage cardiac disease

For all NYHA III-IV patients, only the proportion of mDCs in peripheral blood leukocytes was higher when compared with NYHA-II patients and controls (p = 0.032) (Table 2). Compared directly to controls, NYHA class III-IV HF patients showed higher absolute blood mDC levels (16 ± 14 vs 9 ± 5 cells/μL, p = 0.031), while mDC numbers were the highest in DCM patients compared to other cardiac disease etiology within the same group [DCM: 34 ± 13; CAD: 11 ± 7; HCM: 11 ± 5; other: 6 ± 2 cells/μL, p < 0.0001] (Figure 3A). To a lesser extent, pDC numbers were also increased in patients with DCM compared to CAD, HCM or other end-stage HF patients [DCM: 13 ± 6; CAD: 8 ± 5; HCM: 7 ± 2; other: 4 ± 3 cells/μL, respectively, p = 0.025] (Figure 3B). Consequently, total circulating DC content in end-stage HF tended to be higher than in controls (25 ± 17 vs 16 ± 9 cells/μL, p = 0.081) due to the significantly raised total DC numbers of DCM patients [DCM: 44 ± 18; CAD: 19 ± 11; HCM: 18 ± 7; other: 10 ± 4 cells/μL, p = 0.0002] (Figure 3C). Among DCM patients, 2 individuals possessed a HLA-phenotype known to be associated with DCM development (HLA-DR4). While cardiac histopathological findings did not differ considerably, patients without known predisposition for disease manifestation (idiopathic DCM) exhibited the highest total circulating DC content due to higher mDC numbers, compared to 2 patients who developed the disease after pregnancy and alcohol abuse, respectively. This effect was also reflected in the mDC/pDC ratio, which was abnormally high in the same patients (Table 3).

Circulating DC levels with systolic and diastolic cardiac dysfunction

As individual DCM patients had elevated absolute mDC levels, we further examined whether hemodynamic impairment influenced circulating DC levels. Within the DCM group, all patients had a depressed LVEF (< 40%). However, no significant correlation was found between the numbers of mDCs (R^2 = 0.002) or pDCs (R^2 = 0.034) and LVEF (Figure 4A and 4B). Associated with the degree of diastolic dysfunction, no significant correlation was found for mDC (R^2 = 0.009) or pDC (R^2 = 0.029) numbers in peripheral blood of NYHA-II and NYHA III-IV HF patients (Figure 4C and 4D).

Table 2: Immune cell parameters in blood of patients and controls

	Controls	Heart Failure patients					
		NYHA II	NYHA III-IV				
	(n = 18)	CAD (n = 12)	All (n = 28)	CAD (n = 9)	HCM (n = 7)	DCM (n = 8)	Other (n = 4)
leukocytes, cells/μL	5933 ± 2678	8207 ± 2645	7850 ± 3094	7811 ± 3233	7600 ± 2440	8688 ± 3110	6700 ± 4474
lymphocytes, %	29.1 ± 9.1	16.5 ± 7.1***	16.6 ± 9.9***	14.2 ± 7.6	17.5 ± 12.1	20.3 ± 11.4	12.8 ± 9.2
monocytes, %	6.5 ± 3.1	4.7 ± 2.1	6.0 ± 2.9	6.7 ± 3.2	5.5 ± 3.6	5.0 ± 2.5	7.5 ± 1.3
granulocytes, %	64.4 ± 9.7	78.9 ± 7.9 ***	77.5 ± 10.1**	79.1 ± 9.6	77.3 ± 12.5	74.6 ± 10.9	79.8 ± 8.7
DC, %	0.3 ± 0.1 (0.1 - 0.4)	0.3 ± 0.1 (0.1 – 0.8)	0.4 ± 0.2 (0.0 – 0.9)	0.3 ± 0.2 (0.1 – 0.8)	0.3 ± 0.1 (0.1 – 0.4)	0.5 ± 0.2¤ (0.2 – 0.9)	0.2 ± 0.2 (0.0 – 0.5)
mDCs, %	0.2 ± 0.1 (0.1 – 0.5)	0.2 ± 0.1 (0.0 – 0.3)	0.3 ± 0.2* (0.0 – 0.7)	0.2 ± 0.1 (0.0 – 0.4)	0.2 ± 0.1 (0.1 – 0.3)	0.4 ± 0.2¤ (0.1 – 0.7)	0.1 ± 0.1 (0.0 – 0.2)
pDCs, %	0.1 ± 0.0 (0.0 – 0.2)	0.1 ± 0.1 (0.0 – 0.3)	0.1 ± 0.1 (0.0 – 0.4)	0.2 ± 0.1 (0.0 – 0.4)	0.1 ± 0.0 (0.1 – 0.2)	0.2 ± 0.1 (0.1 – 0.3)	0.1 ± 0.1 (0.0 – 0.2)

DCs, dendritic cells; m, myeloid; p, plasmacytoid; CAD, coronary artery disease; HCM, hypertrophic cardiomyopathy; DCM, non-ischaemic dilated cardiomyopathy; Other, 3 patients with valvular disease and 1 with giant-cell myocarditis; percentages (%) were calculated from total leukocyte count; range for % DC, %mDC and %pDC is depicted in parenthesis * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$ from one-way ANOVA with Bonferroni's Multiple Comparison Test between controls, NYHA I-II and all NYHA III-IV patients; ¤ $p < 0.05$ from one-way ANOVA with Bonferroni's Multiple Comparison Test between NYHA III-IV patients of different HF etiologies

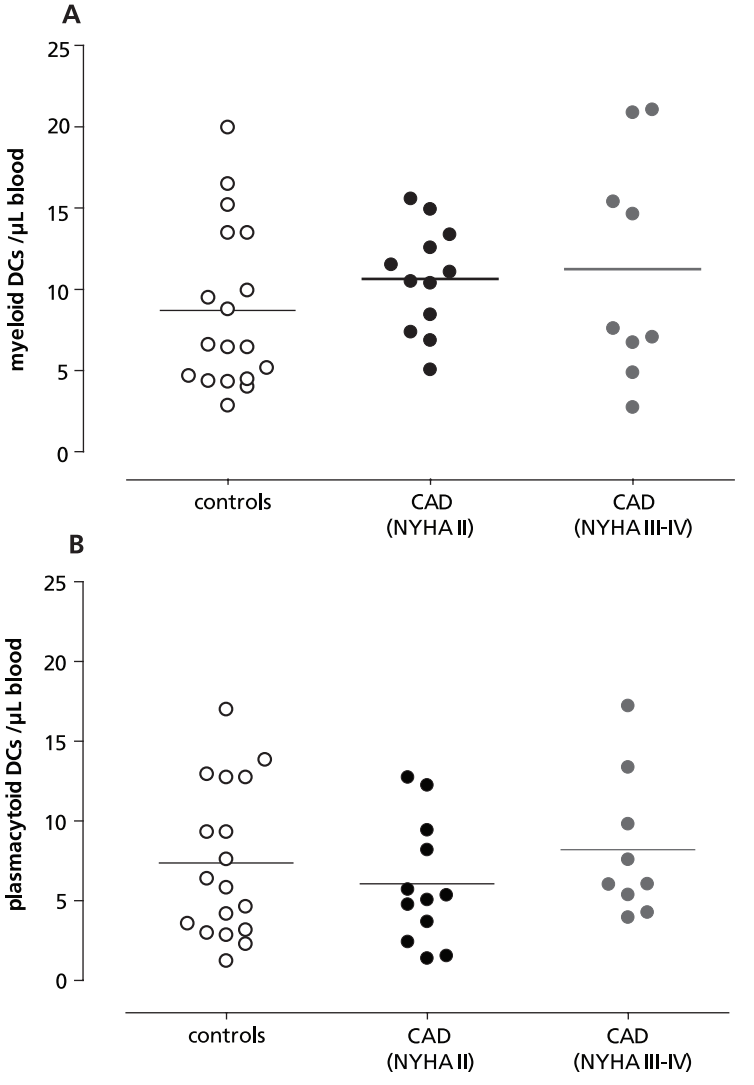

Figure 2: Absolute levels of circulating (A) myeloid and (B) plasmacytoid dendritic cells in 21 HF patients with coronary artery disease (CAD) as a function of symptom severity according to NYHA functional classification (NYHA II, n = 12; NYHA III-IV, n = 9), and 18 healthy control subjects.

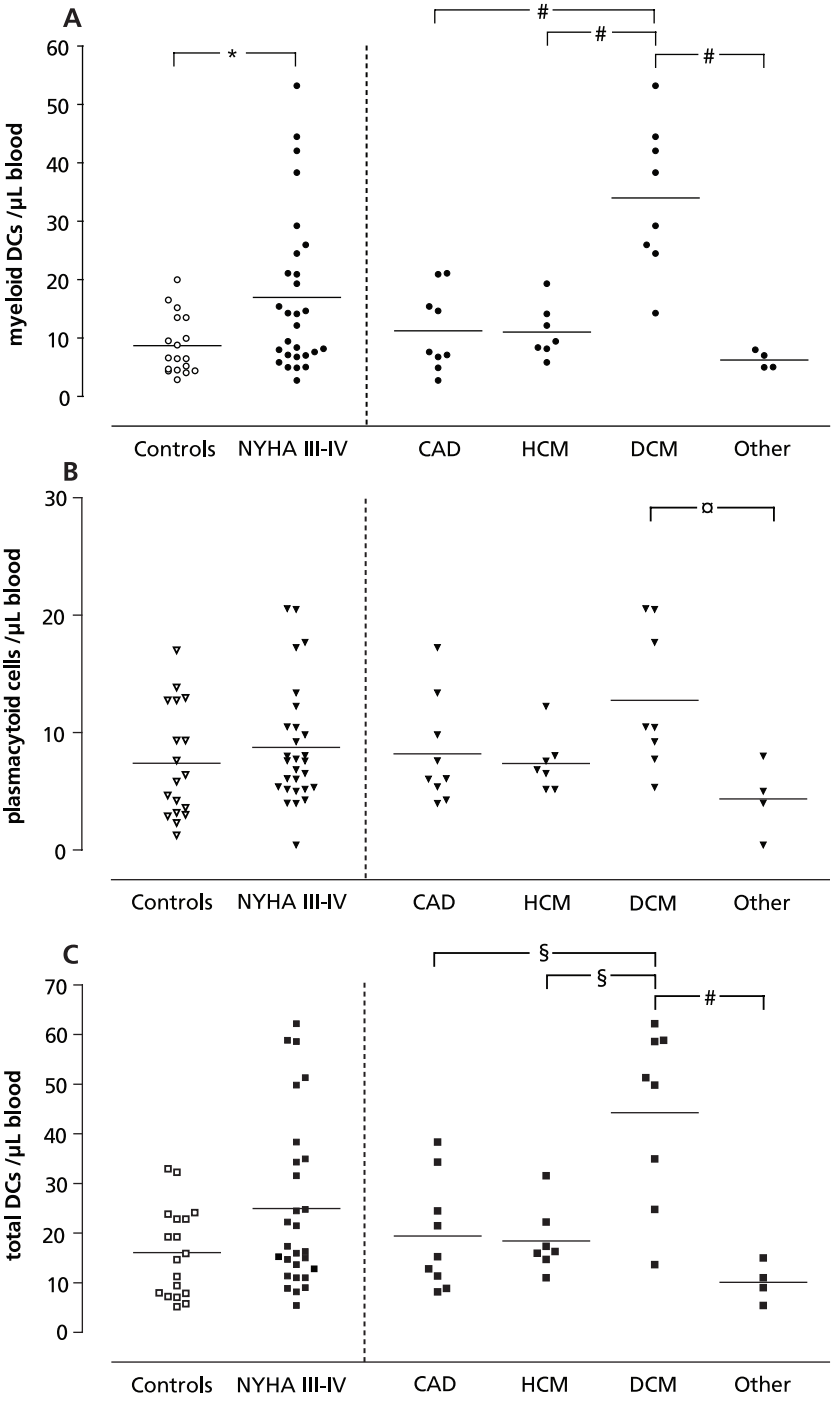

Figure 3: Peripheral blood levels of (A) myeloid, (B) plasmacytoid and (C) total dendritic cell numbers from 28 end-stage HF patients according to etiology of cardiac disease (CAD, n = 9; HCM, n = 7; DCM, n = 8, other, n = 4). *$p < 0.05$ (Mann-Whitney *U*-test); $^{¤}p < 0.05$, $^{§}p < 0.01$, $^{\#}p < 0.001$ (one-way ANOVA with Bonferroni's Multiple Comparisons).

Table 3: Individual characteristics and circulating dendritic cell parameters for the patients with dilated cardiomyopathy

Pt No.	Gender, Age (yrs)	NYHA Class	Duration HF (yrs)	LVEF (%)	HLA-DR	HLA-DQ	Contributing factors	Histological findings	mDC (cells/µL)	pDC (cells/µL)	mDC/pDC (ratio)
1	M, 63	IV	9	15	13(6), 7	6(1), 9(3)	alcohol abuse	LV: IF, vacuolar dystrophy	14	10	1.4
2	F, 40	IV	5	16	13(6), 7	6(1), 9(3)	idiopathic	LV: IF, focal liposis	53	5	10.6
3	F, 59	IV	3	19	1, 17(3)	5(1), 2	idiopathic	LV: IF	42	9	4.7
4	M, 57	III	3	25	4, 13(6)	6(1), 8(3)	idiopathic	LV & RV: IF	29	21	1.4
5	M, 55	IV	4	16	17(3), 10	5(1), 2	idiopathic	n.a.	45	18	2.5
6	F, 44	IV	2	16	4, 10	5(1), 8(3)	post-partum	n.a.	25	11	2.3
7	M, 60	III	5	30	1, 15(2)	5(1), 6(1)	idiopathic	LV & RV: IF	26	8	3.3
8	M, 38	IV	4	19	15(2), 7	6(1), 2	idiopathic	n.a.	38	20	1.9

M, male; F, female; NYHA, New York Heart Association functional class; HF, heart failure; LVEF, left ventricular ejection fraction; HLA, human leukocyte antigen (broad shown in parenthesis); LV, left ventricle; RV, right ventricle; IF, interstitial fibrosis; mDC, myeloid dendritic cell; pDC, plasmacytoid dendritic cell; n.a., not available

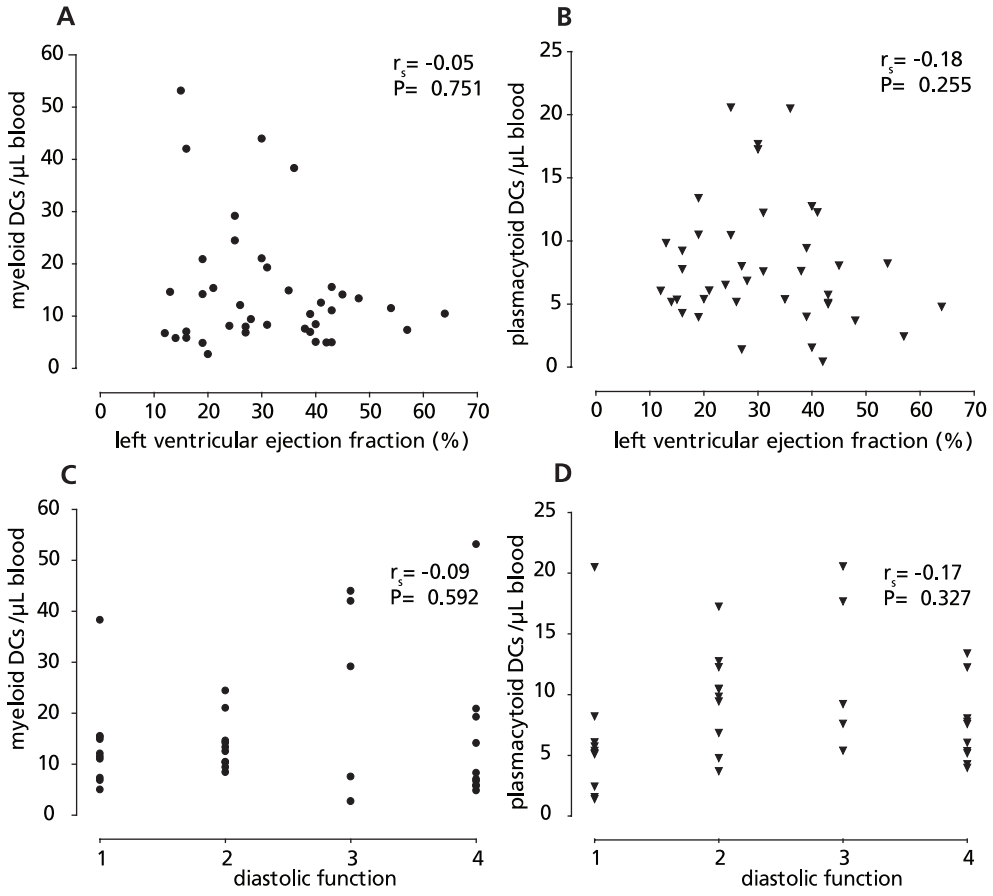

Figure 4: Circulating levels of (A) myeloid and (B) plasmacytoid DCs as a function of left ventricular ejection fraction in 40 HF patients. The degree of cardiac diastolic dysfunction was also associated to the levels of (C) myeloid and (D) plasmacytoid DCs. Diastolic heart function was defined as normal (grade 1), with impaired relaxation (grade 2), pseudonormal (grade 3) or restrictive (grade 4).

DC subset maturation marker and lymphoid chemokine receptor expression with etiology of end-stage cardiac disease

Upon comparison of blood CD83 or CCR7 positive mDCs and pDCs between NYHA III-IV HF patients and controls, $CD83^+$ mDC and $CCR7^+$ mDC frequency were increased in the patients [% $CD83^+$ mDC: 22.1 ± 21.3 vs 8.7 ± 3.8, p = 0.018; % $CCR7^+$ mDC: 24.2 ± 18.7 vs 17.4 ± 16.8, p = 0.011]. Surface expression of both molecules was also increased on patient mDCs [CD83: 1939 ± 1733 vs 830 ± 424 MESF, p = 0.016; CCR7: 1648 ± 791 vs 985 ± 346 MESF, p = 0.0002] (Figure 5A and 5B). However, only circulating mDCs of individuals with DCM showed higher CD83 levels [2785 ± 2147 MESF] compared to patients with other etiology of cardiac disease [CAD: 1232 ± 1100; HCM: 1363 ± 1302; other: 1878 ± 1449

MESF, p = 0.016] (Figure 5A). Similarly CCR7 expression on mDCs from DCM patients was elevated, compared to other HF pathophysiology [DCM: 2323 ± 1076; CAD: 1272 ± 193; HCM: 1223 ± 259; other: 1547 ± 817 MESF, p = 0.043] (Figure 5B). Expression of CD83 and CCR7 on blood pDCs from patients was similar to control pDCs [p = 0.159 and p = 0.258, respectively]. No substantial differences on CD83 or CCR7 expression levels were found on patient pDCs with underlying disease [p = 0.815 and p =0.461] (Figures 5C and 5D).

Discussion

We have previously reported that raised blood levels of mDCs, but not pDCs, in a pre-transplant cohort when compared with age and sex-matched healthy individuals, could be part of the inflammatory response seen in end-stage HF [33]. Here, we demonstrate that within end-stage HF patients, individuals with the most advanced disease (NYHA-IV), did not exhibit significantly different DC levels than their NYHA-III counterparts. Conceptually, homogeneous cardiac disease etiology (CAD) and older age of NYHA-II patients, might have affected DC subset content through interference with their leukocyte counts. However, CAD patients with different symptom severity (NYHA-II or NYHA III-IV) exhibited comparable mDC and pDC levels to healthy individuals. Furthermore, in adults, only pDCs are known to decline gradually (1-1.5% per year) with age [34]. Except for elevated leukocyte levels in patients with acute coronary syndromes, no significant covariance of leukocyte counts with increasing age or symptom severity has been reported in the past [35]. Our findings show that altered absolute DC content is a phenomenon independent of symptoms or white blood cell numbers in individual HF patients.

Cardiac endothelium activation and neurohumoral dysregulation are known to promote chronic immune activation and to induce impaired diastolic distensibility and cardiac stiffness in end-stage HF [36]. Longer mean HF duration and deterioration of cardiac output in the NYHA III-IV group, may account for a higher state of immune activation in relation to systolic dysfunction [37]. However, in our study neither mDC nor pDC levels were correlated with parameters of systolic or diastolic cardiac dysfunction for the whole group of HF patients. Presumably, blood absolute DC content, although a very small entity within circulating leukocytes, reflects the pathogenesis of end-stage HF in humans irrespective of impairment in systolic or diastolic function of one or both ventricles.

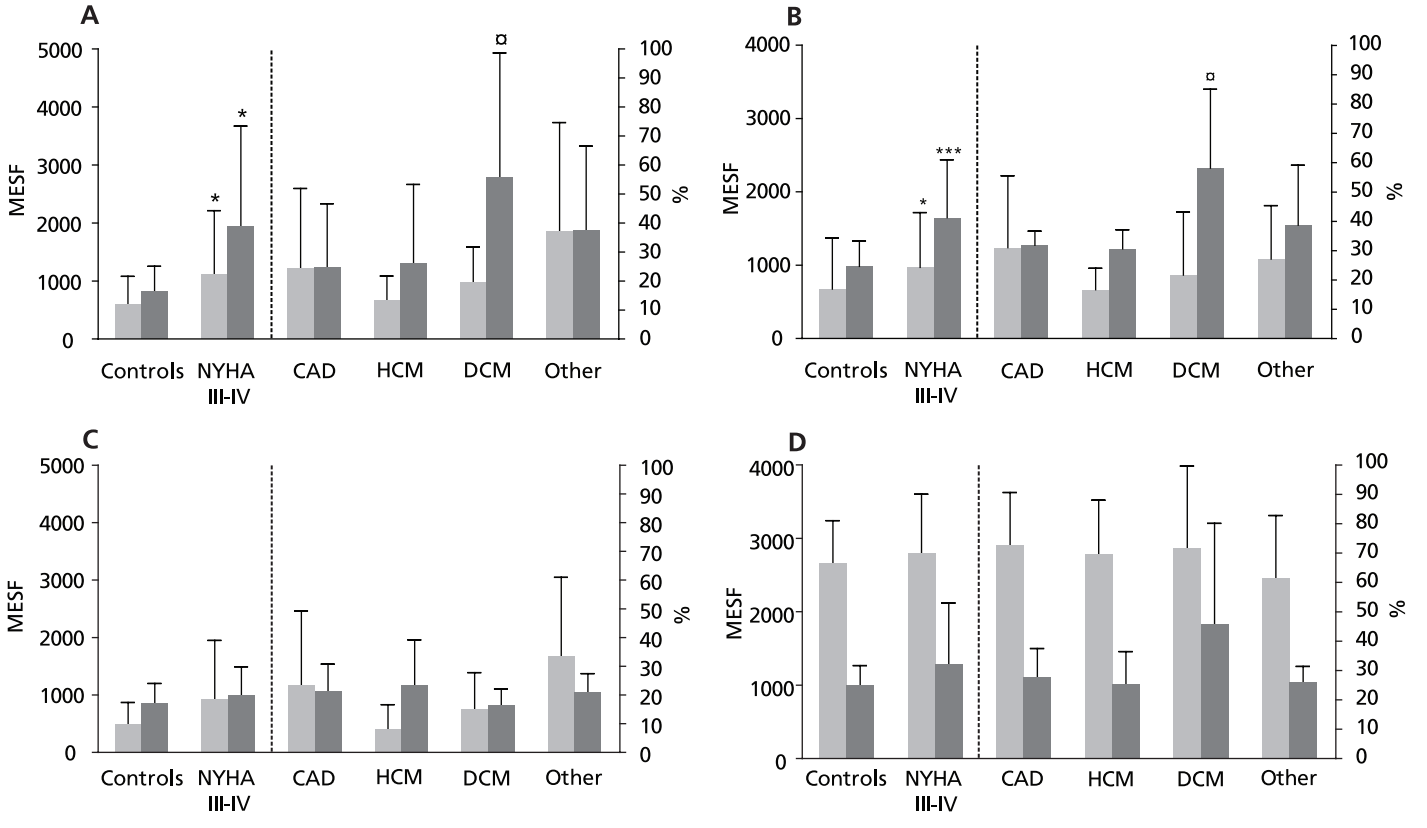

Figure 5: Frequency (open bars) and surface expression (hatched bars) profile of peripheral blood (A) CD83$^+$ mDC, (B) CCR7$^+$ mDC, (C) CD83$^+$ pDC and (D) CCR7$^+$ pDC subsets in 18 healthy controls and 28 end-stage HF patients. Comparisons in the patients according to cause of cardiac disease are depicted accordingly. * $p < 0.05$, *** $p < 0.001$ (Mann-Whitney *U*-test); $^{\text{¤}}p < 0.05$ (Kruskal-Wallis with Dunn's Multiple Comparisons).

The novel and important finding of the present study is a strong association of DC levels with DCM as etiology of end-stage HF. Patients with DCM exhibited elevated circulating mDC levels when compared to patients with cardiac dysfunction of other origins (CAD, HCM, VD, or gianT-cell myocarditits). Except for their elevated levels, blood mDCs from DCM patients also showed increased CD83 and CCR7 surface expression compared with mDCs from the other groups of end-stage HF patients. In our opinion the parallel enhancement of maturation marker and lymphoid homing chemokine receptor expression levels only on mDCs from DCM patients, accounts for a state of functional mDC response to immunogenic stimuli related with the pathogenesis of DCM. More importantly, the imbalance in the two blood DC subsets with a skewing towards mDCs in patients with idiopathic DCM (IDCM), suggests that these patients may have a reduced ability to generate peripheral tolerance to a number of auto-antigens. These results support the notion that immunologic and inflammatory processes are important features of IDCM. However, the exact cause of raised blood absolute mDC levels in patients with IDCM particular, is at present unclear.

Phenotypic characterization of myocardial infiltrate cell populations in patients with IDCM has substantiated the involvement of immune cell-mediated inflammation. Onset of IDCM is thought to be triggered by diverse causes of heart injury, leading to activation of antigen-specific auto-reactive cells [38]. Except for mimicry with known viral or bacterial proteins, IDCM may also be preceded by sub-clinical infections with a wide variety of pathogens not sharing any antigenic similarity with cardiac antigen components [39]. Under normal conditions intracellular auto-antigens can be seen by the immune system and maintain tolerance but in the presence of appropriate triggers (e.g. inflammatory stimuli and genetic predisposition), tolerance could break up due to activation of mDCs and autoimmunity to these self-intracellular auto-antigens might ensue.

Endogenous peptides derived from the intracellular processing of myocardial cytoplasmic proteins are also associated with IDCM etiology. Resident DCs in the normal mouse heart process and express myosin in the context of MHC molecules. It has already been demonstrated that expression of these myosin complexes is increased by the induction of autoimmune myocarditis [40]. Similarly, autoantibodies to cardiac troponin-I induce heart dysfunction and dilatation in a mouse model of dilated cardiomyopathy by chronic stimulation of Ca^{2+} influx in cardiomyocytes [41]. In addition, stress molecules like heat shock proteins (HSPs) have been implicated in the pathophysiology of IDCM.

Myocardial HSP-27, -60, -70, -72 mRNA expression and considerable amounts of serum antibodies against HSP-60 and HSP-70 have been detected in a high proportion of IDCM patients [42-44].
Interestingly, both HSP-60 and HSP-70 have been shown to activate DCs and polarise towards potent T_H1 responses [45,46]. Co-culture of mDCs and pDCs with pro-inflammatory cytokines (IL-3, TNF-α) and HSP-70 up-regulates CD83 expression only in mDCs, but not pDCs [47]. As abnormal recruitment to inflamed tissue and the retention of antigen-loaded DCs in blood and ectopic lymphoid tissue within target organs are known to contribute to the chronicity of inflammation [48], we suggest that CD83 and CCR7 upregulation on mDCs is functional and reflects IDCM progression. This $CD83^+CCR7^+$ circulating mDC population may represent a fraction of mDCs capable to present non-specific auto-antigens and lead to the production of cardiac auto-antibodies [49].
In conclusion, circulating DC subsets in patients with end-stage HF are differentially regulated upon etiology of human HF. Still, the precise mechanism of DCM pathogenesis is unknown, but the association of altered blood mDC levels and phenotypic characteristics with DCM is intriguing. The absence of patients with DCM in the NYHA-II group does not allow us to consolidate if this observation is exclusive for NYHA III-IV HF patients. However, by lack of autoimmune markers and little evidence for cellular involvement in late IDCM [50], whole blood DC characterization may lead to new insights into the pathophysiology of cardiac decompensation in IDCM patients. Further studies are needed to clarify the possible pathogenic role of blood and myocardial DCs in the development of human IDCM.

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References

1. Bristow MR. Why does the myocardium fail? Insights from basic science. Lancet 1998;352 Suppl 1:SI8-14.
2. Mobini R, Maschke H, Waagstein F. New insights into the pathogenesis of dilated cardiomyopathy: possible underlying autoimmune mechanisms and therapy. Autoimmun Rev 2004;3(4):277-84.
3. Stevenson LW, Perloff JK. The dilated cardiomyopathies: clinical aspects. Cardiol Clin 1988;6(2):187-218.
4. McKenna CJ, Codd MB, McCann HA, Sugrue DD. Idiopathic dilated cardiomyopathy: familial prevalence and HLA distribution. Heart 1997;77(6):549-52.
5. Braz JC, Gregory K, Pathak A, Zhao W, Sahin B, Klevitsky R, Kimball TF, Lorenz JN, Nairn AC, Liggett SB, Bodi I, Wang S, Schwartz A, Lakatta EG, DePaoli-Roach AA, Robbins J, Hewett TE, Bibb JA, Westfall MV, Kranias EG, Molkentin JD. PKC-alpha regulates cardiac contractility and propensity toward heart failure. Nat Med 2004;10(3):248-54.
6. Matsumoto Y, Tsukada Y, Miyakoshi A, Sakuma H, Kohyama K. C protein-induced myocarditis and subsequent dilated cardiomyopathy: rescue from death and prevention of dilated cardiomyopathy by chemokine receptor DNA therapy. J Immunol 2004; 173 (5):3535-41.
7. Haghighi K, Gregory KN, Kranias EG. Sarcoplasmic reticulum Ca-ATPase-phospholamban interactions and dilated cardiomyopathy. Biochem Biophys Res Commun 2004;322(4): 1214-22.
8. Holzinger C, Schollhammer A, Imhof M, Reinwald C, Kramer G, Zuckermann A, Wolner E, Steiner G. Phenotypic patterns of mononuclear cells in dilated cardiomyopathy. Circulation 1995;92(10):2876-85.
9. Noutsias M, Pauschinger M, Schultheiss H, U Kh. Phenotypic characterization of infiltrates in dilated cardiomyopathy - diagnostic significance of T-lymphocytes and macrophages in inflammatory cardiomyopathy. Med Sci Monit 2002;8(7):CR478-87.
10. Fujioka S, Kitaura Y, Deguchi H, Shimizu A, Isomura T, Suma H, Sabbah HN. Evidence of viral infection in the myocardium of American and Japanese patients with idiopathic dilated cardiomyopathy. Am J Cardiol 2004;94(5):602-5.
11. Fairweather D, Kaya Z, Shellam GR, Lawson CM, Rose NR. From infection to autoimmunity. J Autoimmun 2001;16(3):175-86.
12. Huber SA, Sartini D, Exley M. Vgamma4(+) T-cells promote autoimmune CD8(+) cytolytic T-lymphocyte activation in coxsackievirus B3-induced myocarditis in mice: role for CD4(+) T_H1 cells. J Virol 2002;76(21):10785-90.
13. Kishimoto C, Hiraoka Y, Takamatsu N, Takada H, Kamiya H, Ochiai H. An in vivo model of autoimmune post-coxsackievirus B3 myocarditis in severe combined immunodeficiency mouse. Cardiovasc Res 2003;60(2):397-403.
14. Ludewig B, Krebs P, Junt T, Bocharov G. Dendritic cell homeostasis in the regulation of self-reactivity. Curr Pharm Des 2003;9(3):221-31.

15. Pulendran B, Palucka K, Banchereau J. Sensing pathogens and tuning immune responses. Science 2001;293(5528):253-6.
16. Eriksson U, Ricci R, Hunziker L, Kurrer MO, Oudit GY, Watts TH, Sonderegger I, Bachmaier K, Kopf M, Penninger JM. Dendritic cell-induced autoimmune heart failure requires cooperation between adaptive and innate immunity. Nat Med 2003;9(12):1484-90.
17. Afanasyeva M, Georgakopoulos D, Belardi DF, Ramsundar AC, Barin JG, Kass DA, Rose NR. Quantitative analysis of myocardial inflammation by flow cytometry in murine autoimmune myocarditis: correlation with cardiac function. Am J Pathol 2004;164(3): 807-15.
18. Kohrgruber N, Halanek N, Groger M, Winter D, Rappersberger K, Schmitt-Egenolf M, Stingl G, Maurer D. Survival, maturation, and function of CD11c- and CD11c+ peripheral blood dendritic cells are differentially regulated by cytokines. J Immunol 1999; 163(6):3250-9.
19. Thomas R, MacDonald KP, Pettit AR, Cavanagh LL, Padmanabha J, Zehntner S. Dendritic cells and the pathogenesis of rheumatoid arthritis. J Leukoc Biol 1999;66(2):286-92.
20. Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S, Antonenko S, Liu YJ. The nature of the principal type 1 interferon-producing cells in human blood. Science 1999;284(5421):1835-7.
21. Mohty M, Jarrossay D, Lafage-Pochitaloff M, Zandotti C, Briere F, de Lamballeri XN, Isnardon D, Sainty D, Olive D, Gaugler B. Circulating blood dendritic cells from myeloid leukemia patients display quantitative and cytogenetic abnormalities as well as functional impairment. Blood 2001;98(13):3750-6.
22. Kuwana M. Induction of anergic and regulatory T-cells by plasmacytoid dendritic cells and other dendritic cell subsets. Hum Immunol 2002;63(12):1156-63.
23. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K. Immunobiology of dendritic cells. Annu Rev Immunol 2000;18:767-811.
24. Sallusto F, Palermo B, Lenig D, Miettinen M, Matikainen S, Julkunen I, Forster R, Burgstahler R, Lipp M, Lanzavecchia A. Distinct patterns and kinetics of chemokine production regulate dendritic cell function. Eur J Immunol 1999;29(5):1617-25.
25. de Leeuw N, Ruiter DJ, Balk AH, de Jonge N, Melchers WJ, Galama JM. Histopathologic findings in explanted heart tissue from patients with end-stage idiopathic dilated cardiomyopathy. Transpl Int 2001;14(5):299-306.
26. Verduyn W, Doxiadis, II, Anholts J, Drabbels JJ, Naipal A, D'Amaro J, Persijn GG, Giphart MJ, Schreuder GM. Biotinylated DRB sequence-specific oligonucleotides. Comparison to serologic HLA-DR typing of organ donors in eurotransplant. Hum Immunol 1993;37(1):59-67.
27. Schreuder GM, Hurley CK, Marsh SG, Lau M, Fernandez-Vina MA, Noreen HJ, Setterholm M, Maiers M. HLA dictionary 2004: summary of HLA-A, -B, -C, -DRB1/3/4/5, -DQB1 alleles and their association with serologically defined HLA-A, -B, -C, -DR, and -DQ antigens. Hum Immunol 2005;66(2):170-210.

28. Senni M, Tribouilloy CM, Rodeheffer RJ, Jacobsen SJ, Evans JM, Bailey KR, Redfield MM. Congestive heart failure in the community: a study of all incident cases in Olmsted County, Minnesota, in 1991. Circulation 1998;98(21):2282-9.
29. Zile MR, Brutsaert DL. New concepts in diastolic dysfunction and diastolic heart failure: Part I: diagnosis, prognosis, and measurements of diastolic function. Circulation 2002; 105(11):1387-93.
30. Athanassopoulos P, Vaessen LM, Balk, AH, Takkenberg JJ, Maat AP, Weimar W, Bogers AJ. Impaired circulating dendritic cell reconstitution identifies rejecting recipients after clinical heart transplantation independent of rejection therapy. Eur J Cardiothorac Surg 2005;27(5):783-789.
31. Athanassopoulos P, Vaessen LM, Maat AP, Zondervan PE, Balk AH, Bogers AJ, Weimar W. Preferential depletion of blood myeloid dendritic cells during acute cardiac allograft rejection under controlled immunosuppression. Am J Transplant 2005;5(4):810-20.
32. Schwartz A, Gaigalas AK, Wang L, Marti GE, Vogt RF, Fernandez-Repollet E. Formalization of the MESF unit of fluorescence intensity. Cytometry B Clin Cytom 2004;57(1):1-6.
33. Athanassopoulos P, Vaessen LM, Maat AP, Balk AH, Weimar W, Bogers AJ. Peripheral blood dendritic cells in human end-stage heart failure and the early post-transplant period: evidence for systemic T_H1 immune responses. Eur J Cardiothorac Surg 2004;25(4):619-26.
34. Shodell M, Siegal FP. Circulating, interferon-producing plasmacytoid dendritic cells decline during human ageing. Scand J Immunol 2002;56(5):518-21.
35. Hung MJ, Cherng WJ. Comparison of white blood cell counts in acute myocardial infarction patients with significant versus insignificant coronary artery disease. Am J Cardiol 2003;91(11):1339-42.
36. Andrew P. Diastolic heart failure demystified. Chest 2003;124(2):744-53.
37. Hasper D, Hummel M, Kleber FX, Reindl I, Volk HD. Systemic inflammation in patients with heart failure. Eur Heart J 1998;19(5):761-5.
38. Fairweather D, Yusung S, Frisancho S, Barrett M, Gatewood S, Steele R, Rose NR. IL-12 receptor beta 1 and Toll-like receptor 4 increase IL-1 beta- and IL-18-associated myocarditis and coxsackievirus replication. J Immunol 2003;170(9):4731-7.
39. Borda E, Pascual J, Cossio P, De La Vega M, Arana R, Sterin-Borda L. A circulating IgG in Chagas' disease which binds to beta-adrenoceptors of myocardium and modulates their activity. Clin Exp Immunol 1984;57(3):679-86.
40. Smith SC, Allen PM. Expression of myosin-class II major histocompatibility complexes in the normal myocardium occurs before induction of autoimmune myocarditis. Proc Natl Acad Sci U S A 1992;89(19):9131-5.
41. Okazaki T, Tanaka Y, Nishio R, Mitsuiye T, Mizoguchi A, Wang J, Ishida M, Hiai H, Matsumori A, Minato N, Honjo T. Autoantibodies against cardiac troponin I are responsible for dilated cardiomyopathy in PD-1-deficient mice. Nat Med 2003;9(12):1477-83.
42. Knowlton AA, Kapadia S, Torre-Amione G, Durand JB, Bies R, Young J, Mann DL. Differential expression of heat shock proteins in normal and failing human hearts. J Mol Cell Cardiol 1998;30(4):811-8.

