Inflammatory Process in Chronic Heart Failure: Impact of Cardiac Resynchronization Therapy

Sílvia Raquel Monteiro Martins

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Orientador: Prof. Doutor Artur Augusto Paiva
Co-orientador: Prof. Doutora Natália Sofia Cláudio António
Co-orientador: Prof. Doutora Cândida Ascensão Teixeira Tomaz

Constutuição do júri
Presidente: Prof. Doutor Paulo Jorge da Silva Almeida
Vogais: Prof. Doutor Joaquim Adelino Correia Ferreira Leite Moreira
Prof. Doutora Maria Teresa Teixeira Cruz Rosete
Doutora Cristina Isabel Gonçalves Grunho Teodósio
Prof. Doutor Samuel Martins Silvestre
Prof. Doutor Artur Augusto Paiva

Covilhã, 1 de Agosto de 2024

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Dedicatory

Especially dedicated to those I love the most…

Dinis, Pedro, Zélita, Xavier, Fernanda and Joaquim

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there to offer comfort and support with his warm hugs. This thesis is dedicated to him with love and appreciation.

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Thesis overview

This thesis is divided into six main chapters, whose content is summarized below.

The first chapter consists of a general introduction and literature review on chronic heart failure and the immune system, with particular emphasis on the role of immune cells in reverse cardiac remodelling and on the impact of cardiac resynchronization therapy on the inflammatory process inherent to heart failure.

The second chapter includes this thesis's global and specific aims and its development purpose.

Chapters three to five contain three original articles published in international peer-reviewed journals, organized as follows:

Chapter III – Role of monocytes and dendritic cells in cardiac reverse remodelling after cardiac resynchronization therapy;

Chapter IV – Impact of cardiac resynchronization therapy on circulating IL-17 producing cells in patients with advanced heart failure;

Chapter V – Reduced numbers of regulatory T cells in chronic heart failure seems not to be restored by cardiac resynchronization therapy;

Finally, the sixth chapter includes an integrated discussion/conclusion summarising the main results of this thesis. Some of the future perspectives are also suggested for complement the important findings achieved in this study.

Publications

Articles in international peer-reviewed journals, included in the thesis:

I. Martins S, António N, Rodrigues R, Carvalheiro T, Tomaz C, Gonçalves L, Paiva A. Role of monocytes and dendritic cells in cardiac reverse remodelling after cardiac resynchronization therapy. BMC Cardiovascular Disorders. 2023; 23(1): 558.

II. Martins S, Carvalheiro T, Laranjeira P, Martinho A, Elvas L, Gonçalves L, Tomaz C, António N, Paiva A. Impact of cardiac resynchronization therapy on circulating IL-17 producing cells in patients with advanced heart failure. Journal of Interventional Cardiac Electrophysiology. 2019; 54, 257-265.

III. Martins S, António N, Carvalheiro T, Laranjeira P, Rodrigues R, Gonçalves L, Tomaz C, Paiva A. Reduced numbers of regulatory T cells in chronic heart failure seems not to be restored by cardiac resynchronization therapy. BMC Cardiovascular Disorders. 2023; 23(1), 1-13.

Resumo

A insuficiência cardíaca crónica (ICC) é um problema de saúde pública global que se caracteriza por uma função cardíaca inadequada, acompanhada de sintomas cardinais e múltiplos sinais e comorbidades. A fisiopatologia da insuficiência cardíaca (IC) não é completamente compreendida, mas, geralmente, culmina em disfunção miocárdica progressiva associada a remodelação ventricular contínua. Atualmente, está bem estabelecido que a ativação do sistema imunitário promove um estado inflamatório sistémico nesta patologia. Doentes com IC, independente da prevalência da disfunção sistólica ou diastólica, apresentam níveis aumentados de diversas citocinas pró-inflamatórias, associados a desfechos clínicos adversos. As células sanguíneas periféricas têm sido sugeridas como uma potencial fonte para a produção sistémica e prolongada de citocinas na ICC. De facto, numerosos estudos propõem a participação destas células na própria patogénese da doença, contribuindo para a remodelação cardíaca progressiva.

A terapia de ressincronização cardíaca (TRC) é um tratamento recomendado pelas diretrizes internacionais para doentes com IC refratária a terapêutica farmacológica, fração de ejeção do ventrículo esquerdo (FEVE) reduzida, e bloqueio de ramo esquerdo (BRE). Esta terapia tem efeitos benéficos sobre os sintomas e remodelação cardíaca em doentes respondedores, incluindo a remodelação cardíaca reversa (com redução dos volumes ventriculares esquerdos e aumento da FEVE), melhoria do estado funcional segundo a *New York Heart Association* (NYHA), dos sintomas e qualidade de vida, redução do peptídeo natriurético cerebral, melhoria no teste de marcha de seis minutos e redução da mortalidade e internamento por IC. Além disso, estudos anteriores também mostraram uma redução nos mediadores inflamatórios em doentes com IC submetidos à TRC. No entanto, a ligação entre a remodelação cardíaca reversa, o potencial efeito anti-inflamatório da TRC e as células imunitárias periféricas está longe de ser compreendida. Neste sentido, os objetivos gerais da presente pesquisa foram compreender o papel das células imunitárias periféricas na ICC, investigar o efeito da TRC na dinâmica destas células e, estudar a sua possível contribuição na remodelação cardíaca reversa. Os objetivos específicos incluíram: primeiro, quantificar e caracterizar funcionalmente células imunitárias inatas circulantes, como monócitos e células dendríticas (DC), e células da imunidade adaptativa, como os diferentes subconjuntos funcionais de linfócitos nos doentes com IC avançada; segundo, estudar o impacto da TRC nessas células, avaliando a sua frequência e atividade funcional nos doentes com IC, antes e 6 meses após a TRC; e terceiro, investigar possíveis diferenças entre

respondedores e não respondedores à TRC. Para isso, esta investigação foi dividida em duas fases. Primeiramente, foram colhidas amostras de sangue de doentes com IC agendados para TRC. Nesse momento, as células foram identificadas, quantificadas e caracterizadas por citometria de fluxo através de protocolos de marcação de membrana e intracitoplasmática. A sua caracterização funcional foi também avaliada por citometria de fluxo, após estimulação *in vitro*. A quantificação da expressão do mRNA foi realizada por *polymerase chain reaction* (PCR) em tempo real. As análises estatísticas foram realizadas para comparar os resultados entre o grupo controlo e o grupo de doentes com IC.

A segunda fase compreendeu uma reavaliação dos doentes 6 meses após a TRC, na qual foram analisadas as mesmas variáveis. Nesse momento, foram realizadas comparações estatísticas não apenas entre o grupo controlo e os doentes, mas também entre a avaliação inicial e o acompanhamento, e entre os doentes respondedores e os não respondedores à TRC.

O grupo de doentes com IC apresentou uma frequência significativamente menor de DC plasmacitóides (pDC) no início do estudo e uma maior proporção de monócitos e DC mielóides (mDC) produtores de citocinas pró-inflamatórias do que os indivíduos normais. Quanto às células da imunidade adaptativa, as células Tc17 circulantes encontravam-se aumentadas nos doentes comparativamente ao grupo controlo. Pelo contrário, as células que medeiam a tolerância imunológica e a homeostasia, as células T reguladoras (Treg), estavam diminuídas em doentes com IC. Estas diferenças caracterizam o estado inflamatório da IC e apoiam a ideia de que as células imunitárias periféricas estão envolvidas neste processo inflamatório.

Após a TRC, ocorreu uma mudança de paradigma nos subconjuntos de monócitos: a frequência de monócitos clássicos (cMo) diminuiu enquanto a de monócitos intermediários (iMo) aumentou em doentes respondedores. Este grupo também apresentou valores mais elevados de monócitos não clássicos (ncMo) no seguimento em comparação ao grupo não-respondedor. Esses achados sugerem uma participação dos subgrupos de monócitos na remodelação cardíaca reversa e na resposta à TRC. Além disso, todos os doentes apresentaram diminuição na expressão de CD86 em todas as subpopulações de monócitos e DC, mostrando um comprometimento na indução da resposta imunitária adaptativa por estas células. Mais, nos doentes não-respondedores, o aumento da frequência de DC produtoras de citocinas pró-inflamatórias persistiu após a TRC.

Em relação às subpopulações de células T, a frequência de células Tc17 diminuiu após a terapia, atingindo níveis semelhantes aos do grupo controlo. Este achado foi observado principalmente nos doentes respondedores. Estas descobertas mostraram que as células T produtoras de IL-17 parecem ser suprimidas após a TRC. Por outro lado, o nível de células Treg não foi restaurado após o tratamento, e a produção de citocinas inflamatórias pelas células T $CD8^+$ aumentou no acompanhamento.
Em conclusão, embora algumas subpopulações celulares tenham permanecido pró-inflamatórias após a TRC, os resultados observados nos respondedores indicam que a TRC pode modular o comportamento das células imunitárias, contribuindo potencialmente para reverter a remodelação cardíaca e melhorar a função cardíaca. Estas descobertas ajudam a elucidar a complexa relação entre o sistema imunitário e a terapia cardíaca, sugerindo que a TRC pode beneficiar os doentes com ICC, não só pela melhoria dos sintomas e remodelação cardíaca reversa, mas também alterando o comportamento das células imunitárias, reduzindo potencialmente as respostas inflamatórias no coração.

Palavras-chave

Insuficiência cardíaca crónica; terapêutica de ressincronização cardíaca; sistema imunitário; monócitos; células dendríticas; linfócitos

Abstract

Chronic heart failure (CHF) is a worldwide public health problem. It consists of a complex condition characterized by inadequate cardiac function, accompanied by cardinal symptoms and multiple signs and comorbidities. The pathophysiology of heart failure (HF) is not completely understood; however, it usually culminates in a progressive myocardial dysfunction associated with continuous ventricular remodelling. Nowadays, it is also well established that immune system activation promotes a systemic inflammatory status in this condition. HF patients, independent from prevalent systolic or diastolic dysfunction, present increased levels of several pro-inflammatory cytokines, associated with adverse clinical outcomes. Peripheral blood cells have been suggested as a potential source for the prolonged systemic production of cytokines in CHF. Numerous studies propose the participation of these cells in the pathogenesis of the disease itself, contributing to progressive cardiac remodelling.

Cardiac resynchronization therapy (CRT) is a guideline-recommended treatment for patients with drug-refractory HF, reduced left ventricle ejection fraction (LVEF), and left bundle branch block (LBBB). This therapy has beneficial effects on symptoms and cardiac remodelling in responder patients, such as reverse cardiac remodelling (with the reduction of left ventricular volumes and increased LVEF), improvement of New York Heart Association (NYHA)-based functional status, symptoms, and quality of life, reduction of brain natriuretic peptide, improvement in the six-minute walk test, and reduction of mortality and hospitalization for HF. In addition, previous studies have shown a reduction in inflammatory mediators in HF patients treated with CRT. Nonetheless, the connection between reverse cardiac remodelling, and the potential anti-inflammatory effect of CRT and peripheral immune cells remains far from fully understood. Therefore, the overall goals of the present research were to elucidate the role of peripheral immune cells in CHF, to investigate the effect of CRT on immune cell dynamics, and to study the possible contribution of those cells to reverse cardiac remodelling. The specific aims included: first, to quantify and functionally characterize circulating innate immune cells, like monocytes and dendritic cells (DC), and cells from adaptive immunity, like the different functional subsets of lymphocytes in patients with advanced HF; second, to study the impact of CRT on those cells by evaluating their frequency of and functional activity in HF patients before and 6 months after CRT; and third, to investigate possible differences between responders and non-responders to CRT. To this end, this research was divided into two phases. First, blood samples were

collected from HF patients scheduled for CRT. Subsequently, cells were identified, quantified, and characterized by flow cytometry through direct immunofluorescence membrane and intracytoplasmic staining protocols. Their functional characterization was also evaluated by flow cytometry, but after in vitro stimulation. The quantification of mRNA expression was conducted by real-time polymerase chain reaction (PCR). Statistical analyses were performed to compare data between the control group and HF patients. The second phase comprised a reassessment of patients 6 months after CRT, in which the same variables were analysed. At this time, statistical comparisons were performed not only between the control group and patients, but also between baseline assessment and follow-up, and between responders and non-responders to CRT.

Patients with HF presented a significantly lower frequency of plasmacytoid DC (pDC) at baseline and a higher proportion of monocytes and myeloid DC (mDC) producing pro-inflammatory cytokines than normal individuals. As for adaptive immunity cells, circulating Tc17 cells tended to be higher in patients than in the control group. On the contrary, the level of cells measuring immune tolerance and homeostasis, regulatory T (Treg) cells, was decreased in HF patients. These differences characterize the inflammatory state of HF and support the idea that peripheral immune cells are involved in this inflammatory process.

After CRT, a paradigm shift occurred within monocyte subsets: the frequency of classical monocytes (cMo) decreased while that of intermediate monocytes (iMo) increased among responder patients. This group also presented higher non-classical monocytes (ncMo) values at follow-up compared to the non-responder group. These findings suggest the involvement of monocyte subsets in reverse cardiac remodelling and CRT response. In addition, all patients presented a reduction in CD86 expression in all monocyte and DC subpopulations, indicating a compromise in the induction of the adaptive immune response by monocytes and DC. Moreover, in non-responders, the increased frequency of pro-inflammatory cytokines-producing DC persisted after CRT.

Regarding T cell subpopulations, the frequency of Tc17 cells decreased after therapy, reaching levels similar to those in the control group. This observation was mainly found among responder patients. Additionally, the expression of IL-17 mRNA was detected in a few responders at initial evaluation and only in one responder at follow-up. These findings showed that IL-17-producing T cells appear to be suppressed after CRT. On the other hand, the level of Treg cells was not restored after treatment, and the production of inflammatory cytokines by $CD8^+$ T cells increased during follow-up.

In conclusion, although some cell subpopulations remained pro-inflammatory after CRT, the findings observed in responders indicate that CRT can modulate the behaviour of immune cells, potentially contributing to reverse cardiac remodelling and improving cardiac function. These findings help elucidate the complex relationship between the immune system and cardiac therapy, suggesting that CRT may benefit patients with CHF by improving symptoms, reversing cardiac remodelling, and altering the behaviour of immune cells, thereby potentially reducing the inflammatory response in the heart.

Keywords

Chronic heart failure; cardiac resynchronization therapy; immune system; monocytes; dendritic cells; lymphocytes.

Index

List of figures

Chapter 1

Chapter 3

Chapter 5

List of Tables

List of Acronyms

A

ACE-I	Angiotensin-Converting Enzyme inhibitors
AF	Atrial fibrillation
AHF	Acute Heart Failure
AHT	Arterial Hypertension
APC	Antigen Presenting Cells
ARBs	Angiotensin-Receptor Blockers
ARNI	Angiotensin Receptor-Neprilysin Inhibitor
ARVC	Arrhythmogenic Right Ventricular Cardiomyopathy
AV	Atrioventricular

B

BCR	B Cell Receptor
BDCA	Blood Dendritic Cells Antigen
BNP	B-type Natriuretic Peptide

C

CAD	Coronary Artery Disease
CCR2	C-C Chemokine Receptor type 2
CCR7	C-C Chemokine Receptor type 7
CD	Cluster of Differentiation
cDC	Conventional DC
CHF	Chronic Heart Failure
CKD	Chronic Kidney Disease
CLPs	Common Lymphoid Cell Progenitors
cMo	Classical Monocytes
CMP	Alcohol-induced Cardiomyopathy
cNK	Conventional NK
CRP	C-reactive protein
CRT	Cardiac Resynchronization Therapy
CTLA-4	Cytotoxic T-Lymphocyte-Associated Antigen 4
CV	Cardiovascular

D

DC	Dendritic Cells
DCM	Dilated Cardiomyopathy
DM	Diabetes Mellitus
DN	Double-Negative
DP	Double-Positive

E

EBF	Early B-Cell Factor
ECG	Electrocardiogram
EF	Ejection Fraction

Eomes	Eomesodermin
EPICA	Epidemiology of Heart Failure and Learning
ESC	European Society of Cardiology
ETP	Early Thymic Progenitors

G

GC	Germinal Centers
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor

H

HF	Heart failure
HFA	Heart Failure Association
HFmrEF	HF with Mid-Range Ejection Fraction
HFpEF	HF with Preserved Ejection Fraction
HFrEF	HF with Reduced Ejection Fraction
HG	Healthy Control Group
HLA	Human Leukocyte Antigen
hs-CRP	High Sensitivity C-Reactive Protein

I

ICAM-1	Intercellular Adhesion Molecule 1
ICD	Implantable Cardioverter-Defibrillator
ICOS	Inducible T Cell Co-Stimulator
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate Lymphoid Cells
iMo	Intermediate Monocytes
iTreg	Inducible Treg cells

L

LBBB	Left Bundle Branch Block
LV	Left Ventricular
LVEDV	Left Ventricular End-Diastolic Volume
LVEF	Left Ventricular Ejection Fraction
LVESV	Left Ventricular End-Systolic Volume
LVH	Left Ventricular Hypertrophy
LTi	Lymphoid Tissue Inducer

M

MCP1	Monocyte Chemotactic Protein 1
mDC	Myeloid Dendritic Cells
MHC	Major Histocompatibility Complex
MI	Myocardial Infarction
MMPs	Matrix Metalloproteinases
MRA	Mineralocorticoid Receptor Antagonists
MR-proANP	Mid-Regional pro-Atrial Natriuretic Peptide
mRNA	Messenger Ribonucleic Acid

N

ncMo	Non-Classical Monocytes
NK	Natural Killer
NLRP	Nod-like receptors (Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing)
NPs	Natriuretic Peptides
NT-proBNP	N-Terminal pro-B-type Natriuretic Peptide
nTreg	Natural Treg cells
NYHA	New York Heart Association

P

PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
pDC	Plasmacytoid Dendritic Cells
PD-L1	Programmed Death-Ligand 1
PGE2	Prostaglandin E2
PPCM	Peripartum Cardiomyopathy

R

RAA	Renin-Angiotensin-Aldosterone
RORγt	Retinoic Acid–Related Orphan Receptor γt
RV	Right Ventricular

S

SD	Standard Deviation
SGLT2	Sodium-Glucose Co-Transporter 2
SP	Single Positive
SR	Sinus Rhythm
STAT	Signalling Transducer and Activator of Transcription

T

Tc	T Cytotoxic
TCR	T Cell Receptor
Tfh	T Follicular Helper Cells
TGF-β	Transforming Growth Factor β
Th	T Helper
TNF-α	Tumor Necrosis Factor - α
TLR	Toll-Like Receptors
Treg	T Regulatory

V

VCAM1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
VHD	Valvular Heart Disease

W

WBC	White Blood Cells

Chapter 1

Introduction

Chapter 1

Introduction

1.1. Chronic heart failure

Chronic heart failure (CHF) is a complex clinical syndrome [1, 2] with different aetiologies and pathophysiology [1-3]. For that reason, it is challenging to define CHF as a specific disease, based solely on diagnostic features and haemodynamic and physiological aspects. Nowadays, the existing definition of heart failure (HF) considers three main elements: the evidence of structural heart impairment, a history of commonly reported symptoms, and objective signs usually observed in HF patients. This definition is described in the Contemporary Clinical Practice Guideline (Table 1.1) [2]. It is essential to note that in HF condition, the heart is unable to generate a sufficient cardiac output to meet the perfusion demands of the body. This is due to ineffective pumping (systolic failure or HF with reduced ejection fraction (HFrEF)) or an ineffective filling (diastolic failure or HF with preserved ejection fraction (HFpEF)) [4].

1.1.1. Terminology of heart failure

HF has been commonly defined according to the left ventricular ejection fraction (LVEF) measurement. In patients with significantly reduced left ventricular (LV) systolic function, i.e., LVEF ≤40%, HF is designated as HFrEF. Patients with LVEF between 41% and 49%, are classified as HF with mildly reduced ejection fraction (HFmrEF). Patients with symptoms and signs of HF, with structural and/or functional cardiac abnormalities and/or elevated natriuretic peptides (NPs), and LVEF ≥50% have HFpEF [5].

Heart failure can also result from right ventricular (RV) dysfunction when pressure-setting elevations or volume overload occurs in the right ventricle. The main cause of chronic RV failure is LV dysfunction-induced pulmonary hypertension. However, other conditions such as myocardial infarction (MI), arrhythmogenic right ventricular cardiomyopathy (ARVC), or valve disease can also cause RV dysfunction [5].

Table 1.1 – The definition of HF in contemporary clinical practice guidelines, (Adapted from Bozkurt et al., 2021 [2]).

American College of Cardiology/ American Heart Association (2013) [6]	HF is a complex clinical syndrome that results from any structural or functional impairment of ventricular filling or ejection of blood. The cardinal manifestations of HF are dyspnoea and fatigue, which may limit exercise tolerance, and fluid retention, which may lead to pulmonary and/or splanchnic congestion and/or peripheral oedema. Some patients have exercise intolerance but little evidence of fluid retention, whereas others complain primarily of oedema, dyspnoea, or fatigue.
European Society of Cardiology (2021) [5]	HF is a clinical syndrome characterized by typical symptoms (e.g., breathlessness, ankle swelling, and fatigue) that may be accompanied by signs (e.g., elevated jugular venous pressure, pulmonary crackles, and peripheral oedema) caused by a structural and/or functional cardiac abnormality, resulting in a reduced cardiac output and/or elevated intracardiac pressures at rest and/or during stress.
Japanese Cardiology Society/ Japanese Heart Failure Society JCS/JHFS (2017) [7]	HF is a clinical syndrome consisting of dyspnoea, malaise, swelling, and/or decreased exercise capacity due to the loss of compensation for cardiac pumping function due to structural and/or functional abnormalities of the heart.

HF can also present itself as CHF or acute heart failure (AHF) [5, 8]. CHF embraces patients with a previously established diagnosis of HF or who have a gradual onset of symptoms. On the other hand, AHF occurs suddenly and is commonly observed in patients with alcohol-induced cardiomyopathy (CMP), viral myocarditis, Takotsubo syndrome, peripartum cardiomyopathy (PPCM), or tachycardiomyopathy. In these cases, patients may fully recover. Other patients with LV systolic dysfunction may have substantial or even complete recovery of LV systolic pressure function after treatment with drugs and device therapy [5].

The simplest classification used to describe the severity of HF is the New York Heart Association (NYHA) functional classification (Table 1.2) [5, 8]. This terminology depends only on symptoms and lacks other indicators of prognosis in HF [5, 8]. It is important to consider that even patients with mild symptoms may have an increased risk of hospitalization and death, and predicting outcomes in those with advanced HF is crucial for selecting appropriate heart transplantation and device therapies [5].

Table 1.2 – Heart failure functional classification according to the New York Heart Association (adapted from McDonagh et al., 2021 [5]).

Class I	No limitation on physical activity. Ordinary physical activity does not cause undue breathlessness, fatigue, or palpitations.
Class II	Slight limitation of physical activity. Comfortable at rest, but ordinary physical activity results in undue breathlessness, fatigue, or palpitations.
Class III	Marked limitation of physical activity. Comfortable at rest, but less than ordinary activity results undue breathlessness, fatigue, or palpitations.
Class IV	Unable to carry on any physical activity without discomfort. Symptoms at rest can be present. If any physical activity is undertaken, discomfort is increased.

1.1.2. Epidemiology

The prevalence of HF ranges between 2% and 3% of the global population, [9, 10] affecting 60 million individuals worldwide [11, 12]. It presents a higher incidence among the elderly [9, 10, 12, 13] with estimates suggesting a continued growth of up to 8.5% by the year 2030 in this population [9, 13]. The prevalence of CHF is also projected to increase by 50% due to factors such as an ageing population, the occurrence of co-morbidities, and the improved survival rates of patients with cardiovascular (CV) diseases and HF itself [10-12].

In 2019, the Heart Failure Association (HFA) Atlas Task Force provided an overview of the epidemiology of HF in Europe. Data from the member countries of the European Society of Cardiology (ESC) were presented and an atlas was designed and populated (Figure 1.1 and 1.2) [14]. The median prevalence of HF per 1000 people was 17.20 cases (ranging from ≤ 12 cases in Greece and Spain, to > 30 cases in Lithuania and Germany – Figure 1.1), while the median annual incidence of HF was 3.20 cases per 1000 person-years, (with Italy presenting the lowest incidence (< 2) and Estonia and Germany presenting the highest (≥ 6) – Figure 1.2) [14].

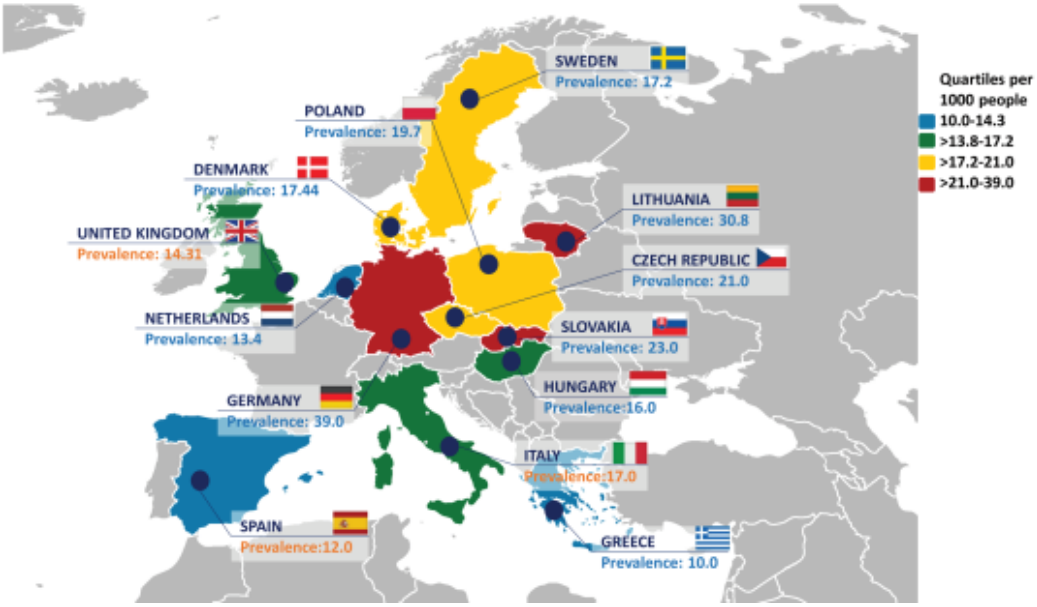


Figure 1.1 – Prevalence of heart failure per 1000 persons. (Data in blue letters derived from national statistics, governmental and/or ministry of health documents, and prospective national registers/surveys. Data in orange letters derived from publications based on defined populations) (adapted from Seferović et al., 2021 [14]).

Figure 1.2 – Incidence of heart failure per 1000 persons. (Data in blue letters derived from national statistics, governmental and/or ministry of health documents, and prospective national registers/surveys. Data in orange letters derived from publications based on defined populations) (adapted from Seferović et al., 2021 [14]).

The overall prevalence of chronic HF in Portugal (between the years 1998 and 2000) was estimated at 4.36% by the Epidemiology of Heart Failure and Learning (EPICA) study [15], ranging from 1.36% in individuals aged 25–49 years to 16.14% in those aged over 80 [12, 15, 16].

Considering current clinical practices, the prevalence of HF in mainland Portugal is expected to increase by approximately 30% in 2035 and 33% in 2060, compared to 2011 (General Population Census of Portugal date) affecting 479921 and 494191 individuals, respectively [16, 17].

The prevalence of different HF phenotypes according to ejection fraction (EF) (HFrEF, HFmrEF, and HFpEF) is limited due to the lack of EF assessment in several large-scale studies. In epidemiological data from Western countries, HFrEF seems to affect approximately 50% of patients, while HFmrEF and HFpEF affect approximately 20–25%. However, with improved treatments and current prevention of ischaemic disease, the prevalence of HFrEF appears to stabilize or even decline, contrary to the HFpEF, which seems to be increasing. Recent data even suggest that HFpEF will soon become the most common HF phenotype [11, 18].

1.1.3. Aetiology

CHF is the final common pathway of a wide spectrum of diseases and is associated with many risk factors. The detection of the aetiology of HF is crucial, not only in its diagnosis and prognosis but also in the institution of treatment and preventive therapies [8].

The causes of HF can be divided into aetiological and non-aetiological causes.

Structural, congenital, or acquired alterations that modify the normal physiology of the heart are considered predisposing aetiological causes. Disarrangement of peripheral vessels, coronary circulation, pericardium, myocardium, endocardium, or heart valves are some examples. The main predisposing aetiological cause is coronary artery disease (CAD). Patients with previous MI carry a risk of HF 10 times greater than that of the normal population in the first year and up to 20 times in the following years. Another important predisposing cause is arterial hypertension (AHT) [8, 19]. The risk of HF doubles in the population with mild AHT and quadruples when blood pressure exceeds 160/95 mmHg. Other factors like left ventricular hypertrophy (LVH) (mainly caused by

hypertension), diabetes mellitus (DM), and a history of rheumatic fever also increase the risk of HF [3, 8, 19, 20].

Non-aetiological predisposing causes such as advanced age, male sex, obesity, cardiomegaly, reduced vital capacity, smoking, proteinuria, and baseline electrocardiogram abnormalities (such as left bundle branch block (LBBB) and changes in ventricular repolarization) do not have a direct causal relation with HF, but are considered as risk indicators [1, 8, 19].

The determining causes of HF are those that modify the regulatory mechanisms of ventricular function, increase the haemodynamic load, and alter heart rate. These can be classified into myocardial alterations (primary or secondary), haemodynamic overload, ventricular filling defects, ventricular dyssynergy, and alterations in rhythm.

Primary myocardial alterations include idiopathic dilated cardiomyopathy (DCM) (characterized by predominant LV systolic dysfunction, with possible dilation of all four heart chambers), hypertrophic cardiomyopathy (characterized by LVH in the absence of secondary causes), and restrictive cardiomyopathy (characterized by a change in cardiac compliance, with rapid early diastolic filling) [8]. HF related to CAD may occur after chronic MI, chronic ischaemia, ventricular aneurysm, and mitral valve dysfunction. Although less frequent, there are also cardiomyopathies of infectious and inflammatory origin, toxic cardiomyopathies (related to alcohol and drug consumption), metabolic cardiomyopathies (associated with DM, hyperthyroidism, hypothyroidism, Cushing's disease, hypocalcaemia, hypophosphataemia), cardiomyopathies associated with neuromuscular diseases (such as Duchenne or Becker dystrophies, among others) and cardiomyopathies associated with nutrient deficits (thiamine, selenium, carnitine) [8].

Haemodynamic overload results from the effect produced by augmented pressure or volume. In cases of hypertension, aortic stenosis, pulmonary artery hypertension, and pulmonary stenosis, the appearance of HF can occur from an increase in afterload that causes a pressure overload in LV. In volume overload-derived HF, the main associated causes are hypervolemia, mitral and aortic insufficiency, interventricular communication, and persistent arterial duct (in the left cavities), as well as tricuspid insufficiency or interatrial communication (in the right cavities) [8].

Alterations in compliance associated with ventricular hypertrophy, obstruction of the ventricular outflow tract, hypovolemia, cardiac obstruction, pericardial constriction, and intracardiac masses are several of the conditions where there is a ventricular filling defect, which can result in HF [8].

Alterations in heart rate such as tachycardia, bradycardia, and loss of atrioventricular (AV) synchrony are also related to the origins of HF.

Finally, there are precipitating causes of HF, associated with structural cardiac deformation, that may decompensate a stable situation in patients with or without a previous diagnosis of HF. They can be cardiac causes, such as arrhythmias or muscle damage, or extracardiac causes such as infections (mainly respiratory), pulmonary embolism, drugs that cause sodium retention, diet, abandonment of treatment, stress, anaemia, surgery, smoking, and alcohol [8].

1.1.4. Diagnosis

The diagnosis of CHF requires the presence of typical symptoms and/or signs of HF, like shortness of breath, ankle fatigue, and swelling, although symptoms and signs alone are not sufficient to determine the diagnosis of HF. Objective evidence of cardiac dysfunction is also needed. Furthermore, at the diagnostic stage, it is of utmost importance to consider that CHF is more common in patients with a history of MI, AHT, CAD, DM, alcohol abuse, chronic kidney disease (CKD), cardiotoxic chemotherapy, and in those with a family history of cardiomyopathy or sudden death [5].

The diagnostic tests recommended for the evaluation of patients with suspected chronic HF are electrocardiogram (ECG), measurement of NPs, measurement of basic analytical parameters, echocardiography, and chest X-ray [5, 10]. The diagnostic algorithm for HF, presented in 2021 ESC guidelines, is shown in Figure 1.3.

ECG is mandatory, as > 90% of individuals with HF have an abnormal ECG [10]. Additionally, this test not only increases the likelihood of diagnosing HF but also helps guide therapy [5]. It may reveal abnormalities such as atrial fibrillation (AF), Q waves, LVH, and a widened QRS complex which may require individualized approaches [5, 10]. A normal ECG makes the diagnosis of HF improbable [5].

Determination of NPs is recommended in patients with symptoms which are suggestive of HF. A concentration value of B-type natriuretic peptide (BNP) < 35 pg/mL, N-terminal pro-B-type natriuretic peptide (NT-proBNP) < 125 pg/mL, or mid-regional pro-atrial natriuretic peptide (MR-proANP) < 40 pmol/L makes the diagnosis of HF unlikely. Elevated concentrations of NPs support the diagnosis of HF and are useful in prognosis stratification and cardiac investigation. Of note is that there are many causes for elevated NP – both CV and non-CV – that can reduce diagnostic accuracy. These causes include

AF, increasing age, and/or chronic kidney disease. In addition, NP concentrations may be disproportionately low in obese patients.

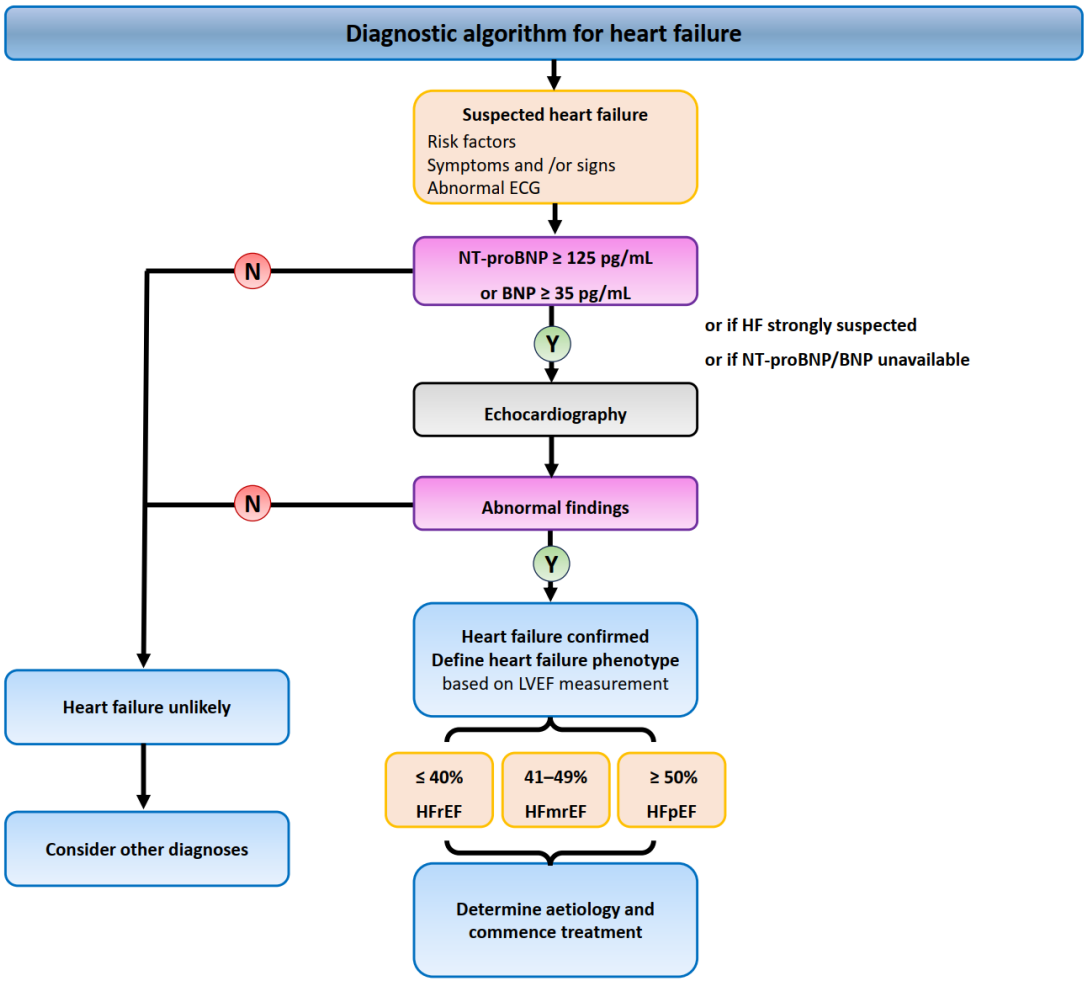


Figure 1.3 – The diagnostic algorithm for heart failure. BNP = B-type natriuretic peptide; ECG = electrocardiogram; HFmrEF = heart failure with mildly reduced ejection fraction; HFpEF = heart failure with preserved ejection fraction; HFrEF = heart failure with reduced ejection fraction; LVEF = left ventricular ejection fraction; NT-proBNP = N-terminal pro-B type natriuretic peptide (adapted from McDonagh et al., 2021 [5]).

Complete blood count and biochemical measurement of serum parameters such as urea, electrolytes, creatinine, liver, and thyroid function are recommended to make a differential diagnosis between HF and other conditions, to establish a prognosis, and to guide potential therapy [5, 10, 19].

Echocardiography is the key to assessing cardiac function [5]. It provides information about chamber size, eccentric or concentric LVH, regional wall motion abnormalities

(which may suggest underlying CAD, Takotsubo syndrome, or myocarditis), RV function, valve function, and markers of diastolic function [5, 10].

Finally, a chest X-ray is recommended to exclude other potential causes of shortness of breath (like lung disease) or to provide supportive evidence of HF (such as lung congestion or cardiomegaly) [5, 10, 19].

1.1.5. Clinical course

Heart failure is a progressive condition with significant morbidity and mortality [21, 22]. The increasing development of signs and symptoms that require administration of intravenous diuretics is associated with a markedly worse prognosis [23, 24]. The one-year mortality rate is 7.2% and the one-year hospitalization rate is 31.9% in patients with CHF, increasing to 17.4% and 43.9%, respectively, in patients with AHF [21, 25].

A subset of patients with HF progresses to advanced HF, which is characterized by persistent and severe symptoms despite optimized guideline-directed medical therapies [22, 26]. Advanced HF therefore represents a clinically significant and vulnerable patient group that may suffer clinical, financial, and psychological concerns and a marked increase in mortality [22, 26].

Patients with HFrEF are at risk of worsening HF, have a high risk of adverse outcomes, and experience higher healthcare use and costs [27]. Prospective multinational data have shown that the mortality rate is higher in HFrEF than in HFpEF and HFmrEF. The mortality rate between HFpEF and HFmrEF is comparable [28].

1.2. Heart failure with reduced ejection fraction

HFrEF comprises a complex and heterogeneous group of patients, and its aetiology is primarily related to comorbidities [29, 30]. These comorbidities result in myocardial injury and significant depression of LV systolic function. Therefore, a higher preload is required to maintain cardiac output, resulting in adverse cardiac remodelling (Figure 1.4). Approximately one-third to one-half of all HF cases are categorized as HFrEF [11, 27, 29, 31].

1.2.1. Pathophysiology of Heart Failure with Reduced Ejection Fraction

HF is characterized by a decrease in cardiac output, in which the functioning heart cannot meet the perfusion demands of the body. CAD, hypertension, cardiomyopathy, valvular heart disease (VHD), heart inflammation and toxic cardiomyopathy are the most common causes of HF (Figure 1.4) [19, 20, 32-34].