43. Tanonaka K, Yoshida H, Toga W, Furuhama K, Takeo S. Myocardial heat shock proteins during the development of heart failure. Biochem Biophys Res Commun 2001;283(2):520-5.
44. Portig I, Pankuweit S, Maisch B. Antibodies against stress proteins in sera of patients with dilated cardiomyopathy. J Mol Cell Cardiol 1997;29(8):2245-51.
45. Wan T, Zhou X, Chen G, An H, Chen T, Zhang W, Liu S, Jiang Y, Yang F, Wu Y, Cao X. Novel heat shock protein Hsp70L1 activates dendritic cells and acts as a T_H1 polarizing adjuvant. Blood 2003.
46. Flohe SB, Bruggemann J, Lendemans S, Nikulina M, Meierhoff G, Flohe S, Kolb H. Human heat shock protein 60 induces maturation of dendritic cells versus a T_H1-promoting phenotype. J Immunol 2003;170(5):2340-8.
47. Gomez J, Borras FE, Singh R, Rajananthanan P, English N, Knight SC, Navarrete CV. Differential up-regulation of HLA-DM, invariant chain, and CD83 on myeloid and plasmacytoid dendritic cells from peripheral blood. Tissue Antigens 2004;63(2):149-57.
48. Sallusto F, Lanzavecchia A. Mobilizing dendritic cells for tolerance, priming, and chronic inflammation. J Exp Med 1999;189(4):611-4.
49. MacLellan WR, Lusis AJ. Dilated cardiomyopathy: learning to live with yourself. Nat Med 2003;9(12):1455-6.
50. de Leeuw N, Melchers WJ, Ruiter DJ, Caforio AL, Balk AH, de Jonge N, Galama JM. Autoimmune markers are undetectable in end stage idiopathic dilated cardiomyopathy. J Clin Pathol 1999;52(10):739-43.

Chapter 5

Preferential Depletion of Blood Myeloid Dendritic Cells during Acute Cardiac Allograft Rejection under Controlled Immunosuppression

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Abstract

Allo-Ag presentation to Ag-specific T-lymphocytes by donor or recipient dendritic cells (DCs) induces acute rejection (AR) after solid organ transplantation. It is postulated that myeloid (mDC) and plasmacytoid (pDC) subsets circulate differentially between bone marrow, heart and lymphoid tissues after cardiac transplantation (HTx). We investigated peripheral blood DC subset distribution, maturation and lymphoid homing properties in relation to endomyocardial biopsy (EMB) rejection grade after clinical HTx. Twenty-one HTx recipients under standard immunosuppression were studied in a 9-month follow-up. mDC and pDC numbers were analyzed by flow-cytometry in fresh venous whole blood samples collected during the EMB procedures and before histological diagnosis of AR. Subsets were further characterized for maturation marker CD83 and lymphoid homing chemokine receptor CCR7. Although numbers of both DC subsets remained low for the whole post-HTx period, we observed a negative association of mDCs with rejection grade. Repeated measurements analysis revealed that only mDCs decreased during AR episodes. Rejectors had lower mDC numbers after 3 months follow-up compared to non-rejectors. Furthermore, patients during AR exhibited low proportions of mDCs positive for CD83 or CCR7. These findings suggest peripheral blood mDC depletion in association with selective lymphoid homing of this subset during AR after clinical HTx.

Introduction

Acute rejection (AR) remains a clinical obstacle in the first year after clinical heart transplantation (HTx) [1]. Allo-specific T-cell activation is the major immunogenic mechanism leading to this condition. The latter is initiated by the presentation of foreign allo-Ag to the TCR complex of naïve or memory T-cells by APCs such as macrophages, B cells and dendritic cells (DCs). In this respect, donor and recipient bone-marrow derived DCs are regarded as the most efficient APCs to elicit rejection post-HTx [2,3].

Human DCs can be divided into 2 functional phenotypes. Plasmacytoid DCs (pDCs) delineate from lymphoid precursors, show plasma cell-like morphology, express high amounts of IL-3 receptor-α chain (CD123) and are regarded as the main IFNα-producing blood cells that induce T-helper 2 (T_H2) immune responses [4]. As pDC primed T-lymphocytes also differentiate into regulatory T-cells, it is hypothesized that pDCs may promote tolerance [5]. On the other hand, myeloid DCs (mDCs) which originate from myeloid precursors, express the β_2 integrin CD11c, CD13 and CD33. *In vitro,* they can be derived from circulating monocytes in response to GM-CSF & IL-4 and are able to polarize naïve T-cells towards a T-helper 1 (T_H1) immune response [6,7]. As mDC primed T-lymphocytes are able to differentiate into effector $CD4^+$ helper or cytotoxic $CD8^+$ cells, it is suggested that mDCs are involved in the induction of immunity [8].

Circulating DC subsets are identified by a distinct surface immunophenotype, that includes non-DC lineage negativity, inflammatory chemokine receptor expression (CCR1, CCR5), constitutive MHC class II Ag expression and low surface levels of maturation marker (CD83) and co-stimulatory molecules (CD40, CD80, CD86) necessary for T-cell activation [9,10,11]. Under homeostatic conditions, immature DCs migrate into blood from the bone marrow and reside in non-lymphoid tissues where they constantly process microbial or viral Ags. Upon Ag encounter mDCs migrate into regional lymph nodes primarily through afferent lymphatics [12,13], while pDCs enter the T-cell lymph node areas directly from the blood stream via high endothelial venules (HEVs) by expressing high levels of L-selectin, or the interaction of chemokine receptor CXCR4 and its ligand SDF-1 (CXCL12) [14]. Migrating donor or recipient DCs lose their ability to capture Ags, increase the expression of MHC class I and II molecules, upregulate CD80, CD83 and CD86 and secrete pro-inflammatory cytokines [15]. With heart allograft lymph drainage being disrupted post-HTx, mature mDCs and pDCs traffic from allograft via the blood, with homing

chemokine receptor CCR7 into draining lymph node T-cell areas where they will present allo-Ag and activate T-cells [16].
The scope of this study was to investigate peripheral whole blood mDCs and pDCs in relation to AR after clinical HTx. Given their differential trafficking pattern between bone marrow, heart and lymphoid tissues, we identified circulating DCs in a patient cohort under standard immunosuppressive therapy. In an attempt to unravel a role for mDCs or pDCs in AR, incidence, maturation and homing properties of circulating DC subsets were associated with the histological diagnosis of AR post-HTx.

Patients and Methods

Study populations

Twenty-one consecutive adult heart transplant (HTx) recipients became eligible for study between May 2002 and November 2003. All patients had experienced a stable medical regimen of ACE inhibitor, statin, β-blocker, diuretic, anti-arrhythmic and anti-coagulant agents for at least 1 month prior to transplantation. One patient had received maintenance immunosuppression pre-HTx as he was re-transplanted due to cardiac allograft failure induced by transplant coronary artery disease. Donor and recipient clinical parameters are depicted in Table 1. Eighteen healthy laboratory co-workers, matched for age and gender, (10 men and 8 women; mean age 48.8 ± 11.5; range 26.7-60.1 years) volunteered to donate blood for this investigation as control subjects. They received no medication and represented a reference for all values obtained throughout our inquiry. None of the patients presented with any severe co-morbidity directly prior to transplantation. The research conformed with the principles outlined by the Declaration of Helsinki. Before entering the study, all patients provided written informed consent in accordance with a protocol approved by the medical ethical committee (MEC 215.732/2002/157) of the Erasmus University Medical Center, The Netherlands.

Endomyocardial biopsies

Scheduled right venticular endomyocardial biopsies (EMB) were performed weekly until week 6, biweekly until week 10, monthly up to 6 months and bimonthly up to the first annual examination, on week 52 post-HTx. Clinical course and prior rejection profile of each individual patient triggered EMB rescheduling in 2- to 4- weekly intervals at later time-points of follow-up. Six

tissue samples were extracted during each procedure and diagnosis was based on the verdict of two independent cardiac pathologists. Multiple sections stained with hematoxylin and eosin were analyzed to exclude Quilty effect as the etiology of lymphocytic infiltration. EMB grading was performed by light microscopy using the standard working formulation of the International Society for Heart and Lung Transplantation (ISHLT) [17]. Histologically, only patients with ISHLT grade ≥ 3A episodes were considered to experience significant acute cellular rejection.

Table 1: Recipient and donor demographic characteristics

Recipients	
age	51.6 ± 9.2
sex, (m/f)	12/9
nº of transplantation	
1st	20
2nd	1
Donors	
age	34.7 ± 11.9
sex, (m/f)	7/14
etiology of death	
intracerebral hematoma	2
post-anoxic encephalopathy	4
subarrachnoidal hemorrhage	8
cerebral trauma	7
CMV D/R combinations	
+/+	4
+/–	9
–/+	5
–/–	3

m = male, f = female, CMV = cytomegalovirus, D = donor, R = recipient. Age in years, is expressed as the mean ± SD.

After transplantation, all HTx patients received anti-thymocyte globulin (ATG) induction and triple therapy in the form of corticosteroids, CsA (Novartis, Basel, Switzerland) or Tacrolimus (FK506, Fujisawa GmbH, Munich, Germany) & MMF (Roche, Basel, Switzerland) as maintenance immunosuppression. Horse-ATG (Imtix Sangstat B.V., Lyon, France) at 3 to 8 i.v. doses daily (mean dose = 3.8 ± 1.1; 1 dose = 212.5 lymphocytotoxic-units/kg/24hrs) was administered for a median of 7 days (range: 5-12) until adequate CsA or FK506 trough

levels were achieved. All patients received 75mg prednisolone i.v. during the first post-transplant day, 50mg/day for 5, 40mg/day for 3 and 30mg/day for 2 consecutive days post-HTx. Thereafter, orally administered prednisone was tapered with 5mg decrements up to 20mg/day for 3 days and then with 2.5mg decrements every 7 days up to 10mg/day. CsA and FK506 were started at 8mg/kg/24hrs and 0.3mg/kg/24hrs oral dosages respectively, divided in 2 doses daily, were titrated further to reach trough levels of 150-250ng/mL and 12-16ng/mL respectively. MMF was started at the end of the induction therapy in doses between 2000 and 3000mg, daily. Rejecting recipients received additional anti-rejection therapy in the form of methyl-prednisolone (Pfizer, Pharmacia & Upjohn, Sandwich, UK) 1000 mg/day i.v. for 3 days upon biopsy-proven AR episodes. All patients were further adjusted to a dosage of 10-20 mg/day pravastatin *per os*.

CMV seronegative (-ve) recipient / seropositive (+ve) donor combinations (Table 1) received prophylaxis with 450 mg/day Valganciclovir (n = 3) or anti-CMV hyper-Ig (Megalotect, Biotest Pharma GmbH, Dreiech, Germany), from 5-7 days post-HTx (n = 6). CMV status (IgG, IgM and PCR) was routinely screened at 14, 30, 60, 90, 120 days post-HTx and at the end of follow-up. CMV disease, as manifested by symptoms and a positive PCR, was treated with ganciclovir (5 mg/kg, 2 times daily) for 10-14 days. Inadequate efficacy of ganciclovir, as determined by ongoing symptoms and positive PCR, was an indication for MMF withdrawal. No patients received anti-CMV globulin as therapy for documented disease.

Monoclonal antibodies

Allophycocyanin (APC)-conjugated CD11c, PE-conjugated anti-IL-3 receptor α chain, PerCP-conjugated anti-HLA-DR, FITC-conjugated CD34 and FITC-lineage cocktail 1 (Lin-1) which contained mAbs: CD3 (T-cells), CD14 (monocytes/macrophages), CD16 (NK cells), CD19 (B cells) & CD56 (NK cells) were all purchased from Becton Dickinson Biosciences (San Jose, CA, USA). PE-conjugated CD83 and PE-conjugated anti-CCR7 (CD197w) were obtained from Serotec (Oxford, UK) and R&D Systems (Abingdon, UK), respectivelly. PE- and PerCP-conjugated isotype control murine mAbs (IgG1 & IgG2a) were provided by Becton Dickinson. CD45 FITC / CD14 PE reagent from Serotec was used to monitor lymphocyte, monocyte and granulocyte presence in each sample.

Whole blood preparation

Peripheral blood cells from patients were collected 4-6 hrs before (pre-) and at 1, 2, 3, 4, 12, 24 and 38 wks post-HTx. Venous blood drawn at wks 1, 2 and 4 within 1-month from controls, was used for comparison. All samples from patients and controls were collected in the morning. Blood from patients post-HTx was obtained during the EMB procedure and before administration of anti-rejection therapy. Heparinized pyrogen-free tubes were used to divide all specimens in 300 μL aliquots for DC characterization. Samples were placed directly into a 5 mL polystyrene tube containing 10μL anti-HLA-DR (PerCP), 20μL Lin1 (FITC), 10μL CD11c (APC) and 10μL CD123 (PE) used for rare event four-color flow cytometric analysis. In separate tubes, CD123 (PE) was replaced by 5μL CD83 (PE) or 5μL CCR7 (PE). For the latter analyses, the acquired $CD11c^{low}$ cells were considered to be pDCs as their numbers were equal to the $CD123^{high}$ cells in the first tube. Negative controls, with irrelevant isotypic antibodies (IgG, IgM), were prepared in each experiment, as appropriate. To minimize selective loss during the preparation procedure, whole blood cells were first incubated in the dark with mAbs for 20 mins, followed by 10-min lysis of the erythrocytes with FACS lyzing solution (BD Biosciences) at room temperature. After washing with FACSflow (BD Immunocytometry Systems), 200,000-300,000 events were acquired. A minimum of 100 events per DC subset was analyzed in a fluorescence-activated cell sorter (FACSCalibur) with the CellQuest Pro software (both supplied by BD Biosciences). White blood cell (WBC) numbers in all samples were determined by an automated cell counter (Sysmex Microcellcounter F-300, Goffin Meyvis, Etten Leur, The Netherlands).

Dendritic cell immunophenotypic analysis

DC characterization was practically censored for occurrence of AR at the time of blood sampling. Whole peripheral blood cells stained with anti-HLA-DR mAb were identified within $CD34^{-}$ $Lin\text{-}1^{-}$ events as discussed in the past [18]. In the gated cells, CD11c and CD123 expression was determined in order to define the two distinct DC subsets. Precursor mDCs and pDCs were delineated as $CD11c^{high}$ $CD123^{low}$ and $CD11c^{low}$ $CD123^{high}$, respectively. Absolute numbers of mDCs and pDCs, expressed in cells/μL, were calculated from the WBC count multiplied by the proportion of each subset within WBCs and represented the incidence of each subset per subject at the time of sampling, as defined by flow cytometric analysis. The acquired mDCs ($CD11c^{high}$) and pDCs ($CD11c^{low}$) were further analyzed for maturation marker CD83 and lymphoid homing chemokine receptor CCR7. Percentage of precursor mDCs or pDCs positive for

CD83 or CCR7 was calculated from the total number of mDCs or pDCs and was used as a means to express the proportion of mature or homing peripheral blood mDCs or pDCs, respectively.

Statistical analysis

Results are expressed as absolute mDC & pDC numbers or mean ± SD of percentage (%) lymphocytes, monocytes, granulocytes, DC, WBC numbers, absolute DC numbers and % positive mDC or pDC for CD83 or CCR7. All data sets were tested before comparisons whether they passed normality. Coefficient of variation (CV) was determined to assess assay reproducibility. The Mann Whitney *U*-test and the paired Wilcoxon samples *t*-test were employed to compare differences between means as appropriate. Associations were calculated using the Spearman (r) correlation coefficient. Continuous data were analyzed by repeated measurements and one-way ANOVA in order to decipher differences within and between groups or individual patients over time. Two-sided tests were performed and 'p' values with an α level of < 0.05 were considered to be significant. SPSS 11.0.1 software (Chicago, IL, USA) and the GraphPad statistical program (San Diego, CA, USA) were applied for analyses and graphics, respectively.

Results

Transplantation outcome

Fourteen patients experienced a total of 20 biopsy-proven AR episodes post-HTx. Between rejecting (R) and non-rejecting (NR) recipients there were no significant differences in clinical variables (Table 2). Infections were the major complications in both groups post-HTx and these were treated with conventional antibiotic or anti-viral therapy according to local protocol. Median time for initiation of statin therapy was 19 days after transplantation (range: 7-115) and was tolerated in 20 of the 21 patients for the whole period post-HTx. Between R and NR patients, h-ATG dosis (mean: 3.7 ± 1.1 vs 4.2 ± 1.0 dosages daily) and duration of therapy [median time: 6.5 (range: 5-12) vs 7.5 (range: 5-11) days] were not different. Maintenance immunosuppressive drug dosages and acquired trough levels were comparable between R and NR recipients for the whole period of follow-up (Table 2). Despite prophylaxis, 11 patients presented with CMV disease post-HTx and for 8 of those MMF therapy was discontinued. One NR patient died at 21 days post-HTx due to brain

stem coning after a severe cerebro-vascular accident. Further complications included groin wound dehiscence, pleural empyema, femoral bone fracture and motoric axonal polyneuropathy.

Table 2: Clinical variables for non-rejecting and rejecting recipients post-HTx

	NR	R	P value
n	7	14	
Recipient			
age	52.0 ± 10.6	50.4 ± 8.1	0.685
sex (m/f)	4/3	8/6	
Donor			
age	38.6 ±12.3	33.4 ±11.9	0.414
sex (m/f)	3/4	4/10	
Ischemic time	150.8 ± 46.5	158.4 ± 38.6	0.697
HLA mismatches			
HLA-A	1.8 ± 0.4	1.2 ± 0.9	0.098
HLA-B	1.8 ± 0.4	1.9 ± 0.4	0.947
HLA-DR	1.5 ± 0.6	1.6 ± 0.7	0.713
Immunosuppression			
Prednisone, dosis			
early	20.0 (15.0 – 40.0)	20.0 (10.0 – 50.0)	0.799
late	10.0 (10.0 – 15.0)	10.0 (5.0 – 12.5)	0.596
FK506, trough levels			
early	10.6 (7.4 – 13.8)	10.3 (6.7 – 21.6)	0.889
late	7.2 (6.5 – 10.5)	10.9 (8.4 – 14.5)	0.095
CsA, trough levels			
early	262.5 (225.0 – 307.9)	327.1 (256.0 – 370.0)	0.073
late	177.5 (173.8 – 226.0)	218.8 (186.7 – 277.0)	0.262
MMF, trough levels			
early	2.0 (1.5 – 2.5)	2.1 (0.9 – 2.9)	0.886
late	1.7 (1.5 – 2.9)	1.5 (0.7 – 3.0)	0.714
MMF, withdrawal	3/6[†]	5/14	

NR = non-rejectors, R = rejectors, HLA = human leukocyte antigen, early = 1-4 weeks post-HTx, late = 12-38 weeks post-HTx. Age (years), ischemic time (minutes) and HLA-mismatches are expressed as mean ± SD. Dosis (mg/day) and trough levels (ng/mL) are expressed as median (range). [†]One of the NR patients died due to brain stem coning after severe cerebro-vascular accident

Table 3: Hematological characteristics of heart transplant recipients

	HTx patients (n=21)							
	pre-	post-HTx, wks (range)						
		1 (0.6 – 1.7)	2 (1.8 – 2.6)	3 (2.8 – 3.4)	4 (3.5 – 5.1)	12 (10.1 – 16.0)	24 (20.4 – 27.8)	38 (36.0 – 40.5)
WBC, (10^3cells/μL)	7.18 ± 2.09	12.22 ± 5.25*	9.81 ± 4.78	8.94 ± 3.48	6.83 ± 2.95	8.18 ± 3.47	7.39 ± 3.3	7.55 ± 2.3
% lymphocytes	17.13 ± 10.19	7.22 ± 3.17**	9.28 ± 3.33*	9.75 ± 4.26*	14.26 ± 8.24	11.85 ± 8.15	21.35 ± 13.46	19.60 ± 12.18
% monocytes	5.75 ± 2.98	5.68 ± 2.22	5.78 ± 2.62	5.10 ± 3.92	5.65 ± 3.49	5.44 ± 2.71	6.48 ± 4.19	4.95 ± 2.19
% granulocytes	77.12 ± 10.61	87.10 ± 14.03*	84.94 ± 4.54	85.15 ± 16.35	80.09 ± 10.43	82.71 ± 8.72	72.17 ± 16.19	75.45 ± 12.72
DCs, (cells/μL)	28.26 ± 10.37	4.29 ± 4.93***	4.56 ± 4.01***	5.85 ± 4.36***	6.27 ± 4.45***	6.19 ± 3.96***	7.77 ± 5.54***	10.79 ± 6.37***

HTx, heart transplantation; WBC, white blood cells; DCs, dendritic cells. *p < 0.05, **p < 0.01, ***p < 0.001; all compared to pre-HTx (Wilcoxon samples *t*-test)

Hematological characteristics of study subjects

Patient WBC counts pre-HTx, were comparable to control leukocytes at 5.93 ± 3.13 x10^3 cells/μL ($p = 0.156$). WBCs were significantly elevated at 1-wk post-HTx, against the pre-HTx condition. This was attributed to a transient increment of 9.9% in granulocytes and an equal albeit more persistent decrement in lymphocytes. WBC counts normalized again at 2 weeks post-HTx and remained within a constant range for the rest of the follow-up. Granulocyte and lymphocyte proportions returned to pre-HTx levels at 2 and 3 weeks post-HTx, respectively (Table 3).

DC numbers and subset distribution in HTx recipients and normal subjects

Sequential profiles of CD11c and CD123 expression by peripheral blood DCs from a healthy control subject, a non-rejecting and a rejecting patient post-HTx, are depicted in Figure 1. For each individual, mDC incidence was greater than pDC incidence while a third, Lineage$^-$ HLA-DR$^+$ CD11c^{low}, CD123^{low} cell population was subtly but consistently present in controls and patients pre- and post-HTx. Control DC sample analysis within 1-mo, confirmed interassay reproducibility as % DC was stable during this period (Figure 2A) and CV for mDC, pDC numbers and the mDC:pDC ratio were all consistently < 15% (13.3%, 14.8% and 12.6%, respectively). Patient total DC numbers decreased significantly at 1-wk post-HTx and remained lower than the pre-HTx condition for the whole post-HTx period (Table 3). Consequently, % DC declined sharply post-HTx and ranged from 0.05-0.13 % of WBC until the completion of follow-up (Figure 2B). Controls exhibited stable mDC and pDC subset incidence during 1-mo follow-up (Figure 2C). For patients 1-wk post-HTx, mDC and pDC numbers at 3.63 ± 2.99 and 0.67 ± 0.29 cells/μL were significantly lower than the pre-transplant and control condition. Further subset distribution analysis revealed that both mDC (range 3.63-7.88 cells/μL) and pDC (range 1.00-2.91 cells/μL) numbers remained consistently lower post-HTx than the pre-HTx condition (Figure 2D).

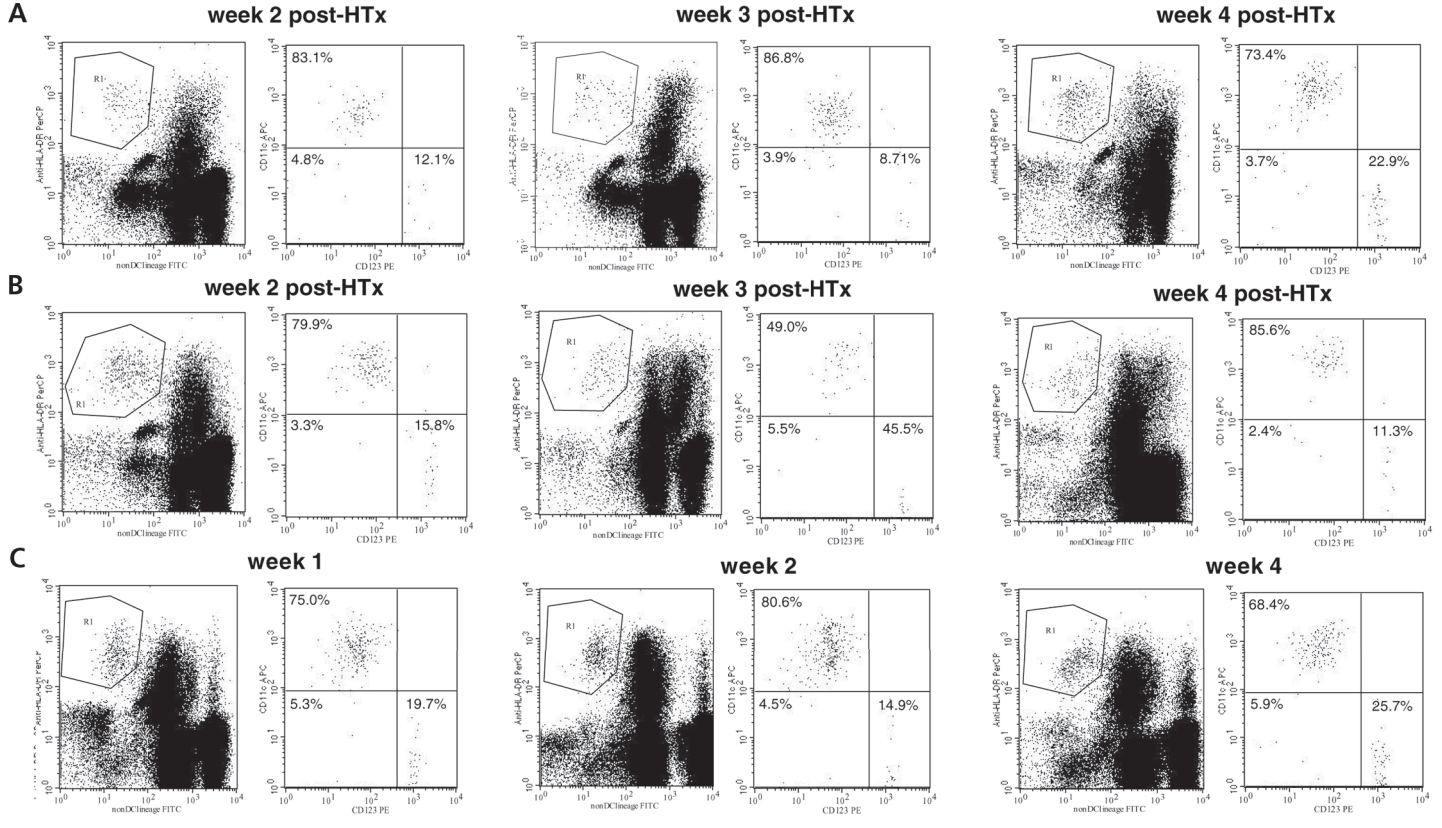

Figure 1: DC subsets in whole peripheral blood of heart transplant recipients and a normal healthy control by rare event, 4 color, flow cytometric analysis. Lin^- $HLA\text{-}DR^+$ events were gated (R1) within $CD34^-$ cells. mDC and pDC incidence was calculated from the % $CD11c^{high}$ $CD123^{low}$ and % $CD11c^{low}$ $CD123^{high}$ cells, respectively. $CD11c^{low}$ $CD123^{low}$ events represented a small fraction of Lin^- $HLA\text{-}DR^+$ events (consistently <6%). Data at wk 2, 3 and 4 weeks post-HTx are shown for a non-rejector (A) and a rejector (B), in addition to sequential data from a normal healthy control at the 1^{st}, 2^{nd} and 4^{th} wk follow-up (C). An AR episode is depicted for the rejecting patient at the 3^{rd} wk post-HTx (B).

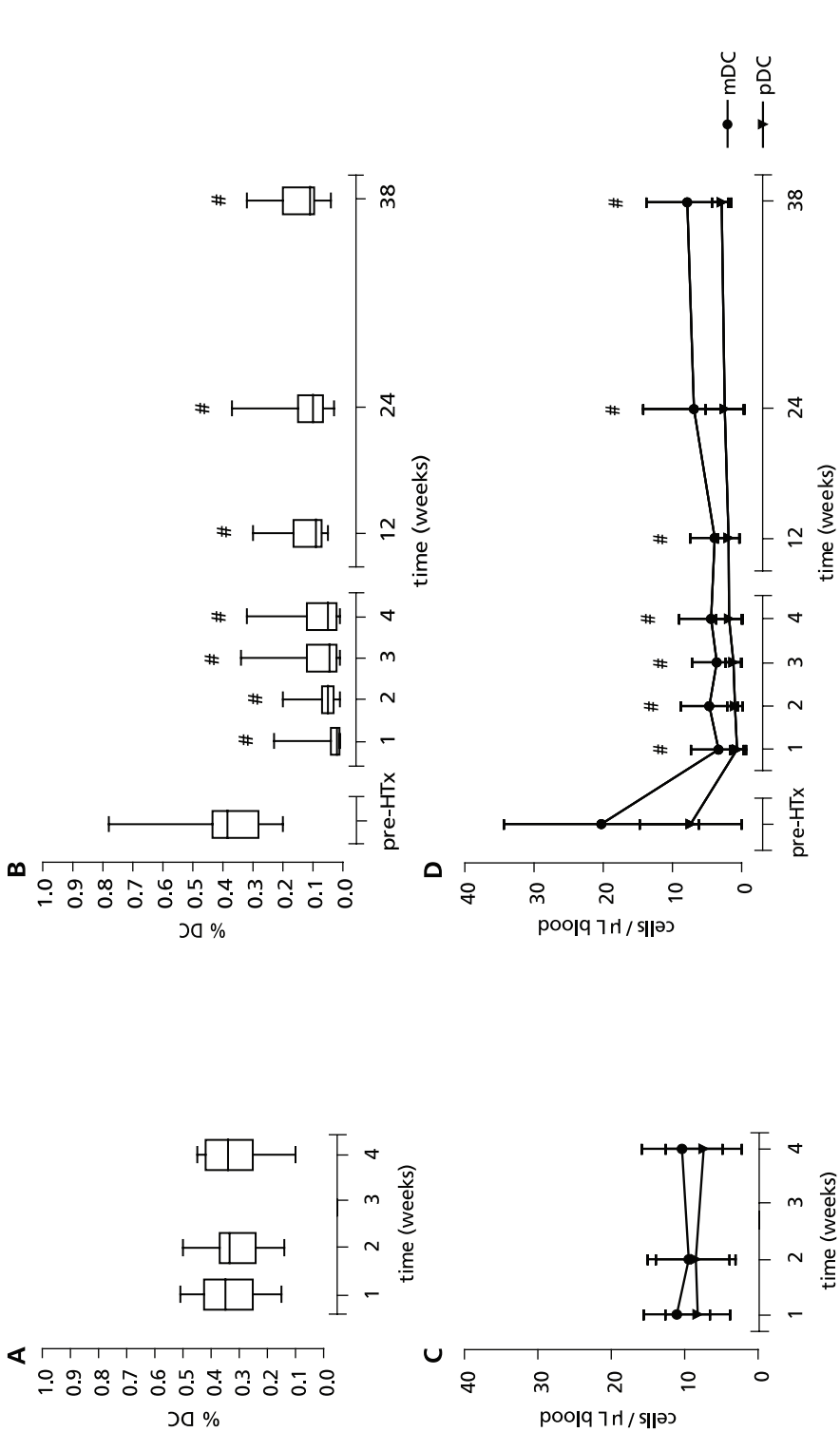

Figure 2: Percentage DCs of whole white blood cells and absolute numbers of mDCs and pDCs in a 1-mo follow-up for 18 controls (A, C) and 21 transplant recipients pre- and up to 9 months post-HTx (B, D). Within subject repeated measures ANOVA analysis revealed: (A) control % DC, $p = 0.755$; (B) HTx recipient % DC, $p < 0.0001^{\#}$; (C) control mDC numbers, $p = 0.583$; control pDC numbers, $p = 0.678$; (D) HTx recipient mDC numbers, $p < 0.0001^{\#}$; HTx recipient pDC numbers, $p < 0.0001^{\#}$.

Association of DC numbers with endomyocardial biopsy ISHLT grade

A total of 142 right ventricular biopsies became available for study. Histological diagnosis could not be certified for 2 biopsies due to shortage of material. No severe (grade 4) AR episodes were documented during follow-up. Upon analysis of blood samples corresponding to 70 grade 0, 39 grade 1A, 2 grade 1B, 16 grade 2, 12 grade 3A and 1 grade 3B biopsies, there was a negative association of absolute mDC numbers with the diagnosed ISHLT infiltration grade observed for the whole period of follow-up (Figure 3A). The difference between 7.0 or 5.2 cells/μL during grades 0 or 1A/B with 2.9 or 1.6 cells/μL during grades 2 or 3A/B, was significant. However, this effect was not seen in the pDCs as this fraction was constitutively present at a low but stable level (< 2 cells/μL) in peripheral blood independent of the pathological grade of rejection (Figure 3B). Correlation with ISHLT grade, although weak, was significant for mDCs ($r = -0.49$, $R^2 = 0.24$; $p = 0.02$) but not for pDCs ($r = -0.38$, $R^2 = 0.14$; $p = 0.33$) at the peak of the AR period (12 weeks post-HTx). From the 13 AR episodes (grade 3A/B) caught in the follow-up, 2 occurred at the 1st, 2 in the 3rd, 2 in the 4th, 4 in the 12th, 2 in the 24th and 1 in the 38th week post-HTx. Complete data before, during and after rejection were available for 10 AR episodes. Paired samples analysis in the rejecting HTx-recipients for all episodes before, during and after AR (n = 13) revealed that mDC, but not pDC, numbers decreased during each episode and the majority increased after resolution of the rejection (Figure 4).

Rejection and non-rejection DC subset maturation and lymphoid homing properties

Characterization of CD83 and CCR7 on mDCs and pDCs has been described before [18]. The % of CD83 or CCR7 positive blood mDCs and pDCs for both R and NR patients were low post-HTx. However, rejecting recipient paired analysis of samples acquired during the biopsy-proven AR episodes (AR+) and non-rejection episodes (AR-) exhibited a nearly significant decrease in CD83 and a significant decrease in CCR7 positive mDCs during AR for the total follow-up period (%CD83+ mDCs: 8.9 ± 4.3 vs 17.3 ± 10.4; $p = 0.062$) (%CCR7+ mDCs 9.7 ± 5.9 vs 21.1 ± 14.7; $p = 0.024$) (Figure 5A). No differences were observed in the % CD83 or CCR7 positive pDCs when blood taken during AR+ was compared to samples taken from the same patients during AR- episodes (%CD83+ pDCs: 11.6 ± 9.1 vs 10.6 ± 9.6; $p = 0.161$) (%CCR7+ pDCs 56.1 ± 22.6 vs 52.1 ± 23.1; $p = 0.580$) (Figure 5B).

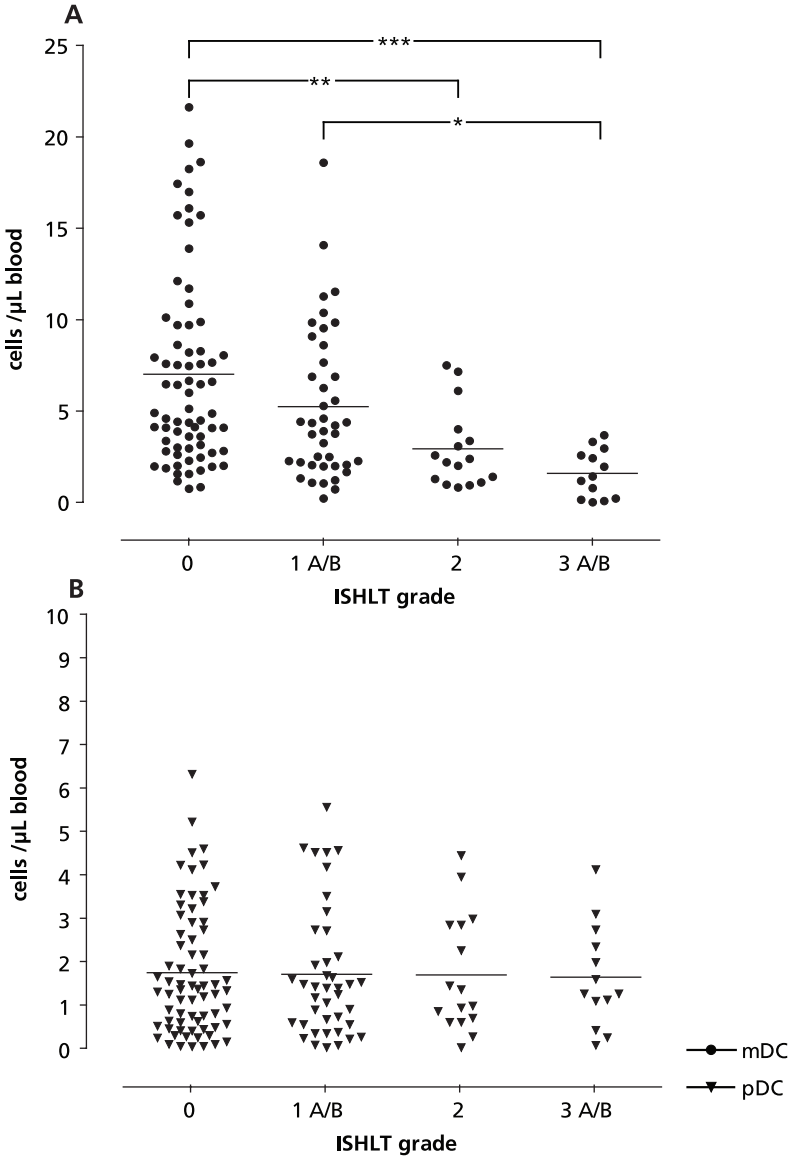

Figure 3: Incidence of peripheral blood (A) mDCs and (B) pDCs with ISHLT grade of 140 endomyocardial biopsies acquired within 9 months post-HTx. p-values were < 0.0001 for mDCs and 0.996 for pDCs (one way ANOVA); *p < 0.05, **p < 0.01, ***p < 0.001 represent outcome of the Tukey's multiple comparisons test for mDCs.