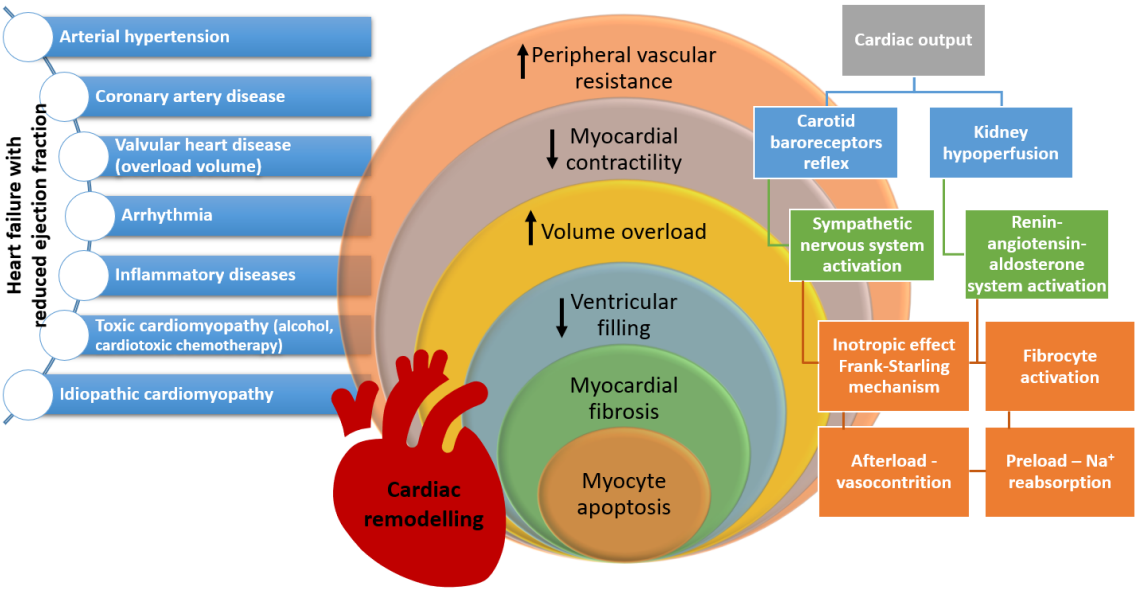


Figure 1.4 – Pathophysiology of heart failure with reduced ejection fraction (adapted from Iovanovici et al., 2022 [34]).

Ventricular dysfunction results from the death or dysfunction of cardiac myocytes and longstanding pressure or volume overload. When myocardial contractility decreases, stroke volume falls, and end-diastolic volume and pressure increase. According to the Frank-Starling law, these events restore myocardial contractility and, consequently, cardiac output. Maintained over the long term, the increase in volume gives rise to cardiac remodelling. This includes myocardial hypertrophy, enlargement of the chambers, and enhanced stress on the ventricular wall, with a consequent increase in oxygen demand. There is also an augment in ventricular stiffness due to increased collagen deposition in the heart, which impairs filling and aggravates the condition. Moreover, the decrease in cardiac output leads to sympathetic activation and stimulation of the renin-angiotensin-aldosterone (RAA) system, accompanied by salt and water retention in the kidney and increased circulating volume (Figure 1.4) [33]. In the beginning, according to the Frank-Starling law, this will restore cardiac output. However,

this mechanism also leads to a vicious cycle of increased and inefficient cardiac energy consumption and reduced myocardial perfusion (particularly in the subendocardial region) [33, 35]. The combination of cardiac remodelling and the vicious RAA cycle results in a myocardium which is vulnerable to ischaemia, and a circulation which depends on sympathetic tone [33].

1.2.2. Treatment for patients with heart failure with reduced ejection fraction

The three main goals of treatment for patients with HFrEF consist of reducing mortality, preventing recurrent hospitalizations due to worsening HF, and improvements in clinical status, functional capacity, and quality of life [5].

According to ESC guidelines [5], pharmacotherapy is the base treatment for HFrEF patients and is usually implemented before considering device therapy and non-pharmacological interventions (Figure 1.5).

Modulation of the RAA and sympathetic nervous systems with angiotensin-converting enzyme inhibitors (ACE-I) or an angiotensin receptor-neprilysin inhibitor (ARNI), beta-blockers, and mineralocorticoid receptor antagonists (MRA) has been shown to improve survival, reduce the risk of HF hospitalizations, and reduce symptoms in patients with HFrEF [5, 36]. This triad of an ACE-I/ARNI, a beta-blocker, and an MRA is recommended as a cornerstone therapy for these patients unless the drugs are contraindicated or not tolerated. Angiotensin-receptor blockers (ARBs) are indicated in those who are intolerant to ACE-I or ARNI. The sodium-glucose co-transporter 2 (SGLT2) inhibitors dapagliflozin and empagliflozin added to therapy with ACE-I/ARNI/beta-blocker/MRA reduce the risk of CV death and worsening HF in patients with HFrEF. Unless contraindicated or not tolerated, dapagliflozin or empagliflozin are recommended for all patients with HFrEF already treated with an ACE-I/ARNI, a beta-blocker, and an MRA, regardless of whether they have diabetes or not [5].

In addition, there are other therapies to consider in selected HFrEF patients to reduce HF hospitalization and mortality. For instance, loop diuretics are recommended to reduce the signs and/or symptoms of congestion, achieving and maintaining euvolaemia. An implantable cardioverter-defibrillator (ICD) is mainly used to treat arrhythmic events and is an effective therapy in correcting potentially lethal ventricular arrhythmias.

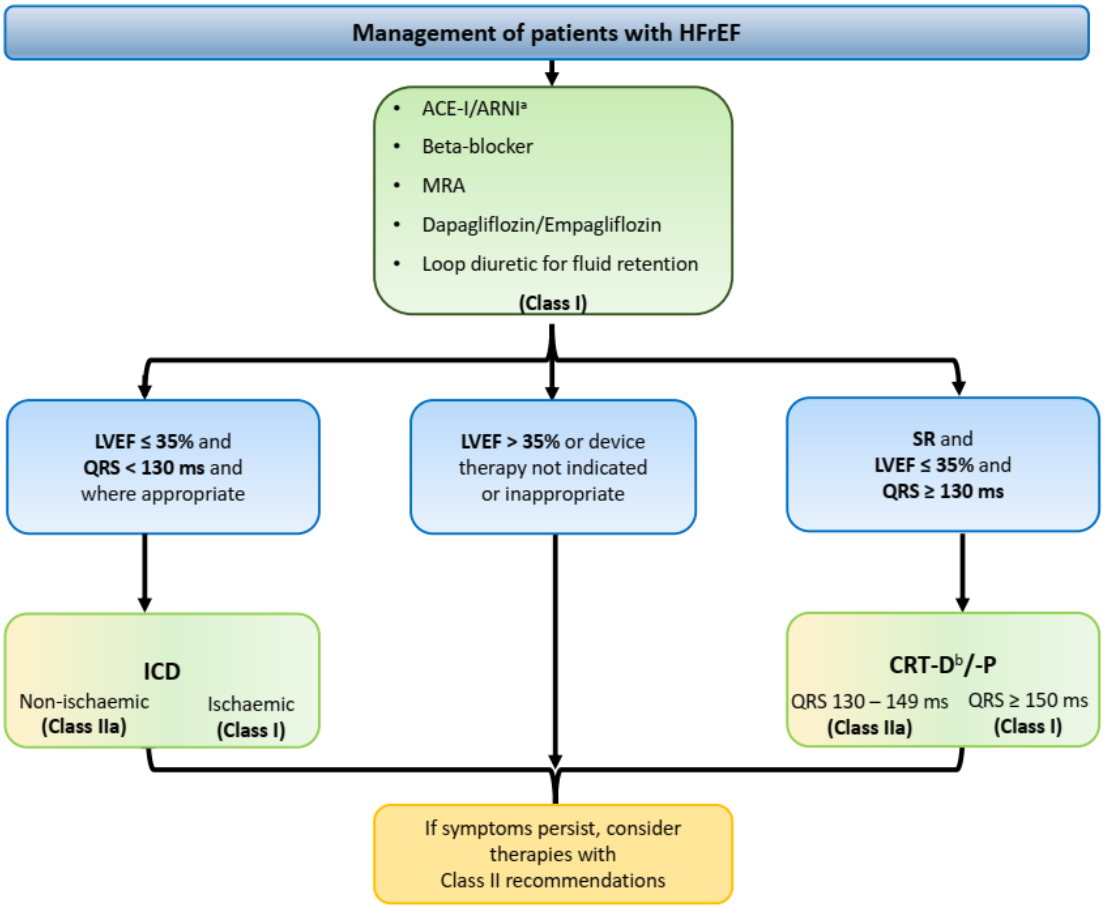


Figure 1.5 – Therapeutic algorithm of Class I Therapy Indications for a patient with heart failure with reduced ejection fraction. ACE-I = angiotensin-converting enzyme inhibitor; ARNI = angiotensin receptor-neprilysin inhibitor; CRT-D = cardiac resynchronization therapy with defibrillator; CRT-P = cardiac resynchronization therapy pacemaker; ICD = implantable cardioverter-defibrillator; HFrEF = heart failure with reduced ejection fraction; MRA = mineralocorticoid receptor antagonist; QRS = Q, R, and S waves of an ECG; SR = sinus rhythm. [a]As a replacement for ACE-I. [b]Where appropriate. Class I = green. Class IIa = Yellow (adapted from McDonagh et al., 2021 [5]).

ICD has a class I recommendation in primary prevention in HF with ischaemic aetiology and class II in non-ischaemic if the LVEF is ≤ 35% (Figure 1.5). Cardiac resynchronization therapy (CRT) is one of the most effective therapies for HFrEF. CRT improves the quality of life by resynchronizing intra-ventricular conduction/contraction and fostering beneficial reverse remodelling, resulting in a reduction of HF hospitalization rates and all-cause mortality [37, 38]. According to the current guidelines, CRT has a class I recommendation in patients with sinus rhythm (SR) with left LBBB ≥ 150 ms, and a class II recommendation in SR with LBBB between 130 and 149 ms or non LBBB ≥ 150 ms (Figure 1.5) [5].

1.3. Immune system activation in heart failure

The immune system is constituted by a collection of cells, chemicals, and processes that act simultaneously to host defence from foreign antigens, which are derived from several microorganisms (such bacteria, fungi and parasites or viruses, tumour cells, and toxins). In addition to structural and chemical barriers to pathogens, the immune system has two major lines of defence: innate immunity and adaptive immunity. Innate immunity is an antigen-independent and non-specific mechanism, with no immunological memory. However, this immune response is rapid, starting within minutes or hours of the pathogen attack. On the other hand, adaptive immunity is specific and antigen-dependent and therefore requires a certain amount of time between the exposure to the antigen and the maximal response. It presents a memory capacity that allows the host to form a rapid and efficient immune response after exposure to a previously recognized antigen. The innate and adaptive immune responses act synergistically and any defect in one of these systems can cause disease or illness, involving inappropriate inflammation, autoimmune and immunodeficiency disorders, and hypersensitivity reactions [39]. Different cells are involved in the innate immunity response, such as monocytes/macrophages, neutrophils, dendritic cells, mast cells, basophils, eosinophils, natural killer (NK) cells, and innate lymphoid cells. Neutrophils and macrophages engulf microbes and exterminate them via multiple bactericidal pathways, being designated as phagocytes. In addition, macrophages also play a key role in presenting antigens to T cells, along with dendritic cells (DC), initiating the acquired immune response and acting as important messengers between innate and adaptive immunity. After professional antigen-presenting cells (APC) operation (macrophages and DC), the acquired response is initiated through the activation and proliferation of the antigen-specific T cells, and B cells that differentiate into plasma cells to produce antibodies [39].

In CHF, irrespectively from aetiology, there is an activation of the immune system (Figure 1.6) [40]. This activation results in the production and release of proinflammatory cytokines, activation of the complement system, and production of autoantibodies.

Immune activation can occur directly by antigenic stimulation, or secondary to cardiac injury which exposes “new antigens” capable of triggering an immune response against the heart [41, 42].

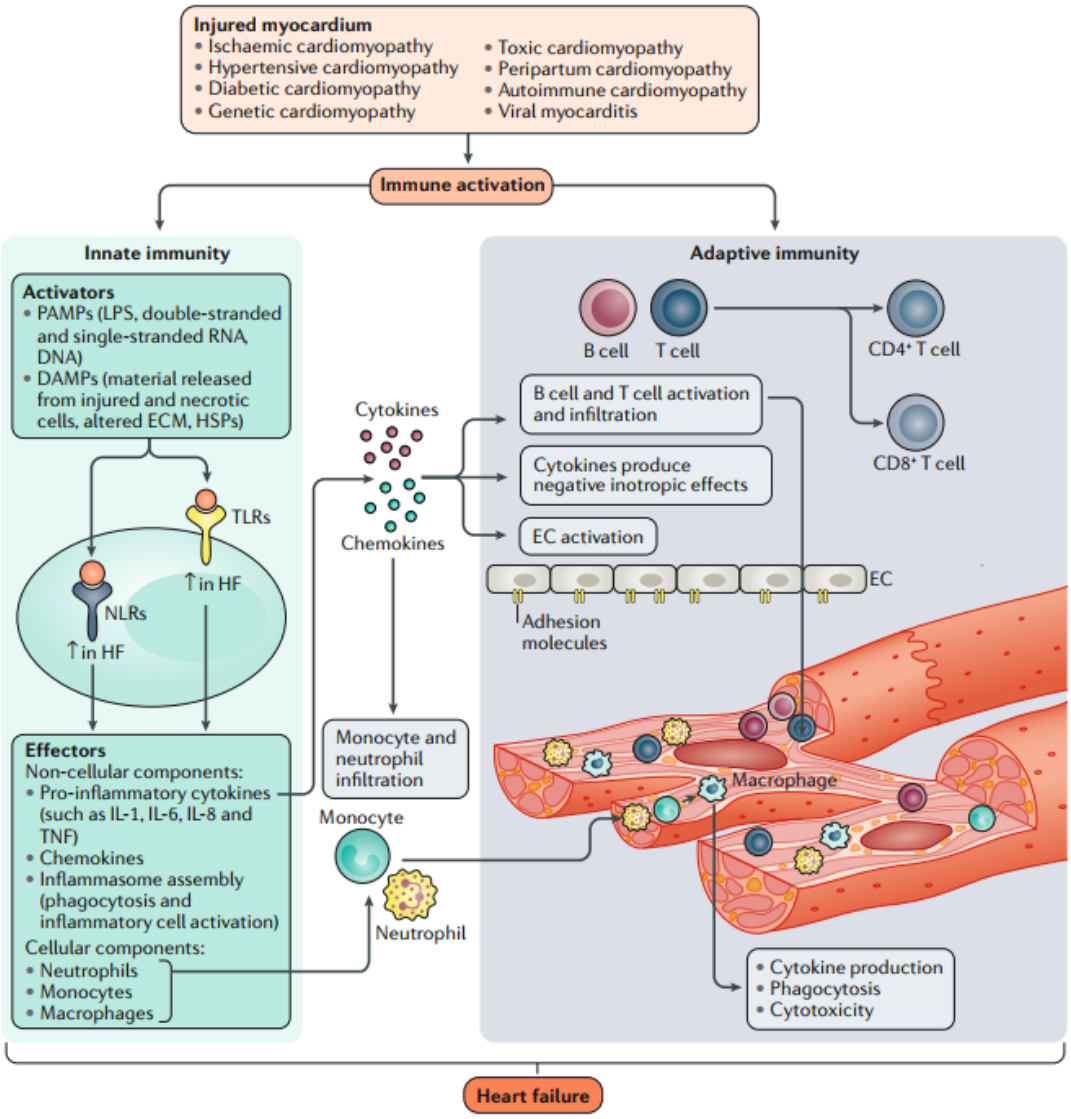


Figure 1.6 – The innate and adaptive immune systems in heart failure. A variety of cardiac disease states that lead to cardiac injury can activate the innate immune response in the heart through binding of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) to pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs) and NOD-like receptors (NLRs), present on cardiomyocytes and tissue-resident immune cells. Activation of PRRs induces a variety of non-cellular effectors in the heart, including pro-inflammatory cytokines and chemokines and activation of the complement system, which lead to endothelial cell activation and recruitment of monocytes and neutrophils. Activation of the innate immune system triggers the activation of the adaptive immune response through the recruitment of B cells and T cells to the injured myocardium. ECM, extracellular matrix; HF, heart failure; HSP, heat shock protein; LPS, lipopolysaccharide; TNF, tumour necrosis factor (adapted from Adamo et al., 2020 [43]).

Other studies also refer the release of cytokines by cardiac cells (from both myocytes and non-myocytes) in response to haemodynamic stress, as essential for the propagation and

magnification of the immune response [42, 44, 45]. Cytokines stimulate the division, proliferation, and differentiation of the recruited cells in the inflammation area [41, 44]. In the pathogenesis of HF, the most relevant cytokines seem to be tumour necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1 [41, 45, 46], being also considered as predictors of worse prognosis in HF [45, 47].

1.4. Immune cells in chronic heart failure

Resident and recruited immune cells, such as macrophages, mast cells, monocytes, neutrophils, eosinophils, B cells, and T cells, play a role in cardiac injury and are present in cardiac tissue early in HF [48, 49]. Initially, these immune cells infiltrate the heart, triggering inflammatory/reparative pathways in order to scavenge dead or dying cardiomyocytes and digest the tissue with proteolytic enzymes. However, the dead cells amplify the inflammatory cascade with consequent release of inflammatory cytokines which intensify inflammation even more through their effects on leukocytes, endothelial cells, and cardiomyocytes [49, 50]. When this protective inflammation is prolonged, immune cells modulate not only cardiomyocyte function but also induce injury responses like scar formation and interstitial fibrosis. In consequence, cardiac remodelling occurs, and cardiac function is affected. The relative balance between pathological inflammation and tissue repair pathways and processes (physiological inflammation) defines the course of HF [49].

1.4.1. Monocytes

Monocytes are components of both innate and adaptive immune systems and play primary roles in immune defence, inflammation, and tissue remodelling [47]. Representing about 2–8% of peripheral leukocytes [51], they protect the host against foreign pathogens in an antigen-independent manner by direct elimination (through interactions between pattern recognition receptors, including toll-like receptors (TLR), the lipopolysaccharide co-receptor CD14 and, scavenger receptors), or by cytokine production (including TNF-α, IL-1, and IL-12). Monocytes are the main cellular source of inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-12 and, at the same time, an important target of these cytokines. Only a small amount of chemokines is required to recruit peripheral blood monocytes into tissues and activate them to differentiate into macrophages and initiate local immune responses [47, 52, 53].

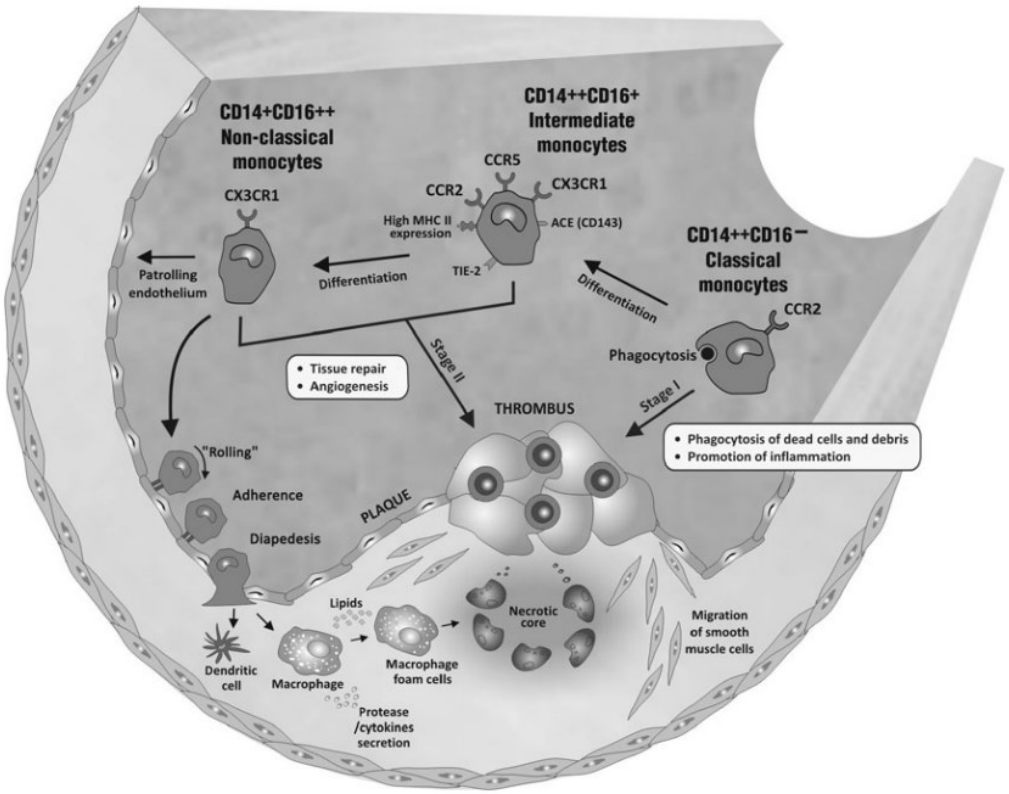


Figure 1.7 – Monocyte differentiation and the major roles of three monocyte subsets in atherosclerosis and acute coronary syndrome. The white boxes show the differential roles of monocyte subsets in different stages of myocardial infarction. At first (stage I), classical monocytes promote inflammation and scavenge necrotic debris. During stage II, non-classical and probably intermediate cells attenuate inflammation and promote healing (adapted from Idzkowska et al., 2015 [54]).

In acute or chronic cardiac damage, myeloid bone marrow-derived mononuclear phagocytes, or splenic monocyte precursors are attracted from the bloodstream through chemotactic signals (mainly through monocyte chemotactic protein 1 (MCP1/CCL2) secreted by the affected endothelium and injured tissue) and migrate through the vessel wall into the damaged tissue [47, 55]. There, monocytes differentiate into macrophages with distinct functional properties under the influence of local cytokine stimuli [47, 52, 56]. In pathological conditions, there is an abrogation of tissue homeostasis resulting in uncontrolled inflammation, which leads to the excessive release of macrophages. Instead of healing the tissue, these macrophages can cause tissue damage and adverse remodelling [57].

Human monocytes are dominated by the "classical" $CD14^{++}CD16^{-}$ (cMo) subset, corresponding to about 85–90% of total monocytes. The other two minor subsets are $CD16^{+}$ and classified as: "intermediate" monocytes (iMo), which represent ~6% of

monocytes and express higher levels of CD14 with a lower expression of CD16 ($CD14^{++}/CD16^{+}$); and "non-classical" monocytes (ncMo) that correspond to ~9% of all monocytes and express lower levels of CD14 coupled with high expression of CD16 ($CD14^{+}/CD16^{++}$) [57-60]. Currently, it is presumed that blood monocyte subsets represent stages in a developmental sequence, with the ncMo subset being considered the most mature monocytes since they share phenotypic characteristics with tissue macrophages [47]. Nonetheless, there are many significant phenotypic and functional differences between monocyte subsets. cMo are highly phagocytic, actively migrate through MCP1/MCP1-receptor gradients to inflamed tissue, have high myeloperoxidase activity and high antibody-dependent cell-mediated toxicity, and produce high levels of IL-1, MCP-1, TNF-α, and IL-6. iMo display a pro-angiogenic behaviour and are low cytokine producers. ncMo constantly survey the endothelium for signs of inflammation or damage and poised to transmigrate rapidly, exhibiting a patrolling behaviour [47, 61].

Following disruption of tissue homeostasis, such as inflammation, sepsis, and cancer, $CD16^{+}$ monocytes have been found to be significantly increased in the bloodstream [61, 62]. Monocyte-derived macrophages (assumed to be late-stage cells in the monocyte-macrophage lineage) have also been detected in almost all tissues and in lymphatic vessels [63, 64]. For instance, they have been detected in the thoracic duct during inflammation, from where lymph drains into the bloodstream [65, 66]. In a general way, these studies suggest that tissue macrophages continuously phagocytose and digest apoptotic cells and tissue debris; and, after completing these functions, they can migrate through the lymphatic vessels to the draining lymph nodes and possibly recirculate in the bloodstream. As resolution of inflammation requires apoptosis of inflammatory cells such as monocytes and macrophages [67], they possibly return to the peripheral blood to die in the spleen.

In heart disease, monocyte activation is visible in both early and late stages, and is associated with inflammation, fibrosis, endothelial damage, oxidative stress, and tissue damage and repair in patients with renovascular disease, pressure overload, myocardial fibrosis, diastolic and systolic dysfunction (Figure 1.7) [47, 56, 68-70]. Nevertheless, some monocyte subsets also present a potential for beneficial effects on HF pathogenesis [57, 59], but the mechanisms involved are still not fully understood.

Studies on circulating monocytes have reported cMo as the major subset in HF (87–48%), followed by iMo (5–44%) and ncMo subsets (7.1–8.4%) [58, 71, 72]. An increase in cMo numbers have also been described during HF decompensation. In contrast, iMo counts have been associated with a better survival, suggesting a protective role of this subset in patients with HF [57, 73], but the mechanisms inducing their possible benefits in HF are

far from clear. Regarding ncMo, depletion or no changes have been described in this condition [59, 72], hindering the understanding of these cells in HF.

1.4.2. Dendritic cells

DC are professional APCs crucial for initiation and orchestration of the immune response [74]. Representing 1–2% of the total leukocytes in peripheral blood, DC are an extremely heterogeneous population. Their main function consists of patrolling various anatomical locations and environmental interphases, capturing antigens, processing and presenting them to the effector cells, and initiating the adaptive immune response [74-76]. DC also regulate immune response and inflammatory processes through the secretion of cytokines and growth factors. They express major histocompatibility complex (MHC) II and some express CD11c similarly to monocytes and macrophages; however, they belong to a distinct cell population [75, 77].

In detail, DC are found in two different functional states, “mature” and “immature”. Before receiving maturation stimuli, DC are called immature. At this stage, they poorly induce naïve T lymphocyte effector responses, as they present low surface expression of costimulatory molecules and chemokine receptors, and do not produce immunostimulatory cytokines. However, immature DC possess a high endocytic capacity, via receptor-mediated endocytosis – including toll-like-lectin receptors, Fc-complement receptors, and macropinocytosis, which makes them very efficient in capturing antigens. Thus, immature DC act as sentinels against invading pathogens, and as scavengers in tissues capturing apoptotic and necrotic cells. This last feature gives the immature DC an essential role in the induction and maintenance of immune tolerance. In the case of natural tissue turnover, for example, emerging apoptotic cells are internalized by DC but do not induce maturation. These antigens are presented to T cells by DC but without the costimulatory activation signals, which results in T cell apoptosis, anergy, or the development of regulatory T (Treg) cells. Designated "tolerogenic", those DC express less costimulatory molecules and pro-inflammatory cytokines but upregulate the expression of inhibitory molecules (such as programmed death-ligand (PD-L) 1 and cytotoxic T-lymphocyte-associated antigen (CTLA)-4), secrete anti-inflammatory cytokines (IL-10) and are essential for preventing responses against healthy tissue [77].

DC maturation depends on disturbances in tissue homeostasis, which are detected by recognizing pathogen-associated molecular patterns (PAMPs) or damage-associated

molecular patterns (DAMPs) [76]. DC maturation activates their metabolic, cellular, and genetic transcription program (with loss of adhesive structures, cytoskeletal reorganization, and increased motility) which allows DC to migrate from peripheral tissues to T-dependent areas in secondary lymphoid organs, where the antigen-presentation and activation of T lymphocyte occurs. In addition, maturation also leads to a decrease in its endocytic activity and an increase in the expression of MHC-II and costimulatory molecules such as CD83, CD80, or CD86 as well as to a higher expression of the chemokine receptor, CCR7, and production of other cytokines essentials for T cell activation (Figure 1.8). When interacting with $CD4^+$ T cells, for instance, DC can induce their differentiation into different subsets of T helper (Th) cells, such as Th1, Th2, and Th17, among others [76, 77].

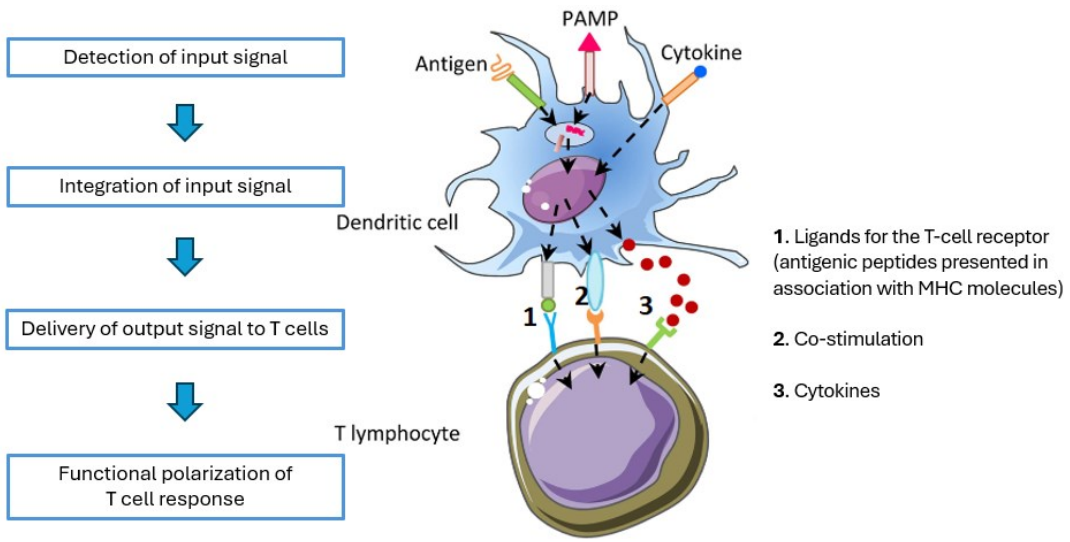


Figure 1.8 – DC inducing T cell immune response. DC deliver three types of output signals to T cells instructing their functional polarization: ligands for the T-cell receptor, co-stimulatory molecules, and cytokines. Co-stimulation and cytokine signals can be either activating (e.g., CD86 and IL-12, respectively) or inhibitory (e.g., PD-L1 and IL-10, respectively). Different cytokines induce distinct types of helper T-cell responses. For example, IL-12 primarily promotes Th1, IL-4 promotes Th2, and IL-23 promotes Th17. (adapted from Vu Manh et al., 2011 [78]).

Another important feature of DC is the fact that they can perform cross presentation. This phenomenon is defined as the presentation of captured antigens from the extracellular environment, using class I MHC molecules, which allows DC to trigger responses against intracellular antigens of other cell types that can avoid the action of professional APCs; and activate $CD8^+$ T cells in the absence of $CD4^+$ T cells. Moreover,

cross-presentation is also related to the induction of tolerance to intracellular autoantigens, thus called cross-tolerance [77].

According to their haematopoietic origin, DC can be classified into myeloid DC (mDC) and plasmacytoid DC (pDC). mDC, also titled conventional DC (cDC), typically express myeloid antigens as CD11c, CD13, CD33, and CD11b, while pDC lack myeloid antigens and are distinguished by expression of CD123 (IL-3Rα), CD303, CD304, CD1c and CD141. (Figure 1.9) [79, 80]. However, the classification of DC into subsets is currently based on specific phenotype, key gene signature, including critical transcription factors, TLRs, and other functionally relevant molecules including chemokine receptors and C-type lectin receptors and ontogeny [74]. Accordingly, within mDC, the cDC1 and cDC2 subtypes emerged and were also considered steady-stage DC [74, 81]. The cDC1 population is characterized by the expression of CD141 (also known as thrombomodulin or blood dendritic cells antigen (BDCA) 3), while human cDC2 cells are defined by the expression of CD1c (BDCA1) (Figure 1.9) [81].

mDC express high levels of molecules involved in antigen presentation to T cells, including class I and class II MHC molecules. After an inflammatory stimulus with antigen recognition and internalization, the mDC maturation process initiates display of that antigen and up-regulation of CCR7. The expression of this chemokine facilitates mDC migration through lymphatics to T-cell-dependent areas of secondary lymphoid organs that express CCR7 ligands. There, activation of $CD4^+$ T cells occurs and the immune response can be polarized into a pro-inflammatory (Th1, Th17), anti-inflammatory (Th2), or immune-regulatory (Tregs) phenotype. The class I MHC-dependent cross-presentation mechanism is also essential for the stimulation of $CD8^+$ T cells [82, 83]. The presence of cDC1 has been confirmed in blood, tonsil, spleen, skin, lung, intestine, ileum, payers patch, liver, and different lymph nodes [74]. BDCA3, highly expressed by the cDC1 subset, acts as a cofactor for thrombin and has been linked to supporting anti-inflammatory functions [84, 85]. The cDC1 subset can also be identified by the expression of C-type lectin receptor 9A (Clec9a), which facilitates the uptake of necrotic cells and in physiological conditions and cross-presenting antigens to $CD8^+$ T cells [86].

Human cDC1 also express a high level of TLR3 and have a unique ability to produce high levels of type III interferon (IFN) upon poly I:C stimulation. In addition, it is also believed that the cDC1 may interact with or activate the pDC through secretion of type III IFNs, improving pDC viability and increasing the type I IFN production [87].

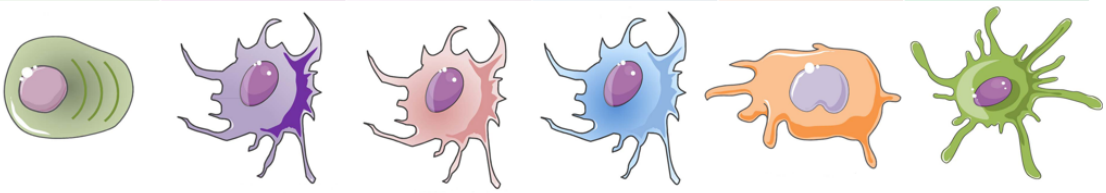
Plasmacytoid DC	Conventional DC1	Conventional DC2	Pre-DC	Monocyte-derived DCs	Langerhans cells
					
Phenotype					
BDCA2$^+$ (CD303), BDCA4$^+$ (CD304), CD123$^+$	BDCA3$^+$ (CD141), CD11c$^+$, CD13$^+$, CD33$^+$, CD11b$^+$	BDCA1$^+$ (CD1c), CD11c$^+$, CD11b^{+v}	CD123$^+$,CD303$^+$, CD304$^+$, AXL$^+$, SIGLEC$^+$	CD14$^+$, CD11c$^+$, DR$^+$, CD1a$^+$, CD1c$^+$, CD11b$^+$	Langerin$^+$ (CD207), CD1a$^+$, E-cadherin$^+$(CD324)
Cytokines profile					
Production of type I and III Interferons, IL-6, IL-12p40, TNF	Production of IL-12 and type III Interferons, TNF-α, CXCL9 CXCL10	TNF-α, IL-8, IL-10, IL-23, IL-12, IL-1	IL-12p40	Production of TNF, IL-1β, IL-6, IL-23	
Functions					
Treg induction Th1, Th17 induction?	Th1 or Treg induction	Th2, Th17, Thf induction	Induce proliferation of naïve CD4 T cells	Th1, Th17 induction Th2 induction?	Th2 induction Treg induction?
	Cross presentation of exogenous antigens to CD8$^+$ cells	Presentation of exogenous antigens to CD4$^+$ cells			
Innate defense against viruses	Tolerance to self/ cellular immunity to intracellular pathogens and tumor	Cellular and humoral immunity to extracellular pathogens	Contribute to homeostasis but also to inflammatory and autoimmune processes?	Innate defence against infections	Skin defence against skin infections

Figure 1.9 – Human dendritic cell subset characterization (adapted from Vu Manh et al., 2011 [78], Segura, 2022 [88], Soltani et al., 2021 [89] and See et al., 2017 [90]).

Relative to cDC2, they are a major population of DC subsets which are present in different human tissues and organs. Expressing an array of TLRs, cDC2 can respond to a variety of danger signals ranging from nucleotides to polysaccharides. Compared to other steady-state DC subsets, they express higher levels of NOD-like receptors (NLRPs) and inflammatory signalling molecules, making them functionally specialized in detecting danger signals [91]. Human blood cDC2 generally secrete higher levels of inflammatory cytokines such IL-12p70, IL-1β, IL-6, and IL-23 in response to TLR agonists and can induce Th1 and Th17 polarization [92].

Considering pDC, they acquire a dendritic morphology, up-regulate human leukocyte antigen (HLA)-DR and costimulatory molecules, and differentiate into functional APC capable of activating naïve CD4$^+$ T cells after stimulation [83]. The most notable feature of pDC is its ability to produce large amounts of type I IFN in response to a viral infection (Figure 1.9). In addition, pDC selectively expresses endosomal TLR7 and TLR9 that detect nucleic acids derived from viruses, bacteria, and dead cells. This TLR binding

triggers a downstream signalling cascade, resulting in the secretion of IFN-α, IFN-β, and IFN-λ [83, 93].

Additionally, Langerhans cells, originally identified by Paul Langerhans in the 19th century, were later classified as a subset of the DC lineage. They are generally restricted to epidermis and closely associated with keratinocytes [74, 94]. Their main functions are tolerance and immune regulation, epithelial homeostasis, induction of Th2 and cytotoxic lymphocyte responses (Figure 1.9) [89].

Furthermore, between $CD34^+$ progenitors and mature DC, there is a population of DC-restricted precursors, known as pre-DC, that do not yet express the full phenotype of mature DC but are distinct from $CD34^+$ progenitors. Several research groups have attempted to characterize the maturation path of these cells; however, it is still not completely clear how these cells transit or relate to mDC and/or pDC [93]. According to See *et al.* [90] pre-DC subset shares surface markers with pDC but has distinct functional properties (Figure 1.9). They define this population as $AXL^+SIGLEC6^+$ cells and describe them as "early pre-DC" with the ability to develop into cDC1 and cDC2. Furthermore, this group observed that the ratio of cDC1:cDC2 production by their pre-DC is proportional to that of mature cells in the blood. However, even though they have been identified in blood and lymphoid organs, whether $AXL^+SIGLEC6^+$ DC represents a genuine DC subset or an intermediate population remains unclear [88].

There is also a subset referred to as monocyte-derived DCs (moDCs) or inflammatory DCs (infDC) in the landscape of human DCs [89]. moDC play a key role in inflammation and infection [95] and share numerous phenotypic markers and functions with monocyte-derived macrophages (Figure 1.9). It becomes challenging to distinguish these two kinds of cell due to the rarity and lack of definitive markers [88, 95]. However, a key feature of moDC is their dendritic morphology, (similar to cDC), whereas macrophages show a large cytoplasm containing numerous phagocytic vacuoles. In addition, moDCs have a superior ability to activate naïve T-cells compared to macrophages [88]. Inflammatory moDC also play a complementary role to cDC, synergizing with them in response to inflammation or infection [95].

Histological studies have been carried out on infiltrated DC in cardiac samples from autopsied HF patients and whole blood DC counts were performed to investigate the role of DC cells in the pathophysiology of HF. Specifically, a destructive effect on myocytes by cardiac DC was suggested in patients with myocarditis [96], and a relation of mature activated $CD11c^+$ $CD11b^+$ DC with the deterioration of left ventricle remodelling was demonstrated in experimental infarcted hearts [97]. Studies on circulating DC have also

revealed a decrease in circulating DC after MI, with an increase in the number of DC in the infarcted myocardium, suggesting a migration of DC from the peripheral blood to the heart [98-100]. In contrast, Pistulli *et al.* found a reduction in the myocardial of all DC subtypes (mDCs, pDCs, mature DCs, and immature DCs). A decrease in maturation markers in endomyocardial biopsies from patients with DCM was also found, along with an inverse correlation of DC with tissue fibrosis [101]. These controversial findings suggest that DC can play both inflammatory and immunoprotective roles in HF.

1.4.3. B lymphocytes

B cells play an important role in the development of adaptive immunity and cell–cell interactions [102]. Their maturation process begins in the bone marrow after exposure of haematopoietic stem cells to specific cytokines, such as CXCL12 and IL-7, and differentiate into common lymphoid cell progenitors (CLPs). Upon expression of the transcription factors, E2A, and early B-cell factor (EBF), CLPs further develop into early pro-B cells. The following maturation phase consists of the formation of late pro-B cells in which the reordering of the immunoglobulin (Ig) heavy chains is developed. This is followed by a reordering of the Ig light chains in the pre-B maturation phase. The consequence of these processes is the generation of immature B cells expressing IgM [103].

Afterward, immature B cells are released into the bloodstream and migrate to the secondary lymphoid tissue and/or spleen. There, they undergo a series of differentiations to reach the transitional (T1 and T2) stages, which are also known as transitional B cells [102, 103]. After that, they finally mature into naive B cells, expressing both IgD and IgM.