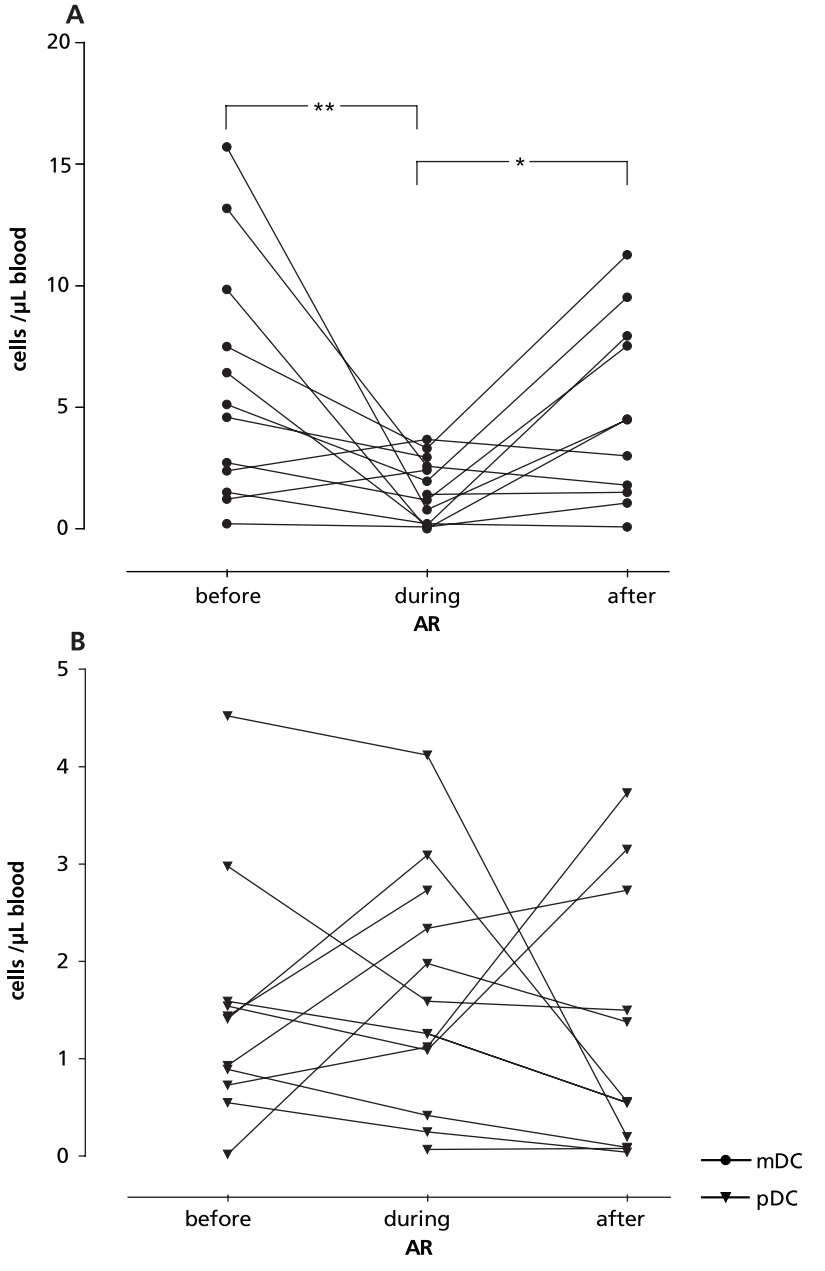

Figure 4: Peripheral blood (A) mDC and (B) pDC absolute numbers of 14 rejecting patients post-HTx before, during and after 13 acute rejection (AR) episodes caught during 9 months follow-up. *$p < 0.05$, **$p < 0.01$ represents the outcome of paired samples analysis for mDCs; pDC numbers did not alter significantly during the same time-intervals ($p = 0.557$ and $p = 0.339$, respectively).

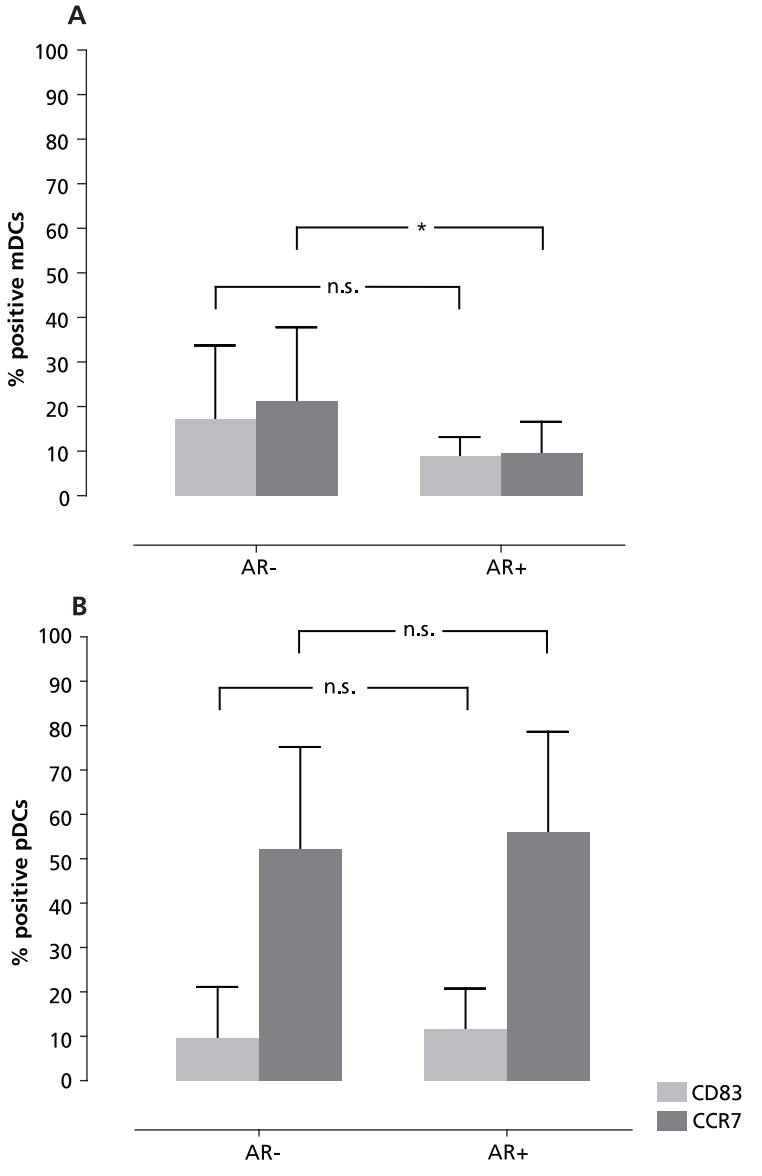

Figure 5: Percentage of positive (a) mDCs and (b) pDCs for CD83 and CCR7 in the peripheral blood of 14 patients which experienced at least one rejection episode during 9 months post-HTx. Episodes with no rejection (AR-: 0, n = 44; 1A/1B; n = 23; 2, n = 10) were compared to 13 acute rejection (AR+) episodes. n.s. not significant; *p < 0.05 paired samples analysis.

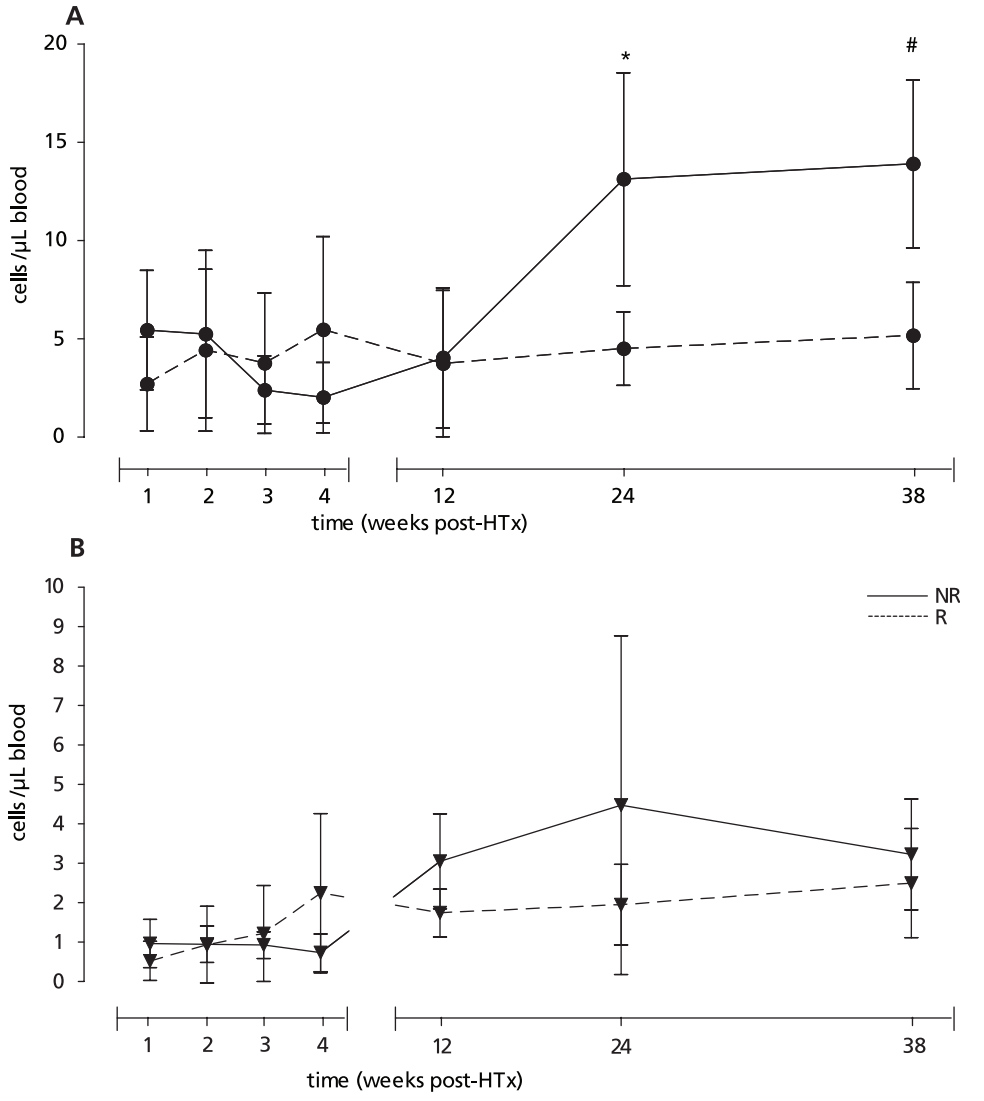

Figure 6: Temporal mDC (A) and pDC (B) subset distribution in peripheral blood of 7 non-rejecting (NR) and 14 rejecting (R) recipients up to 9 months post-HTx. *$p < 0.05$, #$p < 0.0001$ represent the outcome of between groups one-way ANOVA comparisons.

Rejector and non-rejector mDC and pDC numbers in time

One patient was diagnosed to reject 3 times during follow-up (wk 1, wk 4 and wk 12). For 3 rejecting HTx recipients no data on AR episodes were caught in our follow-up. Sequential analysis performed for the whole period designated lower mDC numbers in time, to patients who had experienced biopsy-proven AR, when compared to NR patients who started recovering in their mDC numbers

after 12 weeks post-HTx (Figure 6A). At that time, it became obvious that this difference could not be seen for pDCs (Figure 6B). However, the respective mDC recovery and depletion observed in NR and R patients, was not influenced by manifestation of infections or withdrawal of immunosuppressants post-HTx. Patients receiving statin therapy post-HTx were found to be distributed equally amongst the NR and R groups (100% and 93%, repectively). Median time of CMV disease onset post-HTx was 79 days (range 40-122) for the NR group and 96 days (range 48-139) for the R group. Consequently, MMF withdrawal for NR and R groups occurred in 130 days (range: 121-182) and 146.5 days (102-212) post-HTx, respectively. Repeated measures ANOVA analysis between R or NR subjects showed that CMV disease development or MMF use were factors that did not affect mDC outcome significantly, up to 38 weeks post-HTx ($p = 0.980$ and $p = 0.900$, respectively).

Discussion

We investigated peripheral blood DC incidence, phenotype and subset distribution in an attempt to reveal a possible mechanism by which circulating bone marrow-derived DCs may be involved in the initiation or progression of AR after clinical HTx. Dendritic cell homeostasis is altered considerably after transplantation as DC incidence and subset distribution differed substantially between the recipients pre- and post-HTx and normal subjects. The overall decrease in circulating mDC and pDC numbers must be ascribed to the controlled immunosuppression (h-ATG, Prednisone, CsA or FK506 and MMF), administered post-HTx. Anti-thymocyte globulin (ATG) has been demonstrated to bind both immature and mature human dendritic cell subsets and induce complement-mediated DC lysis, *in vitro* [19]. Prednisone reduces circulating pDC numbers [20] and inhibits their function *in vivo* [21], an effect which may explain the abrupt and sustained pDC shortage for the whole period post-HTx. Evidence suggests that corticosteroids and calcineurin inhibitors not only restrain immature DC differentiation from their hemopoietic progenitors [22,23] but can also inhibit DC migration to lymphoid tissues [24] by competitive inhibition of the lipid transporters Abcb1 (p-glycoprotein, MDR1) and Abcc1 (multidrug resistance protein 1, MRP1) [25]. Specifically, CsA may interfere with DC recirculation also through cyclooxygenase 2 (COX2) inhibition or prostaglandin E2 (PGE2) uncoupling with CCR7 [26-28]. On the one hand, statins are known to impair DC maturation by down-regulating CD83 and CCR7 and inhibit both antigen

capture capacity and T-stimulatory activity as part of their anti-inflammatory potential [29-30]. On the other hand, FK506 and the anti-proliferative MMF seem to be of minor influence to DC differentiation, maturation or migration, although both agents suppress DC allo-co-stimulatory capacity by decreasing TNFα and IL12 secretion, respectively [31].

During each AR episode mDC numbers decreased markedly and we observed that mDCs were negatively associated with EMB pathological grade of rejection, while pDCs remained constantly low. Rejecting patients did not exhibit the mDC recovery that non-rejectors experienced after 3 months post-HTx. Despite convincing evidence of immunosuppressive drugs affecting blood DC trafficking, to our knowledge no reports exist in supporting differential *in vivo* DC subset kinetics during AR after clinical HTx. Conceptually, the mDC profiles of rejectors and non-rejectors in time might be explained by quantitative differences in their immunosuppressive regimen. However, in our patient cohort, rejectors and non-rejectors received comparable prednisone dosages and experienced similar trough levels of CsA, FK506 and MMF for the whole period of follow-up. Additionally, MMF therapy was discontinued for a comparable number of rejectors and non-rejectors. Both groups were further comparable with respect to age, which might influence DC subset distribution substantially [32]. Donor age was also similar between the R and NR groups. Manifestation and onset time of a major infectious complication post-HTx such as CMV disease, which induces a severe but transient immunosuppression in the host [33], was comparable between rejectors and non-rejectors. Furthermore, patients receiving statin therapy post-HTx were found to be equally distributed amongst the NR and R groups. Thus, rejecting HTx recipients undergo circulating mDC depletion over time, independent of infection onset, statin treatment or tapering of maintenance immunosuppression. Nonetheless, additional anti-rejection therapy (methyl-prednisolone) cannot be excluded when accounting for the long-term disparity between mDC numbers of rejectors and non-rejectors post-HTx. However, such a prolonged effect *in vivo* is highly unlikely as steroids, the only form of anti-rejection therapy administered to this cohort, are implicated in DC redistribution by selectively reducing only circulating pDC numbers [20].

The immunostimulatory capacity of DCs has been shown to depend on membrane-bound CD83 [34,35]. In our patients, the percentage $CD83^+$ circulating mDCs or pDCs declined significantly post-HTx. Analyzed sequentially, no significant differences were found between rejectors and non-rejectors during follow-up. Except for immunosuppression, this effect may reside on CMV as mature DCs have been shown to downregulate CD83 expression upon

infection with human CMV (hCMV) [36]. On the other hand, monocyte derived DCs infected with hCMV do not attain different CCR7 expression, but only an altered expression of CD11c, CD33, CD54 and CD58 [37]. Thus, it is conceivable that the decrease of circulating %CCR7$^+$ mDCs relies only on CsA use post-HTx. However, it is interesting that rejectors experienced a decreased % of CCR7$^+$ mDCs in their blood during AR. Seeing blood purely as a DC trafficking module post-HTx, uniformly altered DC kinetics must reflect an inherent mechanism of circulating mDC depletion during AR. We speculate that low % CCR7$^+$ mDCs suggest selective mDC homing to secondary lymphoid tissues in order to induce T-cell clonal expansion and differentiation into CD4$^+$ helper or cytotoxic CD8$^+$ cells and therefore initiate AR.

The limitation of this study is in the nature of the chosen follow-up, which inadvertently lead to some loss of AR episodes due to a large variance in the time-intervals between measurements before, during and after rejection for some rejectors. However, we feel that censoring for AR grade was useful as follow-up time was the same for all patients. Looking at the chosen time for venous puncture (during routine EMB procedure and before histological diagnosis of AR), we have avoided any adverse effects caused by circadian rhythm or administration of anti-rejection therapy at the time of blood sampling.

Experience on DC reconstitution after allogeneic stem cell transplantation dictates that low circulating mDC and pDC numbers for up to 6 months can be imposed by the administration of lymphocyte depleting antibodies [38]. However, as such protocols exert profound inhibitory effects on the bone marrow, they cannot be compared to the effects of polyclonal ATG, which are restricted to the intravascular and lymphoid compartments [39]. Induction analogous to ATG, via Campath-1G antibody (CD52), causes early depletion of both circulating mDCs and pDCs but late defective reconstitution for pDCs only, at 1 year after stem cell transplantation [40]. Comparable effects were described recently for both induction via ATG or CD52, after human lung transplantation [41,42]. In our patients, we speculate that both mDCs and pDCs regenerated in an abberant fashion due to h-ATG induction, early post-HTx. However, since the intensity of this therapy did not differ between rejecting and non-rejecting recipients, we conclude that the long-term differences observed in mDCs post-HTx are not caused by the induction therapy. Furthermore, non-rejecting patients on maintenance immunosuppression experienced lower DC levels, but exhibited comparable DC subset distribution to normal adult controls after 6 months post-HTx. This suggests further that a normal peripheral blood DC reconstitution late post-HTx, is associated with allograft non-responsiveness

independent of induction therapy. Moreover, it is intriguing that low circulating DC counts have been demonstrated to characterize patients at risk for relapse and graft-versus-host disease late after stem cell transplantation [43] or acute rejection shortly after kidney transplantation [44]. To us, these findings imply a basic immunological phenomenon, occurring irrespective of the transplanted organ and independent of induction therapy.

One may further reckon that the differences in mDC numbers between rejectors and non-rejectors post-HTx could be based on the reappearance of circulating donor derived DCs. Indeed, late blood APC microchimerism has been demonstrated to arise due to the survival of a few donor-type hematopoietic stem cells originating from the organ graft [45]. However, in clinical heart transplantation, allograft derived circulating DC progenitors appear only after cessation of therapeutic immunosuppression [46]. As their frequency in cardiac allografts has been shown to be extremely low under induction with antibody depleting protocols [47], we do not believe that donor derived mDCs are the explanation for the overall elevation of mDC numbers for the non-rejector group after 6 months post-HTx.

Identifying potential causes of low blood DC counts post-HTx warrants further investigation as adequate reconstitution of mDCs but not pDCs appears to be important for improved outcomes within the 1st year post-HTx. DC profiling might reflect the status of HTx recipients with respect to risk for AR after 3 months post-HTx, but extended prospective follow-up studies of both rejector and non-rejector cohorts are needed to identify patients dependent on anti-rejection therapy long post-HTx. Understanding the role of chemokines in the recruitment and activation of DCs post-HTx are likely, with time, to yield important new targets for efforts to promote tolerance induction.

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References

1. Hosenpud JD, Bennett LE, Keck BM, Boucek MM, Novick RJ. The Registry of the International Society for Heart and Lung Transplantation: eighteenth Official Report-2001. J Heart Lung Transplant 2001;20(8):805-15.
2. O'Connell PJ, Burlingham WJ. Donor dendritic cell persistence in organ allograft recipients in the absence of immunosuppression. J Leukoc Biol 1999;66(2):301-5.
3. Maksymowicz M, Olszewski WL, Cybulska E. Repopulation of donor heart by recipient bone marrow-derived dendritic cells prior to transplantation causes acute rejection by both the allogeneic and syngeneic recipient. Transplant Proc 2003;35(6):2374-5.
4. Grouard G, Rissoan MC, Filgueira L, Durand I, Banchereau J, Liu YJ. The enigmatic plasmacytoid T-cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. J Exp Med 1997;185(6):1101-11.
5. Kuwana M. Induction of anergic and regulatory T-cells by plasmacytoid dendritic cells and other dendritic cell subsets. Hum Immunol 2002;63(12):1156-63.
6. Zou W, Borvak J, Marches F, Wei S, Galanaud P, Emilie D, et al. Macrophage-derived dendritic cells have strong T_H1-polarizing potential mediated by beta-chemokines rather than IL-12. J Immunol 2000;165(8):4388-96.
7. Tanaka H, Demeure CE, Rubio M, Delespesse G, Sarfati M. Human monocyte-derived dendritic cells induce naïve T-cell differentiation into T helper cell type 2 (T_H2) or T_H1/T_H2 effectors. Role of stimulator/responder ratio. J Exp Med 2000;192(3):405-12.
8. Liu YJ, Kanzler H, Soumelis V, Gilliet M. Dendritic cell lineage, plasticity and cross-regulation. Nat Immunol 2001;2(7):585-9.
9. Romani N, Gruner S, Brang D, Kampgen E, Lenz A, Trockenbacher B, et al. Proliferating dendritic cell progenitors in human blood. J Exp Med 1994;180(1):83-93.
10. Zhou LJ, Tedder TF. Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. J Immunol 1995;154(8):3821-35.
11. Kohrgruber N, Halanek N, Groger M, Winter D, Rappersberger K, Schmitt-Egenolf M, et al. Survival, maturation, and function of CD11c- and CD11c+ peripheral blood dendritic cells are differentially regulated by cytokines. J Immunol 1999;163(6):3250-9.
12. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, et al. Immunobiology of dendritic cells. Annu Rev Immunol 2000;18:767-811.
13. von Andrian UH, Mempel TR. Homing and cellular traffic in lymph nodes. Nat Rev Immunol 2003;3(11):867-78.
14. Penna G, Vulcano M, Sozzani S, Adorini L. Differential migration behavior and chemokine production by myeloid and plasmacytoid dendritic cells. Hum Immunol 2002;63(12):1164-71.
15. Hancock WW, Wang L, Ye Q. Chemokine-directed dendritic cell trafficking in allograft rejection. Current Opinion in Organ Transplantation 2003;8:35-39.

16. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E, et al. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell 1999;99(1):23-33.
17. Billingham ME, Cary NR, Hammond ME, Kemnitz J, Marboe C, McCallister HA, et al. A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group. The International Society for Heart Transplantation. J Heart Transplant 1990;9(6):587-93.
18. Athanassopoulos P, Vaessen LM, Maat AP, Balk AH, Weimar W, Bogers AJ. Peripheral blood dendritic cells in human end-stage heart failure and the early post-transplant period: evidence for systemic T_H1 immune responses. Eur J Cardiothorac Surg 2004; 25(4):619-26.
19. Monti P, Allavena P, Di Carlo V, Piemonti L. Effects of anti-lymphocytes and anti-thymocytes globulin on human dendritic cells. Int Immunopharmacol 2003;3(2):189-96.
20. Moser M, De Smedt T, Sornasse T, Tielemans F, Chentoufi AA, Muraille E, et al. Glucocorticoids down-regulate dendritic cell function in vitro and in vivo. Eur J Immunol 1995;25(10):2818-24.
21. Shodell M, Siegal FP. Corticosteroids depress IFN-alpha-producing plasmacytoid dendritic cells in human blood. J Allergy Clin Immunol 2001;108(3):446-8.
22. Piemonti L, Monti P, Allavena P, Sironi M, Soldini L, Leone BE, et al. Glucocorticoids affect human dendritic cell differentiation and maturation. J Immunol 1999;162(11):6473-81.
23. Woltman AM, Massacrier C, de Fijter JW, Caux C, van Kooten C. Corticosteroids prevent generation of CD34+-derived dermal dendritic cells but do not inhibit Langerhans cell development. J Immunol 2002;168(12):6181-8.
24. Cumberbatch M, Dearman RJ, Kimber I. Inhibition by dexamethasone of Langerhans cell migration: influence of epidermal cytokine signals. Immunopharmacology 1999;41(3):235-43.
25. Randolph GJ. Dendritic cell migration to lymph nodes: cytokines, chemokines, and lipid mediators. Semin Immunol 2001;13(5):267-74.
26. Luft T, Jefford M, Luetjens P, Toy T, Hochrein H, Masterman KA, et al. Functionally distinct dendritic cell (DC) populations induced by physiologic stimuli: prostaglandin E(2) regulates the migratory capacity of specific DC subsets. Blood 2002;100(4):1362-72.
27. Chen T, Guo J, Yang M, Han C, Zhang M, Chen W, et al. Cyclosporin A impairs dendritic cell migration by regulating chemokine receptor expression and inhibiting cyclooxygenase-2 expression. Blood 2004;103(2):413-21.
28. Scandella E, Men Y, Legler DF, Gillessen S, Prikler L, Ludewig B, et al. CCL19/CCL21-triggered signal transduction and migration of dendritic cells requires prostaglandin E2. Blood 2004;103(5):1595-601.
29. Sun D and Fernandes G. Lovastatin inhibits bone marrow-derived dendritic cell maturation and upregulates proinflammatory cytokine production. Cell Immunol 2003; 223(1): 52-62.

30. Yilmaz A, Reiss C, Tantawi O, Weng A, Stumpf C, Raaz D, Ludwig J, Berger T, Steinkasserer A, Daniel WG, Garlichs C D. HMG-CoA reductase inhibitors suppress maturation of human dendritic cells: new implications for atherosclerosis Atherosclerosis 2004; 172(1): 85-93.
31. Lagaraine C, Lebranchu Y. Effects of immunosuppressive drugs on dendritic cells and tolerance induction. Transplantation 2003;75(9 Suppl):37S-42S.
32. Teig N, Moses D, Gieseler S, Schauer U. Age-related changes in human blood dendritic cell subpopulations. Scand J Immunol 2002;55(5):453-7.
33. Lautenschlager. Cytomegalovirus and solid organ transplantation: an update. Curr Opin Organ Transplant 2003;8:269-275.
34. Lechmann M, Berchtold S, Hauber J, Steinkasserer A. CD83 on dendritic cells: more than just a marker for maturation. Trends Immunol 2002;23(6):273-5.
35. Lechmann M, Zinser E, Golka A, Steinkasserer A. Role of CD83 in the immunomodulation of dendritic cells. Int Arch Allergy Immunol 2002;129(2):113-8.
36. Beck K, Meyer-Konig U, Weidmann M, Nern C, Hufert FT. Human cytomegalovirus impairs dendritic cell function: a novel mechanism of human cytomegalovirus immune escape. Eur J Immunol 2003;33(6):1528-38.
37. Senechal B, Boruchov AM, Reagan JL, Hart DN, Young JW. Infection of mature monocyte-derived dendritic cells with human cytomegalovirus inhibits stimulation of T-cell proliferation via the release of soluble CD83. Blood 2004;103(11):4207-15.
38. Damiani D, Stocchi R, Masolini P, Michelutti A, Geromin A, Sperotto A, Skert C, Michieli M, Baccarani M, Fanin R. CD34+-selected versus unmanipulated autologous stem cell transplantation in multiple myeloma: impact on dendritic and immune recovery and on complications due to infection. Ann Oncol 2003; 14(3): 475-80
39. Mueller TF. Thymoglobulin: an imunologic review. Current Opinion in Organ Transplantation 2003; 8(4): 305-312.
40. Klangsinsirikul P, Carter GI, Byrne JL, Hale G, Russell NH. Campath-1G causes rapid depletion of circulating host dendritic cells (DCs) before allogeneic transplantation but does not delay donor DC reconstitution. Blood 2002; 99(7): 2586-91.
41. Zeevi A, Girnita AL, Spichty K, Zaldonis D, McDade K, Metes D, Iacono AT, Yousem SA, McCurry KR. The impact of T-cell depletion on immune recovery in lung transplant recipients (abstract). Transplantation 2004; 78(2): (O 477), 187-188.
42. McCurry KR, Zeevi A, Iacono A, Zaldonis DB, Bentlejewski C, Spichty K, Metes D, Murase N, Starzl TE. Outcomes following pretransplant T-cell depletion with thymoglobulin or campath in human lung transplantation (abstract). Transplantation 2004; 78(2): (O 478), 188.
43. Reddy V, Iturraspe JA, Tzolas AC, Meier-Kriesche HU, Schold J, Wingard JR. Low dendritic cell count after allogeneic hematopoietic stem cell transplantation predicts relapse, death, and acute graft-versus-host disease. Blood 2004;103(11):4330-5.
44. Saresella M, Marini M, Guerini F, Ferrante P, Della Bella S, Chiaramonte S, et al. Peripheral blood dendritic cells increase in kidney-transplant patients without rejection. Clin Immunol 2004;110(2):191-3; author reply 194.

45. Larsen CP, Morris PJ and Austyn JM. Migration of dendritic leukocytes from cardiac allografts into host spleens. A novel pathway for initiation of rejection. J Exp Med 1990;171(1): 307-14
46. O'Connell PJ and Burlingham WJ. Donor dendritic cell persistence in organ allograft recipients in the absence of immunosuppression. J Leukoc Biol 1999; 66 (2): 301-5.
47. Ko S, Deiwick A, Jager MD, Dinkel A, Rohde F, Fischer R, Tsui TY, Rittmann KL, Wonigeit K and Schlitt HJ. The functional relevance of passenger leukocytes and microchimerism for heart allograft acceptance in the rat. Nat Med 1999; 5(11): 1292-7.

Chapter 6

Impaired Circulating Dendritic Cell Reconstitution Identifies Rejecting Recipients after Clinical Heart Transplantation Independent of Rejection Therapy

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Abstract

Objective: Dendritic cell (DC) mediated allo-antigen presentation to host antigen specific T-lymphocytes initiates acute allograft rejection. We investigated peripheral blood DC (PBDC) incidence and DC subset reconstitution in relation to histological diagnosis of acute cellular rejection (AR) and administration of rejection therapy after clinical heart transplantation (post-HTx).

Methods: Venous blood from 20 HTx recipients under standard immunosuppression was collected during serial endomyocardial biopsy (EMB) prior to administration of rejection therapy in a 9-month follow-up post-HTx. Echocardiographic assessment of allograft function during EMB was performed to distinguish clinical necessity for rejection therapy within histologically rejecting patients (R). Myeloid (mDC) and plasmacytoid (pDC) subsets identified by flow cytometry were analyzed for different ISHLT rejection grades. Circulating PBDC incidence and mDC/pDC ratio were compared sequentially between non-rejecting (NR) recipients and R patients treated ($3A^+$) or not-treated ($3A^-$) with rejection therapy during follow-up.

Results: Eleven samples from biopsy-proven AR episodes (AR^+: ISHLT ≥ 3) were compared to 89 samples from non-rejection episodes (AR^-: ISHLT grade 0, n = 52; grade 1, n = 29; grade 2, n = 8). We observed an inverse correlation of mDCs ($p < 0.05$) but not pDCs with increasing rejection grade. PBDC incidence and mDC/pDC ratio were low in blood samples obtained during AR ($p < 0.05$ and $p < 0.01$, respectively). Both PBDCs and mDC/pDC ratio decreased during each AR episode ($p < 0.05$). Comparison of $3A^+$ and $3A^-$ rejectors with NR patients after 12 weeks post-HTx revealed lower PBDC incidence ($p < 0.01$) and mDC/pDC ratio ($p < 0.05$) for R patients, independent of rejection therapy.

Conclusions: Defective DC subset reconstitution by dendritic cell profiling identifies patients at risk for AR after 3 months post-HTx. This finding may contribute to further optimization of immunosuppressive treatment strategies after clinical heart transplantation.

Introduction

Macrophages, neutrophils and natural killer (NK) cells are intrinsic components of early non-specific allograft damage induced by ischaemia-reperfusion injury after heart transplantation (HTx) [1,2]. Acute rejection (AR) through allo-specific T-cell graft infiltration remains the leading cause of cardiac allograft dysfunction in the first months post-HTx [3]. The latter is initiated by the presentation of foreign allo-antigen (allo-Ag) to T-lymphocytes by antigen presenting cells (APCs) such as donor or recipient dendritic cells (DCs) [4].

Two major DC subsets have been identified in human circulation [5]. The $CD11c^+$ subset belongs to the myeloid lineage, whereas the $CD11c^-$ subset, recognized as plasmacytoid cells, expresses the IL-3 receptor-α chain (CD123) and consists of the direct precursors of lymphoid DCs [6]. Peripheral blood DCs (PBDCs) emerge in an immature state from bone marrow progenitors and traffic via blood to non-lymphoid tissues, where they exhibit high phagocytic capacity for antigens (Ags). Upon Ag encounter, DC subsets undergo multi-step maturation and present Ags in the lymph nodes. In this process, DC chemokine receptor expression is altered from an inflammatory to a lymphoid homing mode [7]. Major histocompatibility class I and II molecules, several adhesion (CD11a, CD18, CD44 & CD54) and co-stimulatory molecules (CD40, CD80, CD86) are increased on their surface, while DCs start secreting pro-inflammatory cytokines like IL-1β, IL-6, IL-12, IL-10 and IL-23 [8].

After transplantation, DCs induce anti-donor responses through the direct or indirect presentation of donor Ags [9]. Myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) enter the T-cell areas of secondary lymphoid organs via the blood and drive the proliferation and differentiation of donor specific T-cells [10]. As mDC primed T-lymphocytes are able to differentiate into effector $CD4^+$ or cytotoxic $CD8^+$ cells [11] it is postulated that mDCs are the primary instigators of allograft rejection post-HTx. On the other hand, as pDC primed T-lymphocytes also differentiate into regulatory T-cells, it is suggested that pDCs may promote allograft tolerance [12]. Accordingly, peripheral blood DC subset balance is believed to be critical for the development of allograft acceptance. However, clinical doses of induction and maintenance immunosuppressive regimens are known to negatively affect peripheral blood DC subset Ag presenting capacity as well as maturation, migration and differentiation from their haemopoietic progenitors after transplantation [13]. Interestingly, patients at risk for AR are known to differ substantially in the mDC/pDC ratio from patients successfully withdrawn from immunosuppression [14].

The aim of this study was to look into circulating DC subset kinetics in relation to histological grading of AR and administration of rejection therapy after heart transplantation. Given that 1 week post-HTx circulating mDC and pDC numbers decrease significantly [15], we investigated peripheral blood DC numbers and the mDC/pDC ratio as a measure of DC subset reconstitution in a prospective 9 month follow-up post-HTx. Non-rejecting recipients were compared to patients with biopsy proven AR episodes in order to explore whether DC content might reflect their status with respect to risk for AR. Rejecting recipients were analyzed according to rejection treatment in order to investigate dependence of DC profile on rejection therapy post-HTx.

Patients and Methods

Patients

We studied 20 adult HTx recipients (11 men and 9 women, mean age 51.6 years, range 37-65 years) operated between June 2002 and December 2003. All patients were classified with chronic symptomatic but clinically stable NYHA III-IV heart failure and had experienced a standardised medical regimen before transplantation [15]. One patient had already received maintenance immunosuppression pre-HTx, as he was re-transplanted due to graft vascular disease of an 11-year old cardiac allograft. None of the patients presented with any major co-morbidity pre-HTx. 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 written informed consent before entering the study.

Endomyocardial biopsies

Scheduled right venticular endomyocardial biopsies (EMB) were performed weekly until week 6, once every two weeks until week 10, monthly up to 6 months and once every two months up to the end of the first year post-HTx. Clinical course and prior rejection profile of each individual patient triggered EMB rescheduling in 2- to 4- weekly intervals late during follow-up. Using light microscopy, rejection grade was evaluated by two cardiac pathologists on 6 tissue samples extracted during each EMB procedure, according to the standard working formulation of the International Society for Heart and Lung Transplantation (ISHLT) [16]. Hematoxylin and eosin stained sections were also

analyzed to exclude Quilty effect as the etiology of lymphocytic infiltration. Patients with ISHLT grade ≥ 3 episodes were considered to experience acute cellular rejection.