The activation and subsequent maturation of B cells continue upon contact and stimulation by a non-self-antigen, triggering a specific immune response in secondary lymphoid organs with the production of antibody-producing plasma cells. This differentiation requires a first signal derived from antigen-coupled B cell receptor (BCR), and a second signal, which may be T cell-dependent through T follicular helper cells (Tfh), or independent of T cells through LPS and glycolipids. T cell-dependent differentiation allows B cells to become rapidly short-lived plasma cells or enter the germinal centers (GC) to differentiate into pre-effector B cells (plasmablasts) or memory B cells with a great affinity for antigens, while T cell-independent differentiation gives rise to short-lived plasma cells that produce low-affinity antibodies. During this specific antigen-dependent maturation, B cell receptor edition, Ig class switching (with the

change of the Ig constant region from one isotype to another), and somatic hypermutation (in the variable regions of the BCR genes) may occur [103]. Along this process, B cells undergo positive and negative selection, in which cells with functional BCR and no self-antigen recognition survive. GC-derived plasmablasts return to the bone marrow and secrete antigen-specific antibodies to become long-lived plasma cells, providing long-term protection against specific antigens [103].

Several studies using mouse models demonstrate that ~10% of B cells are present in healthy hearts [104-106], and that they are involved in myocardial immune cell trafficking modulation, as well as left ventricular structure and function [106]. The presence of B cells in the intravascular space and the close contact with the endothelium indicate the important roles of these cells in HF [107]. Interaction with T cells, specifically Th cells, stimulates the production of circulating cytokines, which can affect contractility and adverse remodelling. Additionally, activated B cells induce direct cardiac injury through the production of cardiac antibodies that bind to target cells, and via apoptotic signalling pathways and complement-mediated cytotoxicity [102, 107]. Furthermore, after encountering the same antigen, memory B cells may form a larger and stronger secondary immune response displaying enhanced damage in the heart. In case of multiple episodes of MI, for instance, the memory B cell response can lead to a persistent inflammatory state, increasing myocardial cell death and injury [102].

Data on B cells in cardiac disease is scarce. In a study by Noutsias *et al.*, no differences were found in B cells between samples from patients with DCM and healthy hearts [108].

Similarly, there are only a few recent studies that investigate the frequency of peripheral B cells in heart disease, and they are mainly performed in some B cell-specific subtypes. Tang *et al.* found that the percentage of B1 cell subtype in the peripheral blood of patients with idiopathic DCM is abnormally decreased, while the percentage of transitional B cells is significantly increased [109]. Jiao *et al.* also found a decrease in regulatory B cells in patients with DCM compared to healthy individuals. In addition, those regulatory B cells also exhibited an impaired IL-10 expression and a decreased ability to suppress the TNF-α production, being considered defective cells [110].

These recent lines of investigation have demonstrated the involvement of B cells in heart disease; however, further work is needed to expand these initial observations and to delve deeper into the role of B cells in these disorders and HF.

1.4.4. T lymphocytes

T lymphocytes are cells of adaptive immunity with an important role in the maintenance of immunological memory and self-tolerance [111].

During T development, progenitor cells migrate from the bone marrow into the bloodstream and subsequently enter the thymus [112]. There, thymocyte maturation is undertaken through a sequential and well-defined stages that can be followed by gene expression and by the presence of cell surface receptors [113].

In the earliest developing thymocytes, the T-cell receptor (TCR) genes are not rearranged, and co-receptor expression is absent. They are termed double negative (DN) cells since they do not express either CD4 or CD8 co-receptor. From DN thymocytes, two lineages of T cells are derived: γδ T cells, which do not express CD4 or CD8 even when mature, and αβ T cells (Figure 1.10).

During the development of αβ T cells, CD4 and CD8 molecules are expressed simultaneously by the same thymocytes, known as double-positive (DP) cells. Thereafter, immature DP thymocytes go through a T cell selection pathway in the thymic cortex and become specific mature $CD4^+$ or $CD8^+$ T cells (Figure 1.10). This selection can be explained by three different models: the first model posits that T cell maturation results from positive selection based on recognition of MHC class I or class II by the TCR; the second proposes a random extinction of the CD4 or CD8 co-receptor in DP cells, and an exclusive survival of cells with appropriate combinations between MHC-I-$CD8^+$ or MHC-II-$CD4^+$; and at last, the third model suggests that the $CD4^+$ or $CD8^+$ lineage commitment is determined by the intensity and duration of the interaction between the TCR and MHC [114]. In addition, the two transcription factors, ThPOK and Runx3, are of high importance during the transition of DP thymocytes to mature single-positive (SP) T cells [112].

Positive and negative selection events including the recognition of self-HLA by DP thymocytes, through interactions with thymic epithelial cells, and the removal of strongly self-reactive clones, through interactions with thymic DC, are also crucial during the development of T cells [111, 114, 115].

Maturation in the thymus ends when DP cells give rise to SP $CD4^+$ or $CD8^+$ thymocytes and finally emerge in the periphery as naïve T cells exhibiting CD45RA+ CCR7+ phenotypes [111, 114-116].

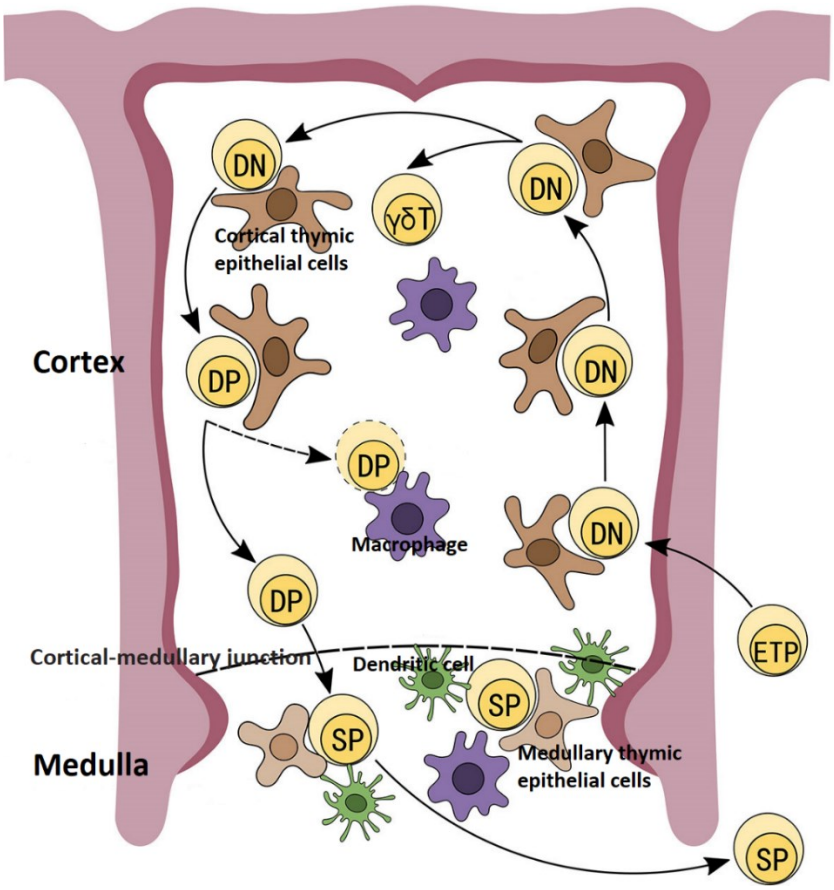


Figure 1.10 – Schematic representation of T cell development in the thymus. Early thymic progenitors (ETP), arriving from the bone marrow, seed the thymus and receive Notch signals from thymic epithelial cells to differentiate into $CD4^{-}CD8^{-}$ double negative (DN) T-lineage cells. DN cells that have undergone successful V(D)J rearrangement at TCRβ gene loci differentiate into $CD4^{+}CD8^{+}$ double positive (DP) cells. After completing TCRα rearrangements and successfully undergoing positive selection, DPs migrate to the thymus medullary region and are subjected to negative selections while DPs that fail positive selection will be programmed for apoptosis. Cells that successfully pass these checkpoints will exit the thymus as CD4 or CD8 single-positive (SP) T cells (adapted from Wang et al., 2022 [117]).

Specific antigen presentation by APC cells in lymphoid organs is mandatory for T naïve cell activation and clonal expansion [75]. It primarily requires the presentation of antigen by the APC's MHC to the TCR and secondarily a signal provided by the interaction of co-stimulatory molecules such as CD80 and CD86 from the APC with CD28 expressed by T cells. The cytokine milieu created by the T cell itself (with the important production of IL-2) and by DC constitute the third instruction necessary for T cell activation [115, 118]. These multiple interactions induce new gene transcription, particularly those involved in cellular survival, proliferation, and differentiation. Afterward, there is a clonal expansion of pathogen-specific T cells that differentiate into effector T cells, mediating the elimination of the pathogen and consequent infection. After pathogen clearance,

most of the effector T cells die by apoptosis; however, a fraction of these primed T cells persist for a long time as memory cells, protecting against subsequent infection [111].

The immune response mediated by T cells originates from two main categories: Th cells and cytotoxic T (Tc) cells. In general, auxiliary functionality is considered for $CD4^+$ T cells, and cytotoxic functionality for $CD8^+$ T cells. $CD4^+$ Th cells produce a robust quantity of cytokines and chemokines that activate other cells to perform specific functions (cytokines) or recruit (chemokines) new subsets of immune cells to pathogen encounter sites. $CD8^+$ Tc cells also produce several cytokines; however, their main function is focused on eliminating host cells infected by pathogens through cytotoxic means. For instance, in the delivery of cytotoxic granules into the cytosol of the infected cell (through TCR binding to peptide/MHC on target cell) [119].

In cardiac disease, Th and Tc cells play a central role in its pathogenesis: either by direct cytotoxicity, by enhancing the inflammatory functions of other cells, or helping B cells to produce pathogenic antibodies [120]. Furthermore, increased mRNA levels of several inflammatory cytokines have been described, as well as an increase in the surface expression of activation markers on T lymphocytes in patients with HF [121]. Also, investigations using animal models demonstrate the involvement of T cells in cardiac inflammation, hypertrophy, fibrosis, and dysfunction. Nonetheless, the participation of T cells in chronic cardiac remodelling has been poorly addressed in the literature [122, 123].

1.4.4.1. $CD4^+$ T cells

$CD4^+$ Th cells are professional effector cytokine-producing cells, playing critical roles in adaptive immune responses. According to their cytokines profile production, Th cells differentiate into different subtypes. Initially, Th cells were thought to be limited to two major subsets, type 1 (Th1) and type 2 (Th2), based on their production of IFN-γ and IL-4, respectively. More recently, a third remarkable population of IL-17-producing $CD4^+$ effector T cells was reported, denominated Th17. Differentiation into Th1, Th2, and Th17 cells comes from the activation of naïve $CD4^+$ T cells through TCR-mediated signalling (Figure 1.11). Interestingly, naïve $CD4^+$ T cells can also differentiate into Tregs, which are essential for immune tolerance maintenance and regulation of immune response magnitude (Figure 1.11) [124].

Other additional subsets of “unconventional” Th cells have been described: Th9 cells designated as IL-9 producers, distinct from Th2 cells producing IL-9; Th22 cells, a subset with exclusive expression of IL-22, distinguishing it from IL-22-producing Th17 cells; and finally, Tfh cells [124, 125]. All T cell subsets maintain a balance stage [125].

1.4.4.1.1. Th1 cells

The differentiation of Th1 effector cells is promoted by the cytokines IL-12 and IFN-γ. Following cytokine stimulation, signalling transducer and activator of transcription (STAT) family proteins like STAT1 and STAT4 are activated, inducing the expression of the major transcription factor T-box expressed in T cells (T-bet). Positive feedback regulation by IFN-γ secreted from these $CD4^+$ Th1 cells supports further Th1 differentiation with the increasing secretion of IFN-γ, IL-2, and TNF-α. At the same time, the transcription factors such as GATA3 and retinoic acid–related orphan receptor γt (RORγt) are inhibited suppressing Th2/Th17 differentiation, respectively [124, 126, 127]. IFN-γ, IL-2, and TNF-α production by these type 1 T cells activates macrophages, making those cells responsible for cell-mediated immunity and phagocyte-dependent protective responses [128].

Nowadays, it is commonly accepted that the Th1/Th2 imbalance, with a shift towards Th1 [125], plays a pathogenic role in the development of CHF [125, 129, 130]. In addition, a positive correlation between inflammatory cytokines potentially produced by Th cells and LV dysfunction has been described in patients with chronic ischaemic or idiopathic DCM [131]. Nonetheless, it remains unclear if it occurs systemically or as a result of Th-cell infiltration in the heart. In the context of infection, heart transplantation, myocardial infarction, and disruption of self-tolerance to antigens in autoimmunity, Th cells can infiltrate the heart, negatively affecting cardiac function. However, the role of T cells in the development of LV hypertrophy, remodelling, and dysfunction in HF, has only recently begun to be investigated [132].

In this context, some recent studies have shown that $CD4^+$ T cells promote cardiac remodelling in mice with HF [132], and demonstrate the important role of IFN-γ–producing Th1 cells in cardiac fibrosis orchestration [123].

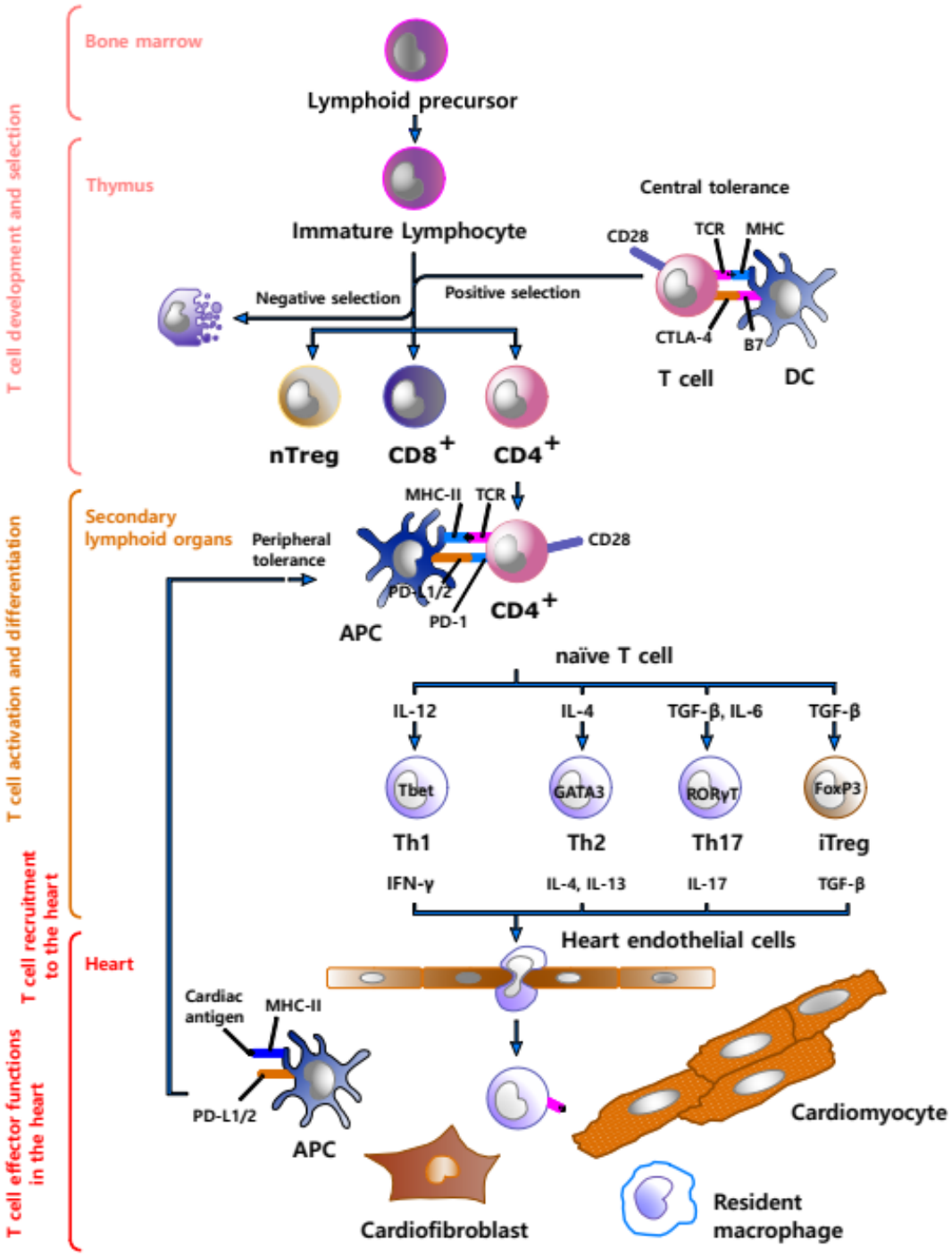


Figure 1.11 – Development, activation, and trafficking of T cells to the heart. Lymphoid precursors originate in the bone marrow and migrate to the thymus, where they develop into $CD4^+$ or $CD8^+$ T cells after a complex selection process to discard autoreactive and dysfunctional immature T cells. This crucial step in "learning" antigen recognition is the basis of the concept of central tolerance, regulated by signals involving the major histocompatibility complex (MHC) and B7 (CD80 and CD86) on the antigen-presenting cell (APC), receptor of T cells (TCR), CD28 and CTLA-4 on the T cell. Positively selected T cell clones' traffic to secondary

lymphoid organs as naïve T cells. If cardiac inflammation is triggered, dendritic cells (DCs) process cardiac antigens and traffic to secondary lymphoid organs for antigen presentation to naïve T cells that differentiate into effector cell subsets upon antigen recognition. PD-1 and CD28 signals from T cells and DC PD-L1/2 and B7 (CD80 and CD86) are an additional mechanism of tolerance in the periphery to antigens that have escaped negative selection in the thymus. Effector T cells acquire a migratory profile during differentiation and will be recruited to the heart in response to signals in the local environment. INF-γ (interferon-γ); TGF-β (transforming growth factor-β); Treg (regulatory T cell); Th (T helper cell) (adapted from Blanton et al., 2019 [133]).

1.4.4.1.2. Th2 cells

The Th2 cell subset plays a critical role in protecting the host against infections by helminth parasites and stimulating the repair of damaged tissues. Nonetheless, the production of cytokines by these cells also underlies the inappropriate immune responses that characterize allergy and allergic asthma. Cytokines produced by Th2 cells include IL-4, IL-5, IL-9, IL10 and IL-13, which drive B cell proliferation and Ig class switching to IgE, eosinophilia and mastocytosis, goblet cell hyperplasia, macrophage polarization to an iMo-like phenotype, and smooth muscle contraction in type 2 immune responses [134, 135].

It is believed that the differentiation of Th2 cells requires the secretion of IL-4 by various cell types including basophils, eosinophils, mast cells, NK cells, or even by previously differentiated Th2 cells. Naïve T cells express the IL-4R, and the combination of TCR along with the costimulatory molecules (CD28 and ICOS), and IL-4R/STAT6 signalling, induce IL-4 transcription and the production of the transcription factors c-Maf and GATA3. While c-Maf helps establish Th2 polarity by promoting IL-4 production and suppressing IFN-γ production, GATA3 promotes the production of IL-5 and IL-13 in addition to the IFN-γ inhibition, becoming critical in the establishment of Th2 cells [135].

Th2 effector cells eventually migrate to inflammatory tissues where they can exert their effector function when exposed to factors in their environment [136]. The production of IL-13 by Th2 cells, for example, promotes the elimination of allergens from the epithelium by inducing the accumulation of mucin in the epithelium. IL-5, in turn, recruits eosinophils to sites of inflammation and supports their survival. On the other hand, the neurotoxin derived from eosinophils in granular proteins activate and attracts DC, which increases Th2 response. Furthermore, eosinophils are sources of Th2-inducing cytokines and can therefore promote Th2 differentiation. Group 2 innate

lymphoid cells (ILC) are also involved in the type 2 inflammation by directly producing Th2 cytokines and activating Th2 cells through MHC-II and IL-13.

Nevertheless, the study of this T cell subtype in HF has been limited. Some investigations relate the imbalance between Th1 and Th2 cells and the shift toward Th1, with the immune dysfunction and the exaggerated production of pro-inflammatory cytokines in HF [129, 137, 138]. Yndestad *et al.* reported an significantly enhanced gene expression of IFN-γ by T cells in patients with CHF [139]. Also, Cheng *et al.*, describe a significantly increase of Th1-related cytokine (IL-12, IL-18, and IFN-γ) in CHF patients compared with control group. On contrary, in this study, patients presented lower serum levels of IL-10 than healthy individuals and no differences on IL-4 [129]. These results are possibly the reason why the role of Th2 cells in HF has not been fully explored.