Echocardiography

All patients underwent trans-thoracic echocardiography in order to assess left ventricular (LV) function within 4 hours of each EMB procedure, using a Hewlett Packard Sonos 5500 utrasonograph with a 3.75 MHz transducer (Hewlett Packard, Andover, MA, USA). LV wall dimensions were obtained by parasternal M-mode recordings combined with an electrocardiogram. Mitral flow velocities were measured within the valve orifice near the leaflet tips by two-dimensional Doppler echocardiography through an apical four-chamber view. M-mode echocardiograms were analyzed for end-diastolic total wall thickness (TWT), calculated by adding posterior left ventricular wall thickness and end-diastolic interventricular septum thickness. Overall changes in systolic function were assessed by "eyeballing" technique. Diastolic function was evaluated by peak early (E) / peak atrial (A) mitral flow velocity (E/A ratio) and deceleration time of E (DT). Recordings were analyzed by a single investigator without knowledge of EMB outcome.

Immunosuppressive regimen

After transplantation, all HTx patients received anti-thymocyte globulin (ATG) and triple therapy consisting of steroids, Cyclosporine A (CsA, Novartis, Basel, Switzerland) or Tacrolimus (FK506, Fujisawa GmbH, Munich, Germany) and Mycophenolate Mofetil (MMF, Roche, Basel, Switzerland) as maintenance immunosuppression, for the whole post-HTx period. Horse-ATG (Imtix Sangstat BV., Lyon, France) at 3 to 8 i.v. dosages daily (1 dosage = 212,5 lymphocytotoxic-units/kg/24hrs) was administered until adequate CsA or FK506 trough levels were achieved. All patients received 75mg prednisolone i.v. in the first post-transplant day, 50mg/day for 5, 40mg/day for 3 and 30mg/day for 2 consecutive days post-HTx. Thereafter, orally administered prednisone was tapered with 5mg decrements up to 20mg/day for 3 days and then with 2.5mg decrements every 7 days up to 10mg/day. CsA and FK506 were started at 8mg/kg/24hrs and 0.3mg/kg/24hrs oral dosages respectively, divided in 2 doses daily which were titrated further according to the corresponding trough levels. MMF was started at the end of the induction therapy in doses between 2000 and 3000mg, daily. CMV syndrome, as manifested by symptoms and a positive PCR, was treated with ganciclovir (5 mg/kg, 2 times daily) for 10-14 days. In case of

resistance to this therapy, MMF was withdrawn from the immunosuppressive regimen. Patients presenting with ISHLT grade ≥ 3A biopsies were considered for treatment of rejection with additional immunosuppression in the form of methyl-prednisolone i.v. for 3 days at 1000 mg/day (Pfizer, Pharmacia & Upjohn, Sandwich, UK). In case of minor infiltrates and when myocardial damage was considered too mild to warrant rejection therapy, echocardiographic assessment was used to decide upon rejection treatment: in such cases rejection therapy was omitted when systolic and diastolic LV function were normal or unchanged compared to earlier measurements.

Monoclonal antibodies

Allophycocyanin (APC)-conjugated CD11c (clone S-HCL-3), phycoerythrin (PE)-conjugated anti-IL-3 receptor α chain (CD123; clone 9F5), peridinin chlorophyll protein (PerCP)-conjugated anti-HLA-DR (clone L243) and fluorescein isothiocyanate (FITC)-conjugated lineage cocktail 1 (Lin 1) were commercially obtained (Becton Dickinson Biosciences, San Jose, CA, USA). The Lin 1 contained monoclonal antibodies (mAbs): CD3 (T-cells; clone SK7), CD14 (monocytes/macrophages; clone MΦP9), CD16 (natural killer cells; clone 3G8), CD19 (B cells; clone SJ25C1), and CD56 (natural killer cells, clone NCAM16.2). Mouse anti-human CD45 FITC (clone F10-89-4) / CD14 PE (clone UHCM-1) reagent (Serotec, Oxford, UK) was used to monitor lymphocyte, monocyte and granulocyte presence in each sample.

Dendritic cell characterization

Patient peripheral blood cells were collected at 1, 4, 12, 24 and 38 weeks post-HTx, during the EMB procedure and before histological diagnosis of AR. Whole blood cells were incubated with mAbs followed by erythrocyte lysis with FACS lyzing solution (BD Biosciences) at room temperature. After washing with FACSflow (BD Immunocytometry Systems), 200000-300000 events were analyzed in a FACSCalibur fluorescence-activated cell sorter with the CellQuest Pro software program (BD Biosciences). White blood cell (WBC) numbers were determined by a Sysmex Microcellcounter F-300 automated cell counter, (Goffin Meyvis, Etten Leur, The Netherlands). DC characterization was blinded for occurrence of AR at the time of blood sampling. CD11c and CD123 expression was determined within Lin^- $HLA\text{-}DR^+$ cells in order to define mDC ($CD11c^{high}$ $CD123^{low}$) and pDC ($CD11c^{low}$ $CD123^{high}$) subsets, as described before [15]. Absolute mDC and pDC numbers were calculated from the WBC count multiplied by the proportion of each subset within WBCs. DC incidence

was calculated by adding absolute mDC and pDC numbers. The mDC/pDC ratio was used as a means to express circulating DC reconstitution post-HTx. Assay reproducibility has been confirmed previously, as DC sample analysis in healthy controls revealed stable % DC, absolute numbers of mDCs and pDCs as well as a constant mDC/pDC ratio, within the period of 1 month [17].

Statistical analysis

Results are expressed as mean ± SEM of absolute white blood cell, lymphocyte, monocyte, granulocyte and total DC numbers. All continuous data sets were tested before comparisons in order to assess whether the assumption of normality was met. The Mann Whitney *U*-test and the paired Wilcoxon samples *t*-test were employed to compare differences between means as appropriate. Correlation of mDC/pDC ratio with ISHLT grade was performed by linear regression analysis after $\log_{10}$ transformation of the data using the Pearson's (r) correlation coefficient. One-way analysis of variance (ANOVA) was used for comparisons of clinical parameters between groups of patients and DC counts or mDC/pDC ratio between different rejection grades. Post-hoc analysis was performed to compare DC counts and mDC/pDC ratio for each rejection grade separately, using the Tukey's test for multiple comparisons. Continuous DC number and mDC/pDC ratio data of different groups of patients were compared by repeated measurements ANOVA. A p-value < 0.05 was considered significant. SPSS 11.0.1 software (Chicago, IL, USA) and the GraphPad statistical program (San Diego, CA, USA) were applied for analyses and graphics, respectively.

Results

Clinical characteristics

After transplantation, 14 of the 20 patients developed rejection in the whole period of 9-months follow-up. Within this group, 11 patients presented with impaired diastolic function during AR as assessed by echocardiography and therefore received rejection therapy ($3A^+$) on one or more occasions during this period. However, 3 of the 14 rejecting patients, revealed once histological signs of AR but did not receive any rejection therapy after normal diastolic heart function assessment at the time of EMB ($3A^-$). Infection was the most prominent complication for both non-rejecting (NR) and rejecting (R) groups of patients post-HTx (Table 1). In total 11 patients presented with CMV

syndrome post-HTx, and for 8 of those MMF therapy was discontinued. Mean time of CMV syndrome onset between non-rejectors (83.4 ± 12.8 days) and the $3A^+$ or $3A^-$ rejectors (96.7 ± 17.7 and 84.0 ± 13.0 days, respectively) was not significantly different (p = 0.79). Similarly, mean time for MMF withdrawal was comparable (p = 0.66) between the NR (147.6 ± 12.7 days) and the $3A^-$ or $3A^+$ rejecting subjects (124.7 ± 21.7 and 158.4 ± 16.8 days, respectively). Seven patients were converted to FK506 after experiencing calcineurin inhibitor related renal insufficiency post-HTx (2 in the 1st, 1 in the 2nd, 2 in the 3rd, 1 in the 13th and 1 in the 18th week follow-up). Maintenance steroid dosages and acquired trough levels of immunosuppressive drugs were comparable between R and NR patients. However, $3A^+$ rejecting patients tended towards higher CsA trough levels (310.0 ± 19.8 ng/mL) than $3A^-$ rejectors (272.3 ± 25.8 ng/mL) and NR patients (233.6 ± 19.5 ng/mL) for the whole period of follow-up (*P* = 0.06). No differences were observed in immunologic or operation-related parameters between the 3 patient groups (Table 2).

Table 1: Complications for rejecting and non-rejecting patients after heart transplantation

	NR	R
Patients (n)	6	14
CNI related nephrotoxicity (%)	5 (83)	2 (14)
Infections		
CMV syndrome, total (%)	3 (50)	8 (57)
primary infection	2	5
reactivation	1	3
Other		
surgical wound	3	1
respiratory	4	7
oropharyngeal	4	7
urogenital	8	2
sepsis	1	1

NR, non-rejectors; R, rejectors; CNI, calcineurin inhibitor; CMV, cytomegalovirus; Percentages (%) are calculated from the total number of NR and R patients. Other post-transplant infection episodes are depicted accordingly

Table 2: Clinical outcomes for non-rejecting and rejecting recipients post-HTx

	NR	R		P value
		3A–	3A+	
n	6	3	11	
Gender (m/f)	4/2	2/1	7/4	
Age, years	56.3 ± 2.4	55.3 ± 2.0	48.9 ± 2.6	0.201
Transplant procedure				
ECC perfusion time (hrs)	1.9 ± 0.3	2.0 ± 0.5	2.1 ± 0.2	0.966
aortic occlusion time (hrs)	1.1 ± 0.1	1.1 ± 0.3	1.0 ± 0.1	0.884
total ischemia time (hrs)	2.6 ± 0.3	2.9 ± 0.4	2.5 ± 0.2	0.568
HLA mismatches				
HLA-A	1.8 ± 0.2	1.3 ± 0.7	1.1 ± 0.3	0.334
HLA-B	2.0 ± 0.1	1.7 ± 0.3	1.9 ± 0.1	0.388
HLA-DR	1.5 ± 0.2	1.7 ± 0.3	1.6 ± 0.2	0.924

NR, non-rejectors; R, rejectors; ECC, extracorporeal circulation; HLA, human leukocyte antigen; All values are expressed as mean ± SEM.

DC numbers after heart transplantation

Table 3 shows the transplant recipient haematologic characteristics pre- and at 1, 4, 12, 24 and 38 weeks post-HTx. Leukocytes were elevated significantly at 1-week post-HTx compared to the pre-HTx condition ($p = 0.02$), as granulocytes increased ($p = 0.04$) early post-HTx. Blood monocyte counts remained unaffected while lymphocyte counts, initially decreased by 41-48% but returned to pre-HTx levels at 12 weeks post-HTx, long after termination of h-ATG therapy. White blood cell counts normalised again at 2 weeks post-HTx and remained within a normal range for the rest of the follow-up while total DC numbers decreased significantly at 1-week post-HTx and remained lower than the pre-HTx condition for the whole post-HTx period ($p < 0.001$).

Circulating DC reconstitution and EMB rejection grade

In total 100 right ventricular biopsies became available for study. Fourteen patients experienced a total of 20 biopsy-proven AR episodes post-HTx. No severe (grade 4) AR episodes were documented during follow-up. Blood samples corresponded to 52 grade 0, 27 grade 1A, 2 grade 1B, 8 grade 2, 10 grade 3A and 1 grade 3B biopsies. Table 4 shows a cross-tabulation of DC counts and rejection grade. We observed a negative association between absolute total DCs and the diagnosed ISHLT rejection grade for the whole period of follow-up ($p = 0.001$). This effect was seen only in the mDC fraction ($p < 0.001$), but not in the pDC fraction ($p = 0.55$). Consequently the mDC/

pDC ratio decreased significantly by increasing rejection grade (p = 0.002). At the peak of the AR period (12 weeks post-HTx), mDC/pDC ratio was inversely correlated with ISHLT grade (r = -0.59, p = 0.006) (Figure 1). From the 11 AR episodes (grade 3A/B) caught in our follow-up, 2 occurred in the 1st, 2 in the 4th, 4 in the 12th, 2 in the 24th and 1 in the 38th week post-HTx. Complete data before, during and after rejection were available for 8 AR episodes. Paired samples analysis in the rejecting HTx-recipients for all episodes before, during and after AR (n = 11) revealed that DC numbers and mDC/pDC ratio decreased during each rejection episode (Figure 2A and 2B).

Circulating DC reconstitution and rejection therapy post-HTx

Figure 3 shows absolute DC counts and the mDC/pDC ratio at 1, 4, 12, 24 and 38 weeks post-HTx. Rejecting patients exhibited impaired DC recovery post-HTx when compared to NR patients. This phenomenon was independent of rejection therapy as in both $3A^+$ and $3A^-$ rejectors circulating DC levels were equally lower than the PBDC counts of NR patients at 24 weeks (p = 0.01) and 38 weeks post-HTx (p = 0.0006) (Figure 3A). Both mDC and pDC numbers for all patients remained significantly lower than the pre-HTx condition (mDCs: range 3.63-7.88 cells/μL; pDCs: range 1.00-2.91 cells/μL) for the whole period of follow-up. Interestingly, the mDC/pDC ratio of NR patients in the 1st week post-HTx appeared to be lower, compared to the ratio of $3A^+$ or $3A^-$ rejecting recipients, but this difference did not reach statistical significance (p = 0.09). For all patients mDC/pDC ratio decreased equally at 4 weeks post-HTx. For the whole period of follow-up, PBDC counts in patients who experienced clinical CMV syndrome were not different from patients that did not have symptomatic CMV infection (p = 0.98). Later during follow-up it became evident that NR patients attained a higher mDC/pDC ratio over time than $3A^+$ or $3A^-$ rejectors patients. Repeated measures ANOVA analysis showed that the difference in mDC/pDC ratio between NR vs $3A^-$ and 3A+ rejector groups became significant at 38 weeks post-HTx (p = 0.04) (Figure 3B).

Table 3: Recipient hematological characteristics after heart transplantation

	HTx patients (n = 20)					
	pre-HTx	post-HTx, weeks				
		1	4	12	24	38
WBCs	7175 ± 467.9	12240 ± 1581*	7370 ± 661.2	6815 ± 776.1	7595 ± 738.0	8220 ± 521.8
lymphocytes	1258 ± 154.1	737.2 ± 169.9**	647.7 ± 136.7**	870.9 ± 268.3	1372 ± 189.8	1584 ± 248.6
monocytes	491.2 ± 75.64	599.6 ± 85.96	336.6 ± 45.90	374.0 ± 55.24	448.1 ± 81.30	411.9 ± 47.14
granulocytes	6457 ± 638.5	9333 ± 1191*	6377 ± 601.8	5956 ± 701.1	5766 ± 722.9	6228 ± 498.1
DCs	28.26 ± 3.750	4.291 ± 1.293***	6.272 ± 1.443***	6.198 ± 0.885***	7.766 ± 1.238***	10.79 ± 1.425**

HTx, heart transplantation; WBCs, white blood cells; DCs, dendritic cells. All values in mean ± SEM of cells/μL. *p < 0.05, **p < 0.01, ***p<0.001; all compared to pre-HTx.

Table 4: Peripheral blood dendritic cells in relation to EMB rejection grade after clinical heart transplantation

	ISHLT grade			
	0 (n = 52)	1A/B (n = 29)	2 (n = 8)	3A/B (n = 11)
cells /μL				
PBDCs	8.2 ± 1.0	7.6 ± 0.9	3.9 ± 1.2	2.5 ± 0.6*
mDCs	6.5 ± 0.7	5.3 ± 0.7	3.1 ± 0.7	1.9 ± 0.5*
pDCs	1.6 ± 0.2	1.5 ± 0.5	1.8 ± 0.3	1.7 ± 0.3
mDC/pDC	4.7 ± 0.7	3.2 ± 1.1	2.1 ± 0.9*	1.0 ± 0.4**

ISHLT, International Society for Heart and Lung Transplantation; PBDCs, peripheral blood dendritic cells; m, myeloid; p, plasmacytoid; mDC/pDC, ratio. Values for total DCs, mDCs and pDCs are given in cells /μL; all values are expressed as the mean ± SEM; * p <0.05, **p < 0.01 (Tukey's test for multiple comparisons).

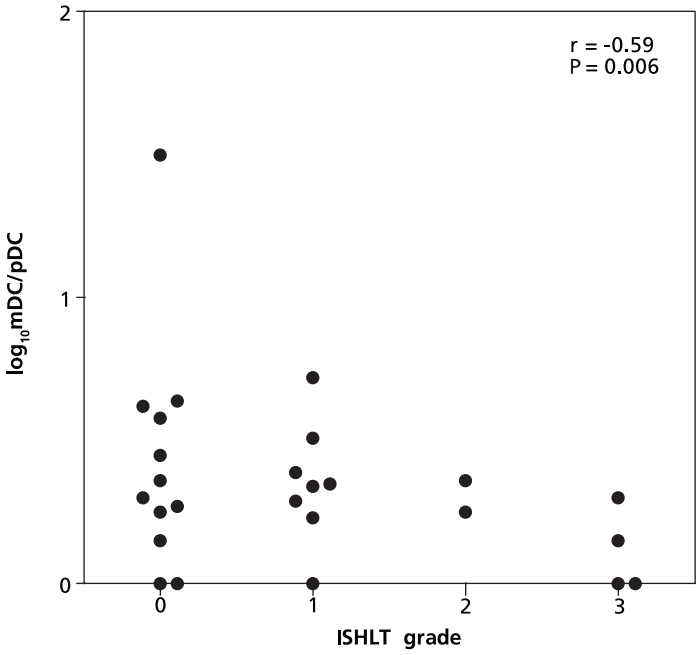

Figure 1: Correlation of mDC/pDC ratio with endomyocardial biopsy ISHLT infiltration grade for 20 heart transplant (HTx) recipients at 12 weeks post-HTx.

Discussion

In the present study, we investigated PBDC counts and DC subset ratio as a means to explore circulating DC reconstitution after HTx. We observed that PBDC numbers do not return to pre-HTx levels for up to 38 weeks post-HTx. In addition, during AR, mDC levels decrease significantly, suggesting selective usage of this subset for induction of AR. The latter finding may be useful for early detection of AR after heart transplantation.

As surgery and stress impose a minimal and transient PBDC increase respectively [18,19] low circulating mDC, pDC and thus PBDC numbers post-HTx, must be ascribed to the administered immunosuppressive regimen. After heart transplantation, circulating mDC kinetics are seemingly affected by the ISHLT rejection grade at the time of the EMB procedure. As pDCs remained constantly low post-HTx, we observed that mDCs, PBDCs and consequently the circulating mDC/pDC ratio were negatively associated with a higher number of infiltrating lymphocytes and presence of myocyte damage diagnosed by ISHLT histological grades ≥ 3. Furthermore, both PBDC counts and mDC/pDC ratio decreased markedly during the majority of AR episodes. We hypothesize

that altered PBDC kinetics reflect an intrinsic mechanism of circulating mDC depletion during AR. We can speculate that selective homing of this subset to secondary lymphoid tissues precedes in order to induce T-cell differentiation into $CD4^+$ helper or cytotoxic $CD8^+$ cells and therefore initiate rejection.

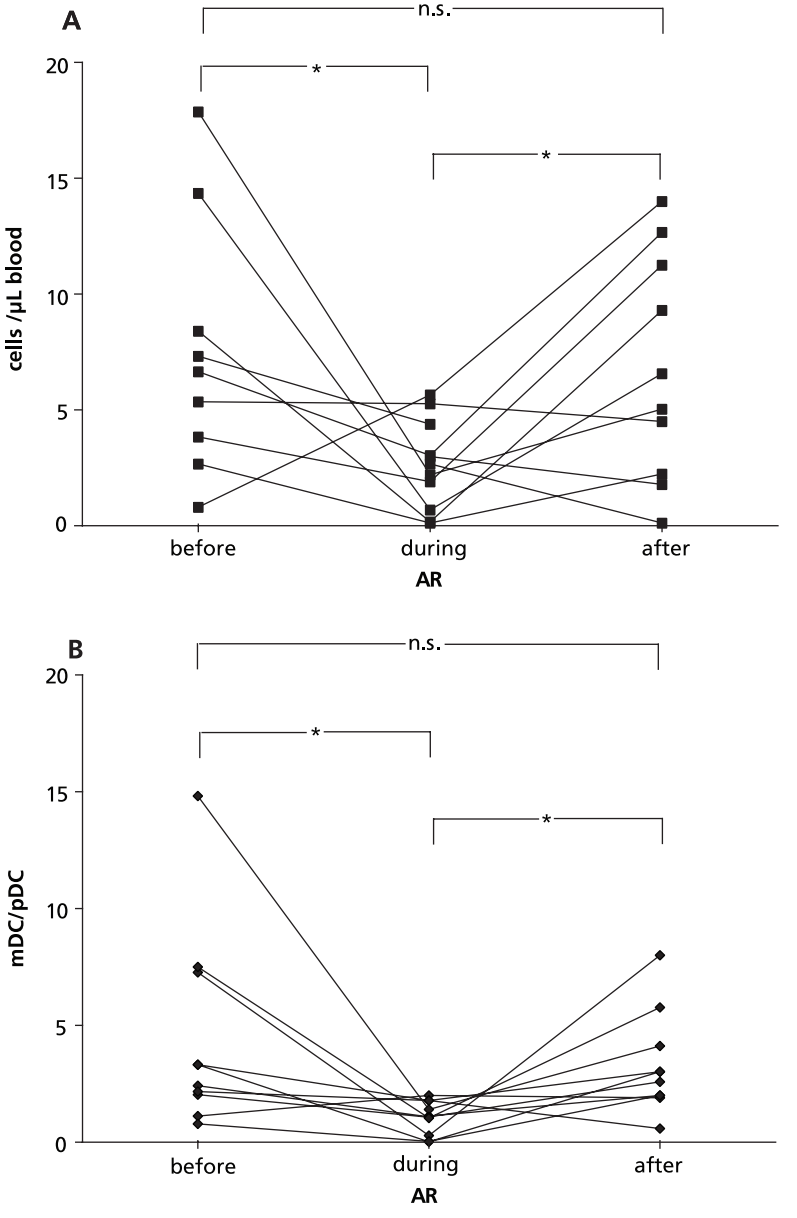

Figure 2: Total peripheral blood DC numbers (A) and circulating mDC/pDC ratio (B) of 14 rejecting patients post-HTx before, during and after 11 acute rejection (AR) episodes caught during 9 months follow-up. * $p < 0.05$ represents the outcome of paired samples analysis for DC numbers or mDC/pDC ratio before vs during and during vs after AR.

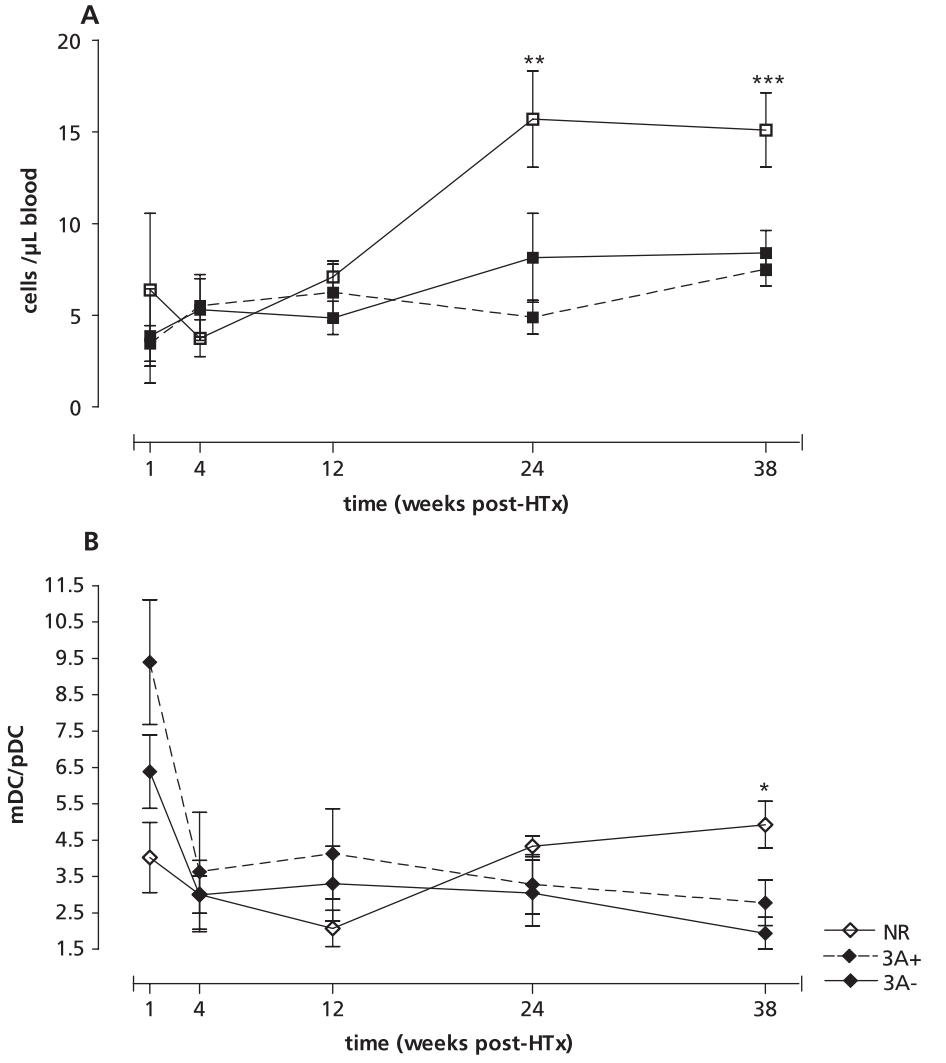

Figure 3: Temporal analysis of absolute DC numbers (A) and mDC/pDC ratio (B) in peripheral blood of 6 non-rejecting (NR) recipients and 14 rejecting (R) patients treated ($3A^+$, n = 11) or not-treated ($3A^-$, n = 3) with rejection therapy up to 9 months post-HTx. *p < 0.05, **p < 0.01, *** p < 0.001 represent the outcome of between groups one-way ANOVA comparisons.

Rejecting patients did not exhibit the PBDC recovery that the NR recipients experienced after 12 weeks post-HTx. Interestingly, within R recipients PBDC numbers remained low in both patients treated ($3A^+$) or not-treated ($3A^-$) with rejection therapy during follow-up. The fact that rejecting HTx recipients undergo circulating mDC depletion over time is supported by the observation

that the mDC/pDC ratio decreases after 24 weeks post-HTx, independent of rejection therapy. This suggests that defective circulating DC reconstitution post-HTx, is inherently related to the immunological process of AR and not to additional immunosuppression administered as rejection treatment post-HTx. On the other hand, it is unlikely that the temporal differences seen between NR and R recipients depend on infection onset or tapering of maintenance immunosuppression post-HTx. Opportunistic infections experienced by both groups of patients, were evenly distributed during follow-up and all recipients were treated according to the same local antibiotic or anti-viral therapeutic protocol. No significant differences were seen in the dosages or the acquired trough levels of maintenance immunosuppressants administered post-HTx, although $3A^+$ rejectors tended towards higher trough levels of CsA than $3A^-$ rejectors and NR recipients for the whole period post-HTx. Nor was the manifestation and onset time of a major post-transplant infectious complication, such as CMV syndrome, different between the patient groups. Furthermore, time of MMF withdrawal from the immunosuppressive regimen for patients with CMV was similar between $3A^+$ or $3A^-$ rejectors and NR recipients.

Emerging evidence suggests that abberant DC reconstitution is indeed related to adverse clinical outcomes after transplantation. For example, PBDCs decrease during acute graft-versus-host disease [20] and low DC counts predict relapse and even death after allogeneic hematopoietic stem cell transplantation [21]. In our hands, cardiac allograft rejectors, exhibited mean total DC numbers of 2.5 cells/μL during AR, for the whole period of follow-up. At 6 and 9 months post-HTx, low mean total DC numbers of 6.5 and 7.7 cells/μL identified patients that had undergone AR independent of rejection therapy, whereas higher mean total DC numbers (15.7 and 15.1 cells/μL, respectively) characterised cardiac allograft non-rejectors. Similarly, shortly after kidney transplantation non-rejecting recipients exhibit higher % PBDCs than their rejecting counterparts [22]. Indeed, in our patient cohort we observed lower DC numbers for rejecting patients at 1 week post-HTx. At the same time DC subset distribution revealed that patients who rejected later during follow-up, had a higher circulating mDC/pDC ratio than NR patients. The differences seen during the early post-transplant period were not significant. However, these observations maybe biased by post-operative infections or influenced by clinical confounders such as induction immunosuppression in the form of h-ATG, high-doses of steroids or normalization of trough levels for CsA, FK506 and MMF, which may interfere with peripheral blood DC data acquired during the first month post-HTx.

The inclusion of small groups of patients and the restricted numbers of rejection episodes examined in this investigation, may limit the extrapolation of our results to clinical outcomes after heart transplantation. However, our long-term findings seem not to be affected by the clinical presentation of the patients after transplantation, with regard to post-operative infections and CMV syndrome manifestation. The analysis of fresh human material, the prospective nature of follow-up and blinding for AR grade during blood sampling, were all advantageous attributes to this study.

In summary, our results show that immunosuppression renders peripheral blood DC numbers decreased after heart transplantation. We have demonstrated that incidence of mDCs as well as the circulating mDC/pDC ratio are negatively associated with ISHLT infiltration grade. Both PBDC numbers and mDC/pDC ratio decreased markedly during AR. Rejecting patients exhibited impaired circulating DC reconstitution after 12 weeks post-HTx when compared to NR patients, independent of rejection therapy. In light of these findings, peripheral blood mDCs may be attributed an important role in eliciting and maintaining allograft rejection post-HTx. Although this technique is prone to bias from immunological complications such as opportunistic infections and CMV syndrome after transplantation, PBDC monitoring may identify patients with high risk for rejection after 3 months post-HTx. This might prove clinically relevant as appropriate adjustments in immunosuppressive regimens may avoid over-immunosuppression of patients at a lower risk for rejection long after clinical HTx.

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References

1. Fairchild PJ, Waldmann H. Dendritic cells and prospects for transplantation tolerance. Curr Opin Immunol 2000;12(5):528-35.
2. Hosenpud JD, Bennett LE, Keck BM, Boucek MM, Novick RJ. The Registry of the International Society for Heart and Lung Transplantation: eighteenth Official Report-2001. J Heart Lung Transplant 2001;20(8):805-15.

3. Mills RM, Naftel DC, Kirklin JK, Van Bakel AB, Jaski BE, Massin EK, Eisen HJ, Lee FA, Fishbein D, Bourge RC. Heart transplant rejection with hemodynamic compromise: a multiinstitutional study of the role of endomyocardial cellular infiltrate. Cardiac Transplant Research Database. J Heart Lung Transplant 1997;16(8):813-21.
4. Morelli AE, Thomson AW. Role of dendritic cells in the immune response against allografts. Curr Opin Nephrol Hypertens 2000;9(6):607-13.
5. Robinson SP, Patterson S, English N, Davies D, Knight SC, Reid CD. Human peripheral blood contains two distinct lineages of dendritic cells. Eur J Immunol 1999;29(9):2769-78.
6. Steinman RM. Some interfaces of dendritic cell biology. Apmis 2003;111(7-8):675-97.
7. Sallusto F, Palermo B, Lenig D, Miettinen M, Matikainen S, Julkunen I, Forster R, Burgstahler R, Lipp M, Lanzavecchia A. Distinct patterns and kinetics of chemokine production regulate dendritic cell function. Eur J Immunol 1999;29(5):1617-25.
8. Almeida J, Bueno C, Alguero MC, Sanchez ML, Canizo MC, Fernandez ME, Vaquero JM, Laso FJ, Escribano L, San Miguel JF, Orfao A. Extensive characterization of the immunophenotype and pattern of cytokine production by distinct subpopulations of normal human peripheral blood MHC II+/lineage- cells. Clin Exp Immunol 1999;118(3):392-401.
9. Game DS, Lechler RI. Pathways of allorecognition: implications for transplantation tolerance. Transpl Immunol 2002;10(2-3):101-8.
10. Hopken UE, Droese J, Li JP, Joergensen J, Breitfeld D, Zerwes HG, Lipp M. The chemokine receptor CCR7 controls lymph node-dependent cytotoxic T-cell priming in alloimmune responses. Eur J Immunol 2004;34(22):461-70.
11. Zou W, Borvak J, Marches F, Wei S, Galanaud P, Emilie D, Curiel TJ. Macrophage-derived dendritic cells have strong T_H1-polarizing potential mediated by beta-chemokines rather than IL-12. J Immunol 2000;165(8):4388-96.
12. Kuwana M, Kaburaki J, Wright TM, Kawakami Y, Ikeda Y. Induction of antigen-specific human CD4(+) T-cell anergy by peripheral blood DC2 precursors. Eur J Immunol 2001;31(9):2547-57.
13. Hackstein H, Thomson AW. Dendritic cells: emerging pharmacological targets of immunosuppressive drugs. Nat Rev Immunol 2004;4(1):24-34.
14. Mazariegos GV, Zahorchak AF, Reyes J, Ostrowski L, Flynn B, Zeevi A, Thomson AW. Dendritic cell subset ratio in peripheral blood correlates with successful withdrawal of immunosuppression in liver transplant patients. Am J Transplant 2003;3(6):689-96.
15. Athanassopoulos P, Vaessen LM, Maat AP, Balk AH, Weimar W, Bogers AJ. Peripheral blood dendritic cells in human end-stage heart failure and the early post-transplant period: evidence for systemic T_H1 immune responses. Eur J Cardiothorac Surg 2004;25 (4):619-26.
16. Billingham ME, Cary NR, Hammond ME, Kemnitz J, Marboe C, McCallister HA, et al. A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group. The International Society for Heart Transplantation. J Heart Transplant 1990; 9(6): 587-93.

17. Athanassopoulos P, Vaessen LM, Maat AP, Zondervan PE, Balk AH, Bogers AJ, Weimar W. Preferential depletion of blood myeloid dendritic cells during acute cardiac allograft rejection under controlled immunosuppression. Am J Transplant in press.
18. Ho CS, Lopez JA, Vuckovic S, Pyke CM, Hockey RL, Hart DN. Surgical and physical stress increases circulating blood dendritic cell counts independently of monocyte counts. Blood 2001;98(1):140-5.
19. Dhabhar FS, McEwen BS. Enhancing versus suppressive effects of stress hormones on skin immune function. Proc Natl Acad Sci U S A 1999;96(3):1059-64.
20. Takebayashi M, Amakawa R, Tajima K, Miyaji M, Nakamura K, Ito T, Matsumoto N, Miyazaki Y, Zen K, Kishimoto Y, Fukuhara S. Blood dendritic cells are decreased in acute graft-versus-host disease. Bone Marrow Transplant 2004;33(10):989-96.
21. Reddy V, Iturraspe JA, Tzolas AC, Meier-Kriesche HU, Schold J, Wingard JR. Low dendritic cell count after allogeneic hematopoietic stem cell transplantation predicts relapse, death, and acute graft-versus-host disease. Blood 2004;103(11):4330-5.
22. Saresella M, Marini M, Guerini F, Ferrante P, Della Bella S, Chiaramonte S, Riva A. Peripheral blood dendritic cells increase in kidney-transplant patients without rejection. Clin Immunol 2004;110(2):191-3; author reply 194.

Chapter 7

Myocardial CCL19 Expression Affects CCR7-Dependent Circulating CD8 T-Cell Homing in Heart Allograft Recipients with Acute Rejection

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Abstract

Inflammatory and homeostatic chemokines (CKs) and chemokine receptors (CKRs) exert differential effects on T-cell homing after heart transplantation (HTx). We investigated circulating T-cell CKR and intragraft CK and CKR levels from cardiac allograft recipients in relation to the histological grade of acute rejection (AR+: ISHLT 3A/B). CCR5, CCR7, CXCR3 and CXCR4 expression on peripheral blood CD4 and CD8 T-cells was measured by flow-cytometry. Intragraft CCR5, CCR7, CXCR3, CXCR4 and CCL5, CCL19, CCL21, CXCL10, CXCL12 mRNA levels were quantified by real-time PCR. Between AR+ and absence of rejection (AR-: ISHLT 0-2), no differences were found in the proportion of $CCR5^+$, $CCR7^+$, $CXCR3^+$ or $CXCR4^+$ cells within circulating CD4 T-cells. For the CD8 subset, during AR+ mean proportions of $CCR5^+$ and $CCR7^+$ cells were 1.5-fold higher and 2.0-fold lower respectively, compared to AR-. Mean intragraft CCR5, CCL5, CCR7 and CCL19 mRNA levels had increased during AR+ compared to time-points before rejection. Additionally, rejecting patients had higher myocardial CCR5 and CXCL10 mRNA levels even before AR+. In these patients, high intragraft CCL19 mRNA was inversely correlated to circulating $CD8^+CCR7^+$ T-cell frequency. This suggests that myocardial CCL19 production affects CD8 T-cell recirculation post-HTx, under influence of homeostatic receptor CCR7. Intragraft homeostatic CK and CKR interactions represent a novel aspect in allograft rejection.

Introduction

Mononuclear leukocyte infiltration is a hallmark in the development of acute cellular rejection (AR+) and a strong predisposing factor for chronic transplant dysfunction [1, 2]. After transplantation of vascularized grafts, both donor- and recipient-derived dendritic cells migrate to lymphoid tissues in order to present allo-antigens and activate T-cells [3]. Consisting of helper and cytotoxic $CD4^+$ and $CD8^+$ subsets, allo-antigen-specific T-cells are continuously recruited into the allograft where they mediate rejection and direct graft loss [4]. Cardiac rejection episodes are dictated by trafficking of allo-activated T-cells from the circulating pool into the engrafted myocardium as a response to production of chemokines by infiltrating mononuclear cells, resident cardiac stromal cells or vascular endothelial cells. Chemotactic gradients have been shown to play a critical role in the influx of T-cells to the transplanted heart during AR+, as they provide signals for activation and intragraft recruitment of effector cells through specific G protein-coupled receptors, the chemokine receptors [5].

Inflammatory chemokines and chemokine receptors amplify cardiac allograft inflammation through integrin activation and endothelial transmigration of allo-activated effector or memory T-cells during rejection [6,7]. Studies on murine models of heart allograft rejection have demonstrated the importance of the interaction between receptors CCR5, CXCR3 and their respective ligands CCL5 (RANTES), CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC), in the outcome of transplantation [8-11]. Additionally, experience from acute cardiac rejection and chronic allograft vasculopathy in humans, has demonstrated predictable temporal and spatial relations between corresponding inflammatory chemokines and chemokine receptor pairs [12-17]. Cognate interactions of homeostatic chemokine receptors with their ligands allow basal trafficking of naïve T-cells to lymphoid tissues in a systematic fashion. In particular, expression of receptors CCR7 and CXCR4 on lymphocytes together with their corresponding chemokines CCL19 (ELC or MIP-3β), CCL21 (SLC) and CXCL12 (SDF-1) on high endothelial venules (HEVs) and marginal zone structures, is thought to be essential for effective migration of naïve T-cells and B lymphocytes into lymph nodes, Peyer's patches and spleen [18]. This classification provides an explanation for the functional compartmentalization of lymphocyte subsets in the immune system, with naïve T-cell homing to lymphoid tissues and allo-reactive T-cell recruitment into the graft, after transplantation [19].

The supporting role of homeostatic chemokines and their receptors within the context of secondary lymphoid organs was confirmed recently, upon

experimentally induced allo-immune responses [20]. Yet, as animals with an abrogated homeostatic receptor (CCR7) phenotype in a fully MHC-disparate combination, exhibit direct negative effects on cardiac allograft cytotoxic T-cell infiltration, it is not clear whether the same paradigm applies to the navigation of different lymphocyte populations under conditions of immnosuppressive therapy after heart transplantation [21]. New insights from models of ongoing inflammation, suggest the necessity of both homeostatic and inflammatory chemokine receptors for local lymphocyte recruitment within inflamed tissues [22,23]. In this model, homeostatic ligand – receptor interactions would support T-lymphocyte re-entry or infiltration within inflammed tissue, while inflammatory ligands and their receptors would regulate T-cell activation and retention within the same target tissue [24-26]. However, the influence of homeostatic chemokines and their corresponding receptors has been ill-defined in the setting of human cardiac allograft rejection. Our goal was to identify the differential contribution of inflammatory and homeostatic chemokines and their receptors on the process of acute rejection under clinical conditions of heart transplantation. In an attempt to dissect markers indicative of AR+, we defined the CCR5, CXCR3 and CCR7, CXCR4 receptor repertoire of peripheral blood $CD4^+$ and $CD8^+$ T-cells, and related their circulating frequencies to the presence or absence of histologically proven AR+ episodes. Gene expression of the corresponding inflammatory (CCL5 and CXCL10) and homeostatic (CCL19, CCL21, CXCL12) chemokines as well as the levels of their receptors were analyzed simultaneously with peripheral blood in endomyocardial biopsy (EMB) specimens from the same individuals, in order to elucidate their intragraft roles during rejection.

Material and Methods

Study Groups and Design

Twenty-one adult heart allograft recipients recruited from the Thoraxcenter (Erasmus MC, Rotterdam, The Netherlands) transplanted between May 2002 and November 2003 were studied. All patients gave informed consent before entering a prospective follow-up of 9 months after transplantation. The study was approved by the local ethics committee (MEC 215.732/2002/157) and was conducted according to the principles outlined by the Declaration of Helsinki. None of the patients presented with any severe co-morbidity directly prior to transplantation. All patients received initial horse-ATG (Imtix Sangstat B.V.,

Lyon, France) induction and triple maintenance immunosuppressive therapy with cyclosporine A (Novartis, Basel, Switzerland) or tacrolimus (FK506, Fujisawa GmbH, Munich, Germany), prednisone and mycophenolate mofetil (Roche, Basel, Switzerland) after transplantation [27]. Oral statin therapy was initiated for all patients after transplantation. One patient died during the course of the investigation. Cytomegalovirus (CMV) seronegative recipient / seropositive donor combinations received prophylaxis in the form of Valganciclovir (Roche, Basel, Switzerland or anti-CMV hyper-Ig (Megalotect, Biotest Pharma GmbH, Dreiech, Germany). CMV disease, as manifested by symptoms and a positive PCR, was treated with ganciclovir for 10-14 days. Inadequate efficacy of ganciclovir, as determined by ongoing symptoms and positive PCR, was an indication for MMF withdrawal.

Sample Collection and Processing

Endomyocardial biopsies

Myocardial samples were collected according to the scheduled surveillance heart biopsy program to monitor histological signs of acute rejection. Right ventricular serial endomyocardial biopsy (EMB) procedures were performed using a central venous access, with a mean of 13 ± 3 procedures per patient. Allograft biopsies were fixed in formalin, embedded in paraffin, and stained with hematoxylin and periodic acid-Schiff. All samples were reviewed by a pathologist who was blinded for the results of all other analyses. Specimens were graded for acute cellular rejection by light microscopy using the criteria outlined by the International Society for Heart and Lung Transplantation (ISHLT) [28]. According to definition of rejection, only patients with ISHLT grade ≥ 3A episodes were considered to experience acute cellular rejection. At the time of EMB procurement, one additional sample was collected for this investigation. Upon biopsy-proven rejection episodes, patients received anti-rejection therapy in the form of methyl-prednisolone (Pfizer, Pharmacia & Upjohn, Sandwich, UK).

Quantitative Real-Time Polymerase Chain Reaction (Q-PCR)

Messenger RNA extraction and cDNA synthesis were performed as described previously [29]. Assay-on-Demand products for the detection and quantification of CCL5 (Hs00174575_m1), CCR5 (Hs00152917_m1), CCL19 (Hs00171149_m1), CCL21 (Hs00171076_m1), CCR7 (Hs00171054_m1), CXCL10 (Hs00171042_m1),

CXCR3 (Hs00171041_m1) and CXCL12 (Hs00171022_m1) mRNA were designed by Applied Biosystems (Foster City, CA, USA). A 5 µL sample of cDNA, 0.625 µL of each specific Primer & Probe-on-Demand mix, 12.5 µL Universal PCR Master Mix (Applied Biosystems), and 6.875 µL H_2O-DEPC were mixed and real-time PCR analysis was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). Sequence-specific PCR primers and probes for CXCR4 were: Taq-Man probe, FAM-5′-ACACTTCAGATAACTACACCGAGGAAATGGG–TAMRA-3′; Forward Primer, 5′-GGTTACCATGGAGGGGATCAGTA- 3′; Reverse Primer, 5′-CAGGGTTCCTTCATGGAGTCATAG-3′ [30]. For CXCR4, 5 µL of cDNA was added to 20 µL of PCR mixture containing 12.5 µL Universal PCR Master Mix (Applied Biosystems), 25 pmol forward primer, 25 pmol reverse primer and 5 pmol probe for the in-house developed PCR. PCR conditions were incubation for 2 min at 50°C, incubation for 10 min at 95°C followed by 40 cycles of 15 sec denaturation at 95°C, 1 min annealing and extension at optimal temperature (60°C). The amount of each target molecule was quantified by measuring threshold cycle (Ct), which was transformed to the number of cDNA copies [$2^{(40-Ct)}$] on a TaqMan™ Real-Time PCR System. Each run contained several negative controls, and three positive reference samples. The obtained values were normalized to the amount of house-keeping gene 18S rRNA (PDAR4319413E; Applied Biosystems) present in each sample and multiplied by 10^6 due to the lower concentration of the target gene as compared to the concentration of 18S.

Whole blood staining

One hundred forty two peripheral venous blood samples were included in this study. Circulating cells from patients were collected in the morning hours at 1, 2, 3, 4, 12, 24 and 38 weeks after transplantation at the time of each EMB procedure and before administration of anti-rejection therapy. All samples were processed within 3 hours of EMB procurement. Whole blood obtained in 7 mL heparinized tubes was divided equally in 100 µL aliquots. Samples were placed directly into 5 mL polystyrene tubes containing 10µL allophycocyanin-conjugated anti-human CD3 (clone SK7), 10µL fluorescein isothiocyanate-conjugated anti-human CD4 (clone S3.5) and 20µL peridinin chlorophyll protein Cy5 CD8 (clone RPA-T8). In separate tubes, 5µL of phycoerythrin (PE)-conjugated anti-CCR5 (CD195; clone 2D7/CCR5), anti-CCR7 (CD197w; clone 150503), anti-CXCR3 (CD183; clone 1C6/CXCR3) and anti-CXCR4 (clone 12G5) were added. Mouse immunoglobulin (Ig) G_1, G_{2a} or G_{2b} PE-conjugated murine isotypic antibodies (BD Biosciences, San Jose, CA, USA) were used as negative

controls. To minimize selective loss during the preparation procedure, cells were first stained at room temperature with the monoclonal antibodies for 20 minutes, followed by lysis of the erythrocytes with FACS lyzing solution (BD Biosciences). After washing with FACSflow (BD Biosciences), 100,000 leukocytes were acquired for chemokine receptor expression analyzis on peripheral blood T-cell subsets.

Flow cytometric analysis

Stained cells were analyzed by a four-color fluorescence-activated cell sorter (FACSCalibur, BD Biosciences). At least 10,000 gated $CD3^+$ lymphocytes were analyzed with the CellQuest Pro software (BD Biosciences). To compare various measurements of chemokine receptor expression in time, the flow cytometer was calibrated using specific RPE fluorescence beads (Calibration Quantum 1000 Beads, Bangs Lab, Fishers, IN, USA). Results were expressed as the frequency (%) of $CD4^+$ or $CD8^+$ T-cells expressing CCR5, CCR7, CXCR3 or CXCR4 in the gated lymphocyte population and were compared according to pathologic grade of rejection. Our laboratory's lymphocyte cytometry performance, is controlled biannually by the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML; http://www.skml.nl).

Statistical analysis

All values are expressed in mean $\pm$ SD unless stated otherwise. The Mann Whitney *U*-test, unpaired Student's *t*-test, and the paired Wilcoxon samples *t*-test were employed to compare differences between means as appropriate. Continuous data were analyzed by repeated measurements and one-way ANOVA analyses in order to decipher differences within and between groups or individual patients over time. Associations were calculated using the Spearman (r) correlation coefficient. Two-sided tests were performed and 'p' values < 0.05 were considered to be significant. SPSS 11.0.1 software (Chicago, IL, USA) and the GraphPad statistical program (San Diego, CA, USA) were applied for analyses and graphics, respectively.

Results

Clinical Characteristics

Demographic and clinical data for all 21 patients have been described previously [31]. Fourteen patients experienced a total of 20 biopsy-proven rejection episodes after transplantation. Histological diagnosis could not be confirmed for 2 biopsies due to shortage of material. Mean values for both non-rejector (NR, n = 7) and rejector (R, n = 14) patient groups were as follows: age of recipients (years), 52 ± 11 vs 50 ± 8; age of donors (years), 39 ± 12 vs 33 ± 12; gender (percentage of males), 57.2 vs 57.1; cold ischemia time (hr), 2.5 ± 0.8 vs 2.6 ± 0.6; HLA mismatches, 4.7 ± 1.1 vs 4.5 ± 1.4. Differences of clinical data between the two groups were not statistically significant. Institution time and intensity of CMV prophylaxis (Valganciclovir, n = 3; Megalotect, n = 6) did not differ between R and NR patients. Similarly, administered dosages and assessed trough levels of maintenance immunosuppressants at the time of biopsy were comparable between the two groups. Triple-maintenance immunosuppressive therapy was not maintained in all study participants, as 11 recipients (3 NR vs 8R) presented with CMV disease post-HTx and for 8 of those (3 NR vs 5R) MMF therapy was tapered. Other post-operative infections in both groups were treated with identical antibiotic or anti-viral therapy protocols. Statin therapy was tolerated by all except for one patient after transplantation.

In peripheral blood only frequency of $CD8^+$ T-cells bearing CCR5 and CCR7 is altered upon rejection

In total, 140 blood samples corresponding to EMB specimens with histological rejection diagnosis were included in this study. Forty-nine percent (70 biopsy specimens) were ISHLT grade 0, 28% (39 biopsy specimens) were grade 1A, 1% (2 biopsy specimens) were grade 1B, 11% (16 biopsy specimens) were grade 2, 9% (12 biopsy specimens) were grade 3A, and < 1% (1 biopsy specimen) was grade 3B. Blood samples collected from the heart transplant patients during the EMB procedure revealed no difference in the frequency of $CD4^+$ T-cells expressing CCR5, CCR7, CXCR3 or CXCR4 with increasing ISHLT grade (*P* values from 0.17 to 0.94; Figure 1A-D). However, we noticed an increase of the % $CD8^+$ T-cells expressing CCR5 (from 42.2 ± 23.9 to 61.6 ± 22.1, $p < 0.05$; Figure 1E) and a statistically significant decrease of the % $CD8^+$ T-cells expressing CCR7 (from 42.1 ± 26.6 to 19.3 ± 10.7, $p < 0.01$; Figure 1F) in the blood of patients experiencing grade 3 rejection, when compared to ISHLT grades with no infiltration or unifocal to multifocal aggressive infiltrates. Similar

observations could not be noted for $CD8^+$ T-cells expressing CXCR3 or CXCR4 (p values at 0.96 and 0.97, respectively; Figure 1G and 1H).

Circulating $CD8^+CCR7^+$ T-cell frequency in relation to rejection and CMV manifestation after transplantation

From the 13 AR+ episodes (ISHLT grade 3A/B) caught in the follow-up, 2 occurred at the 1st, 2 in the 3rd, 2 in the 4th, 4 in the 12th, 2 in the 24th and 1 in the 38th week post-HTx. Sequential analysis of peripheral blood T-cell subsets revealed that the proportion of $CD4^+$ and $CD8^+$ T-cells did not differ significantly between R and NR patients during follow-up (Table 1). Compared to recipients who did not experience any biopsy-proven AR+ for the whole post-HTx period, rejecting patients showed a significantly lower mean % of $CD8^+CCR7^+$ T-cells at 38 weeks post-HTx ($p = 0.007$), irrespective of the presence or absence of AR+ (data not shown). At the same time, both absolute $CD4^+$ and $CD8^+$ T-cell numbers of the rejectors were significantly lower (Table 1). However, the percentage of $CD8^+$ T-cells in patients who presented with CMV disease increased in time when compared to the recipients who did not manifest with the disease (Table 2). Median time of CMV disease onset post-HTx was 79 days (range 40-122) for the NR group and 96 days (range 48-139) for the R group. The $CD8^+CCR7^+$ T-cell decrease at 38 weeks post-HTx in R patients, was independent of CMV disease manifestation (Table 2). Discontinuation of mycophenolate mofetil (MMF) for NR and R patients occurred at 130 days (range: 121-182) and 146.5 days (102-212) post-HTx respectively, but $CD8^+CCR7^+$ T-cell frequency was not influenced by MMF withdrawal post-HTx (data not shown).

Inflammatory and homeostatic myocardial chemokine and chemokine receptor RNA intragraft expression is increased upon rejection

From histologic analysis of the 140 EMB specimens used for chemokine and chemokine receptor gene expression evaluation, 42 biopsy specimens were excluded from the study, as 25 specimens originated from grafts that had evidence of ischemia and 17 specimens were from grafts with Quilty lesions. Additionally, 15 biopsy specimens from 8 individuals in which the 18S rRNA concentration was below the reliable detection limit, were considered as insufficient material and subsequently excluded. The intragraft expression of the inflammatory chemokines CCL5 and CXCL10 as well as their corresponding receptors (CCR5 and CXCR3) was increased during AR+ (Figure 2A & 2B). Compared with grades 0 or 1A/B-2, high expression levels of CCL5 and CCR5 during rejection grade 3 were statistically significant ($p < 0.001$ and $p < 0.0001$,

respectively). The same was observed for CXCL10 and its receptor CXCR3, when compared with grade 0 ($p < 0.05$). With regard to homeostatic chemokines, a higher expression of CCL19 and its receptor CCR7 was observed during AR+. When compared with grade 0, increased expression of both CCL19 (Figure 2A) and CCR7 (Figure 2B) during rejection grade 3, were statistically significant ($p < 0.05$). Expression levels of the other CCR7 ligand (CCL21), homeostatic chemokine CXCL12 and its receptor CXCR4, were not significantly different between any rejection grade.

Intragraft chemokine and chemokine receptor expression is altered already before first biopsy-proven rejection

Next, we studied intragraft chemokine and chemokine receptor gene expression in rejectors during AR- episodes (ISHLT grades 0-2) and NR patients. Within R patients we also compared intragraft chemokine and chemokine receptor gene expression before AR+, as well as at the time-point of AR+. In order to avoid possible influence of prior anti-rejection treatment to the myocardial expression profiles both for chemokines and their receptors, only first AR+ episodes were studied ($n = 11$). Intracardial CCL5 mRNA expression in R patients was significantly increased during AR+, compared to the time-points before AR+ ($p = 0.042$). No difference in mean CCL5 mRNA levels between R and NR patients for ISHLT grades 0-2 was observed (Figure 3A). During AR+, higher mRNA levels of the corresponding receptor CCR5 were observed, when compared with the time-points before AR+ ($p = 0.009$). Mean CCR5 intragraft expression for ISHLT grades 0-2 within R patients was higher than the CCR5 expression in the NR patients ($p = 0.011$; Fig. 4A). For CXCL10 and its receptor CXCR3, we saw higher expression levels of both genes during AR+ ($p = 0.024$; Figure 3B & $p = 0.002$; Figure 4B, respectively). For ISHLT grades 0-2, CXCL10 expression in R patients was higher than the corresponding levels from the NR patients ($p = 0.0005$; Figure 3B). Furthermore, for the CCR7 ligands, only CCL19 ($p = 0.003$) but not CCL21 ($p = 0.206$) expression was higher during AR+ than before AR+ (Figure 3C & 3D, respectively). Additionally, CCR7 expression was higher during AR+, compared to time-points before biopsy-proven AR+ ($p = 0.002$; Figure 4C). Expression of the other homeostatic chemokine ligand (CXCL12) and its receptor (CXCR4) did not reveal any significant differences in R recipients, during as well as before AR+, when compared to the pattern seen by the NR patients (Figure 3E & Figure 4D, respectively).

Myocardial CCL19 expression affects circulating frequency of $CD8^+CCR7^+$ T-cells in rejecting patients

We also investigated whether myocardial CCL19 gene expression differentially affected peripheral blood frequency of $CD8^+CCR7^+$ T-cells in NR and R patients. Complete data (n = 83) on simultaneous intragraft CCL19 mRNA levels and CCR7 expression on peripheral blood $CD8^+$ T-cells during AR+ and AR- episodes were available for 60 and 23 time-points in the R and NR recipients, respectively. Upon association of the two parameters for the whole period of follow-up, we found a significant inverse correlation between intragraft CCL19 mRNA levels and circulating $CD8^+CCR7^+$ T-cell frequency in the R patients ($p < 0.001$; Figure 5A) but not the NR patients ($p = 0.553$; Figure 5B). During the AR+ time-points, no significant correlation between the two parameters was found ($r_S = -0.298$, $R^2 = 0.089$, $p = 0.323$; Figure 5A). Similarly, no association was observed in patients who manifested with CMV disease ($r_S = -0.079$, $R^2 = 0.006$, $p = 0.638$; data not shown).

Intragraft CXCL10 and CCR5 mRNA levels before rejection correlate to CCL19 expression during rejection

During all AR+ episodes, we found strong positive correlations between inflammatory chemokines (CCL5 & CXCL10) and their corresponding receptors (CCR5 & CXCR3). At the same period, we also observed positive correlations for the homeostatic chemokines (CCL19, CCL21 & CXCL12) and their corresponding receptors (CCR7 & CXCR4) (Table 3). Intrigued by the high expression of CCL19 during AR+ we sought to associate CCL19 mRNA levels during AR with chemokine and chemokine receptor driven events before AR+. We found that the increased CCL19 levels during AR+ correlated strongly with the mRNA levels of one inflammatory chemokine CXCL10 ($p < 0.01$) before AR+ (Figure 6A). Also, CCL19 expression during AR+ correlated positively with the levels of inflammatory chemokine receptor CCR5 ($p < 0.05$) before AR+ (Figure 6B).

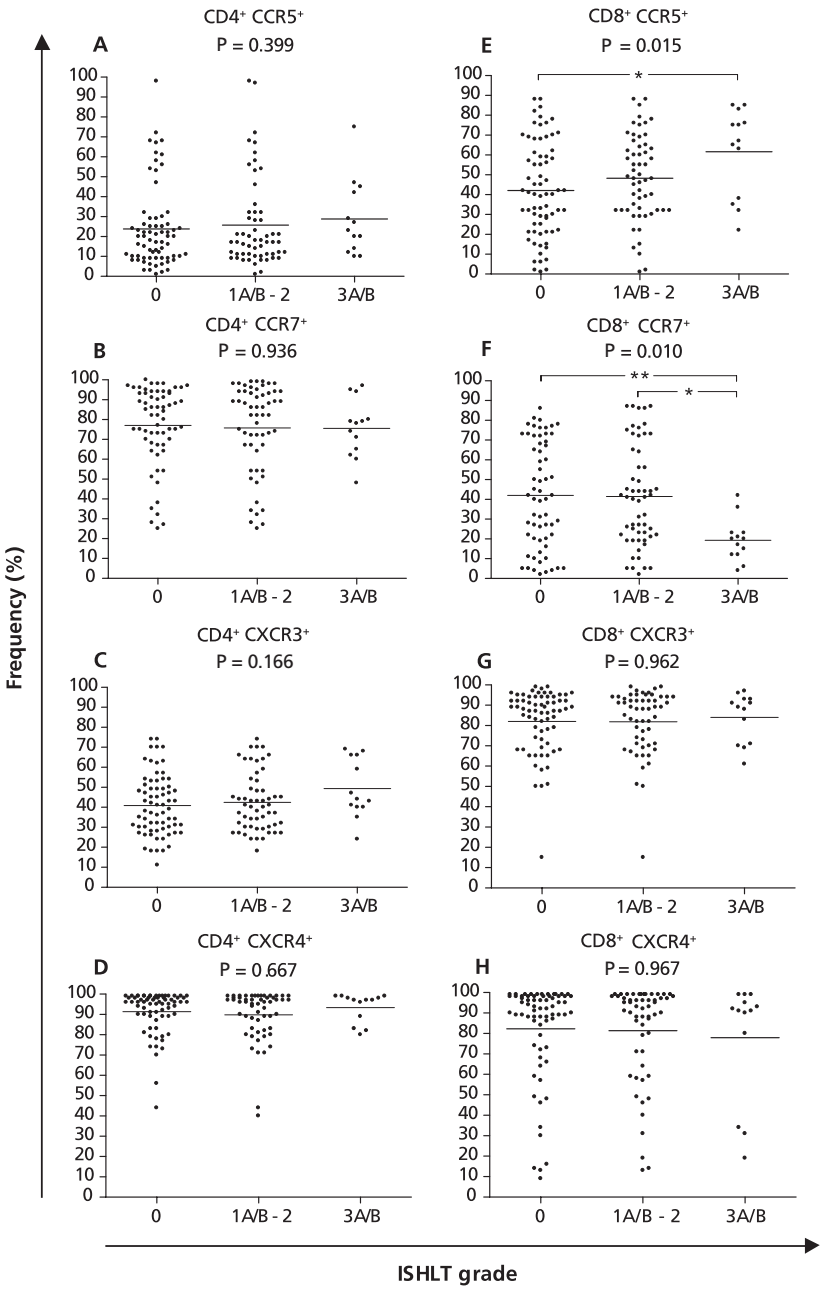

Figure 1: Percentage of CCR5, CCR7, CXCR3 and CXCR4 expressing (A to D) $CD4^+$ and (E to H) $CD8^+$ cells in peripheral blood T-lymphocytes of cardiac transplant patients. The rejection grade of recipient EMB specimens is shown at the time each blood sample was procured (ISHLT 0, n = 70; ISHLT 1A/B-2, n = 57; ISHLT 3A/B, n = 13). Overall statistical results from one-way ANOVA analyses (p) are depicted accordingly; *p < 0.05, **p < 0.01 represent the outcome of Tukey's multiple comparisons test.

Table 1: Circulating $CD4^+$ and $CD8^+$ T-cells in rejecting and non-rejecting heart transplant recipients

	Post-HTx, weeks (range)						
	1 (0.6 – 1.7)	**2 (1.8 – 2.6)**	**3 (2.8 – 3.4)**	**4 (3.5 – 5.1)**	**12 (10.1 – 16.0)**	**24 (20.4 – 27.8)**	**38 (36.0 – 40.5)**
Non-rejectors (n = 7)†							
CD4+ T-cell numbers	120 ± 124	247 ± 179	95 ± 87	46 ± 37	160 ± 128	277 ± 297	1005 ± 553
% CD4+	61.6 ± 10.4	65.2 ± 10.6	69.3 ± 14.2	69.9 ± 7.4	69.9 ± 17.4	43.4 ± 29.9	45.6 ± 35.9
% CD4+CCR7+	89.0 ± 16.8	93.3 ± 15.3	84.0 ± 12.8	90.0 ± 15.6	82.3 ± 13.5	79.2 ± 11.6	77.0 ± 9.6
CD8+ T-cell numbers	90 ± 80	111 ± 80	42 ± 39	15 ± 12	63 ± 42	788 ± 1116	1536 ± 1317
% CD8+	38.4 ± 10.2	34.9 ± 10.6	30.7 ± 14.7	30.1 ± 7.6	30.1 ± 18.0	56.6 ± 31.0	54.5 ± 32.8
% CD8+CCR7+	71.2 ± 5.8	66.0 ± 5.9	63.5 ± 14.5	39.5 ± 11.5	38.2 ± 10.3	35.0 ± 13.2	43.8 ± 9.9
Rejectors (n = 14)							
CD4+ T-cell numbers	160 ± 118	245 ± 167	233 ± 177	41 ± 49	133 ± 92	253 ± 290	199 ± 230 §
% CD4+	61.0 ± 16.5	76.6 ± 11.2	71.8 ± 11.5	67.4 ± 13.9	55.4 ± 20.5	45.0 ± 20.1	44.3 ± 20.5
% CD4+CCR7+	82.4 ± 17.0	94.8 ± 15.0	84.2 ± 20.5	69.8 ± 31.8	66.7 ± 22.2	69.0 ± 22.1	76.5 ± 17.6
CD8+ T-cell numbers	71 ± 41	69 ± 45	110 ± 72	54 ± 35	91 ± 76	279 ± 296	386 ± 414*
% CD8+	39.0 ± 16.3	23.4 ± 11.9	28.2 ± 11.0	32.6 ± 14.0	44.6 ± 20.1	55.1 ± 19.8	55.7 ± 19.6
% CD8+CCR7+	58.0 ± 17.5	71.3 ± 11.8	54.2 ± 18.9	53.3 ± 21.1	29.0 ± 21.3	19.6 ± 22.6	15.4 ± 12.7 §

Absolute $CD4^+$ and $CD8^+$ T-cell numbers in cells /μL blood. Percentage $CD4^+$ or $CD8^+$ cells calculated from total $CD3^+$ cells. %$CCR7^+$ cells are given from total $CD4^+$ or $CD8^+$ cells. Data are shown in mean ± SD. * $p < 0.05$, § $p < 0.01$ (Mann Whitney U-test between non-rejectors and rejectors). †One non-rejecting patient died due to brain stem coning after severe cerebro-vascular accident at the end of the 2nd week post-HTx.

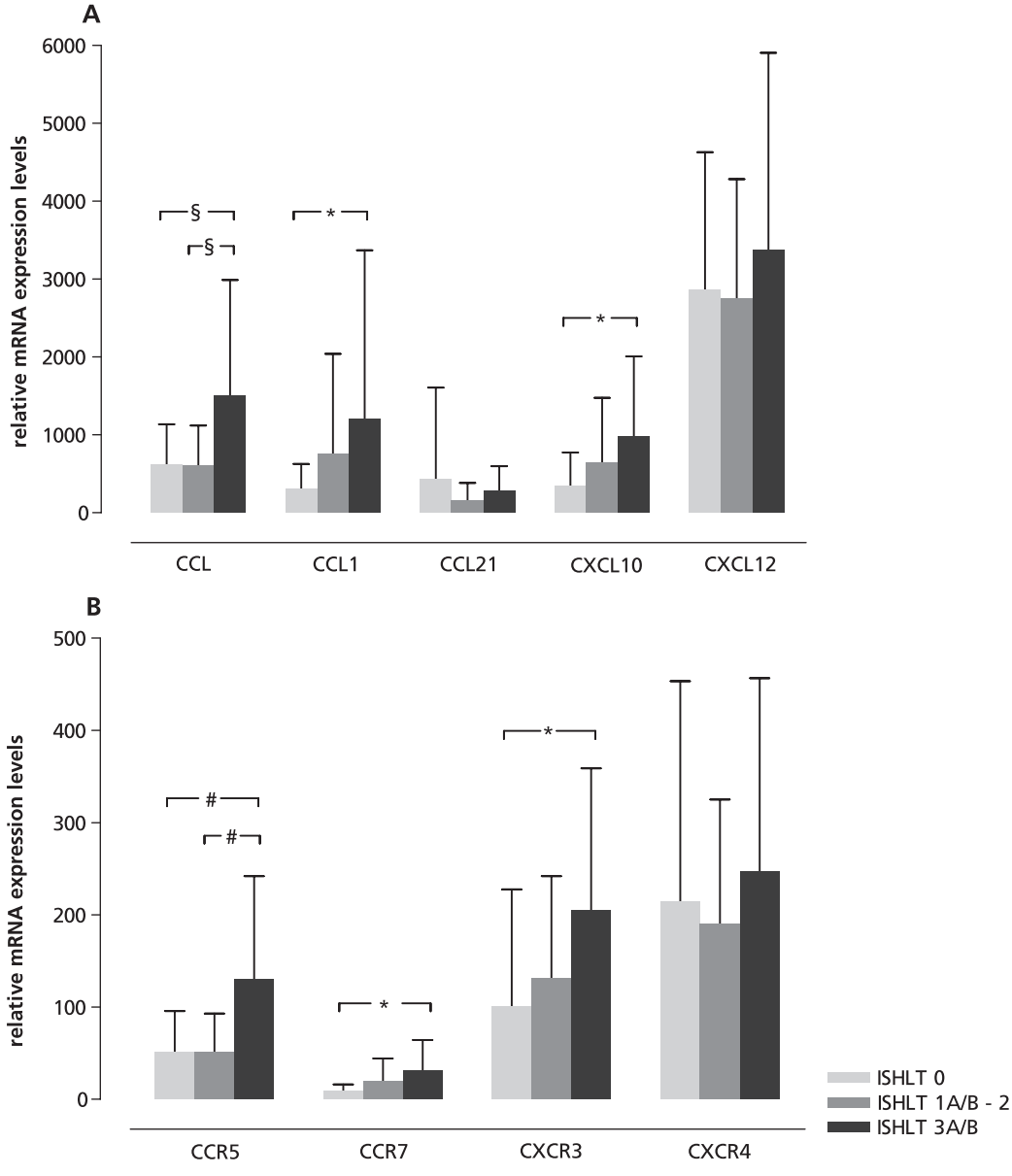

Figure 2: Compilation of (A) chemokine and (B) chemokine receptor gene expression compared with pathological grades of rejection, in the 83 endomyocardial biopsies included in the study during the whole period of follow-up (ISHLT 0, n = 42; ISHLT 1A/B-2, n = 28; ISHLT 3A/B, n = 13). Results from one-way ANOVA analyses for chemokines were: CCL5, $p < 0.001$; CCL19, $p = 0.031$; CCL21, $p = 0.096$; CXCL10, $p = 0.0005$ and CXCL12, $p = 0.569$. For their receptors one-way ANOVA analysis results were: CCR5, $p = 0.001$; CCR7, $p = 0.024$; CXCR3, $p = 0.047$ and CXCR4, $p = 0.598$. *$p < 0.05$, §$p < 0.01$, #$p < 0.001$ represent the outcome of Tukey's multiple comparisons test.

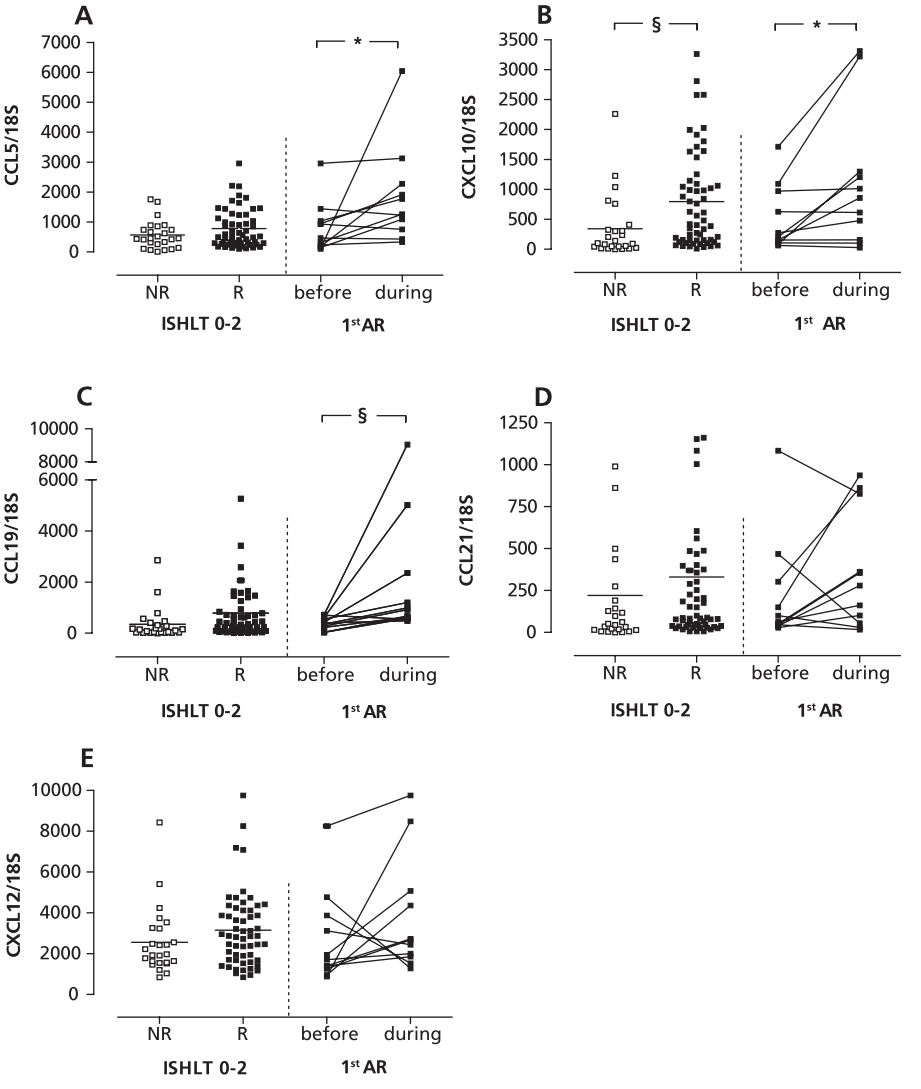

Figure 3: Rejecting patient (R) intragraft gene expression before and at the time of first AR+ (n = 11), in comparison to all non-rejecting time points (ISHLT 0-2) from the rejectors (n = 47) as well as the non-rejectors (n = 23), for the chemokines (A) CCL5, (B) CXCL10, (C) CCL19, (D) CCL21 and (E) CXCL12. *$p < 0.05$, §$p < 0.01$ and #$p < 0.001$ represent results from comparisons within R patients (Wilcoxon matched pairs test) and between the R and NR patients (Mann-Whitney *U*-test).

Table 2: Circulating $CD4^+$ and $CD8^+$ T-cells of cardiac allograft recipients according to presentation with CMV syndrome after transplantation

	Post-HTx, weeks (range)						
	1 (0.6 – 1.7)	2 (1.8 – 2.6)	3 (2.8 – 3.4)	4 (3.5 – 5.1)	12 (10.1 – 16.0)	24 (20.4 – 27.8)	38 (36.0 – 40.5)
CMV syndrome – (n = 10)†							
% $CD4^+$ T-cells	52.9 ± 25.7	64.6 ± 9.9	68.7 ± 10.1	56.9 ± 11.2	62.8 ± 20.8	60.1 ± 23.9	62.4 ± 13.9
% $CD4^+CCR7^+$	85.4 ± 14.1	92.0 ± 5.9	85.5 ± 5.0	85.0 ± 12.7	75.2 ± 22.0	75.5 ± 25.9	86.6 ± 8.9
% $CD8^+$ T-cells	47.0 ± 14.1	35.5 ± 9.8	31.3 ± 9.9	43.1 ± 10.9	37.2 ± 19.6	39.9 ± 11.8	37.6 ± 13.4
% $CD8^+CCR7^+$	69.5 ± 10.5	64.5 ± 12.3	59.0 ± 26.9	32.0 ± 12.7	53.0 ± 31.0	23.6 ± 25.4	28.3 ± 27.1
CMV syndrome + (n = 11)							
% $CD4^+$ T-cells	67.9 ± 10.1	77.2 ± 11.0	72.1 ± 11.5	71.7 ± 10.8	44.5 ± 15.4	39.9 ± 25.3	40.8 ± 30.9
% $CD4^+CCR7^+$	85.3 ± 20.9	96.0 ± 2.9	83.6 ± 20.4	71.5 ± 32.5	68.5 ± 21.4	70.1 ± 16.7	71.1 ± 15.7
% $CD8^+$ T-cells	32.1 ± 10.0	22.8 ± 9.8	28.0 ± 11.1	28.3 ± 9.4	55.5 ± 12.5	60.1 ± 21.2*	63.0 ± 23.1*
% $CD8^+CCR7^+$	62.0 ± 19.6	72.8 ± 9.9	56.0 ± 17.6	49.2 ± 26.0	24.8 ± 12.3	25.7 ± 25.7	24.5 ± 15.3

Percentage $CD4^+$ or $CD8^+$ cells calculated from total blood $CD3^+$ cells. $\%CCR7^+$ cells are given from total $CD4^+$ or $CD8^+$ cells. Data are shown in mean ± SD. $p < 0.05$ (Mann Whitney U-test between patients who presented with CMV syndrome or did not manifest with the disease during 9 months follow-up).
†One patient died due to brain stem coning after severe cerebro-vascular accident at the end of the 2nd week post-HTx.