1.4.4.1.3. Th17 cells

Th17 polarization occurs in three stages, starting with transforming growth factor (TGF)-β and IL-6 driving Th17 differentiation [126], followed by the activation of the STAT3 pathway, and finally with expression of its master transcription factor RORγt [126, 127]. RORγt directly regulates a selected set of specific Th17 genes and the expression of IL-17A and IL-17F [124, 127]. The autocrine amplification by the production of IL-21 and secretion of IL-23 from APC stabilizes the Th17 lineage [126].

TGF-β is important to Th17 differentiation; however, high concentrations can result in the activation of STAT5 signalling and upregulation of the Foxp3 transcription factor, driving Treg differentiation. In this sense, Th17 cells display plasticity during immune response and can induce immune regulatory functions [126].

Functionally, Th17 cells mainly produce IL-17A-F, IL-21, IL-22, IL-10, IL23, and CCL20, [126] playing a critical role in promoting and enhancing inflammation, including autoimmune and inflammatory tissue injury [140]. The secretion of IL-17 by Th17 cells can become pathogenic as this cytokine induces the recruitment of neutrophils, activates innate immune cells, increases the function of B cells, and induces the release of pro-inflammatory cytokines including TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6 and IL-1β. Moreover, IL-17 signalling induces the expression and/or release of chemokines and other inflammatory mediators, such as intercellular adhesion molecule 1 (ICAM-1) and prostaglandin E2 (PGE2)) and promotes tissue damage through the induction of matrix metalloproteinases (MMPs) and antimicrobial

peptides. During these events, several positive feedback loops occur, further increasing IL-17 production, and sustaining a pro-inflammatory environment [140].

Recent studies show that Th17 cells and IL-17 are linked to the pathogenesis of several cardiovascular diseases [141], including atherosclerosis [142-145], hypertension [146], viral myocarditis [147], myocardial ischaemia/reperfusion injury [148], and DCM [149]. Furthermore, the infiltration of Th17 cells in the LV increases significantly in animal models with HF [150], and IL-17, secreted essentially by Th17 cells, participates in cardiac ventricular remodelling in some heart diseases [151], such as ischaemic HF [150, 152], and DCM [153]. Nevertheless, studies focusing on circulating Th17 cells in patients with HF presented inconsistent results [130, 141].

1.4.4.1.4. Tfh cells

Tfh cells constitute a distinct subset of $CD4^+$ T cells [154]. They were initially described in the GC of secondary lymphoid tissues and have been identified by the expression of CXCR5 and by their ability to provide help in selecting high-affinity B cells [154, 155]. Tfh cells have been described in peripheral blood and comprise 15% to 25% of memory $CD4^+$ T cells in humans [156] and are composed of different and heterogeneous subpopulations with distinct phenotypes and functional properties [155, 157]. Considering the expression of CXCR3 and CCR6 chemokines receptors, circulating Tfh cells ($CD3^+CD4^+CXCR5^+$) are classified into Tfh1 ($CXCR3^+CCR6^-$), Tfh2 ($CXCR3^-CCR6^-$), and Tfh17 ($CXCR3^-CCR6^+$) cells [158]. Each subtype (Tfh1, Tfh2, Tfh17) shares the same transcription factors as Th1, Th2, and Th17 cells, respectively. In the same order, Tfh1 also produce IFN-γ, while Tfh2 secrete IL-4, IL-5, and IL-13, and Tfh17 produce IL-17A and IL-22 [155, 159].

Circulating Tfh2 and Tfh17 are efficient helper cells providing B-cell help, whereas Tfh1 are associated with poor antibody responses. Alteration in the homeostasis circulating Tfh cells subtypes is related to the pathogenesis of autoimmune [160, 161], inflammatory [159, 162], and infectious diseases [155, 163] and cancer [164].

Until the present moment, nothing is known about the role of these cells in HF, even considering HF different aetiologies, by themselves. Due to the involvement of these cells in inflammatory diseases, such as juvenile dermatomyositis [159] and inflammatory bowel disease [162], the study of Tfh cells in the inflammatory process in HF should be considered as a future direction of investigation.

1.4.4.1.5. Regulatory T cells

Representing 5-10% of $CD4^+$ T cells in whole blood, the Treg cells have a significant impact on the immune system. They regulate other leukocyte functions and avoid excessive immune activation and its detrimental effects [165]. According to their origin, Treg cells can be classified as natural Treg cells (nTreg), originating in the thymus; and inducible Treg cells (iTreg) generated in certain peripheral tissues in response to nonself antigens [140, 165]. Defined as $CD4^+CD25^+$ cells [111, 166] and characterized through the combined expression of $CD4^+CD25^+CD127^{low/-}$ [167], their signature transcription factor Foxp3 is expressed by both nTreg and iTreg and is critical for their development, lineage commitment, and regulatory functions [140]. Later, iTreg are further discriminated into $Foxp3^+$ cells and $Foxp3^-$ cells.

Mechanisms of Treg-mediated immune suppression consist of secretion of anti-inflammatory cytokines, expression of inhibitory receptors, and cytokine deprivation. TGF-β and IL-10 are the two main cytokines associated with Tregs. TGF-β is produced by B cells, macrophages, DC, and many other non-immune cells. It is imperative for the generation of iTreg by the induction of Foxp3's expression in a paracrine feedback loop, compelling differentiation of naïve T cells (Th0) into iTreg, ensuing a critical role in peripheral tolerance and generation and maintenance of Tregs. Notably, TGF-β is also secreted by Treg themselves and used to exert their suppressive function [140].

Concerning IL-10, this cytokine is expressed by cells of both innate and adaptive immune system, including $CD4^+$ and $CD8^+$ T cells, macrophages, mast cells, NK cells, eosinophils, and neutrophils. However, the main source of IL-10 is iTreg $Foxp3^-$ cells, which are crucial for the generation and maintenance of these cells through an autocrine process. The immunosuppressive effects of IL-10 by Treg cells include the negative regulation of the expression of MHC-II and costimulatory molecules (CD80/CD86 and CD28) on APCs, the reduction of pro-inflammatory cytokines release by mast cells and macrophages, as well as the suppression of its function and activation [140].

Numerous studies performed on animal HF models have reported that Tregs play a protective role in the early stage of cardiac injury [75, 166]. Tissue repair functions, through suppression of inflammatory processes and activation of fibrosis, and improvement of cardiac tissue remodelling via direct stimulation of cardiomyocyte proliferation have been described in MI models [75, 168-170]. Furthermore, artificial depletion of Tregs has been linked to unfavourable left ventricular remodelling, the development of apical aneurysms, and cardiac ruptures [170, 171]. In the same line, patients with HF present a decreased proportion of circulating Treg cells [125, 130] and

a lower amount of IL-10 [125] compared to healthy individuals. On the other hand, the proportion of Th17 cells increases, exacerbating the severity of inflammatory response and rejecting the pathway and production of Treg cells [125]. These Treg/Th17 and IL-10/IL-17 imbalances have been reported in ischaemic and non-ischaemic patients [125] with normal and reduced EF [130], suggesting an important participation of these cells in modulating inflammation in CHF.

1.4.4.2. $CD8^+$ T cells

T $CD8^+$ cells, or Tc cells, constitute about 6% of the white blood cells found in the bloodstream. They become activated when their TCR binds to the MHC-I of the APC and the co-stimulatory molecules connect in the presence of inflammatory cytokines. As effector cells, Tc cells can eliminate other cells that have the same MHC-I/peptide complex, and release type I cytokines such as IFN-γ and TNF-α to support the cell-mediated immune response. These abilities make Tc cells crucial in managing viral and other intracellular infections [172].

In fact, within T $CD8^+$ cells, the Tc1 and Tc2 subsets exist, characterized by the same hallmark cytokines as Th1 and Th2 (INF-γ and IL-4, respectively). As Th17 cells, the subpopulation of $CD8^+$ T cells producing IL-17 was also recently discovered, denominated Tc17 cells [173-175]. The T-box transcription factors, Eomesodermin (Eomes) and T-bet are important in the development of effector and memory Tc1 cells, and regulate the expression of IFN-γ, granzyme B and, perforin. Tc17 cells are generated in a similar way to Th17 cells, requiring the expression of STAT3 and RORγt, in the absence of Eomes and T-bet [175, 176].

Effector Tc1 cells have the ability to destroy their targets, releasing cytotoxic molecules, such as IFN-γ, perforin, and granzymes. Tc17 cells do not present the same toxicity as Tc1, but they contribute to the generation of Th17 cells and are producers of cytokines such as IL-21 and IL-22. While the production of IL-21 by Tc17 cells (in synergy with TGF-β) may promote Th17 cells development in a positive autocrine loop, the production of IL-17 and IL-22 together are considered as highly aggressive and pro-inflammatory [175].

The role of $CD8^+$ T cells in heart disease has received less attention than $CD4^+$ T cells. Most of the published works focus only on type I Tc cells and were performed exclusively in patients and animal models with HF post-MI. They report abundant and activated Tc1

cells in ischaemic failing hearts [177-179], which secreted large amounts of IFN-γ, granzyme B, and, perforin [177, 178]. Granzyme B, in collaboration with perforin, has been considered an important mediator of cellular apoptosis [177-179], and has been directly associated with cardiac fibrosis [177]. Depletion of Tc cells decreased apoptosis of cardiomyocytes, hampered inflammatory response, limited myocardial injury, and improved heart function [178]. Furthermore, elevated circulating levels of granzyme B in patients with acute MI were found to predict an increased risk of death at 1-year follow-up [178].

The effects of $CD8^+$ T cells on cardiac remodelling post-MI have been proven to be mediated by a cell-specific mechanism and appear to be both beneficial and detrimental [180].

Considering Tc17 cells, there is a single study investigating 80 young Indian patients with ST-segment elevation myocardial infarction. Patients with MI presented a higher proportion of Th17 and Tc17 and elevated plasma levels of IL-6 and IL-17, with a significant reduction in circulating Tregs, compared to healthy control.

In summary, Tc1 and Tc17 cells are involved in pathological T-cell immune response in ischaemic failing hearts which may contribute to the progression of HF. $CD8^+$ T cells are associated with the pro-inflammatory/anti-inflammatory imbalance observed in MI and appear to play an important role in cardiac remodelling. However, further studies are needed to investigate the role of these cells in HF resulting from different causes and pathophysiology.

1.4.4.3. $CD4^+CD8^+$ T cells

DP T cells were primarily considered as a developmental stage in the thymus, before maturation as $CD4^+$ or $CD8^+$ T cells [181]. However, the existence of $CD4^+CD8^+$ cells in peripheral blood defines DP cells as a specific mature T cell subpopulation. The origin of these cells is suspected to be thymic [114, 181]; however it has also been suggested that $CD4^+CD8^+$ T cells may arise from SP CD4 or CD8 T cells. During a productive immune response, SP cells can acquire the other co-receptor in response to the stimulus, becoming CD4/CD8 DP cells [114].

Peripheral blood $CD4^+CD8^+$ T cells constitute a small heterogeneous population [114], representing 1–2% of circulating human T lymphocytes [182]. The role of this T cell

subpopulation remains controversial, with conflicting investigations describing either cytotoxic or suppressive functions.

The frequency of $CD4^+CD8^+$ cells has been found to increase in patients with viral (HIV and hepatitis) [183-185], tumour (melanoma, breast cancer) [186, 187] and autoimmune (rheumatoid arthritis) [188] and parasitic (Chagas disease) [189] diseases [182]. It has also been demonstrated that $CD4^+CD8^+$ T cells are highly activated cells, producing pro-inflammatory cytokines, inducing cytotoxicity, and capable of exhibiting a memory phenotype [112, 182]. On the other hand, findings in animal models and studies in some human cancers have also attributed immunosuppressive and regulatory properties to $CD4^+CD8^+$ T lymphocytes [112].

Taken together, there does not appear to be a consistent trend that can explain CD4/CD8 DP T cell function. It seems likely that CD4/CD8 DP T cells are heavily influenced by as-yet-unidentified mechanisms and/or local environmental conditions [112]. Due to their pleiotropic functions, DP cells need to be investigated in the context of each specific disease. In the case of HF and its aetiological conditions, no previous studies have been carried out in humans or animal models on $CD4^+CD8^+$ T cells.

1.4.4.4. γδ T cells

The expression of αβ or γδ TCR defines two different T cell subsets: αβ and γδ T cells. Despite sharing many effector capabilities with αβ T cells, such as cytokine production and cytotoxicity, γδ T cells exhibit different biological properties. They can produce TRAIL, Fas/Fas-L, granzyme B, and perforin and cytokines such as IFN-γ, TNF-α, and IL-17 as αβ T cells, but they differ in thymus-dependent or independent development, MHC restriction, and recognition of soluble proteins and non-protein antigens of endogenous origin [190-192]. In fact, γδ T cells constitute part of the “unconventional” T-cell subset and function in unique roles, such as stress surveillance [190].

Deriving from CD4/CD8 DN thymocytes, γδ T cells functionally develop different stages along with varying TCR pairs [190, 193]. In humans, they comprehend a minor subpopulation of T lymphocytes in the peripheral blood, comprising only 1–5% of circulating lymphocytes. Nonetheless, they are abundant at barrier sites such as the skin, gut, lung, and reproductive tract [192, 194].

Immunologically, they are considered innate immune cells that function in an MHC-unrestricted manner [190, 195, 196], recognizing surface proteins such as butyrophilin and CD1 molecules [196]. Nonetheless, they are also important cells in adaptive immune response. The interaction with B cells through multiple co-stimulatory pathways, including CD40/CD40 ligand, inducible T cell co-stimulator (ICOS/ICOS ligand) or CD86/CD28, and their cytokine production, can drive the differentiation of B cells into antibody-secreting plasma cells [195].

Few studies have focused the role of γδ T cells in heart diseases. Li *et al.* demonstrated that the frequencies and absolute numbers of total γδ T cells were significantly decreased in CAD patients as well as the levels of immunosuppressive molecules (PD-1 and CTLA-4) when compared to healthy individuals [191]. Another study in acute MI patients which evaluated the gene expression levels of Foxp3, IL-17A and TCR Vγ subfamilies reported that γδ T cells play a key role in the pathological progress of acute MI and may be associated with the IL-17A-mediated pathway [197]. Moreover, a case study of a patient with DCM, presented by Takeda *et al.*, describes a moderate infiltration of mononuclear cells in an endomyocardial biopsy specimen. Interestingly, those mononuclear cells were mainly lymphocytes expressing γδ TCR and a cytolytic factor perforin. In this specific case, the confirmed diagnosis was DCM mediated by γδ T-cells [198]. Lastly, a study conducted on virally infected myocyte cultures (isolated from mice) revealed that T cells expressing the γδ TCR are more efficient than αβ T cells in triggering myocyte apoptosis. This suggests that these effectors may be primarily responsible for myocardial injury associated with DCM-like signs during coxsackievirus B3-induced myocarditis [199].

In summary, it appears that γδ T cells are associated with worse outcomes in cardiac disease; however, complementary studies are necessary to understand their role in HF.

1.4.4.5. Other innate lymphoid cells

ILC are a group of innate lymphocytes that play important functions in immune defence against viruses, tumours, intra- and extracellular bacteria, and parasites, and in regulation of adaptive immunity, tissue remodelling, and repair and homeostasis of haematopoietic and non-haematopoietic cell types [200-203].

The ILC family encompasses the classic cytotoxic NK cells (previously discovered in 1975 and which perform an important function in the protection against viruses and tumours); the lymphoid tissue inducer (LTi) cells (discovered in 1997, which promote

the formation of lymph nodes during embryogenesis); and the most recently described: non-cytotoxic ILC populations [200].

All cells of the ILC family presents a classic lymphoid morphology; however there is a lack of expression of cell-surface molecules that usually identify other immune cell types. Thus, they are defined as cell lineage marker negative (Lin^-) cells [200, 201, 203]. ILCs express some IL receptors such as IL-2Rα (also called CD25) and IL-7Rα (also known as CD127), but unlike adaptive T and B cells, ILCs do not express TCR and BCR and therefore do not exhibit any degree of antigen specificity [200, 202]. Notwithstanding, just like the well-known pattern of separation between $CD4^+$ and $CD8^+$ T cells, ILCs have been divided into helper-like cells (ILC1, ILC2, and ILC3) and cytotoxic-like conventional NK (cNK) cells, based on their typical cytokine production and respective transcription factors which dictate their development. Finally, LTi cells form a fifth subset of ILC, which does not appear to have an adaptive counterpart [201, 202].

ILC1 development is dependent on the transcription factor T-bet, and like Th1 cells, they release type I cytokines, such as IFN-γ and TNF, in response to viruses and intracellular bacteria. On the other hand, ILC2 depend on GATA3 and promote a Th2-like immune response by producing IL-5 and IL-13 in response to allergens and parasites. Similar to Th17 cells, ILC3 require the transcription factor RORγT and release IL-17 and IL-22 in response to extracellular bacteria. LTi cells also depend on RORγT, but, unlike ILC3, they regulate the development of secondary lymphoid organs [200, 201]. Regarding the cytotoxic-like cNK cell, their development requires the transcription factors Eomes and T-bet, and are capable of destroying tumour or virally infected cells and inducing inflammatory responses through the production of IFN-γ [201]. Interestingly, NK cells and conventional ILCs belong to distinct developmental lineages. This finding is emphasized by the unique cytotoxic capabilities of NK cells. On the other hand, ILCs share the same committed parent [203].

Several clinical studies have reported lower values of NK cells in patients with CAD and ischaemic heart disease [204-206]. Furthermore, these circulating NK cells presented a lower capacity for cytotoxic and IFN-γ production [205]. Notably, at 12-month follow-up, patients in whom circulating NK cells were restored showed little or no cardiac inflammation. On the contrary, continued NK cell deficiency was correlated with cardiac inflammation [206, 207].

In myocarditis and DCM, the scenario is similar. The levels of NK cells and its own cytotoxicity are lower, pointing to defects in both frequency and function [208].

NK cells also appear to play a protective role against the development of cardiac fibrosis, as they directly limit collagen formation in cardiac fibroblasts and prevent the accumulation of certain inflammatory populations in the heart. IFN-γ, produced by NK cells, induces an anti-inflammatory chemokine milieu in the heart, protecting it against fibrosis through downregulation of pro-fibrotic cell types such as eosinophils [203, 209]. Furthermore, studies using mouse models showed that NK cells prevent right ventricular hypertrophy and right ventricular systolic pressure growth [210, 211]. Altogether, these considerations suggest that NK cells display beneficial effects on cardiac remodelling and HF, acting as modulators on the inflammatory response.

Regarding ILC cells, little is known about the role of these cells in HF. Li *et al.* [212] studied the levels of these circulating cells after MI for several consecutive days. During this period, they detected an increase in ILC1 compared to controls, while the proportion of ILC2 decreased significantly. In addition, during the 23-month follow-up they found that ILC1s were independent predictors of major adverse cardiovascular events. Furthermore, IFN-γ, TNF-α and vascular cell adhesion molecule 1 (VCAM1) and matrix metallopeptidase 9 also increased.

The study performed by Yu *et al.* [213] in experimental MI in mice reported that ILC2s could benefit cardiac healing and recovery of ventricular function after MI. Chen *et al.* [214] also reported that cardiac ILC2 confer protection from cardiac fibrosis and improve myocardial function.

In conclusion, ILCs appear to have important roles in MI. However more research is needed to further explore the mechanisms of ILC in HF.

1.5. Impact of cardiac resynchronization therapy on heart failure

Cardiac resynchronization therapy (CRT) is an established treatment for patients with severe HF [215, 216], belonging to NYHA classes II to IV and who present reduced LVEF (≤ 35%), prolonged QRS (> 150 ms) and ventricular mechanical dyssynchrony manifesting as LBBB, despite the optimal medical therapy [216]. CRT-induced biventricular pacing can help restore left ventricular (LV) systolic function, by correcting electromechanical dyssynchrony. There is strong evidence that CRT improves exercise capacity, and reduces rehospitalization due to HF and mortality [215, 216].

The main mechanism of action of CRT involves the reversal of the abnormal pattern of ventricular activation observed in patients with left LBBB conduction delay. Biventricular pacing generates two ventricular activation wavefronts, which lead to a more normal pattern of ventricular activation. As a result, left ventricular contraction becomes more efficient, and stroke volume improves without increasing myocardial oxygen consumption [217, 218].