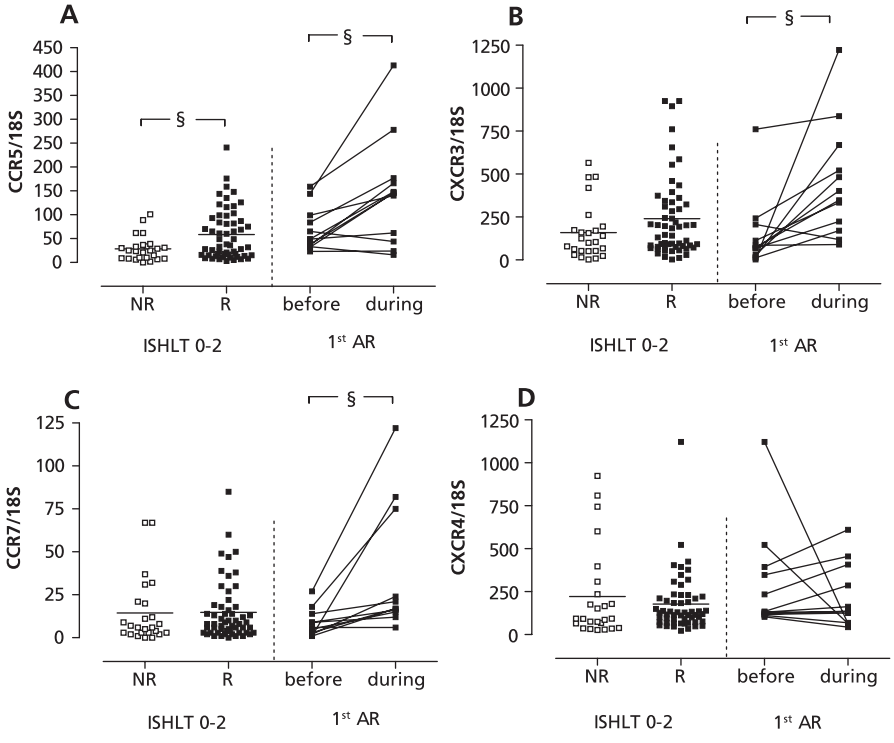

Figure 4: Rejecting patient (R) intragraft gene expression before and at the time of first AR+ (n = 11), in comparison to all non-rejecting time points (ISHLT 0-2) from the rejectors (n = 47) as well as the non-rejectors (n = 23), for the chemokine receptors (A) CCR5, (B) CXCR3, (C) CCR7 and (D) CXCR4. *$p < 0.05$, §$p < 0.01$ and #$p < 0.001$ represent significant differences within R patients (Wilcoxon matched pairs test) and between the R and NR patients (Mann-Whitney *U*-test).

Table 3: Correlation of intragraft chemokine and chemokine receptor gene expression during 13 AR+ episodes

Chemokine	Chemokine receptor	r_S (95% C.I.)	R^2	P value
CCL5	CCR5	0.84 (0.57 – 0.95)	0.71	< 0.0001
CXCL10	CXCR3	0.60 (0.11 – 0.86)	0.36	0.018
CCL19	CCR7	0.68 (0.24 – 0.89)	0.46	0.006
CCL21	CCR7	0.55 (0.04 – 0.83)	0.30	0.033
CXCL12	CXCR4	0.66 (0.21 – 0.88)	0.44	0.007

r_S = Spearman's rank correlation coefficient; C.I. = confidence interval; $R^2 = r_S$ squared

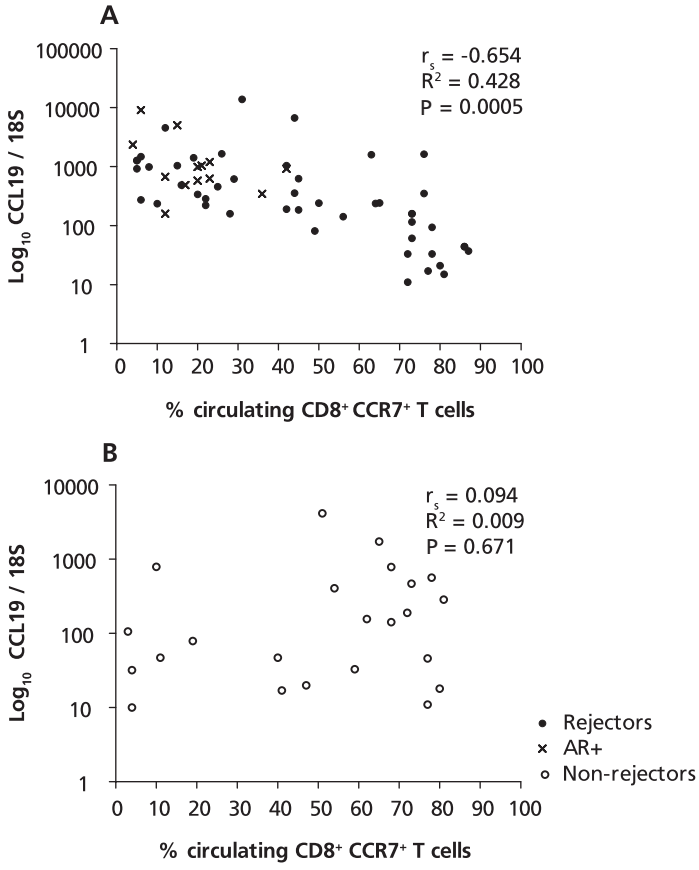

Figure 5: Correlation of simultaneous myocardial CCL19 mRNA expression and the frequency of circulating $CD8^+CCR7^+$ T-cells at (A) 47 time-points from R patients, (AR-: ISHLT 0, n = 31.; ISHLT 1A/1B, n = 10; ISHLT 2, n = 6) and (B) 23 time-points from NR patients (ISHLT 0, n = 11 ; ISHLT 1A/1B, n = 8; ISHLT 2, n = 4). Acute rejection (AR+) episodes (ISHLT ≥ 3A, n = 13) for the R patients are depicted accordingly.

Discussion

The present study analyzed circulating $CD4^+$ and $CD8^+$ T-cell chemokine receptor expression in parallel with intragraft chemokine and chemokine receptor gene expression of cardiac allograft biopsy specimens, from a 9-month follow-up of 21 patients after heart transplantation. No differences were observed in

the frequency of circulating $CD4^+$ cells positive for the chemokine receptors questioned, in relation to the ISHLT classification for histological infiltration grade. Within the $CD8^+$ subset, mean percentage of $CCR5^+$ cells was raised while the percentage of $CCR7^+$ cells was reduced during rejection. This reciprocal regulation of $CCR5^+$ and $CCR7^+$ cells only within circulating $CD8^+$ T-lymphocytes could be explained as proportionally more $CD8^+$ than $CD4^+$ cells, with a predominantly effector or memory $CD45RO^+$ phenotype, are known to infiltrate rejecting human cardiac allografts [32,33]. However, no $CXCR3^+CXCR4^-$ effector/memory phenotype could be confirmed for the peripheral blood $CD8^+$ pool, as both mean frequency of CXCR3- and CXCR4- bearing $CD8^+$ cells were constantly high, irrespective of the ISHLT grade. Furthermore, low percentage of $CCR7^+$ and high percentage of $CCR5^+$ cells within $CD8^+$ T-cells were not specific for ISHLT grade 3. Therefore, no satisfactory values could be obtained by peripheral blood analysis to reliably monitor AR+, as both rejecting and non-rejecting recipients presented with a variable frequency of $CCR5^+$ (0-88%) and $CCR7^+$ (2-87%) $CD8^+$ cells at ISHLT grades 0 to 2, for the whole follow-up period. Nonetheless, decreased mean circulating $CD8^+CCR7^+$ T-lymphocyte frequency during acute cardiac allograft rejection is a novel finding, which suggests either efflux of circulating $CD8^+CCR7^+$ T-cells, or enrichment of blood with $CD8^+CCR7^-$ T-cells, as part of the rejection process.

In endomyocardial biopsies, gene expression indicated that apart from a strong association of intragraft levels from inflammatory chemokines (CCL5 & CXCL10) and their corresponding receptors (CCR5 & CXCR3) with the appearance of AR+ episodes, also intracardial mRNA levels of a homeostatic chemokine (CCL19) and its receptor (CCR7) were significantly elevated upon rejection. Immunohistochemical studies on sections from heart biopsy specimens have shown that elevated expression of chemokine and chemokine receptor genes correlates with the presence of their respective protein levels during rejection [34]. Consequently, differential CCR5 and CXCL10 gene expression profile between rejectors during AR- episodes (ISHLT grades 0-2) and non-rejectors, must represent altered intracardial chemokine and chemokine receptor micro-environment in the rejecting patients, even prior to histologically proven rejection. Furthermore, the significant positive correlations between all inflammatory and homeostatic chemokines with their corresponding receptors during AR+, support the notion that the mRNA quantities from all chemokine ligands measured in our study could be functional in the recruitment of cells positive for the respective chemokine receptors in question.

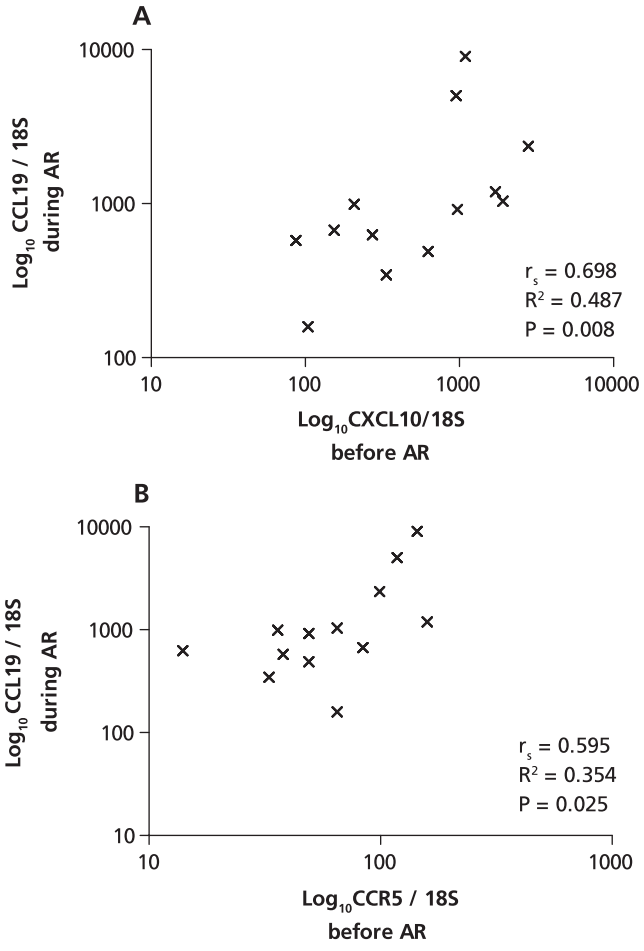

Figure 6: Correlation of intragraft CCL19 mRNA expression during all AR+ episodes (ISHLT ≥ 3) caught in our follow-up (n = 13) with myocardial (A) CXCL10 and (B) CCR5 mRNA levels before AR+ (ISHLT 0-2) for the rejectors.

Indeed, during cardiac allograft rejection, CCL5 and CXCL10 have been known to drive local intracardial recruitment of effector $CCR5^+$ and $CXCR3^+$ cells, respectively [35]. However, the elevated intragraft levels of CCL19 and CCR7 during AR+ shown by our study, suggest that intragraft homeostatic chemokine – chemokine receptor interactions are also involved in rejection. Both CCL19 and CCR7 have been suggested to participate in the trafficking of a broad spectrum of lymphocytes and especially activated T-cells [36]. Additionally, CCR7 mediated T-cell homing has been implicated in naïve and

activated $CD8^+$ T-cell recruitment to non-lymphoid tissues upon fulminant inflammatory responses and exacerbations of autoimmune diseases [37-41]. In our study, myocardial CCL19 expression was shown to negatively affect circulating $CD8^+CCR7^+$ T-cell frequency in rejecting patients. We postulate that high intragraft CCL19 production influences peripheral blood $CD8^+$ T-cell recirculation by facilitating intracardial $CD8^+CCR7^+$ T-cell infiltration in these patients. This phenomenon may potentially reflect a reduction in the homing specificity of $CD8^+$ cells in rejecting patients, driven by high myocardial CCL19 expression after heart transplantation. Such a mechanism could explain the high post-transplant incidence of non-donor specific cytotoxic T-lymphocytes in the grafts of rejecting patients, as described previously [42,43].

Nevertheless, this effect seems to be time-dependent as, in contrast to the non-rejecting patients, circulating $CD8^+CCR7^+$ T-cell frequency in rejectors decreased more markedly during the course of follow-up. Although viral – and specifically CMV – infections are known to interfere with CCR7 expression on $CD8^+$ T-cells [44], no substantial effects of CMV syndrome manifestation were noted on $CD8^+CCR7^+$ T-cell frequency during follow-up. As CMV-specific $CCR7^+$ cells are known to account for 0.5-4% of $CD8^+$ cells [45], they could be practically undetectable by our technique within the bulk of peripheral blood T-cells studied. Alternatively, circulating CMV-specific $CD8^+$ T-cells that do not express CCR7 may not be able to recirculate through blood to peripheral lymph nodes [46]. Furthermore, as immunosuppressants are known to affect CCR7-dependent T-cell migration *in vivo* [47], decreased frequency of $CD8^+CCR7^+$ T-cells in rejectors with time, could be ascribed to additional influence on chemokine receptor expression from anti-rejection therapy. However, while absolute numbers of both $CD4^+$ and $CD8^+$ T-cell subsets decreased in the rejecting patients towards the end of follow-up (possibly due to the effects of methyl-prednisolone treatment), low $CCR7^+$ cell frequency was observed only for the $CD8^+$ and not the $CD4^+$ subset. In our opinion, a reduced $CCR7^+$, or alternatively raised $CCR7^-$, proportion within circulating $CD8^+$ T-cells, may reflect the increasing magnitude of allo-antigen-specific responses that develop with time after transplantation.

Tissue specific endothelial chemokine expression within target organs has been demonstrated to be crucial for the recruitment of immune cells with a certain chemokine receptor repertoire [48]. After heart transplantation, generation of a single inflammatory chemokine CXCL10 through allograft vascular endothelium in response to IFN-γ secretion, oxidant stress or ischemia-reperfusion injury can initiate progressive graft infiltration and amplification

of multiple effector pathways [49,50]. Targetting the graft, infiltrating neutrophils, macrophages, monocytes and NK cells are capable of producing inflammatory chemokines like CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES) and CXCL8 (IL-8) [51]. In our study, increased intragraft expression of CXCL10 and CCR5 in rejecting patients already before first biopsy-proven acute rejection, may represent endothelial activation and $CCR5^+$ monocyte/macrophage influx before cardiomyocyte damage. Considering that increased CCL19 expression during AR+ was positively correlated to raised CXCL10 and CCR5 levels before AR+, it is tempting to speculate that monocyte/macrophage infiltration and inflammatory chemokine expression before AR+ potentiates vascular endothelial or cardiomyocyte homeostatic chemokine production with a tropism for CCL19. Conceptually, this abberant chemokine microenvironment might not only cause intracardial effector T-cell influx, but may also lead to intragraft naïve lymphocyte activation, dendritic cell maturation and homing [52]. Such a process may support local lymphoid follicle formation and contribute to persistence of risk for cardiac allograft loss, as described by late acute and chronic rejection after heart transplantation [53].

Our findings suggest that T-cell subset heart allograft infiltration is regulated differentially by inflammatory and homeostatic chemokines and their respective chemokine receptors. Here, we provide evidence that CCL19 and CCR7 interactions are involved in the onset and chronicity of cardiac rejection by a systemic effect on $CD8^+$ T-cell homing and recirculation after heart transplantation. In this manner, homeostatic and inflammatory chemokines may act synergistically in $CD8^+$ T-cell infiltration during acute cardiac allograft rejection [54]. Further analyses are required to consolidate this view-point: immunohistochemical investigation of cardiac allograft CCL19 production and *in situ* $CCR7^+$ cell localization on biopsies from rejecting and non-rejecting patients in order to dissect the nature of intracardial tissue segments or cells producing CCL19, is pending. Functional roles of CCL19 could be unraveled for endothelial dysfunction during rejection and accelerated coronary artery disease after heart transplantation.

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References

1. Cotts WG, Johnson MR. The challenge of rejection and cardiac allograft vasculopathy. Heart Fail Rev 2001;6(3):227-40.
2. Deng MC. Cardiac transplantation. Heart 2002;87(2):177-84.
3. Hancock WW, WLaYQ. Chemokine-directed dendritic cell trafficking in allograft rejection. Current Opinion in Organ Transplantation 2003;8:35-39.
4. Arakelov A, Lakkis FG. The alloimmune response and effector mechanisms of allograft rejection. Semin Nephrol 2000;20(2):95-102.
5. Hancock WW, Gao W, Faia KL, Csizmadia V. Chemokines and their receptors in allograft rejection. Curr Opin Immunol 2000;12(5):511-6.
6. Fairchild R, Kobayashi, H., Miura, M. Chemokines and the recruitment of inflammatory infiltrates into allografts. Graft 2000;3(3):s24-s31.
7. Hancock WW, Turka LA. Transplantation immunobiology. Immunol Rev 2003;196:5-6.
8. Hancock WW, Lu B, Gao W, Csizmadia V, Faia K, King JA, et al. Requirement of the chemokine receptor CXCR3 for acute allograft rejection. J Exp Med 2000;192(10):1515-20.
9. Gao W, Faia KL, Csizmadia V, Smiley ST, Soler D, King JA, et al. Beneficial effects of targeting CCR5 in allograft recipients. Transplantation 2001;72(7):1199-205.
10. Miura M, Morita K, Kobayashi H, Hamilton TA, Burdick MD, Strieter RM, et al. Monokine induced by IFN-gamma is a dominant factor directing T-cells into murine cardiac allografts during acute rejection. J Immunol 2001;167(6):3494-504.
11. Hancock WW, Gao W, Csizmadia V, Faia KL, Shemmeri N, Luster AD. Donor-derived IP-10 initiates development of acute allograft rejection. J Exp Med 2001;193(8):975-80.
12. Melter M, Exeni A, Reinders ME, Fang JC, McMahon G, Ganz P, et al. Expression of the chemokine receptor CXCR3 and its ligand IP-10 during human cardiac allograft rejection. Circulation 2001;104(21):2558-64.
13. Zhao DX, Hu Y, Miller GG, Luster AD, Mitchell RN, Libby P. Differential expression of the IFN-gamma-inducible CXCR3-binding chemokines, IFN-inducible protein 10, monokine induced by IFN, and IFN-inducible T-cell alpha chemoattractant in human cardiac allografts: association with cardiac allograft vasculopathy and acute rejection. J Immunol 2002;169(3):1556-60.
14. Yun JJ, Fischbein MP, Whiting D, Irie Y, Fishbein MC, Burdick MD, et al. The role of MIG/CXCL9 in cardiac allograft vasculopathy. Am J Pathol 2002;161(4):1307-13.
15. Kao J, Kobashigawa J, Fishbein MC, MacLellan WR, Burdick MD, Belperio JA, et al. Elevated serum levels of the CXCR3 chemokine ITAC are associated with the development of transplant coronary artery disease. Circulation 2003;107(15):1958-61.
16. Fahmy NM, Yamani MH, Starling RC, Ratliff NB, Young JB, McCarthy PM, et al. Chemokine and chemokine receptor gene expression indicates acute rejection of human cardiac transplants. Transplantation 2003;75(1):72-8.
17. Fahmy NM, Yamani MH, Starling RC, Ratliff NB, Young JB, McCarthy PM, et al. Chemokine and receptor-gene expression during early and late acute rejection episodes in human cardiac allografts. Transplantation 2003;75(12):2044-7.

18. Butcher EC, Picker LJ. Lymphocyte homing and homeostasis. Science 1996;272(5258):60-6.
19. Moser B, Loetscher P. Lymphocyte traffic control by chemokines. Nat Immunol 2001;2(2):123-8.
20. Beckmann JH, Yan S, Luhrs H, Heid B, Skubich S, Forster R, et al. Prolongation of allograft survival in ccr7-deficient mice. Transplantation 2004;77(12):1809-14.
21. Hopken UE, Droese J, Li JP, Joergensen J, Breitfeld D, Zerwes HG, et al. The chemokine receptor CCR7 controls lymph node-dependent cytotoxic T-cell priming in alloimmune responses. Eur J Immunol 2004;34(22):461-70.
22. Buckley CD, Amft N, Bradfield PF, Pilling D, Ross E, Arenzana-Seisdedos F, et al. Persistent induction of the chemokine receptor CXCR4 by TGF-beta 1 on synovial T-cells contributes to their accumulation within the rheumatoid synovium. J Immunol 2000;165(6):3423-9.
23. Nanki T, Hayashida K, El-Gabalawy HS, Suson S, Shi K, Girschick HJ, et al. Stromal cell-derived factor-1-CXC chemokine receptor 4 interactions play a central role in CD4+ T-cell accumulation in rheumatoid arthritis synovium. J Immunol 2000;165(11):6590-8.
24. Kunkel EJ, Boisvert J, Murphy K, Vierra MA, Genovese MC, Wardlaw AJ, et al. Expression of the chemokine receptors CCR4, CCR5, and CXCR3 by human tissue-infiltrating lymphocytes. Am J Pathol 2002;160(1):347-55.
25. Yoneyama H, Narumi S, Zhang Y, Murai M, Baggiolini M, Lanzavecchia A, et al. Pivotal role of dendritic cell-derived CXCL10 in the retention of T helper cell 1 lymphocytes in secondary lymph nodes. J Exp Med 2002;195(10):1257-66.
26. Burman A, Haworth O, Hardie DL, Amft EN, Siewert C, Jackson DG, et al. A chemokine-dependent stromal induction mechanism for aberrant lymphocyte accumulation and compromised lymphatic return in rheumatoid arthritis. J Immunol 2005;174(3):1693-700.
27. Athanassopoulos P, Vaessen, L.M.B., Balk, A.H.M.M., Takkenberg, J.J.M., Maat, A.P.W.M., Weimar, W., Bogers, A.J.J.C. Impaired circulating dendritic cell reconstitution identifies rejecting recipients after clinical heart transplantation independent of rejection therapy. Eur J Cardiothorac Surg *2005*;27(5):783-789.
28. Billingham ME, Cary NR, Hammond ME, Kemnitz J, Marboe C, McCallister HA, et al. A working formulation for the standardization of nomenclature in the diagnosis of heart and lung rejection: Heart Rejection Study Group. The International Society for Heart Transplantation. J Heart Transplant 1990;9(6):587-93.
29. Baan CC, Balk AH, van Riemsdijk IC, Vantrimpont PJ, Maat AP, Niesters HG, et al. Anti-CD25 monoclonal antibody therapy affects the death signals of graft-infiltrating cells after clinical heart transplantation. Transplantation 2003;75(10):1704-10.
30. Waehre T, Damas JK, Gullestad L, Holm AM, Pedersen TR, Arnesen KE, et al. Hydroxymethylglutaryl coenzyme a reductase inhibitors down-regulate chemokines and chemokine receptors in patients with coronary artery disease. J Am Coll Cardiol 2003;41(9):1460-7.

31. Athanassopoulos P, Vaessen LM, Maat AP, Zondervan PE, Balk AH, Bogers AJ, et al. Preferential depletion of blood myeloid dendritic cells during acute cardiac allograft rejection under controlled immunosuppression. Am J Transplant 2005;5(4):810-20.
32. Rose ML, Gracie JA, Fraser A, Chisholm PM, Yacoub MH. Use of monoclonal antibodies to quantitate T lymphocyte subpopulations in human cardiac allografts. Transplantation 1984;38(3):230-4.
33. Morita K, Miura M, Paolone DR, Engeman TM, Kapoor A, Remick DG, et al. Early chemokine cascades in murine cardiac grafts regulate T-cell recruitment and progression of acute allograft rejection. J Immunol 2001;167(5):2979-84.
34. Yun JJ, Fischbein MP, Laks H, Fishbein MC, Espejo ML, Ebrahimi K, et al. Early and late chemokine production correlates with cellular recruitment in cardiac allograft vasculopathy. Transplantation 2000;69(12):2515-24.
35. Burns WR, Wang Y, Tang PC, Ranjbaran H, Iakimov A, Kim J, et al. Recruitment of CXCR3 and CCR5 T-cells and Production of Interferon-gamma-Inducible Chemokines in Rejecting Human Arteries. Am J Transplant 2005;5(6):1226-36.
36. Yoshida R, Nagira M, Imai T, Baba M, Takagi S, Tabira Y, et al. EBI1-ligand chemokine (ELC) attracts a broad spectrum of lymphocytes: activated T-cells strongly up-regulate CCR7 and efficiently migrate toward ELC. Int Immunol 1998;10(7):901-10.
37. Alt C, Laschinger M, Engelhardt B. Functional expression of the lymphoid chemokines CCL19 (ELC) and CCL 21 (SLC) at the blood-brain barrier suggests their involvement in G-protein-dependent lymphocyte recruitment into the central nervous system during experimental autoimmune encephalomyelitis. Eur J Immunol 2002;32(8):2133-44.
38. Weninger W, Carlsen HS, Goodarzi M, Moazed F, Crowley MA, Baekkevold ES, et al. Naïve T-cell recruitment to nonlymphoid tissues: a role for endothelium-expressed CC chemokine ligand 21 in autoimmune disease and lymphoid neogenesis. J Immunol 2003;170(9):4638-48.
39. Columba-Cabezas S, Serafini B, Ambrosini E, Aloisi F. Lymphoid chemokines CCL19 and CCL21 are expressed in the central nervous system during experimental autoimmune encephalomyelitis: implications for the maintenance of chronic neuroinflammation. Brain Pathol 2003;13(1):38-51.
40. Masopust D, Vezys V, Usherwood EJ, Cauley LS, Olson S, Marzo AL, et al. Activated primary and memory CD8 T-cells migrate to nonlymphoid tissues regardless of site of activation or tissue of origin. J Immunol 2004;172(8):4875-82.
41. Christopherson KW, 2nd, Hood AF, Travers JB, Ramsey H, Hromas RA. Endothelial induction of the T-cell chemokine CCL21 in T-cell autoimmune diseases. Blood 2003;101(3):801-6.
42. Vaessen LM, Baan CC, Ouwehand AJ, Balk AH, Jutte NH, Mochtar B, et al. Differential avidity and cyclosporine sensitivity of committed donor-specific graft-infiltrating cytotoxic T-cells and their precursors. Relevance for clinical cardiac graft rejection. Transplantation 1994;57(7):1051-9.

43. Wagoner LE, Zhao L, Bishop DK, Chan S, Xu S, Barry WH. Lysis of adult ventricular myocytes by cells infiltrating rejecting murine cardiac allografts. Circulation 1996;93(1):111-9.
44. Chen G, Shankar P, Lange C, Valdez H, Skolnik PR, Wu L, et al. CD8 T-cells specific for human immunodeficiency virus, Epstein-Barr virus, and cytomegalovirus lack molecules for homing to lymphoid sites of infection. Blood 2001;98(1):156-64.
45. Ravkov EV, Myrick CM, Altman JD. Immediate early effector functions of virus-specific CD8+CCR7+ memory cells in humans defined by HLA and CC chemokine ligand 19 tetramers. J Immunol 2003;170(5):2461-8.
46. Gamadia LE, Rentenaar RJ, Baars PA, Remmerswaal EB, Surachno S, Weel JF, et al. Differentiation of cytomegalovirus-specific CD8(+) T-cells in healthy and immunosuppressed virus carriers. Blood 2001;98(3):754-61.
47. Yopp AC, Krieger NR, Ochando JC, Bromberg JS. Therapeutic manipulation of T-cell chemotaxis in transplantation. Curr Opin Immunol 2004;16(5):571-7.
48. Campbell DJ, Kim CH, Butcher EC. Chemokines in the systemic organization of immunity. Immunol Rev 2003;195:58-71.
49. Kobayashi H, Koga S, Novick AC, Toma H, Fairchild RL. T-cell mediated induction of allogeneic endothelial cell chemokine expression. Transplantation 2003;75(4):529-36.
50. El-Sawy T, Miura M, Fairchild R. Early T-cell response to allografts occurring prior to alloantigen priming up-regulates innate-mediated inflammation and graft necrosis. Am J Pathol 2004;165(1):147-57.
51. Hancock WW, Wang L, Ye Q, Han R, Lee I. Chemokines and their receptors as markers of allograft rejection and targets for immunosuppression. Curr Opin Immunol 2003;15(5):479-86.
52. Luther SA, Bidgol A, Hargreaves DC, Schmidt A, Xu Y, Paniyadi J, et al. Differing activities of homeostatic chemokines CCL19, CCL21, and CXCL12 in lymphocyte and dendritic cell recruitment and lymphoid neogenesis. J Immunol 2002;169(1):424-33.
53. Baddoura FK, Nasr IW, Wrobel B, Li Q, Ruddle NH, Lakkis FG. Lymphoid neogenesis in murine cardiac allografts undergoing chronic rejection. Am J Transplant 2005;5(3):510-6.
54. Colvin BL, Wang Z, Nakano H, Wu W, Kakiuchi T, Fairchild RL, et al. CXCL9 antagonism further extends prolonged cardiac allograft survival in CCL19/CCL21-deficient mice. Am J Transplant 2005;5(9):2104-13.

Chapter 8

General Discussion

Discussion

Circulating dendritic cell phenotype in end-stage heart failure

In this thesis, two distinct human dendritic cell subsets, myeloid (m) DCs and plasmacytoid (p) DCs, were directly enumerated in peripheral blood of patients with end-stage (NYHA III-IV) heart failure (HF) before and after transplantation, using four-color 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 HF. This polarized mDC/pDC balance towards mDCs suggests immune responses with a T_H1 predisposition under such conditions [1]. We also demonstrated that in a pathological state like HF, according to their chemokine receptor profile, DC subsets exhibit altered migratory properties. Differential chemokine expression has been shown to affect hemopoietic cell migration and induce stem cell homing to injured myocardium in animal models of HF [2]. In our studies, both mDCs and pDCs encountered in the blood of the HF patients were phenotypically activated, according to the upregulation of $CD83^+$ cells. The concurrent increase of $CCR7^+$ mDCs, must also account for increased potential of the mDCs encountered in HF patients to migrate towards secondary lymphoid organs.

Whereas patients with the most advanced disease (NYHA-IV), did not exhibit significantly different DC levels than their NYHA-III counterparts, we found a strong association of DC levels with dilated cardiomyopathy (DCM) as etiology of end-stage HF. Blood mDCs from patients with DCM, except for their elevated levels also showed increased CD83 and CCR7 surface expression compared to mDCs from groups with different cardiac dysfunction etiology. We propose that parallel enhancement of maturation marker and lymphoid homing chemokine receptor expression levels only on mDCs from DCM patients, accounts for a state of functional response of mDCs to immunogenic stimuli related with the pathogenesis of DCM. More importantly, the imbalance in the two blood DC subsets with a skewing towards mDCs in patients with idiopathic DCM, suggests that these patients may have a reduced ability to generate peripheral tolerance to a number of auto-antigens [Chapter 4].

Circulating dendritic cell phenotype in acute rejection after clinical heart transplantation

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. antithymocyte globulin, corticosteroids, calcineurin inhibitors or mycophenolate mofetil) are able to induce immunological quiescence. 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 exerts a potent effect not only on the maturation status but also on the migration characteristics of both mDCs and pDCs [1].

Peritransplant ischemic injury induces complex immune responses through differential chemokine and chemokine receptor gene induction [3]. After heart transplantation (HTx), processes such as stem cell homing and tissue regeneration are based on altered chemokine receptor expression on circulating hematopoietic cells [4]. Similarly, we believe that a shifted balance between blood mDCs and pDCs towards mDCs shortly post-HTx reflects a mechanism based on immune deviation by acute heart injury, due to trauma or ischemia/reperfusion injury during the transplantation procedure. Our results suggest that both donor and recipient DCs are programmed to drive the differentiation of T_H0 to T_H1 cells and to initiate acute rejection (AR) after transplantation. Indirectly, this is confirmed as only blood mDC numbers decreased markedly during each AR episode. Furthermore, we observed that mDCs were negatively associated with endomyocardial biopsy (EMB) pathological grade of rejection (ISHLT), while pDCs remained constantly low. Interestingly, rejecting patients experienced a decreased frequency of $CCR7^+$ mDCs in their blood during AR. Uniformly altered DC kinetics must reflect an inherent mechanism of circulating mDC depletion during AR. We speculate that low $CCR7^+$ mDC frequency must suggest selective mDC homing to secondary lymphoid tissues in order to induce $CD4^+$ and $CD8^+$ T-cell clonal expansion and differentiation into helper or cytotoxic T-cells and therefore initiate AR. In this context, it is interesting that rejectors experienced a decreased % of $CCR7^+$ mDCs in their blood during AR. Uniformly altered DC kinetics must reflect an inherent mechanism of circulating mDC depletion during AR [5].

However, rejecting patients did not exhibit the mDC recovery that non-rejectors experienced after 3 months post-HTx. Furthermore, in rejecting HTx recipients the defective circulating mDC reconstitution over time was independent of infection onset (e.g. CMV), statin treatment or tapering of maintenance immunosuppression. Moreover, within rejecting recipients mDC numbers remained low in both patients treated or not-treated with rejection therapy during follow-up. This phenomenon suggests that defective circulating DC reconstitution post-HTx, is inherently related to the immunological process

of AR and not to additional immunosuppression administered as rejection treatment post-HTx [6].

Circulating T-cell phenotypic characteristics in end-stage heart failure

We also assessed blood from heart transplant recipients before transplantation and healthy individuals in order to clarify whether circulating leukocyte chemokine receptor expression profile is modified during NYHA III-IV heart failure. Peripheral blood T-cells from HF patients exhibited upregulated expression of the chemokine receptor CXCR4, which was reflected primarily on $CD8^+$ cells, independent of etiology of the disease. Elevated CXCR4 expression on both circulating $CD4^+$ and $CD8^+$ cells in the same patients, must reflect enhanced migration of both T-cell subsets with a naïve phenotype towards lymphoid organs in end-stage HF. These results concur with the upregulation of CCR7 expression on the surface of both circulating $CD4^+$ and $CD8^+$ cells. Interestingly, patients with coronary artery disease and idiopathic DCM had higher CCR7 expression on $CD8^+$ cells than patients with hypertrophic cardiomyopathy and valvular disease. Parallel increased homeostatic chemokine receptor (CCR7 and CXCR4) expression on naïve peripheral blood T-cell subsets could result in accelerated homing of $CD4^+$ or $CD8^+$ cells to lymphoid tissues for T_H1 or T_H2 priming during end-stage HF. In this state, lymphoid tissue could play a central role in T-cell and dendritic cell recirculation.

In peripheral blood, we did not see any clear polarization of $CD4^+$ cells towards a T_H1 or T_H2 phenotype. On the contrary, $CD8^+$ $CXCR3^+$ and $CD8^+$ $CXCR1^+$ T-cell frequency in the blood of patients was increased. As CXCR3 is known to be induced on effector-memory T-cells and CXCR1 expression has been shown on human effector $CD8^+$ T-cells, our results provide the evidence for a small fraction of $CD8^+$ cells with a T_H1 type inflammatory inducing capacity in the peripheral blood of end-stage HF patients. Conceptually, these cells should exhibit affinity for homing into inflammatory sites through CXCL-1, CXCL-5 or CXCL-8, and in the case of our patients this should be the myocardium [7].

Circulating T-cell phenotype in relation to endomyocardial biopsy chemokine production by acute rejection after clinical heart transplantation

By analyzing circulating $CD4^+$ and $CD8^+$ T-cell chemokine receptor expression in parallel with intragraft chemokine and chemokine receptor gene expression of cardiac allograft biopsy specimens, in relation to the ISHLT classification for histological infiltration grade after transplantation, no differences were

observed in the frequency of circulating $CD4^+$ cells positive for the chemokine receptors questioned. Within the $CD8^+$ subset, mean percentage of $CCR5^+$ cells was raised while the percentage of $CCR7^+$ cells was reduced during AR. Reciprocal regulation of $CCR5^+$ and $CCR7^+$ cells only within circulating $CD8^+$ but not $CD4^+$ T-lymphocytes could be explained as proportionally more $CD8^+$ than $CD4^+$ cells, with an effector/memory $CD45RO^+$ phenotype, are known to infiltrate rejecting human cardiac allografts. However, no satisfactory values could be obtained by peripheral blood analysis to reliably monitor AR, as both rejecting and non-rejecting recipients presented with a variable frequency of $CCR5^+$ and $CCR7^+$ $CD8^+$ cells at ISHLT grades 0 to 2, for the whole follow-up period after transplantation. Decreased mean circulating $CD8^+CCR7^+$ T-lymphocyte frequency during acute cardiac allograft rejection suggests either efflux of circulating $CD8^+CCR7^+$ T-cells, or alternatively enrichment of blood with $CD8^+CCR7^-$ T-cells, as part of the rejection process.

Endomyocardial biopsy gene expression indicated a strong association of intragraft levels from inflammatory chemokines (CCL5 & CXCL10) and their corresponding receptors (CCR5 & CXCR3) with the appearance of AR episodes. Intracardial mRNA levels of the homeostatic chemokine (CCL19) and its receptor (CCR7) were significantly elevated upon AR. In our opinion, differential CCR5 and CXCL10 gene expression profiles between rejectors during non-rejection episodes (ISHLT grades 0–2) and non-rejectors, must represent altered intracardial chemokine and chemokine receptor micro-environment in the rejecting patients, prior to histologically proven rejection.

It is known that during cardiac allograft rejection, CCL5 and CXCL10 drive local intracardial recruitment of effector $CCR5^+$ and $CXCR3^+$ cells, respectively. However, the elevated intragraft levels of CCL19 and CCR7 during AR, suggest that intragraft homeostatic chemokine – chemokine receptor interactions are also involved in the process of rejection. As myocardial CCL19 expression negatively affects circulating $CD8^+CCR7^+$ T-cell frequency in rejecting patients, high intragraft CCL19 production must influence peripheral blood $CD8^+$ T-cell recirculation by facilitating intracardial $CD8^+CCR7^+$ T-cell infiltration in these patients. This phenomenon must reflect a reduction in the migratory specificity of $CD8^+$ cells in rejecting patients, driven by high myocardial CCL19 expression after heart transplantation. Such a mechanism may explain the high post-transplant incidence of non-donor specific cytotoxic T-lymphocytes in the grafts of rejecting patients.

This effect seems to be time-dependent as circulating $CD8^+CCR7^+$ T-cell frequency in rejectors decreased more markedly, independent of CMV syndrome

manifestation during the course of follow-up. As immunosuppressants are known to affect CCR7-dependent T-cell migration *in vivo*, decreased $CD8^+CCR7^+$ T-cell frequency in rejectors with time, could be ascribed to additional influence on chemokine receptor expression from anti-rejection therapy. Reduced $CCR7^+$, or alternatively raised $CCR7^-$, proportion within circulating $CD8^+$ T-cells, may reflect the increasing magnitude of allo-antigen-specific responses that develop with time after transplantation.

Considering the positive correlation between CCL19 expression during AR+ and raised CXCL10 and CCR5 levels before AR, we speculate that monocyte/macrophage infiltration and inflammatory chemokine expression before AR potentiates vascular endothelial or cardiomyocyte homeostatic chemokine production with a tropism for CCL19. This chemokine microenvironment might not only facilitate intracardial effector T-cell influx, but may also lead to intragraft naïve lymphocyte activation, dendritic cell maturation and homing. Such a process may support local lymphoid follicle formation and contribute to persistence of risk for cardiac allograft loss, as described by late acute and chronic rejection after heart transplantation. In this manner, homeostatic and inflammatory chemokines may act synergistically in $CD8^+$ T-cell infiltration during cardiac allograft rejection [Chapter 7].

Prospects

Chemokines and their receptors by DC and T-cell recirculation in heart failure

Despite recent improvements in cardiovascular treatment, HF is a progressive disease with high mortality and morbidity, suggesting that important pathogenic mechanisms like persistent immune activation and inflammation, remain active and unmodified by the present treatment modalities [8]. Indeed, neither of the already existing drugs seem to have any profound effects on inflammatory cytokines in HF patients [9]. Different forms of immunomodulatory therapy, such as pentoxyfylline, intravenous immunoglobulin, thalidomide and statins in addition to conventional cardiovascular treatment regimens, have therefore emerged as possible new and promising treatment modalities for end-stage HF patients.

Following tissue damage, infection, or inflammation, mDCs become activated and migrate via afferent lymphatic vessels or blood to secondary lymphoid organs and to inflamed myocardium [1]. Enhanced myocardial expression of

chemokines and their corresponding receptors on both cardiomyocytes and infiltrating T-cells in various forms of myocardial failure suggests a central role for chemokine-related interactions in the pathogenesis of end-stage HF. In this context, alterations of chemokine receptor expression may not only be a new parameter of enhanced immune activation, but may also reflect an important mechanism contributing to the progression of HF with underlying etiology [7].

The capacity to control activation and movement of DC and T-cell subsets, suggests that chemokines and their receptors might provide novel targets for therapeutical intervention in chronic myocardial failure. A series of chemokine receptor antagonists have been found to prevent the development of several inflammatory diseases in animal models [10]. In depth knowledge of the possible pathogenic role of chemokines in human heart failure suggests that modulation of the chemokine network may represent interesting alternative therapeutic modalities in this disorder.

Chemokines and their receptors by DC and T-cell recirculation in rejection after heart transplantation

Innate and adaptive immune responses in allograft rejection are linked through chemokine-chemokine receptor directed DC and T-cell trafficking. For example, certain chemokines are important in directing exit of DCs from peripheral tissues to secondary lymphoid organs [11]. In this context, peripheral blood mDCs may be attributed an important role in eliciting and maintaining allograft rejection post-HTx [5]. Monitoring of peripheral blood DC subsets may identify patients at high risk for rejection after 3 months post-HTx. This might prove clinically relevant as appropriate adjustments in immunosuppressive regimens may avoid over-immunosuppression of patients at a lower risk for rejection long after clinical HTx [6].

Chemokines and their receptors, are also pivotal mediators in allograft rejection by recruiting alloantigen-primed T-cells into allografts through their activity as regulators of T-cell movement, adhesion, and effector function [12]. Because the regulation of effector T-cell infiltration is complex, it is difficult to dissect the relative role of each chemokine in the inflammatory processes leading to allograft rejection, especially since many chemokines and their receptors are seemingly redundant [13]. Nevertheless, it is clear that specific chemokine and/or chemokine receptor blockades may influence various aspects of the rejection process. Experimental data point to the chemokine receptors CXCR3 and CCR5, which are induced upon activation of T-cells, as key mediators of

effector cell recruitment in response to the local elaboration of chemokines by graft endothelial and parenchymal cells [14].

Most researchers in the transplantation field have so far concentrated on chemokines rather than receptors and on a rather few chemokines at that, especially some of the original chemokines described (RANTES, MCP-1 and IL-8). Tools are now becoming available to analyze these roles in a meaningful manner, including commercially available anti-chemokine and anti-chemokine-receptor mAbs and gene knockout animals. It is a reasonable expectation that within the coming years the key primary data as to which receptors and ligands are of fundamental significance for therapeutic targeting will become available from experimental systems. Clinical applications are still several years away, with the exception that use of mAbs and cDNA arrays for monitoring purposes is likely to yield new diagnostic and prognostic application in transplantation for knowledge of this unique family of genes [15].

Clinical therapeutic interventions aimed at chemokines and/or their receptors may prove useful in treatment of transplant rejection and/or induction of immunologic tolerance. Therapeutic effects will likely differ, depending upon the stage of rejection and the other therapeutics administered. As to now, data from clinical studies suggests that certain pathways are also active in human recipients of solid organ allografts. The marked enhancement of long-term heart transplant survival in patients homozygous for a CCR5 null allele is very encouraging for future therapies directed at CCR5-positive mononuclear cells. Results with blockades of other chemokine receptors expressed by effector T-cells CCR1, CXCR3, and CX_3CR1 are also remarkable [16]. Clearly, interruption of the afferent pathway leading to allosensitization – e.g. through inhibition of the CCL19/CCR7 interaction – might also be of potential therapeutic importance [17]. For this, immunohistochemical analyses are required to consolidate CCL19 production and *in situ* $CCR7^+$ cell localization on allograft biopsies from rejecting and non-rejecting patients. In this manner, functional roles of CCL19 and CCR7 could be unraveled for endothelial dysfunction during rejection and accelerated coronary artery disease after heart transplantation [Chapter 7]. In this context, anti-CCR7 antibodies as well as the drug Ciglitazone and other selective peroxisome proliferative activated receptor-γ agonists known to decrease expression of CCR7, may have the potential to become important therapeutics in treatment of acute and chronic allograft rejection [18]. The extent to which these processes can be usefully targeted to the beneficial effect of an allograft recipient, constitutes one of the most exciting areas of transplant immunobiology at the present time.

Although it is becoming apparent that inhibiting DC and T-cell trafficking is a very effective strategy for inhibiting rejection after heart transplantation, other checkpoints in leukocyte trafficking can be potential targets. For example, T-cell exit from lymphoid tissue requires sphingosine 1-phosphate (S1P) and its G- Protein Coupled Receptor $S1P_1$,[19] whereas exit of DCs and T- cells from peripheral tissue requires CCR7 [20, 21]. Pharmacological inhibition of this pathway with drugs such as FTY720 traps T-cells in lymph nodes and primary organs, leading to peripheral T-cell depletion [22, 23]. FTY720 and other $S1P_1$ antagonists are being evaluated as new immunosuppressants for organ transplantation. Other examples include targeting proteases secreted by leukocytes, which facilitate leukocyte movement through basement membrane and the ECM in tissue, and targeting signaling molecules that link GPCR activation to changes in integrin affinity and to directed cell migration. The challenge for the future is to identify the key DC or T-cell subset that initiates rejection and to identify the trafficking molecule(s) that will most specifically inhibit that subset of cells while leaving most leukocytes unaffected to avoid untoward infectious complications. An unavoidable consequence of inhibiting leukocyte trafficking will be that the host is left more susceptible to certain infections. It is clear, then, that better understanding of the consequences of inhibiting the trafficking of specific leukocyte subsets is needed, and patients must be screened for latent infections and/or provided with prophylaxis against the relevant potential pathogens before therapy is initiated. The goal, then, is to minimize infectious risk by identifying the trafficking molecules whose inhibition will result in the narrowest range of cellular inhibition while still allowing the therapy to be efficacious. Furthermore, regional delivery of this inhibition (such as the heart or lymph nodes) and precise targeting of pathogenic DC or T-cell subsets will minimize systemic immunodeficiency and improve the therapeutic 'window' of treatments aimed at dampening the inflammatory response by inhibiting DC or T-cell trafficking.

References

1. Athanassopoulos P, Vaessen LM, Maat AP, Balk AH, Weimar W, Bogers AJ. Peripheral blood dendritic cells in human end-stage heart failure and the early post-transplant period: evidence for systemic T_H1 immune responses. Eur J Cardiothorac Surg 2004;25 (4):619-26.
2. Askari AT, Unzek S, Popovic ZB, Goldman CK, Forudi F, Kiedrowski M, et al. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. Lancet 2003;362(9385):697-703.
3. Chen D, Ding Y, Schroppel B, Zhang N, Fu S, Zhang H, et al. Differential chemokine and chemokine receptor gene induction by ischemia, alloantigen, and gene transfer in cardiac grafts. Am J Transplant 2003;3(10):1216-29.
4. Yamani MH, Ratliff NB, Cook DJ, Tuzcu EM, Yu Y, Hobbs R, et al. Peritransplant ischemic injury is associated with up-regulation of stromal cell-derived factor-1. J Am Coll Cardiol 2005;46(6):1029-35.
5. Athanassopoulos P, Vaessen LM, Maat AP, Zondervan PE, Balk AH, Bogers AJ, et al. Preferential depletion of blood myeloid dendritic cells during acute cardiac allograft rejection under controlled immunosuppression. Am J Transplant 2005;5(4):810-20.
6. Athanassopoulos P, Vaessen, L.M.B., Balk, A.H.M.M., Takkenberg, J.J.M., Maat, A.P.W.M., Weimar, W., Bogers, A.J.J.C. Impaired circulating dendritic cell reconstitution identifies rejecting recipients after clinical heart transplantation independent of rejection therapy. Eur J Cardiothorac Surg *2005*;27(5):783-789.
7. Athanassopoulos P, Vaessen LM, Balk AH, Weimar W, Sharma HS, Bogers AJ. Altered Chemokine Receptor Profile on Circulating Leukocytes in Human Heart Failure. Cell Biochem Biophys 2006;44(1):83-101.
8. Aukrust P, Damas JK, Gullestad L, Froland SS. Chemokines in myocardial failure -- pathogenic importance and potential therapeutic targets. Clin Exp Immunol 2001;124(3):343-5.
9. Gullestad L, Kjekshus J, Damas JK, Ueland T, Yndestad A, Aukrust P. Agents targeting inflammation in heart failure. Expert Opin Investig Drugs 2005;14(5):557-66.
10. Johnson Z, Schwarz M, Power CA, Wells TN, Proudfoot AE. Multi-faceted strategies to combat disease by interference with the chemokine system. Trends Immunol 2005; 26(5):268-74.
11. el-Sawy T, Fahmy NM, Fairchild RL. Chemokines: directing leukocyte infiltration into allografts. Curr Opin Immunol 2002;14(5):562-8.
12. Cascieri MA, Springer MS. The chemokine/chemokine-receptor family: potential and progress for therapeutic intervention. Curr Opin Chem Biol 2000;4(4):420-7.
13. Yopp AC, Krieger NR, Ochando JC, Bromberg JS. Therapeutic manipulation of T-cell chemotaxis in transplantation. Curr Opin Immunol 2004;16(5):571-7.
14. Hancock WW, Wang L, Ye Q, Han R, Lee I. Chemokines and their receptors as markers of allograft rejection and targets for immunosuppression. Curr Opin Immunol 2003;15(5):479-86.

15. Hancock WW, Gao W, Faia KL, Csizmadia V. Chemokines and their receptors in allograft rejection. Curr Opin Immunol 2000;12(5):511-6.
16. Nelson PJ, Krensky AM. Chemokines, chemokine receptors, and allograft rejection. Immunity 2001;14(4):377-86.
17. Wang L, Han R, Lee I, Hancock AS, Xiong G, Gunn MD, et al. Permanent survival of fully MHC-mismatched islet allografts by targeting a single chemokine receptor pathway. J Immunol 2005;175(10):6311-8.
18. Fainaru O, Shseyov D, Hantisteanu S, Groner Y. Accelerated chemokine receptor 7-mediated dendritic cell migration in Runx3 knockout mice and the spontaneous development of asthma-like disease. Proc Natl Acad Sci U S A 2005;102(30):10598-603.
19. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. Nature 2004;427(6972):355-60.
20. Bromley SK, Thomas SY, Luster AD. Chemokine receptor CCR7 guides T-cell exit from peripheral tissues and entry into afferent lymphatics. Nat Immunol 2005;6(9):895-901.
21. Debes GF, Arnold CN, Young AJ, Krautwald S, Lipp M, Hay JB, et al. Chemokine receptor CCR7 required for T lymphocyte exit from peripheral tissues. Nat Immunol 2005;6(9):889-94.
22. Rosen H, Goetzl EJ. Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. Nat Rev Immunol 2005;5(7):560-70.
23. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annual Review of Immunology 2005;23:127-159.

Chapter 9

Summary and Conclusions

Summary

Heart failure (HF) is a complex clinical syndrome characterized by immune activation in its chronic stages. By different forms of cardiac dysfunction, infiltration of failing human myocardium with effector leukocytes through low-grade inflammation, leads to deterioration of clinical condition to end-stage HF. The only available permanent therapeutic option for patients with end-stage cardiac disease is heart transplantation (HTx). Allo-antigen dependent immune cell activation and recirculation determine graft outcome, with heart allograft rejection remaining a severe complication after HTx. For years the international transplantation community attempts to improve patient survival and prevent graft loss. In this context, identifying patients at high risk for rejection or even predicting acute cardiac rejection (AR) would be important, as appropriate adjustments in immunosuppressive regimens may avoid over-immunosuppression related complications after clinical HTx. In this thesis, we attempted to clarify the role of chemokines and chemokine receptors in leukocyte recirculation during end-stage HF and rejection after HTx. Trafficking of dendritic cell (DC) and T-cell subsets received our particular attention, because their interaction critically determines the type of immune reactivity elicited in chronic disease and transplantation.

Chapter 1, provides a general introduction on the migratory routes followed by DCs and T-cells, based on chemokines and their receptors. Known chemotactic pathways of leukocyte activation and infiltration as well as their role in the pathogenesis of HF and heart allograft rejection are presented. A description of the objectives of our studies is also included.

Chapter 2 describes alterations in CC- and CXC- chemokine receptor expression as well as circulating frequency of peripheral blood $CD4^+$ and $CD8^+$ T-cell subsets, monocytes and granulocytes positive for the chemokine receptors questioned, from patients with end-stage HF. The results were outlined for both non-ischemic and ischemic forms of cardiomyopathy. No clear polarization of circulating T-cells towards a T_H1 or T_H2 phenotype was seen. Circulating T-cells from HF patients exhibited upregulated CCR7 and CXCR4 expression, while $CD8^+$ cells from patients with coronary artery disease (CAD) and dilated cardiomyopathy (DCM) had higher CCR7 expression than patients with hypertrophic cardiomyopathy (HCM) and valvular disease (VD). In CAD patients, frequencies of $CCR1^+$ and $CCR2^+$ monocytes were decreased, while on granulocytes, CXCR1 and CXCR2 receptors were downregulated in all patients, irrespective of cardiac disease etiology.

In **chapter 3**, changes in absolute numbers and frequency of phenotypically mature peripheral blood myeloid (m) and plasmacytoid (p) DCs are discussed in end-stage HF as well as early after HTx. Patients with NYHA III-IV HF had higher DC numbers compared to controls, due to enrichment of their mDC fraction. According to the upregulation of $CD83^+$ cells, both DC subsets exhibited an activated phenotype, while following the upregulation of CCR7, mDCs possessed an increased potential to migrate towards secondary lymphoid organs. After transplantation, numbers of circulating mDCs and pDCs were decreased drastically, suggesting "immunological quiescence" on the basis of immunosuppression. The mDC/pDC balance shifted in favour of mDCs, while both circulating DC subsets were also rendered into a non-activated, immature state.

Chapter 4 is concerned with circulating DC numbers and phenotype according to etiology of end-stage HF. While NYHA III-IV HF patients had comparable circulating DC subset levels to NYHA-II patients and controls, total blood DC levels in end-stage HF were elevated in patients with DCM. This was due to a marked increase of mDCs and to a lesser extent of pDCs in idiopathic DCM patients, independent of systolic or diastolic cardiac dysfunction. Blood mDCs from patients with DCM, except for their elevated levels also showed increased CD83 and CCR7 surface expression compared to mDCs from groups with different etiology of cardiac dysfunction.

Chapter 5 describes circulating DC subset distribution, maturation and lymphoid homing properties in relation to histological diagnosis of AR early and late after clinical HTx. Overall circulating numbers of both DC subsets remained low for the whole post-HTx period, while a negative association of mDCs was observed with rejection grade. Only mDCs decreased during AR episodes while compared to non-rejectors, rejectors had lower mDC numbers after 3 months post-HTx. This defective circulating mDC reconstitution over time was independent of infection onset (e.g. CMV), statin treatment or tapering of maintenance immunosuppression. Rejecting patients during AR exhibited low proportions of mDCs positive for CCR7, suggesting that circulating mDC depletion is associated with selective lymphoid homing of this subset in the process of rejection.

In **chapter 6**, total peripheral blood DC incidence and DC subset reconstitution was analyzed in time for both rejecting and non-rejecting recipients considering administration of rejection therapy after transplantation. Both mDC incidence as well as circulating mDC/pDC ratio – representing DC subset reconstitution – were negatively associated with ISHLT infiltration grade. Rejecting patients

exhibited impaired circulating DC reconstitution after 12 weeks post-HTx when compared to NR patients. Within rejectors, mDC numbers remained low in both patients treated or not-treated with rejection therapy during follow-up after HTx.

Chapter 7, investigated the differential contribution of inflammatory and homeostatic chemokines and their receptors on $CD4^+$ and $CD8^+$ T-cell recirculation in relation to corresponding intragraft chemokine and chemokine receptor gene expression during the process of rejection. While no differences were observed in the frequency of circulating $CD4^+$ cells positive for the chemokine receptors questioned, during AR mean proportions of $CCR5^+$ and $CCR7^+$ $CD8^+$ cells were altered. Mean intragraft CCR5, CCL5, CXCL10, CXCR3, CCR7 and CCL19 mRNA levels had increased during AR, while rejecting patients presented with higher myocardial CCR5 and CXCL10 mRNA levels even before AR. In these patients, high intragraft CCL19 mRNA was inversely correlated to circulating $CD8^+CCR7^+$ T-cell frequency, suggesting that myocardial CCL19 production affects $CD8^+$ T-cell recirculation under influence of CCR7 after HTx.

Finally, **chapter 8** embodies a comprehensive discussion of the aforementioned studies in the perspective of the international literature. The most important results and conclusions of the manuscripts comprising this thesis are reviewed for possible clinical implications and future directions in this field of research.

Conclusions

Heart Failure

The whole blood studies on chemokine receptor mediated DC and T-cell recirculation presented in this thesis, provided us with a new perspective in the immunological basis of human end-stage myocardial failure. Here, we present evidence for altered migratory potential of circulating T-cells in HF patients, based on their lymphoid homing chemokine receptor expression. Through CCR7 and CXCR4 upregulation, peripheral blood $CD4^+$ and $CD8^+$ T-cell subsets from such patients, may experience accelerated homing to lymphoid tissues for T_H1 or T_H2 priming. This suggests a new pathway completing the pathogenic loop for recruitment and activation of these cells under such conditions. Modulation of the chemokine network with inflammatory or lymphoid homing chemokine receptor antagonists, might provide novel targets for therapeutical intervention in chronic HF.

Blood DC characterization may also lead to new insights into the etiology of end-stage HF in humans. Elevated circulating DC levels confirm the altered migration patterns of bone marrow derived cells in this state. Moreover, the shifted mDC/pDC balance towards mDCs with a mature phenotype, suggests immune responses with a T_H1 predisposition in end-stage HF. In particular, parallel enhancement of CD83 and CCR7 expression only on mDCs from DCM patients, must account for functional mDC responses to immunogenic stimuli related with the pathogenesis of DCM. We propose that further skewing of the balance between the two blood DC subsets towards mDCs in patients with idiopathic DCM, is part of their reduced ability to generate peripheral tolerance to a number of auto-antigens. Further studies are needed to clarify the possible pathogenic role of blood and resident DCs in the development of human DCM.

Heart Transplantation

Involvement of chemokine – chemokine receptor interactions in DC and T-cell recirculation was also confirmed for rejection after clinical HTx. Circulating DC numbers decreased drastically, suggesting immunological quiescence on the basis of immunosuppression administered after transplantation. Thus, with DC homeostasis altered considerably, a shifted balance between blood mDCs and pDCs towards mDCs shortly post-HTx must reflect immune deviation due to trauma or ischemia/reperfusion injury during the transplantation procedure. During AR, peripheral blood mDC depletion was associated with selective mDC homing to secondary lymphoid tissues through CCR7, inducing T-cell clonal expansion and differentiation into effector-cytotoxic T-cells. In this context, circulating mDCs may be attributed an important role in eliciting and maintaining allograft rejection post-HTx. Except for providing insight into *in vivo* mechanisms of heart allograft rejection, peripheral blood DC monitoring may contribute to further optimization of immunosuppressive treatment strategies after clinical HTx. Although the technique is prone to bias from immunological phenomena such as opportunistic infections or cytomegalovirus syndrome after transplantation, DC monitoring may identify patients with high risk for rejection after 3 months post-HTx. This might prove clinically relevant as appropriate adjustments in immunosuppressive regimens may avoid over-immunosuppression of patients at a lower risk for rejection long after clinical HTx.

By the analysis of chemokine receptor expression on peripheral blood T-cells, no satisfactory values to reliably monitor AR could be obtained. However,

decreased mean circulating $CD8^+CCR7^+$ T-lymphocyte frequency during AR suggests either efflux of circulating $CD8^+CCR7^+$ T-cells, or enrichment of blood with $CD8^+CCR7^-$ T-cells, as part of the rejection process. Except for the confirmed associations of intragraft gene expression for inflammatory chemokines (CCL5 & CXCL10) and their corresponding receptors (CCR5 & CXCR3) with the appearance of AR, intracardial mRNA levels of the homeostatic chemokine (CCL19) and its receptor (CCR7) were also elevated upon AR. As myocardial CCL19 expression negatively affects circulating $CD8^+CCR7^+$ T-cell frequency in rejecting patients, high intragraft CCL19 production must influence peripheral blood $CD8^+$ T-cell recirculation by facilitating intracardial $CD8^+CCR7^+$ T-cell infiltration in these patients. Involvement of intragraft homeostatic chemokine – chemokine receptor interactions represents a novel aspect in the process of allograft rejection. Such a chemokine microenvironment may lead to intragraft naïve lymphocyte activation, dendritic cell maturation and homing. This could support local lymphoid follicle formation and contribute to persistent risk for cardiac allograft loss, by late acute and chronic rejection after HTx. Functional roles for CCL19 and CCR7 should be further investigated. Anti-CCR7 antibodies or agents known to decrease CCR7 expression and therefore inhibit DC or T-cell trafficking, may become important therapeutics in the treatment of acute and chronic allograft rejection in the future.

Chapter 10

Samenvatting
&
Περίληπση
(samenvatting in het Grieks)

Samenvatting

Hartfalen (HF) is een complex klinisch syndroom dat in haar chronische vorm gekarakteriseerd wordt door activatie van het afweersysteem. Bij verschillende uitingen van cardiale dysfunctie zien we dat infiltratie van het falende menselijke myocard door effector leukocyten, tot verslechtering van de klinische conditie naar eind-stadium HF leidt, via een laag-gradige ontstekingsproces. Vaak blijft harttransplantatie (HTx) als de enige beschikbare therapeutische optie over, voor patiënten met eind-stadium HF. Terwijl allo-antigeen afhankelijke immuuncel activatie en recirculatie de transplantaat overleving bepalen, blijft transplantaatafstoting een van de belangrijkste complicaties na HTx. De internationale transplantatie gemeenschap probeert de overleving van patiënten te bevorderen, tegelijkertijd met het verhinderen van het verlies van het transplantaat. Daarom probeert men patiënten met een hoog risico op afstoting te identificeren of zelfs acute afstoting (AR) te voorspellen, met als doel de toegediende immunosuppressie zodanig aan te passen om de daaraan gerelateerde complicaties te minimaliseren. In dit proefschrift, probeerden we de rol van chemokinen en chemokine receptoren te ontcijferen bij de recirculatie van leukocyten bij eind-stadium HF en afstoting na HTx. We richtten speciaal ons aandacht op dendritische cellen (DCs) en T-cel subtypen, aangezien hun interactie bepalend is voor immuunreacties bij zowel chronische ziekten als transplantatie.

Hoofdstuk 1 geeft een algemene introductie in de migratie van DCs en T cellen, op basis van chemokinen en hun receptoren. De rol hiervan op de pathogenese van HF en afstoting na HTx worden besproken samen met bekende chemotactische routes van activatie en infiltratie van leukocyten. In dit hoofdstuk wordt ook het doel van dit onderzoek besproken.

In **hoofdstuk 2** worden de veranderingen beschreven in zowel de expressie van CC- en CXC- chemokine receptoren als de frequentie van circulerende $CD4^+$ and $CD8^+$ T-cell subtypen, monocyten en granulocyten die positief zijn voor de genoemde receptoren, in patiënten met eind-stadium HF. De resultaten werden zowel voor non-ischemische en ischemische vormen van cardiomyopathie weergegeven. We vonden geen duidelijke polarisatie van circulerende T cellen naar een T_H1 of T_H2 fenotype. Wel lieten de circulerende T cellen van patiënten met eind-stadium HF een toegenomen CCR7 en CXCR4 expressie zien, terwijl de $CD8^+$ cellen van patiënten met coronairlijden (CAD) en gedilateerde cardiomyopathie (DCM) hogere expressie van CCR7 hadden dan patiënten met hypertrophische cardiomyopathie (HCM) of kleplijden

(VD). Patiënten met CAD, lieten een lager percentage van $CCR1^+$ en $CCR2^+$ monocyten zien, terwijl de CXCR1 en CXCR2 receptoren minder tot expressie kwamen op granulocyten van alle patiënten, onafhankelijk van de etiologie van hartlijden.

In **hoofdstuk 3,** worden het absoluut aantal en de frequentie van fenotypisch rijpe myeloïde (m) en plasmacytoïde (p) DCs in het perifeer bloed besproken, zowel tijdens eind-stadium HF als vroeg na HTx. Vergeleken met gezonde vrijwilligers, presenteerden patiënten met "New York Heart Association" (NYHA) Klasse III-IV HF zich met een hoger aantal DCs door een verrijking van de mDC fractie. Door een toename van $CD83^+$ cellen, lieten de mDCs en pDCs van patiënten een geactiveerd fenotype zien, terwijl de toename van $CCR7^+$ mDCs op een verhoogd migratie potentiaal van mDCs naar secundaire lymfoïde organen duidde. Na transplantatie, was het aantal circulerende mDCs and pDCs drastisch gedaald, een fenomeen dat "immunologische rust" suggereert waarschijnlijk tengevolge van de toegediende immunosuppressie. De balans tussen mDCs en pDCs (mDC/pDC) verschoof in de richting van mDCs, en beide circulerende DC subtypen verkeerden in een fenotypisch onrijpe staat.

Hoofdstuk 4 behandelt circulerende DCs en hun fenotype op basis van de etiologie van eind-stadium HF. Hoewel, het aantal circulerende mDCs en pDCs vergelijkbaar was tussen patiënten met NYHA III-IV, NYHA-II HF en controles, was het totaal aantal bloed DCs tijdens eind-stadium HF verhoogd in patiënten met DCM. Dit kwam door een toename van voornamelijk mDCs in patiënten met idiopathische DCM, onafhankelijk van systolische of diastolische dysfunctie van het hart. Wanneer er een onderscheid werd gemaakt op basis van het ontstaan van cardiale dysfunctie, toonden de mDCs van patiënten met DCM ook een hogere CD83 en CCR7 expressie.

Hoofdstuk 5 beschrijft de distributie, maturatie en eigenschappen voor lymfoïde "homing" van circulerende DC subtypen, in relatie tot vroege en late afstoting na klinische HTx. Het aantal circulerende mDCs en pDCs bleef laag voor de gehele periode na transplantatie. Bovendien, was het aantal mDCs op een negatieve manier geassocieerd met de afstotingsgraad en tijdens AR waren de mDCs het laagst. Drie maanden na HTx was het aantal mDCs bij patiënten die een afstoting hadden doorgemaakt lager dan bij patiënten zonder afstotingsverschijnselen. Dit was onafhankelijk van het optreden van infecties (zoals Cytomegalovirus), therapie met statines of vermindering van de immunosuppressieve onderhoudstherapie. Bij AR, waren lage percentages van $CCR7^+$ mDCs waar te nemen, hetgeen suggereert dat depletie van circulerende mDCs geassocieerd is met selectieve "homing" van mDCs naar lymfoïde organen ten tijde van de afstoting.

Hoofdstuk 6, behandelt de invloed van afstotingsbehandeling op het totaal aantal van bloed DCs en onderzoekt het herstel van mDCs en pDCs na transplantatie. Zowel het aantal circulerende mDCs als de mDC/pDC ratio waren op een negatieve manier geassocieerd met de afstotingsgraad volgens de richtlijnen van de "International Society for Heart and Lung Transplantation". Twaalf weken na HTx lieten de afstoters geen herstel in het aantal circulerende DCs zien in tegenstelling tot de niet-afstoters. Binnen de groep van de afstoters bleef het aantal mDCs laag, niet alleen bij patiënten die een afstotingsbehandeling ondergingen, maar ook bij patiënten die tijdens een periode met afstoting niet extra werden behandeld.
Hoofdstuk 7, beschrijft zowel inflammatoiïre als homeostatische chemokinen en hun receptoren op $CD4^+$ en $CD8^+$ T cellen in de circulatie in relatie tot hun corresponderende chemokine en chemokine receptor gen expressie in het transplantaat tijdens afstoting. Er werden geen verschillen waargenomen in het percentage circulerende $CD4^+$ cellen die positief waren voor de onderzochte chemokine receptoren. De gemiddelde percentages van $CCR5^+$ en $CCR7^+$ $CD8^+$ cellen tijdens AR waren echter veranderd. De mRNA expressie van CCR5, CCL5, CXCL10, CXCR3, CCR7 en CCL19 was tijdens AR hoger in het transplantaat, vergeleken met momenten zonder afstoting. Patiënten met afstoting hadden daarentegen hogere hoeveelheden CCR5 en CXCL10 mRNA dan patiënten zonder afstoting, zelfs vóór AR. Bij dezelfde patiënten, was de CCL19 mRNA expressie in het transplantaat negatief gecorreleerd met de hoeveelheid circulerende $CD8^+CCR7^+$ T-cellen. Dit suggereert dat CCL19 produktie door donor myocard de ontvanger $CD8^+$ T-cell recirculatie beïnvloedt via de chemokine receptor CCR7 .
Ten slotte, worden in **hoofdstuk 8** de resultaten van de voorgenoemde studies in het perspectief van de internationale literatuur besproken in de vorm van een algemene discussie. De belangrijkste conclusies van dit proefschrift worden in kaart gebracht om de klinische implicaties hiervan en de verdere mogelijkheden binnen dit onderzoeksveld te evalueren.

Conclusies

Hartfalen

De studies op humane DC en T-cel recirculatie in volbloed die in dit proefschrift beschreven worden, geven een nieuw perspectief in de immunologische mechanismen, die betrokken zijn bij eind-stadium HF. Met de eiwit-expressie van chemokine receptoren, die een rol spelen bij "homing" van leukocyten

naar lymfoïde organen, geven we hier een bewijs voor veranderde migratie patronen van circulerende T cellen in patiënten met HF. Door de verhoogde expressie van CCR7 en CXCR4, kunnen perifeer bloed $CD4^+$ en $CD8^+$ T-cellen van patiënten met HF versneld aangetrokken worden naar lymfoïd weefsel hetgeen leidt tot de inductie van T_H1 of T_H2 reacties. Het proces van migratie en activatie van deze cellen maakt deel uit van mechanismen die ten grondslag liggen aan de pathologische conditie van HF. Mogelijk kan modulatie van het netwerk van chemokinen met antagonisten van zowel inflammatoïre als lymfoïde chemokine-receptoren in de toekomst voor nieuwe therapeutische interventies bij patiënten met chronische HF zorgen.
Het karakteriseren van humane perifeer bloed DCs biedt ook nieuwe inzichten in het ontstaan van eind-stadium HF. Ten tijde van HF bevestigt het verhoogd aantal van circulerende DCs de veranderde migratie patronen van cellen die afkomstig uit het beenmerg zijn. Overigens, suggereert de verschuiving van de mDC/pDC balans naar mDCs met een rijpe fenotype, een predispositie naar T_H1 immuunrereacties bij patiënten met eind-stadium HF. De verhoogde expressie van zowel CD83 en CCR7 vooral op de mDCs van patiënten met DCM, zal mogelijk een onderdeel van de immunogene stimuli zijn die gerelateerd zijn aan de pathogenese van DCM. We suggereren dat de verdere verschuiving van de mDC/pDC balans naar mDCs bij patiënten met idiopathische DCM, ervoor zorgt dat minder perifere tolerantie tegen auto-antigenen optreedt. In de toekomst zijn meer studies nodig om de mogelijke rol van circulerende en weefsel DCs in de ontwikkeling van DCM te achterhalen.

Harttransplantatie

In dit proefschrift werd ook de betrokkenheid van interacties tussen chemokinen en chemokine-receptoren in de recirculatie van DCs en T-cellen bevestigd bij afstoting na klinische HTx. Het aantal perifeer bloed DCs was drastisch verminderd mogelijk op basis van immunosuppressie-gerelateerde "rust" van het afweersysteem na transplantatie. De homeostase van DCs was aanzienlijk veranderd en de verschuiving in de mDC/pDC balans naar mDCs kort na transplantatie, reflecteert mogelijk een deviatie van het afweerapparaat veroorzaakt door trauma of ischemie/reperfusie schade gedurende de transplantatie procedure. De depletie van perifeer bloed mDCs tijdens AR, was geassocieerd met CCR7 afhankelijke selectieve "homing" van mDCs naar lymfoïde organen, die mogelijk clonale expansie en differentiatie van T cellen naar effector-cytotoxische T cellen veroorzaken. In deze context, spelen circulerende mDCs misschien een belangrijke rol in het tot stand brengen van transplantaatafstoting na HTx. Behalve het verdiepen van ons inzicht naar

in vivo mechanismen van harttransplantaat afstoting, kan het vervolgen van perifeer bloed DCs na klinische HTx tevens bijdragen aan het verder verfijnen van de immunosuppressieve therapie. Het vervolgen van DCs in de tijd kan patiënten met een verhoogd risico op afstoting langer dan 3 maanden na HTx identificeren. Dit zou klinisch relevant kunnen zijn omdat het afbouwen van de immunosuppressieve medicatie naar aanleiding van het aantal DCs gericht zou kunnen worden op patiënten met een relatief hoger aantal DCs, die een verlaagd risico op afstoting blijken te hebben langer na transplantatie.
Het ontstaan van AR na transplantatie kon niet worden voorspeld door de analyse van expressie van chemokine-receptoren op perifeer bloed T-cellen. Desalniettemin, suggereert de daling in het gemiddelde percentage van bloed $CD8^+CCR7^+$ T-cellen tijdens AR het uittreden van circulerende $CD8^+CCR7^+$ T-cellen of het verrijken van het bloed met $CD8^+CCR7^-$ T-cellen, als onderdeel van het proces van de afstoting. Weliswaar zijn onze resultaten op gen expressie niveau in het donor myocard een bevestiging voor de betrokkenheid van inflammatoïre chemokinen (CCL5 & CXCL10) en hun receptoren (CCR5 & CXCR3) bij AR, maar de gemiddelde mRNA expressie niveau's van de homeostatische chemokine (CCL19) en haar receptor (CCR7) waren echter ook hoger ten tijde van AR. Het feit dat verhoogde CCL19 mRNA expressie in het transplantaat negatief gecorreleerd was met het percentage circulerende $CD8^+CCR7^+$ T-cellen in de afstoters, suggereert dat hoge CCL19 produktie in het donor myocard van deze patiënten de recirculatie van perifeer bloed $CD8^+$ T-cellen beïnvloedt door infiltratie van $CD8^+CCR7^+$ T-cellen in het transplantaat. De betrokkenheid van interacties tussen homeostatische chemokinen en lymfoïde chemokine-receptoren in het transplantaat geeft een nieuw inzicht in het proces van transplantaatafstoting. Een dusdanige chemokine micromilieu in het transplantaat zou in staat zijn om niet alleen activatie van naïeve lymfocyten maar ook intragraft maturatie en "homing" van DCs te veroorzaken. Dit zou vorming van lokale lymfoïde follikels bevorderen en bijdragen aan een continu risico op verlies van het harttransplantaat bij late acute en chronische afstoting. Verder onderzoek is nodig om de functionele rol van CCL19 en CCR7 in dit proces te achterhalen. Anti-CCR7 antilichamen of medicijnen die de DC of T-cel recirculatie verhinderen door vermindering van CCR7 expressie, kunnen in de toekomst onderdeel worden van de therapeutische mogelijkheden ter voorkoming van acute en chronische afstoting na transplantatie.