Furthermore, studies have shown that biventricular pacing stimulation improves acute haemodynamic variables, enhances systolic efficiency, and optimizes diastolic function. These improvements occur without increasing heart rate or oxygen consumption [217]. In addition, an investigation performed by Kass *et al.*, has also revealed that biventricular pacing results in a reversal of biochemical abnormalities, including a reduction in cellular apoptosis and stress kinase activation, demonstrating the involvement of several cellular response mechanisms [219].

There are three categories to consider when defining the response to CRT, and the response rate can vary according to these categories [218, 220, 221]. One of these categories is based on clinical measurements, such as patient symptoms and functional assessment. The improvement of NYHA functional class and quality of life, as well as the six-minute walk test, exercise duration, and metabolic exercise tests, are some typical clinical measures used for this purpose [218]. The second category is based on LV reverse remodelling assessment [218]. This response, designated echocardiographic definition, is described as a reduction in the LVESV equal to or greater than 15% or an improvement in the LVEF equal to or greater than 5% [220]. The final category involves the assessment of outcome measures, which include reductions in hospitalization for HF and mortality from all causes. These measures are crucial in determining the effectiveness of CRT treatment, and they play a significant role in the management of HF [218]. In fact, the class I indication for CRT in HF is based not on the improvement of symptoms or exercise capacity, but on its effect on mortality or morbidity. The final response to CRT should therefore be a decrease in mortality and morbidity, that is, fewer HF events. All other clinical, echocardiographic, or laboratory improvements are only surrogate markers of actual response [221]. Thus, large clinical trials use event-driven primary endpoints to define CRT response. Secondary endpoints generally assess cardiac function and functional status. However, it has been challenging to incorporate the results of these trials into daily practice. For instance, it would be difficult to use mortality as an outcome measure to evaluate CRT response in an individual patient due to the lack of before-and-after comparison [218]. On the other hand, a disadvantage of clinical criteria is the

subjective nature of their measurement [221]. Here, echocardiographic evaluation ends up gaining an advantage in its use in defining response to CRT.

CRT is a valuable additional tool to the pharmacological treatment of patients with severe HF; however, even if adequately selected for CRT based on criteria recommended by international guidelines, a considerable portion of patients (~30%) do not respond to it [216, 222].

Inflammation and dysregulation of the immune response are important factors in the progression of HF, regardless of the initial form of cardiac injury [216]. Interactions between different immune cell subsets and inflammatory cytokines after myocardial injury promote cardiac repair, eccentric cardiac remodelling, and ventricular dysfunction. In addition to local pro-inflammatory immune activation, patients with HF also present increased levels of systemic inflammatory mediators with activation of peripheral pro-inflammatory immune cells [216, 223]. In fact, the injured myocardium sends signals to other organs, triggering the proliferation of leukocytes and increasing their number in the peripheral circulation [224-226]. Those circulating immune cells are therefore messengers linking systemic inflammation and local inflammation and reflecting local pathophysiological changes [226].

CRT has been associated with favourable changes in circulating levels of neurohormones and inflammatory cytokines in HF patients, but the real impact of CRT on the progression of the systemic inflammatory status associated with CHF has not been sufficiently investigated [215, 227, 228]. At the same time, the effective role of immune cells in reverse cardiac remodelling after CRT is still poorly understood.

Several small and medium-sized trials have described a marked reduction in inflammatory mediators after CRT, such as C-reactive protein (CRP) [229-231], IL-6 [215, 229, 232-234], TNF [232, 234, 235], IL-8 and MCP-1 [215, 233], and in some of these studies, this reduction was only observed in responders to CRT [229-231]. However, there has been some research where no changes in serum markers were detected after CRT, even in the responder group [215, 235-238]. Such differences could be explained in part by limited sample size, but also by heterogeneity between patient groups, such as the aetiology of HF and differences in the technical success in left ventricular lead positioning [227].

Nonetheless, the reduction of "inflammatory load" resulting from resynchronization in HF may be related to the improvement of cardiac contraction, in which the reduction of wall tension may decrease the local production of inflammatory mediators [227].

Another possibility through which CRT reduces systemic inflammation may be related to the reduction of oedema secondary to the improvement in cardiac output and peripheral circulation. The minimization of oedema decreases the leakage of LPS from the gut to the circulation, reducing the cardio-inhibitory effects and the production of several cardiotoxic cytokines [227]. CHF is also associated with microcirculation impairment and systemic ischaemia, which may contribute to an increase in the systemic inflammatory reaction. CRT improves cardiac functional status, circulation, and oxygen consumption, can reduce systemic ischaemia, and therefore decrease the synthesis of inflammatory mediators in skeletal muscle. However, there is still limited data demonstrating that ameliorated microcirculation may contribute to an improved inflammatory profile in CRT patients [227].

Taken together, it is not completely perceived whether the reduction in immune activation post-CRT results from improved heart function or if it is an effect of the immunomodulating therapy as such. The study of circulating innate and adaptive immune cells in patients with HF will allow us to verify whether the improvement in left ventricular function, observed after CRT, is in fact associated with a reduction in the inflammatory response. On the other hand, it may bring forward novel perspectives in predicting CRT response.

1.6. References

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Chapter 2

Aims

Chapter 2

Aims

Global aims

CRT is an effective treatment for patients with severe HF and some evidence suggests that CRT may reduce inflammatory mediators in HF patients. However, the relation between the response to CRT, immune cells, and cardiac reverse remodelling in HF is far from being understood.

The main goals of this thesis were to study the possible contribution of circulating immune cells to reverse cardiac remodelling and response to CRT in HF patients; and to investigate the impact of CRT on these cells' homeostasis, by comparing their circulating levels and functional activity between baseline assessment and post-CRT.

In this context, several hypotheses were raised during this study. First, patients with HF exhibit changes in the number/frequency and functional activity of peripheral blood immune cells. Secondly, CRT either disrupts or restores the balance of cellular functions. And third, patients who respond to CRT present lower cellular inflammatory signs than non-responders.

Specific aims

The present research was divided into two phases: 1) quantification and functional characterization of immune cells in HF patients, and 2) evaluation of the impact of CRT on peripheral immune cell homeostasis.

In the first phase of this research, the specific aims were to:

1. Quantify and functionally characterize circulating monocyte and DC subsets in HF patients proposed for CRT and compare these results with those obtained in healthy subjects;

2. Identify and quantify circulating lymphocyte subpopulations particularly, $CD4^+$ T cells and $CD8^+$ T cells, in the same patients and to compare the obtained results with those acquired in the healthy control group;
3. Characterize circulating Th17 and Tc17 cells in HF patients scheduled for CRT and to compare these results with those obtained in healthy individuals; and
4. Functionally characterize circulating Th1, Tc1, and Treg cells in HF patients proposed for CRT and to compare these results with those obtained in the control group.

In the second phase of this research, the specific aims were to:

1. Study the impact of CRT on monocyte and dendritic cells subsets, by evaluating the frequency of and functional activity of cMo, iMo, ncMo, mDC, and pDC in HF patients, before and 6 months after CRT;
2. Evaluate the potential role of those peripheral innate immune cells on reverse cardiac remodelling and response to CRT by comparing responder with non-responder patients;
3. Analyse the impact of CRT on IL-17-producing T cell homeostasis, by quantifying and functionally characterizing Th17 and Tc17 cells in HF patients at baseline and at 6 months follow-up;
4. Evaluate the differences in Th17 and Tc17 cells between responders and non-responders to CRT;
5. Quantify IL-17 mRNA expression in circulating leukocytes in HF patients before and post-CRT, assessing the differences in IL-17 mRNA levels between responders and non-responders;
6. Study the influence of CRT on the frequency of Th1, Tc1, and Treg cells and their intracellular cytokine production in HF patients, by comparing baseline and post-CRT patterns; and
7. Investigate possible divergences between responders and non-responders to CRT considering Th1/Tc1 and Treg cells and their phenotypic profile.

Chapter 3

Role of monocytes and dendritic cells in cardiac reverse remodelling after cardiac resynchronization therapy

Martins S, António N, Rodrigues R, Carvalheiro T, Tomaz C, Gonçalves L, Paiva A.
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Chapter 3

3.1. Abstract

Background and Aims: Monocytes and dendritic cells (DC) are both key inflammatory cells, with recognized effects on cardiac repair. However, there are distinct subsets of monocytes with potential for beneficial or detrimental effects on heart failure (HF) pathogenesis. The connection between reverse cardiac remodelling, the potential anti-inflammatory effect of cardiac resynchronization therapy (CRT) and monocytes and DC homeostasis in HF is far from being understood. We hypothesized that monocytes and DC play an important role in cardiac reverse remodelling and CRT response. Therefore, we aimed to assess the potential role of baseline peripheral levels of blood monocytes and DC subsets and their phenotypic and functional activity for CRT response, in HF patients. As a secondary objective, we aimed to evaluate the impact of CRT on peripheral blood monocytes and DC subsets, by comparing baseline and post-CRT circulating levels and phenotypic and functional activity.

Methods: Forty-one patients with advanced HF scheduled for CRT were included in this study. The quantification and phenotypic determination of classical (cMo), intermediate (iMo) and non-classical monocytes (ncMo), as well as of myeloid (mDC) and plasmacytoid DC (pDC) were performed by flow cytometry in a FACSCanto™II (BD) flow cytometer. The functional characterization of total monocytes and mDC was performed by flow cytometry in a FACSCalibur flow cytometer, after in vitro stimulation with lipopolysaccharide from Escherichia coli plus Interferon (IFN)-γ, in the presence of Brefeldina A.

Comparisons between the control and the patient group, and between responders and non-responders to CRT were performed.

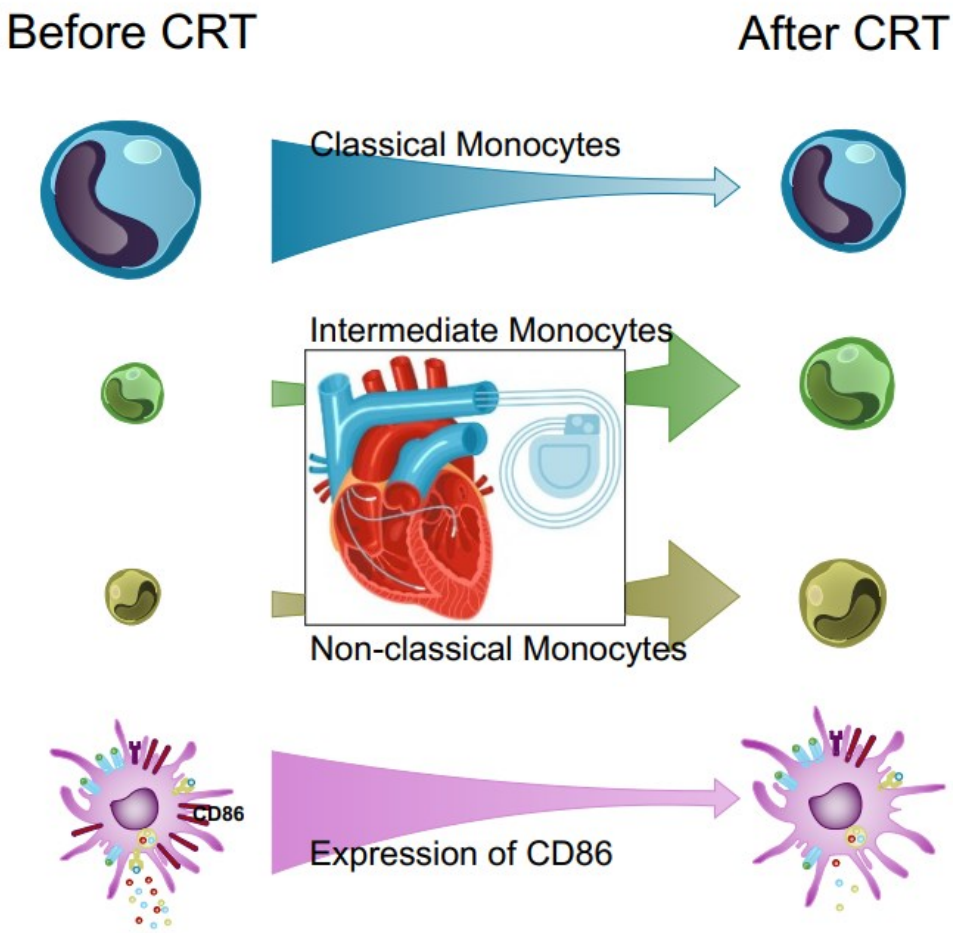
Results: Compared to the control group, the HF population presented a significantly lower frequency of pDC at baseline and a higher proportion of monocytes and mDC producing IL-6 and IL-1β, both before and 6 months after CRT (T6). There was a remarkable decrease in cMo and an increase in iMo after CRT, only in responders. The responder group also presented higher ncMo values at T6 compared to the non-responder group. Both responders and non-responders presented a decrease in the expression of CD86 in all monocyte and DC populations after CRT. Moreover, in non-responders, the increased frequency of IL-6-producing DC persisted after CRT.

Conclusion: Our study provides new knowledge about the possible contribution of pDC and monocyte subsets to cardiac reverse remodelling and response to CRT. Additionally, CRT is associated with a reduction on CD86 expression by monocytes and DC subsets and in their potential to produce pro-inflammatory cytokines, contributing, at least in part, to the well described anti-inflammatory effects of CRT in HF patients.

Keywords

Chronic heart failure, Cardiac resynchronization therapy, Immune response, Monocytes, Dendritic Cells, CD86

3.1.1. Graphical abstract



3.2. Introduction

Chronic heart failure (HF) is a complex and systemic disease [1, 2] characterized by an anomalous structure and function of the heart, resulting in a ventricular filling and/or systolic function impairment [1–4]. Immunological process and inflammation are considered important factors in pathophysiology of heart failure [1, 5, 6], portending a worse functional capacity and a poor prognosis [1, 7].

Cardiac resynchronization therapy (CRT) is a key guideline-recommended treatment for patients with drug refractory HF, reduced left ventricle ejection fraction (LVEF) and left bundle branch block [8, 9]. Several definitions of CRT response have been used in the literature. Improvement in cardiac function, mainly based on echocardiography demonstration of reverse remodelling, is one of the most widely used definitions. A reduction in left ventricular end-systolic volume (LVESV) greater than or equal to 15% is the most accepted echocardiographic CRT response criterion, given the correlation with clinical outcomes [10]. The beneficial effects of CRT in responders include reverse cardiac remodelling (reduced left ventricular volumes and increased LVEF), improvement of New York Heart Association (NYHA)-based functional status, symptoms and quality of life, reduction of brain natriuretic peptide (BNP), improvement in the six-minute walk test (6MWT), and reduction of mortality and HF hospitalization [11, 12]. In fact, CRT can improve clinical outcomes even in high-risk patients, such as those with type 2 diabetes mellitus [13], when added to the beneficial effects caused by antidiabetic therapies with pleiotropic effects on inflammation and HF [14]. Additionally, the reverse remodelling induced by CRT is related to alterations in the expression of genes and microRNAs (miRs), which regulate cardiac processes involved in cardiac apoptosis, fibrosis, hypertrophy, and angiogenesis, and membrane channelling ionic currents [12, 15].

Previous reports also describe a beneficial effect of CRT on inflammation [16–18], however the relationship between the outcome of CRT-treated patients, cardiac remodelling and immune system response, is not clearly understood. Monocytes and dendritic cells (DC) are pivotal cells in innate and adaptative immune response [19, 20]. While monocytes play a crucial role in host defence, immune regulation, inflammation and tissue repair [5, 19, 21, 22], DC orchestrate T cells response and maintain immune tolerance through different antigens presentation [23, 24]. In fact, there are three different subsets of monocytes – the classical monocytes (cMo) ($CD14^{++}/CD16^{-}$) which represent about 90% of the total, and two minor $CD16^{+}$ subsets: the intermediate monocytes (iMo) which express higher levels of CD14 with a lower

expression of CD16 ($CD14^{++}/CD16^{+}$); and non-classical monocytes (ncMo) that express lower levels of CD14 coupled with high expression of CD16 ($CD14^{+}/CD16^{++}$) [19, 25, 26], each one with distinct phenotypes and functions [25, 26]. Monocytes seem to be linked to the genesis and development of various cardiovascular events [6, 19, 27]. However, they can also be beneficial, through the production of interleukin (IL)-10, stimulation of angiogenesis and tissue repair [25]. Concerning DC, they can be divided in two major subpopulations according to their haematopoietic origin: myeloid dendritic cells (mDC) and plasmacytoid dendritic cells (pDC) [23, 24, 28]. The role of these antigen presenting cells in HF is not well elucidated [20]. Several human and animal studies in viral and autoimmune myocarditis, myocardial infarction (MI) and dilated cardiomyopathy have described DC as both key inflammatory cells and immunoprotective regulatory cells [20, 24, 28–32].

Given the role of monocytes and DC in tissue repair [33, 34], their contribution in the reverse cardiac remodelling process is conceivable. We hypothesized that monocytes and DC play an important role in cardiac reverse remodelling and CRT response. Therefore, we aimed to assess the potential role of baseline peripheral levels of blood monocytes and DC subsets and their phenotypic and functional activity for CRT response, in HF patients. As a secondary objective, we aimed to evaluate the impact of CRT on peripheral blood monocytes and DC subsets, by quantifying and functionally characterizing cMo, iMo, ncMo, mDC and pDC and comparing the baseline with post CRT results.

3.3. Methods

3.3.1. Patient population

This is a prospective study enrolling forty-one consecutive patients with advanced HF, undergoing CRT. Implantation of CRT occurred in the tertiary Cardiology Department, Coimbra University Hospital Centre.

Inclusion criteria were defined according to the guideline's criteria for CRT. Therefore, we restricted patients to those with a class I recommendation for CRT: belonging to class II or III or IV NYHA class; presenting a LVEF ≤ 35%; a QRS ≥ 120 ms with left bundle branch block morphology; and normal sinus rhythm.

The exclusion criteria combined several conditions that could influence or interfere with the inflammatory immune response and bias the results, such as: clinical or biochemical manifestation of concomitant inflammatory disease; regular use of nonsteroidal anti-inflammatory drugs or anticoagulants; active infections; known autoimmune or malignant diseases; severe valvular disease or congenital heart disease; cardiogenic shock; continuously or intermittently intravenous inotropic therapy; pregnancy; deep vein thrombosis or pulmonary embolism; severe peripheral arterial occlusive disease; severe and non-controlled arterial hypertension (systolic blood pressure > 180 mmHg or diastolic > 110 mmHg); comorbidities associated with a life expectancy less than 1 year; recent trauma or surgery (< 1 month); recent major bleeding (< 6 months) requiring blood transfusion; renal insufficiency (creatinine >2.0 mg/dl); anaemia (haemoglobin < 8.5 g/dl) or thrombocytopenia (< 100000/L); atrial fibrillation; prior arterial coronary bypass surgery; acute coronary syndrome, or percutaneous coronary intervention within three months; previously implanted CRT system; and excessive alcohol consumption or illicit drug abuse [18].

The selection of patients was performed at baseline before the implantation of TRC (T0), with the clinical evaluation and echocardiographic assessment.

At the time of inclusion, all patients were under stable, optimal pharmacological therapy for chronic HF [35, 36].

After six months of follow-up (T6), patients were reevaluated to assess clinical profile, haematological and chemistry parameters, echocardiographic and inflammatory biomarker changes.

3.3.2. Echocardiographic evaluation

Standard echocardiography was performed at T0 and T6, using a Vivid 7 (GE Healthcare, Oslo, Norway) and 1.7/3.4-MHz tissue harmonic transducer. Loops and three cardiac cycles were stored digitally and analysed offline using a customized software package (EchoPAC, GE Healthcare). Left ventricular end-diastolic volume (LVEDV), LVESV and LVEF were calculated by the biplane Simpson's equation in apical four-chamber and two-chamber views [37, 38].

3.3.3. Definition of response to CRT

The echocardiographic evaluation performed at 6 months follow-up was used to classify the response to CRT. Patients who were still alive and showed at least a 15% reduction in LVESV at 6-month follow-up compared to baseline were considered responders to CRT.