Περίληπση

Η καρδιακή ανεπάρκεια είναι ένα κλινικό σύνδρομο το οποίο στην χρόνια μορφή του χαρακτηρίζεται από την ενεργοποίηση του ανοσοποιητικού συστήματος. Υπό συγκεκριμένες συνθήκες δυσλειτουργίας της καρδιάς, η διείσδυση δραστικών λευκοκυττάρων στο μυοκάρδιο μπορεί να οδηγήσει σε υποτροπή της κλινικής εικόνας του ασθενούς προς ανεπάρκεια τελικού σταδίου εν μέσω μίας γενικευμένης φλεγμονώδους αντιδράσεως χαμηλού βαθμού. Συχνά, η μεταμόσχευση καρδιάς είναι η μόνη θεραπευτική διέξοδος για τους πάσχοντες από καρδιακή ανεπάρκεια τελικού σταδίου. Ωστόσο, η απόρριψη του μοσχεύματος παραμένει μια από τις σημαντικότερες επιπλοκές μετά τη μεταμόσχευση. Παράγοντες οι οποίοι καθορίζουν την έκβαση του μοσχεύματος σε ένα δέκτη οργάνου είναι η αλλο-αντιγονική ενεργοποίηση και η επανακυκλοφορία των λευκοκυττάρων στο αίμα του ίδιου του ασθενούς. Επί αρκετά χρόνια η διεθνής κοινότητα μεταμοσχεύσεων προσπαθεί να βελτιώσει την επιβίωση των ασθενών και παράλληλα να αποτρέψει την απώλεια του μοσχεύματός τους. Σε αυτό το πλαίσιο γίνεται προσπάθεια να προβλεφθούν οι περιπτώσεις άμεσης απόρριψης αλλά και να αναγνωρισθούν οι ασθενείς με αυξημένο κίνδυνο απώλειας του μοσχεύματος ώστε να γίνει αναπροσαρμογή στην δοσολογία των ανοσοκατασταλτικών φαρμάκων με σκοπό να ελαχιστοποιηθούν οι παρενέργειές τους. Σε αυτή την διατριβή, προσπαθήσαμε να διευκρινίσουμε τον ρόλο των χημειοκινών και των ειδικών υποδοχέων τους στην επανακυκλοφορία των λευκών αιμοσφαιρίων κατά την καρδιακή ανεπάρκεια τελικού σταδίου και την απόρριψη του μοσχεύματος μετά από μεταμόσχευση καρδιάς. Ιδιαίτερα, εφιστήσαμε την προσοχή μας σε τύπους δενδριτικών κυττάρων και Τ-λεμφοκυττάρων, η αλληλεπίδραση των οποίων καθορίζει την έκβαση των ανοσολογικών αντιδράσεων τόσο σε χρόνιες ασθένειες όσο και στις μεταμοσχεύσεις.

Το **πρώτο κεφάλαιο** παρέχει ένα εισαγωγικό σημείωμα όσον αφορά στην μετανάστευση των δενδριτικών κυττάρων και των Τ-λεμφοκυττάρων του αίματος, βασισμένο στις χημειοκίνες και τους υποδοχείς τους. Οι ρόλοι των χημειοκινών και των υποδοχέων τους, τόσο στην παθογέννεση της καρδιακής ανεπάρκειας όσο και στην απόρριψη της καρδιάς μετά από μεταμόσχευση, συζητούνται περιεκτικά μαζί με γνωστές χημειοτακτικές οδούς ενεργοποίησης και διείσδυσης των λευκοκυττάρων στο μόσχευμα. Στο ίδιο κεφάλαιο παρουσιάζονται και οι στόχοι της έρευνάς μας.

Στο **δεύτερο κεφάλαιο** περιγράφονται τροποποιήσεις τόσο στην έκφραση των CC- και CXC- υποδοχέων χημειοκινών όσο και στα ποσοστά κυκλοφορούντων $CD4^+$ και $CD8^+$ Τ-λεμφοκυττάρων, μονοκυττάρων και πολυμορφοπύρηνων κυττάρων

θετικών για τους προαναφερθείς υποδοχείς χημειοκινών, στο αίμα ασθενών με καρδιακή ανεπάρκεια τελικού σταδίου. Τα αποτελέσματα εξετάστηκαν τόσο για ισχαιμικούς όσο και για μή ισχαιμικούς τύπους μυοκαρδιοπάθειας. Τα κυκλοφορούντα Τ-λεμφοκύτταρα δεν έδειξαν κάποια πόλωση προς ένα συγκεκριμένο T_H1 ή T_H2 ανοσοφαινότυπο. Ωστόσο, στα Τ-λεμφοκύτταρα όλων των ασθενών βρέθηκε αυξημένη έκφραση των υποδοχέων CCR7 και CXCR4, ενώ τα $CD8^+$ Τ-λεμφοκύτταρα των ασθενών με στεφανιαία νόσο και διατατική μυοκαρδιοπάθεια είχαν υψηλότερη έκφραση του υποδοχέα χημειοκίνης CCR7 απ'ότι τα $CD8^+$ Τ-λεμφοκύτταρα ασθενών με υπερτροφική μυοκαρδιοπάθεια ή νόσο των καρδιακών βαλβίδων. Παρεπιπτόντως, στο αίμα των ασθενών με στεφανιαία νόσο βρέθηκαν μικρότερα ποσοστά $CCR1^+$ και $CCR2^+$ μονοκυττάρων, ενώ η έκφραση των υποδοχέων χημειοκίνης CXCR1 και CXCR2 ήταν περιορισμένη στα πολυμορφοπύρηνα κύτταρα όλων των ασθενών, ανεξάρτητα από τα αίτια της καρδιακής τους παθολογίας.

Στο **τρίτο κεφάλαιο** παρουσιάζονται οι απόλυτοι αριθμοί και τα ποσοστά των ανοσοφαινοτυπικά ωρίμων δενδριτικών κυττάρων μυελώδους και πλασμακυτώδους τύπου κατά την διάρκεια καρδιακής ανεπάρκειας τελικού σταδίου αλλά και σύντομα μετά από μεταμόσχευση καρδιάς. Σε σύγκριση με υγιείς εθελοντές αιμοδότες, οι ασθενείς με καρδιακή ανεπάρκεια τάξης III-IV, σύμφωνα με την Καρδιολογική Ένωση της Νέας Υόρκης (NYHA), παρουσίασαν υψηλότερο αριθμό δενδριτικών κυττάρων στο αίμα τους λόγω εμπλουτισμού του με κύτταρα μυελώδους τύπου. Από τη μία πλευρά, λόγω της αύξησης των $CD83^+$ κυττάρων και στους δυο τύπους δενδριτικών κυττάρων, τόσο τα κύτταρα μυελώδους τύπου όσο και τα κύτταρα πλασμακυτώδους τύπου έδειξαν έναν ενεργοποιημένο ανοσοφαινότυπο. Από την άλλη πλευρά, ο υψηλότερος αριθμός των $CCR7^+$ κυττάρων μυελώδους τύπου καταδεικνύει την διέγερση των κυττάρων αυτών ως προς την μετακίνηση τους προς τα δευτερογενή λεμφικά όργανα. Μετά τη μεταμόσχευση καρδιάς, οι αριθμοί τόσο των κυττάρων μυελώδους τύπου όσο και των κυττάρων πλασμακυτώδους τύπου μειώθηκαν δραστικά, φαινόμενο το οποίο αποδίδεται σε ανοσολογική ηρεμία λόγω των παρεχόμενων ανοσοκατασταλτικών φαρμάκων. Κάτω από τις ίδιες συνθήκες, η ισορροπία μεταξύ των κυκλοφορούντων κυττάρων μυελώδους / πλασμακυτώδους τύπου έτεινε προς τα κύτταρα μυελώδους τύπου, καθώς και οι δύο τύποι δενδριτικών κυττάρων στο αίμα έδειχναν ανοσοφαινοτυπικά ανώριμοι.

Το **τέταρτο κεφάλαιο** ασχολείται με τους τύπους κυκλοφορούντων δενδριτικών κυττάρων καθώς και τον φαινότυπό τους σε σχέση με την αιτιολογία καρδιακής ανεπάρκειας τελικού σταδίου. Ενώ οι αριθμοί των κυττάρων μυελώδους και πλασμακυτώδους τύπου στο αίμα ασθενών με καρδιακή ανεπάρκεια τάξης

ΝΥΗΑ III-IV ή τάξης ΝΥΗΑ II και υγειών εθελοντών ήταν συγκρίσιμοι μεταξύ τους, ο ολικός αριθμός κυκλοφορούντων δενδριτικών κυττάρων στους ασθενείς με καρδιακή ανεπάρκεια τελικού σταδίου ήταν υψηλότερος σε εκείνους που έπασχαν από διατατική μυοκαρδιοπάθεια. Το φαινόμενο αυτό προήλθε από την αύξηση κυττάρων μυελώδους τύπου στο αίμα των ασθενών με ιδιοπαθή διατατική μυοκαρδιοπάθεια, ανεξάρτητα από δυσλειτουργία της καρδιάς κατά τη συστολή της ή τη διαστολή της. Επίσης, σημειώνεται ότι τα κύτταρα μυελώδους τύπου των ασθενών με διατατική μυοκαρδιοπάθεια έδειξαν μεγαλύτερη έκφραση του μορίου ενεργοποίησης CD83 και του υποδοχέα χημειοκίνης CCR7, συγκριτικά με ασθενείς των οποίων η καρδιακή ανεπάρκεια βασιζόταν σε άλλα αίτια.

Το **πέμπτο κεφάλαιο** περιγράφει την ανακατανομή, ωρίμανση και στάδιο διέγερσης των δύο διαφορετικών τύπων δενδριτικών κυττάρων, όσον αφορά στη μετακίνησή τους προς τα δευτερογενή λεμφικά όργανα σε σχέση με την απόρριψη της καρδιάς, μετά τη μεταμόσχευση. Οι μέσοι αριθμοί των κυττάρων μυελώδους τύπου και πλασμακυτώδους τύπου παρέμειναν καθηλωμένοι καθ'όλη την περίοδο μετά τη μεταμόσχευση. Συγκεκριμένα, τα κύτταρα μυελώδους τύπου συσχετίζονταν αρνητικά με τον βαθμό απόρριψης του μοσχεύματος, ενώ ο απόλυτος αριθμός τους ήταν πιο χαμηλός κατά τη διάρκεια της ίδιας της απόρριψης. Τρεις μήνες μετά τη μεταμόσχευση, ο αριθμός των κυττάρων μυελώδους τύπου στους ασθενείς που είχαν ήδη υποστεί επεισόδια απόρριψης, ήταν χαμηλότερος απ'ότι σε ασθενείς χωρίς συμπτώματα απόρριψης. Το φαινόμενο αυτό της ατελούς κυτταρικής ανακατανομής ήταν ανεξάρτητο από την εμφάνιση λοιμώξεων (π.χ. λόγω κυτταρομεγαλοϊού), την θεραπεία με στατίνες ή την μείωση της δοσολογίας των ανοσοκατασταλτικών φαρμάκων μετά τη μεταμόσχευση. Κατά τη διάρκεια άμεσης απόρριψης του οργάνου, τα ποσοστά $CCR7^+$ κυττάρων μυελώδους τύπου στο αίμα ήταν μειωμένά, γεγονός το οποίο συνάγει στην άποψη πως ένας μηχανισμός απεμπλουτισμού κυκλοφορούντων κυττάρων μυελώδους τύπου είναι ενεργός και σχετίζεται με επιλεκτική μεταφορά των κυττάρων αυτών στους λεμφικούς ιστούς κατά τη διάρκεια της απόρριψης.

Το **έκτο κεφάλαιο** ασχολείται με την επιρροή της θεραπείας κατά της απόρριψης του μοσχεύματος στον ολικό αριθμό κυκλοφορούντων δενδριτικών κυττάρων αλλά και στην επαναφορά των κυττάρων μυελώδους και πλασμακυτώδους τύπου στο αίμα των ασθενών μετά τη μεταμόσχευση. Σύμφωνα με τις οδηγίες της Διεθνούς Οργάνωσης Μεταμοσχεύσεως Καρδιάς και Πνευμόνων (ISHLT), τόσο ο αριθμός κυκλοφορούντων κυττάρων μυελώδους τύπου όσο και η αναλογία μυελώδους / πλασμακυτώδους τύπου κυττάρων συσχετίστηκαν αρνητικά με τον βαθμό απόρριψης του μοσχεύματος. Μετά από δώδεκα εβδομάδες από τη μεταμόσχευση, και σε αντίθεση με ασθενείς δίχως συμπτώματα απόρριψης, οι

ασθενείς με επεισόδια απόρριψης δεν έδειξαν δείγματα ολικής επαναφοράς των δενδριτικών κυττάρων στο αίμα τους. Εντός της ομάδας των ασθενών με επεισόδια απόρριψης, ο αριθμός κυττάρων μυελώδους τύπου παρέμεινε χαμηλός όχι μόνο σε αυτούς που δέχθηκαν φάρμακα κατά της απόρριψης μοσχεύματος αλλά και σε εκείνους τους ασθενείς οι οποίοι δεν έλαβαν ποτέ τέτοια θεραπεία.

Στο **έβδομο κεφάλαιο** περιγράφουμε τα αποτελέσματα της έρευνάς μας για τον ρόλο των χημειοκινών και των υποδοχέων τους στην επανακυκλοφορία των $CD4^+$ και $CD8^+$ Τ-λεμφοκυττάρων σε σχέση με την αντίστοιχη γονιδιακή έκφραση των χημειοκινών και των υποδοχέων τους μέσα στο μόσχευμα κατά τη διάρκεια της απόρριψης. Αφ'ενός, στα κυκλοφορούντα Τ-λεμφοκύτταρα δεν βρήκαμε κάποιες διαφορές όσον αφορά τα ποσοστά $CD4^+$ κυττάρων θετικών για τους υποδοχείς των χημειοκινών. Αφ'ετέρου, τα μέσα ποσοστά των $CCR5^+CD8^+$ και $CCR7^+CD8^+$ Τ-λεμφοκυττάρων κατά τη διάρκεια της απόρριψης ήταν διαφοροποιημένα. Επίσης, η έκφραση mRNA των χημειοκινών CCL5, CXCL10 & CCL19 αλλά και των υποδοχέων τους CCR5, CXCR3 & CCR7, ήταν υψηλότερη μέσα στο μόσχευμα κατά τη διάρκεια της απόρριψης. Στους ασθενείς με επεισόδια απόρριψης υπήρξε υψηλότερη έκφραση των CCR5 mRNA και CXCL10 mRNA απ'ότι στους ασθενείς χωρίς συμπτώματα απόρριψης, ακόμα και πρίν την απόρριψη του μοσχεύματος. Επιπλέον, στην ομάδα των ασθενών με επεισόδια απόρριψης, η έκφραση CCL19 mRNA σχετιζόταν αρνητικά με τα ποσοστά κυκλοφορούντων $CD8^+CCR7^+$ Τ-λεμφοκυττάρων. Αυτό συνάγει στην άποψη ότι η παραγωγή της χημειοκίνης CCL19 από το μυοκάρδιο του δότη επηρρεάζει την επανακυκλοφορία των $CD8^+$ Τ-λεμφοκυττάρων του δέκτη της καρδιάς, μέσω του υποδοχέα χημειοκίνης CCR7.

Τέλος, στο **όγδοο κεφάλαιο** ενσωματώσαμε την έρευνά μας σε μία γενική συζήτηση με υπόβαθρο τη διεθνή βιβλιογραφία. Τα πιο σημαντικά αποτελέσματα και συμπεράσματα της διατριβής αυτής αναθεωρούνται με τρόπο τέτοιο ώστε να αναλογισθούμε τις δυνατότητες κλινικής αξιοποίησής τους και τις επιπτώσεις τους στην περαιτέρω έρευνα πάνω σε αυτόν το τομέα.

Συμπεράσματα

Καρδιακή Ανεπάρκεια

Οι μελέτες που παρουσιάζονται σε αυτή τη διατριβή και αφορούν στην επανακυκλοφορία των δενδριτικών κυττάρων και Τ-λεμφοκυττάρων στο αίμα ασθενών, προσδίδουν νέα προοπτική στην έρευνα που ασχολείται με μηχανισμούς του ανοσοποιητικού συστήματος οι οποίοι εμπλέκονται στην καρδιακή ανεπάρκεια τελικού σταδίου. Εδώ, με την κατ'ευθείαν ανάλυση της πρωτεϊνικής έκφρασης των υποδοχέων χημειοκινών που παίζουν ρόλο στη μεταφορά των λευκοκυττάρων προς

τα δευτερογενή λεμφικά όργανα, παρουσιάζουμε στοιχεία για τροποποιημένες μεταναστευτικές δυνατότητες των κυκλοφορούντων Τ-λεμφοκυττάρων στο αίμα ασθενών με καρδιακή ανεπάρκεια. Θεωρητικά, η υψηλή έκφραση των υποδοχέων CCR7 και CXCR4, θα μπορούσε να σημάνει την επιταγχυμένη έλξη των κυκλοφορούντων $CD4^+$ και $CD8^+$ Τ-λεμφοκυττάρων προς τους λεμφικούς ιστούς ώστε να δώσουν T_H1 ή T_H2 ανοσολογικές αντιδράσεις. Η αλλαγή αυτή στη μετανάστευση και στην ενεργοποίηση των Τ-λεμφοκυττάρων στο αίμα, ίσως αποτελεί μέρος των μηχανισμών που προκαλούν την παθολογική κατάσταση της καρδιακής ανεπάρκειας. Καθώς κάποιες φαρμακολογικές παρεμβάσεις στο δίκτυο των χημειοκινών – βασισμένες σε ανταγωνιστές των υποδοχέων τους – είναι ήδη εφικτές, αναμένεται πως μελλοντικά με αυτόν τον τρόπο ίσως επιτευχθούν νέες αποτελεσματικές θεραπευτικές διέξοδοι για τους ασθενείς με χρόνια καρδιακή ανεπάρκεια.

Τα αποτελέσματα της έρευνάς μας από τον απ'ευθείας χαρακτηρισμό δενδριτικών κυττάρων στο αίμα των ασθενών, προσδίδουν επίσης ένα νέο τρόπο αντίληψης όσον αφορά την καρδιακή ανεπάρκεια τελικού σταδίου. Ο αυξημένος αριθμός των κυκλοφορούντων δενδριτικών κυττάρων υπό συνθήκες καρδιακής ανεπάρκειας επιβεβαιώνει τις διαφοροποιημένες μεταναστευτικές ιδιότητες των κυττάρων αυτών, καθώς προέρχονται από τον μυελό των οστών του ασθενούς. Επιπλέον, η μετακίνηση της ισορροπίας των κυττάρων μυελώδους / πλασμακυτώδους τύπου προς τα κύτταρα μυελώδους τύπου με ενεργοποιημένο ανοσοφαινότυπο, προδίδει την προδιάθεση του ανοσοποιητικού συστήματος των ασθενών με καρδιακή ανεπάρκεια τελικού σταδίου προς αντιδράσεις T_H1 τύπου. Η υψηλή έκφραση του μορίου ενεργοποίησης CD83 παράλληλα με την ενίσχυση των επιπέδων του υποδοχέα χημειοκίνης CCR7 στην επιφάνεια κυττάρων μυελώδους τύπου αποκλειστικά στους ασθενείς με διατατική μυοκαρδιοπάθεια, πρέπει να αποτελεί μέρος των ανοσολογικών διεργασιών που συντελούνται κατά την παθογέννεση της διατατικής μυοκαρδιοπάθειας. Θεωρούμε πως η περαιτέρω μετατόπιση της ισορροπίας μεταξύ των κυκλοφορούντων κυττάρων μυελώδους / πλασμακυτώδους τύπου προς τα κύτταρα μυελώδους τύπου στους ασθενείς με ιδιοπαθή διατατική μυοκαρδιοπάθεια, συνάγει σε μειωμένη δυνατότητα ανοχής του ανοσοποιητικού συστήματος των ασθενών αυτών προς αυτο-αντιγόνα. Περισσότερη έρευνα επιβάλλεται στο μέλλον ώστε να διευκρινισθεί πλήρως ο ρόλος των κυκλοφορούντων δενδριτικών κυττάρων στην παθογέννεση της διατατικής μυοκαρδιοπάθειας στον άνθρωπο.

Μεταμόσχευση Καρδιάς

Σε αυτή της διατριβή επιβεβαιώθηκε επίσης και ο ρόλος της αλληλεπίδρασης μεταξύ χημειοκινών και των υποδοχέων τους στην επανακυκλοφορία των δενδριτικών κυττάρων και Τ-λεμφοκυττάρων κατά την απόρριψη του μοσχεύματος, μετά από μεταμόσχευση καρδιάς. Ο αριθμός των δενδριτικών κυττάρων στο αίμα των ασθενών μειώθηκε δραστικά λόγω της καταστολής του ανοσοποιητικού συστήματος μέσω των δοσολογούμενων φαρμάκων μετά από τη μεταμόσχευση. Η βιολογική κατάσταση των δενδριτικών κυττάρων άλλαξε ουσιαστικά, γεγονός που αντιπροσωπεύει την απόκλιση του ανοσοποιητικού συστήματος λόγω του χειρουργικού τραύματος και της ισχαιμικής/επαναιμακτικής βλάβης που επέρχεται κατά τη μεταμόσχευση και αντικατοπτρίζεται στην άμεση μεταβολή της ισορροπίας κυττάρων μυελώδους / πλασμακυτώδους τύπου προς τα κύτταρα μυελώδους τύπου μετά τη μεταμόσχευση. Κατά την απόρριψη του μοσχεύματος, ο απεμπλουτισμός των κυκλοφορούντων κυττάρων μυελώδους τύπου σχετίστηκε με την επιλεκτική μεταφορά των κυττάρων αυτών στους λεμφικούς ιστούς μέσω του υποδοχέα χημειοκίνης CCR7, γεγονός που μπορεί να προκαλέσει την κλωνική ενδυνάμωση και διαφοροποίηση των Τ-λεμφοκυττάρων προς δραστικά κυτταροτοξικά Τ-κύτταρα. Ίσως, με αυτές τις ιδιότητές τους, τα κυκλοφορούντα κύτταρα μυελώδους τύπου να αποτελούν την απαρχή της απόρριψης του μοσχεύματος μετά από τη μεταμόσχευση καρδιάς. Πιστεύουμε πως ο έλεγχος των κυκλοφορούντων δενδριτικών κυττάρων υπό συνθήκες μεταμόσχευσης καρδιάς, εκτός από την απόκτηση διορατικότητας για τους *in vivo* μηχανισμούς απόρριψης του μοσχεύματος, μπορεί να οδηγήσει και σε αναπροσαρμογή στην δοσολογία των ανοσοκατασταλτικών φαρμάκων ώστε να ελαχιστοποιηθούν οι παρενέργειές τους στους ασθενείς μετά τη μεταμόσχευση. Ειδικότερα, μέσω του χαρακτηρισμού και της παρακολούθησης των δενδριτικών κυττάρων στο αίμα των ασθενών μετά τους πρώτους τρεις μήνες από τη μεταμόσχευση καρδιάς, μπορεί να αναγνωρισθούν οι δέκτες του μοσχεύματος με αυξημένο κίνδυνο απόρριψής του, ανεξάρτητα από φαινόμενα του ανοσοποιητικού συστήματος όπως είναι η εμφάνιση λοιμώξεων. Μία τέτοια μέθοδος θα μπορούσε να έχει σημαντικές κλινικές επιπτώσεις, καθώς θα μπορούσε να προτιμηθεί η μείωση στην δοσολογία των ανοσοκατασταλτικών φαρμάκων μόνο στους ασθενείς με δείγματα ολικής επαναφοράς των δενδριτικών κυττάρων στο αίμα τους - μέσω του συγκριτικά μεγαλυτέρου αριθμού κυκλοφορούντων δενδριτικών κυττάρων μετά τη μεταμόσχευση – οι οποίοι διατρέχουν μικρότερο κίνδυνο απόρριψης του μοσχεύματός τους.

Δυστυχώς, η απόρριψη του μοσχεύματος δεν ήταν δυνατόν να προβλεφθεί μέσω της ανάλυσης της έκφρασης των υποδοχέων χημειοκίνης στα κυκλοφορούντα

Τ-λεμφοκύτταρα. Ωστόσο, η μείωση του ποσοστού κυκλοφορούντων $CD8^+CCR7^+$ Τ-λεμφοκυττάρων κατά τη διάρκεια της απόρριψης, συντείνει στο γεγονός είτε της διαρροής $CD8^+CCR7^+$ κυττάρων από το αίμα, είτε της διήθησης του αίματος με $CD8^+CCR7^+$ κύτταρα, ως μέρος του μηχανισμού της απόρριψης. Εκτός της επιβεβαίωσης του συσχετισμού της υψηλής γονιδιακής έκφρασης των χημειοκινών CCL5 & CXCL10 και των αντίστοιχων υποδοχέων τους CCR5 & CXCR3 εντός του μοσχεύματος με την εμφάνιση επεισοδίων απόρριψης, η γονιδιακή έκφραση mRNA της χημειοκίνης CCL19 και του υποδοχέα της (CCR7) ήταν επίσης αυξημένη στο μυοκάρδιο του δότη κατά τη διάρκεια της απόρριψης. Εφόσον στην ομάδα των ασθενών με επεισόδια απόρριψης, η έκφραση CCL19 mRNA σχετιζόταν αρνητικά με τα ποσοστά κυκλοφορούντων $CD8^+CCR7^+$ Τ-λεμφοκυττάρων, θεωρούμε πως η υψηλή έκφραση της χημειοκίνης CCL19 εντός του μοσχεύματος επηρρεάζει την επανακυκλοφορία των $CD8^+$ Τ-λεμφοκυττάρων του δέκτη της καρδιάς με τρόπο τέτοιο ώστε να διευκολύνει την διείσδυση $CD8^+CCR7^+$ Τ-λεμφοκυττάρων στην καρδιά του δότη. Εν πάση περιπτώσει, η συμμετοχή της χημειοκίνης CCL19 και του υποδοχέα της (CCR7) στην απόρριψη καρδιάς, αποτελεί μια νέα πτυχή στην έρευνα για τους μηχανισμούς της απόρριψης οργάνων. Η παραγωγή χημειοκινών όπως της CCL19 από το μυοκάρδιο του δότη, δημιουργεί ένα περιβάλλον τέτοιο που μπορεί να οδηγήσει τοπικά σε ενεργοποίηση λεμφοκυττάρων αλλά και σε ωρίμανση και έλξη δενδριτικών κυττάρων. Με αυτόν τον τρόπο θα μπορούσαν να δημιουργηθούν τοπικά θυλάκια λεμφικού ιστού εντός του μυοκαρδίου, τα οποία θα συνέβαλλαν σε συνεχή κίνδυνο απώλειας του μοσχεύματος μέσω οξείας ή χρονίας απόρριψής του μετά τη μεταμόσχευση. Ωστόσο, ο λειτουργικός ρόλος των CCL19 και CCR7, έπεται ακόμη να αποκαλυφθεί. Σε αυτό το πλαίσιο, συνθετικά αντισώματα κατά του υποδοχέα CCR7 ή φαρμακευτικά παρασκευάσματα τα οποία είναι γνωστό ότι δύνανται να ελαττώσουν την έκφραση του υποδοχέα CCR7, θα μπορούσαν να επιφυλάσσουν νέες θεραπευτικές λύσεις για όλες τις μορφές απόρριψης μοσχεύματος στο μέλλον, παρεμποδίζοντας την μετανάστευση των κυκλοφορούντων δενδριτικών και Τ-λεμφοκυττάρων.

Appendices

Dankwoord

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Dankwoord

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Athene – Den Haag, mei 2006

Presentations

Oral

4th Joint Meeting of the American Society of Transplantation & The American Society of Transplant Surgeons, *May 30th – June 4th 2003, Washington DC, USA*

2nd Joint Meeting of The European Association of Cardio-thoracic Surgery & The European Society of Thoracic Surgeons, *October 12th – 15th 2003, Vienna, Austria*

Bootcongres of The Dutch Society for Transplantation, *March 17th – 19th 2004, Texel, The Netherlands*

24th Annual Meeting of The International Society for Heart and Lung Transplantation, *April 21st – 24th 2004, San Fransisco, CA, USA*

24th Annual Meeting of The International Society for Heart and Lung Transplantation, *April 21st – 24th 2004, San Fransisco, CA, USA* – Nominee for the 'Caves' Young Investigator Award

3rd Joint Meeting of The European Association of Cardio-thoracic Surgery & The European Society of Thoracic Surgeons, *September12th – 15th 2004, Leipzig, Germany*

Bootcongres of The Dutch Society for Transplantation, *March 9th – 11th 2005, Kerkrade, The Netherlands*

Joint Scientific Meeting of The Netherlands Society of Cardiology and The Netherlands Society of Thoracic Surgery, *April 21st – 22nd 2005, Amsterdam, The Netherlands*

Joint Scientific Meeting of The Netherlands Society of Cardiology and The Netherlands Society of Thoracic Surgery, *October7th 2005, Nieuwegein, The Netherlands*

26th Annual Meeting of The International Society for Heart and Lung Transplantation, *April 5 th – 8th 2006, Madrid, Spain*

Poster

Annual Meeting of The Dutch Society for Immunology, *December 18th – 20th 2002, Veldhoven, The Netherlands*

Bootcongres of The Dutch Society for Transplantation, *March 25th – 27th 2003, Rockanje, The Netherlands*

23rd Annual Meeting of The International Society for Heart and Lung Transplantation, *April 9th – 12th 2003, Vienna, Austria*

11th Congress of The European Society for Organ Transplantation, *September 20th – 24th 2003, Venice, Italy*

Annual Meeting of The Dutch Society for Immunology, *December 18th – 19th 2003, Noordwijkerhout, The Netherlands*

5th Joint Meeting of the American Society of Transplantation & The American Society of Transplant Surgeons, *May 14th – 19th 2004, Boston, MA, USA*

20th International Congress of the Transplantation Society, *September 5th – 10th 2004, Vienna, Austria*

8th International Symposium on Dendritic Cells, *October 17th – 21st 2004, Brugge, Belgium*

25th Annual Meeting of The International Society for Heart and Lung Transplantation, *April 5th – 9th 2005, Philadelphia, PA, USA*

9th Basic Science Symposium of The Transplantation Society, *June 19th – 22nd 2005, Nantes, France*

1st World Transplant Congress, *July 23rd – 27th 2006, Boston, MA, USA*

List of Publications

Papers

Varro A, Athanassopoulos P, Dockray GJ. Physiological regulation of GABA uptake by rat pyloric antral mucosa. *Exp Physiol 1996, 81(1): 151-154*

Gussenhoven EJ, Honkoop J, Athanassopoulos P, Goedbloed Y, Hagenaars T, Bom N, Van Essen JA. Intravasculaire echografie als kijkdraad. *Vaatdiagnostiek 1999, 3: 16-20*

Hagenaars T, Gussenhoven EJ, Athanassopoulos P, Pattynama PM, van Sambeek MR. Intravascular ultrasound evidence for the stabilization of compensatory enlargement of femoropopliteal artery segment after endograft placement. *J Endovasc Ther 2001, 8(3): 308-314*

Athanassopoulos P, Vaessen LMB, Maat APWM, Balk AHMM, Weimar W, Bogers AJJC. Peripheral blood dendritic cells in end-stage heart failure and the early post-transplant period: evidence for systemic Th1 immune responses. *Eur J Cardiothorac Surg 2004, 25(4): 619-626*

Athanassopoulos P, Vaessen LMB, Maat APWM, Zondervan PE, Balk AHMM, Bogers AJJC, Weimar W. Preferential depletion of blood myeloid dendritic cells during acute cardiac allograft rejection under controlled immunosuppression. *Am J Transplant 2005, 5(4): 810-820*

Athanassopoulos P, Vaessen LMB, Balk AHMM, Takkenberg JJM, Maat APWM, Weimar W, Bogers AJJC. Impaired circulating dendritic cell reconstitution identifies rejecting recipients after clinical heart transplantation independent of rejection therapy. *Eur J Cardiothorac Surg 2005, 27(5): 783-789*

Hesselink DA, Betjes MG, Verkade MA, Athanassopoulos P, Baan CC, Weimar W. The effects of chronic kidney disease and renal replacement therapy on circulating dendritic cells. *Nephrol Dial Transpl 2005, 20(9): 1868-1873*

Athanassopoulos P, Vaessen LMB, Balk AHMM, Weimar W, Sharma HS, Bogers AJJC. Altered chemokine receptor profile on circulating leukocytes in human heart failure. *Cell Biochem Biophys 2006, 44(1): 83-101*

Athanassopoulos P, Balk AHMM, Vaessen LMB, Caliskan K, Takkenberg JJM, Weimar W, Bogers AJJC. Blood dendritic cell levels and phenotypic characteristics in relation to etiology of human heart failure: implications for idiopathic dilated cardiomyopathy. *Submitted*

Athanassopoulos P, Peeters AM, Vaessen LMB, Velthuis JH, Balk AHMM, Baan CC, Maat APWM, Bogers AJJC, Weimar W. Myocardial CCL19 expression affects CCR7-dependent circulating CD8 T-cell homing in heart allograft recipients with acute rejection. *Submitted*

Gerrits JH, Athanassopoulos P, Vaessen LMB, Klepper M, Weimar W, van Besouw NM. Peripheral blood manipulation influences dendritic cell subsets and maturation markers. *Submitted*

Abstracts

Athanassopoulos P, Vaessen LMB, Weimar W, Balk AHMM, Maat APWM, Bogers AJJC. Elevated CXCR4 levels on peripheral blood T-cells in heart transplantation patients. *J Heart Lung Transpl 2003, 22(1); suppl 1: S169-S170*

Athanassopoulos P, Vaessen LMB, Balk AHMM, Maat APWM, Bogers AJJC, Weimar W. Preferential depletion of myeloid type peripheral blood dendritic cells during acute rejection of human heart allografts. *J Heart Lung Transpl 2004, 23(2); suppl 1: S114*

Athanassopoulos P, Vaessen LMB, Balk AHMM, Maat APWM, Bogers AJJC, Weimar W. Peripheral blood dendritic cell phenotype indicates immune predisposition towards Th-1 responses in human end-stage heart failure. *J Heart Lung Transpl 2004, 23(2); suppl 1: S174-S175*

Hesselink DA, Verkade MA, Athanassopoulos P, Betjes MGH, Hop WCJ, van Dam T, Schoordijk W, Vaessen LMB, Ijzermans JNM, Baan CC, Weimar W. In vivo effects of immunosuppression on dendritic cell kinetics in peripheral blood of patients undergoing renal transplantation. *Transplantation 2004, 78(2): S83-S84*

Athanassopoulos P, Vaessen LMB, Balk AHMM, Takkenberg JJM, Weimar W, Bogers AJJC. Association of blood dendritic cell levels and phenotype with the etiology of human end-stage heart failure: implications for idiopathic dilated cardiomyopathy. *Neth Heart J 2005, 13; suppl 1: 21*

Athanassopoulos P, Peeters AM, Vaessen LMB, Maat APWM, Baan CC, Balk AHMM, Weimar W, Bogers AJJC. Myocardial lymphoid chemokine expression affects CD8+ T-cell recirculation in rejecting recipients after clinical heart transplantation. 2005. *Neth Heart J 2005, 13; suppl 3: 16*

Athanassopoulos P, Vaessen LMB, Balk AHMM, Maat APWM, Bogers AJJC, Weimar W. Accelerated naïve CD8 T-cell homing during acute cellular rejection after clinical heart transplantation. *J Heart Lung Transpl 2005, 24(2); suppl 1: S161*

Athanassopoulos P, Peeters AM, Vaessen LMB, Balk AHMM, Baan CC, Maat APWM, Bogers AJJC, Weimar W. Myocardial CCL19 expression affects CCR7-dependent circulating CD8 T-cell homing in heart allograft recipients with acute rejection after clinical heart transplantation. *2006. J. Heart Lung Transpl 2006, 25(2S): S73*