3.3.4. Healthy control group

The healthy control group (HG) was composed of 11 active healthy volunteers who apparently did not present comorbidities. Inclusion criteria were established considering available and recent analytical results and cardiac exams: history of normal lipid profile and history of normal cardiac evaluation. Exclusion criteria were family history of heart disease and/or cardiomyopathy; active infections, inflammatory processes; autoimmune, neoplastic, and allergic diseases; consumption of any drugs or medications that could alter the immune system homeostasis; consumption of alcohol; and inability to understand informed consent.

3.3.5. Blood samples

Just before implantation of the device, peripheral blood samples were collected in HF patients to determine haematological parameters and for chemistry assessment (including high sensitivity C-reactive protein (hs-CRP), BNP and uric acid). In addition, venous samples were taken from patients to analyse inflammatory parameters at T0 and T6.

The same analysis of inflammatory parameters was performed in the HG.

3.3.6. Quantification and immunophenotypic characterization of circulating dendritic cells and monocyte subsets

Quantification and immunophenotypic characterization of circulating DC and monocytes subsets was assessed using eight-colour combinations of mouse anti-human antibodies: CD11c-allophycocyanin (APC); CD33-peridinin chlorophyll protein (PerCP); CD86-fluorescein isothiocyanate (FITC); CD123-Phycoerythrin (PE); HLA-

DR-PE-Cyanine7 (PE-Cy7); CD14 APC-H7; CD16-Pacific Blue (PB) and CD45-Pacific Orange (PO), detailed in Supplementary Table 3.1.

Briefly, monoclonal antibodies were added to 100 µl of peripheral blood (collected in K3-EDTA) and incubated for 15 min in darkness, at room temperature. Red cell lysis and wash procedures were performed, and the remaining cell pellet was resuspended in 0.5 ml of phosphate-buffered saline (PBS) (Gibco, Paisley, Scotland).

Supplementary Table 3.1 – Monoclonal antibody reagents used for the immunophenotypic and functional characterization of monocytes and dendritic cells.

Monoclonal antibodies	Fluorochromes	Clone	Commercial Source
CD11c	APC	SHCL-3	BDB
CD33	PerCP	P67.6	BDB
CD86	FITC	2331 (FUN-1)	BD Pharmingen
CD123	PE	9F5	BDB
HLA-DR	PE-Cy7	L243	BDB
CD14	APC-H7	MφP9	BDB
CD16	PB	3G8	BD Pharmingen
CD45	PO	HI30	Invitrogen
Dendritic Cell Exclusion Kit (Mixture of anti-CD3, CD56, CD19, and CD14)	FITC		Cytogonos, Salamanca, Spain
HLA-DR	PerCP	L243	BDB, San Jose, USA
CD33	APC	clone P67.6	BDB, San Jose, USA
TNF-α	PE	Mab11	Pharmingen, San Diego, USA
IL-6	PE	clone MQ2- 6A	Pharmingen, San Diego, USA
IL-1β	PE	clone AS10	BDB, San Jose, USA

3.3.7. Flow cytometry data acquisition and analysis

Data acquisition was performed in a FACSCanto™II (BD) flow cytometer equipped with FACSDiva software (version 6.1.2: BD). Samples were acquired with established standardized instrument settings recommended by the Euroflow consortium [39]. Data

acquisition was performed in two consecutive steps: in the first step, a total of 1×10^5 events, corresponding to all nucleated cells in the sample, were acquired and results were stored; in the second step, information was stored exclusively for those cells included in a live gate containing HLA-DR$^+$ events, with a minimum of 1×10^5 events. Absolute counts were calculated using a dual platform methodology (flow cytometry and haematological cell analyser). Results illustrate the percentage of positive cells within each subset.

The DC subsets were identified according to the following phenotypes: mDC as HLA-DR$^{+bright}$CD33$^{+bright}$CD14$^-$CD123$^+$CD11c$^+$ and pDC as HLADR$^+$CD123$^{+bright}$CD33$^-$CD16$^-$ CD14$^-$ [40–42]. The monocytes were identified based on their characteristic FSC/SSC light dispersion properties, strong positivity for CD33, high CD45 expression and CD14 and/or CD16 expression without resorting to the expression of HLA-DR. The cMo were identified as CD33^{+++}CD14$^+$CD16$^-$, iMo as CD14$^+$CD16$^+$ and ncMo as CD14$^\pm$CD16$^+$ [22, 41, 43]. The strategy used for the identification and characterization of DC and monocyte subsets is represented in Figure 3.1. The mean fluorescence intensity (MFI) of CD86 was determined in mDC and monocyte subsets and the percentage of CD86+ cells, as well as the MFI, in pDC.

3.3.8. Functional characterization of myeloid dendritic cells and monocytes

In vitro stimulation to evaluate of cytokine production by DC and monocytes was performed as described by Paiva A. *et al.* [44, 45]. Briefly, a total of 500 µl of each PB sample was diluted l/l (vol/vol), in duplicate, in RPMI-1640 medium (Gibco; Paisley, Scotland, UK), supplemented with 2 mM L-glutamine and incubated at 37 °C in a sterile environment with a 5% CO2 humid atmosphere for 6h, in the presence of 10 lg/ml of Brefeldin A (Sigma, St. Louis, MO). In addition, 100 ng/ml of lipopolysaccharide (LPS) from Escherichia coli (serotype 055:B5 (Sigma)) plus 100 U/ml of interferon (IFN)-γ; Promega, Madison, WI) were added to one of the tubes (stimulated samples). The other tube only with Brefeldin A was used to evaluate the basal cytokine production by the different subpopulations of monocytes and dendritic cells.

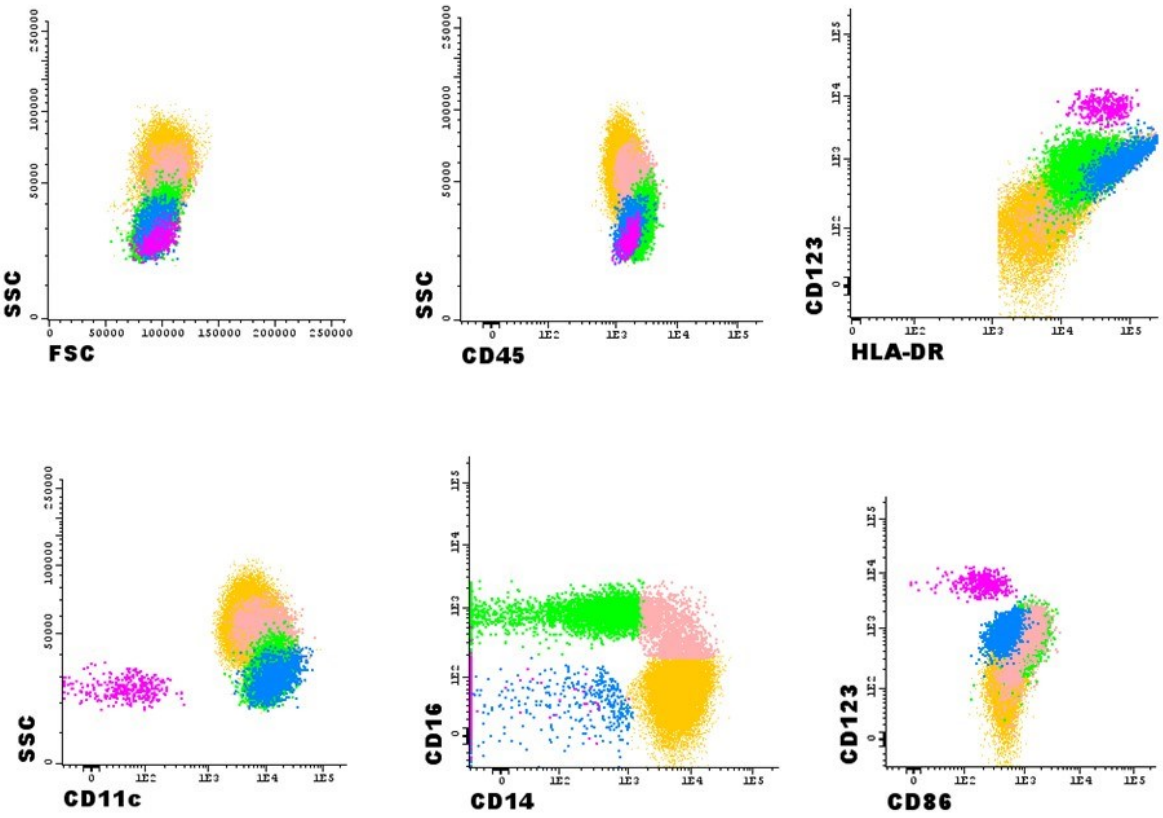


Figure 3.1 – Representative dot plots illustrating the identification of plasmacytoid dendritic cells (pDC) (in pink – based on the bright expression of CD123 and HLA-DR), myeloid dendritic cells (mDC) (in blue – based on the bright expression of CD33 and HLA-DR), classical monocytes (cMo) (in yellow – based on the positive expression of CD14 and negative expression of CD16), intermediate monocyte (iMo) (in light pink – based on the positive expression of both CD14 and CD16), and non-classical monocytes (ncMo) (in green – based on the positive expression of CD16 and dim/negative expression of CD14) in peripheral blood samples, using a combination of eight-colour mouse anti-human antibodies.

3.3.9. Immunofluorescent staining

After incubation period, both stimulated and unstimulated samples were aliquoted in different tubes (200 µl/tube) in order to analyse the expression of each cytokine by monocytes and DC. Dendritic Cell Exclusion Kit-FITC combined with anti-HLA-DR-PerCP and anti-CD33-APC was added to each tube, to identify DC. After gentle mixing, cells were sequentially incubated for 15 min at room temperature, in darkness and washed once in with 2 ml of PBS, (5 min at 540 x g). After discarding the supernatant, cells were fixed and permeabilized with FIX&PERM (Caltag, Hamburg, Germany) according to manufacturer's instructions and stained with PE-conjugated mAb directed

against different human intracytoplasmic cytokines: anti-TNF-α, anti-IL-6 and anti-IL-1β (monoclonal reagents are detailed in Supplementary Table 3.1). Each anti-cytokine mAb reagent was placed in a separate tube containing either, the stimulated or the unstimulated samples. The tubes were incubated for 15 min at room temperature in darkness. Then, cells were washed and resuspended in 0.5 ml of PBS until they were analysed in a flow cytometer.

3.3.10. Flow cytometry data acquisition and analysis

The data acquisition was performed in two consecutive steps in a FACSCalibur flow cytometer (BD, San Jose, USA) equipped with an argon ion laser and a red diode laser. A first acquisition of 2 x 10^4 events, (corresponding to all nucleated cells present in the sample) was performed, followed by an acquisition on an electronic HLA-DR gate. Data were analysed using the Infinicyt™ software, V.1.5 (Cytognos SL, Salamanca, Spain) and absolute counts were determined using two different instrumentation platforms (flow cytometer and haematological cell analyser).

3.3.11. Statistical analysis

Statistical analysis was performed using R Core Team (2017). (R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/), (version 3.4.1). A non-parametric Mann Whitney *U* test was used to compare quantitative independent variables. The Wilcoxon signed-rank test was used to compare T0 vs T6 [46]. Results were expressed as median (range). The values used to establish the effect size were 0.20; small, 0.60; moderate, 1.20; large and 2.00; very large [47]. Differences were considered to be statistically significant when *P* value was < 0.05. To calculate the sample size, the software G*Power 3.1 was used [48]. Prior analysis was performed determining that 35 subjects would be needed for the study (effect size dz:0.7, α error probability:0.05, power:0.80). Additionally, six elements were added to the sample as a matter of convenience.

3.4. Results

3.4.1. Baseline characteristics

Twenty-eight patients were male and thirteen were female, with ages ranging from 34 to 83 years (mean 61.4 ± 10.4). Regarding chronic medication before CRT, 72.2% of the patients were under angiotensin-converting enzyme (ACE) inhibitors, 19.4% under angiotensin type 1-receptor blockers (ARB), 94.4% under beta-blockers, 66.7% under spironolactone, 97.2% under furosemide, 27.8% under digoxin, 50% under statins, and 13.9% under ivabradine. The rate of diabetic patients was 14.6%. The HG consisted of eight males and three females with a mean age of 43.4 ± 10.8 years. The average BMI of the HG was 22.8 ± 1.3, and the lipid profile values were 184.3 ± 16.1 mg/dL for cholesterol; 64 ± 8.6 mg/dL for HDL cholesterol; 96.5 ± 11.4 mg/dL for LDL cholesterol and 119.1 ± 24.0 mg/dL for triglycerides.

3.4.2. Clinical evolution of responders and non-responders to CRT

The clinical characterization of the HF population is detailed in Supplementary Table 3.2. Most HF patients were in class III according to NYHA classification and mean LVEF was 24.9 ± 6.9%. Through echocardiographic definition, the proportion of responders to CRT was 54%. No patient died or was transplanted in the 6-month follow-up period. After CRT, responders presented significantly lower BNP levels compared to non-responders to CRT.

3.4.3. Comparison between heart failure patients and healthy group

- **Frequency of monocytes and dendritic cells**

The frequency of total monocytes and monocyte subsets expressed by HF patients and the HG is presented in Table 3.1. Considering the frequency and absolute number of total monocytes, no significant differences were found between HG and HF patients in either moment of evaluation. Regarding monocyte subsets, HF patients presented a significantly lower frequency of cMo at follow-up evaluation, compared to the HG (*P* =

0.006). No other differences were found between the overall HF patient population and the HG in monocytes subsets at either time of evaluation (cMo: 81.00% in the HG versus 79.42% in HF patients (HFP)(T0), *P* = 0.275; iMo: 11.09% in the HG versus 10.81% in HFP(T0), *P* = 0.695 and versus 15.14% in HFP(T6), *P* = 0.065; ncMo: 8.55% in the HG versus 7.52% in HFP(T0), *P* = 0.747 and 11.35% in HFP(T6) *P* = 0.096).

Supplementary Table 3.2 – Clinical characterization of responders and non-responders to CRT.

	Global Population Mean ± SD (n = 41)	Responders Mean ± SD (n = 22)	Non-Responders Mean ± SD (n = 19)	*P Value Responders vs Non-responders*
Baseline assessment				
Gender (Male/Female)	28 / 13	15 / 7	13 / 6	1
Aetiology (Non-Ischemic/Ischemic)	30 / 11	18 / 4	12 / 7	0,418
NYHA (II/III/IV)	8/ 29/ 4	4/ 17/ 1	4/ 12 /3	0,465
Age (years)	61.4 ± 10.5	**65.2 ± 9.6**	**56.9 ± 9.8**	**0.015**
LVEF (%)	24.9 ± 6.9	23.8 ± 6.5	26.3 ± 7.4	0.309
LVESV (mL)	190.2 ± 84.9	180.1 ± 55.3	202.1 ± 111.0	0.903
LVEDV (mL)	244.2 ± 83.9	233.3 ± 58.9	257.1 ± 106.8	0.583
QRS	148.4 ± 30.6	144.5 ± 22.1	151.4 ± 36.6	0.866
Total Leukocytes (x10^3 µl)	8.4 ± 1.7	8.1 ± 1.6	8.7 ± 1.8	0.242
hs-CRP (mg/L)	5.6 ± 6.0	5.2 ± 5.1	5.9 ± 6.9	0.934
BNP (pg/mL)	362.8 ± 358.7	264.3 ± 214.8	461.3 ± 448.1	0.362
Glucose (mg/dL)	109.9 ± 41.9	97 ± 31.9	100 ± 50.8	0.286
Uric Acid (mg/dL)	6.0 ± 1.7	5.6 ± 1.5	6.5 ± 1.9	0.231
After CRT				
Total Leukocytes (x10^3 µl)	8.3 ± 1.8	8.1 ± 1.5	8.6 ± 2.1	0.220
LVEF (%)	33.9 ± 10.8	**39.1 ± 9.8**	**27.6 ± 8.4**	**0.001**
LVESV (mL)	151.4 ± 96.0	**100.4 ± 36.7**	**215.1 ± 109.6**	**< 0.001**
LVEDV (mL)	220.2 ± 108.5	**168.9 ± 50.3**	**284.4 ± 127.9**	**0.001**
hs-CRP (mg/L)	4.1 ± 4.6	2.6 ± 1.8	6.2 ± 6.4	0.288
BNP (pg/mL)	245.3 ± 334.6	**139.6 ± 164.1**	**403.9 ± 456.0**	**0.043**

SD: standard deviation; *NYHA*: New York Heart Association; *LVEF*: Left Ventricular Ejection Fraction; *LVESV*: Left Ventricular End-Systolic Volume; *LVEDV*: Left Ventricular End-Diastolic Volume; *hs-CRP*: High Sensitivity C-Reactive Protein; *BNP*: B-type natriuretic peptide.

Regarding total DC and their subsets (Table 3.1), HF patients presented a significantly lower baseline percentage of pDC compared to the HG (P = 0.023). No differences were found in mDC compartment.

- **CD86 expression by monocytes and dendritic cells subpopulations**

In both HF and HG, all monocyte subpopulations and mDC expressed the co-stimulatory molecule CD86. Therefore, only the amount of CD86 per cell (MFI) was measured (Figure 3.2a, b, c and d).

Since not all pDCs express CD86, the frequency of this cell population expressing CD86 is presented in Table 3.1 and the MFI in Figure 3.2e.

At T6, the overall HF population presented a significantly lower MFI of CD86 on cMo (Figure 3.2a) and mDC (Figure 3.2d), compared with HG.

Considering the pDC subset, HF patients presented a significantly higher percentage of pDC expressing CD86 compared with the HG, at 6-month follow-up (Table 3.1). However, in the same comparison, the amount of CD86 per cell expressed by pDC was lower at follow-up (Figure 3.2e) as reported for cMo and mDC.

- **Functional characterization of monocytes and myeloid dendritic cells**

After cell stimulation with LPS and IFN-γ, we only determined the frequency of total monocytes producing the cytokines under study, and not among each monocyte subpopulation. HF patients presented a higher proportion of monocytes and mDC producing IL-6 and IL-1β both before and 6 months after CRT (Figure 3.3b, c, e and f), compared with HG. No significant differences were found regarding the frequency of TNF-α-producing monocytes (Figure 3.3a) and mDC (Figure 3.3d).

Table 3.1 – Comparative analysis of the overall monocytes and dendritic cells and their respective subsets in healthy individuals and patient groups.

			HG (n = 11)	HFP (n = 39)		Responders (n = 21)		Non-Responders (n = 18)	
				HFP-To	HFP-T6	HFP-To	HFP-T6	HFP-To	HFP-T6
Total Monocytes		%	8.12 (3.35 - 12.91)	6.48 (1.82 - 13.25)	6.52 (3.36 - 11.17)	6.48 (3.62 - 13.25)	6.59 (3.36 - 11.17)	6.21 (1.82 - 9.50)	5.97 (3.43 - 9.39)
		Cells/µl	449.60 (263.18 - 958.59)	529.11 (239.17 - 1040.04)	545.25 (253.93 - 1004.99)	518.31 (239.17 - 1040.04)	545.25 (288.74 - 1004.99)	533.69 (242.23 - 826.52)	553.25 (253.93 - 819.94)
	***Classical Monocytes** *	%	**81.00** [d, e] **(68.24 - 88.14)**	**79.42** [j] **(43.35 - 88.66)**	**75.95** [d, j] **(42.58 - 84.90)**	**79.73** [m] **(53.54 - 88.66)**	**71.67** [e, m] **(42.58 - 83.05)**	77.50 (43.35 - 85.66)	76.24 (57.41 - 84.90)
	***Intermediate Monocytes** *	%	11.09 (4.69 - 26.76)	**10.81** [k] **(5.09 - 25.22)**	**15.14** [k] **(6.04 - 45.24)**	**10.35** [n] **(5.98 - 16.32)**	**15.36** [n] **(6.04 - 45.24)**	12.61 (5.09 - 25.22)	14.82 (7.95 - 40.12)
	***Non-classical Monocytes** *	%	**8.55** [f] **(0.49 - 13.95)**	7.52 (0.68 - 51.56)	11.35 (1.47 - 23.16)	9.46 (0.68 - 31.56)	**12.97** [f, q] **(3.37 - 23.16)**	7.08 (1.36 - 51.56)	**8.68** [q] **(1.47 - 16.62)**
Myeloid Dendritic Cells		%	0.21 (0.06 - 0.43)	0.19 (0.05 - 2.87)	0.17 (0.00 - 0.39)	0.19 (0.07 - 2.87)	0.17 (0.06 - 0.39)	0.16 (0.05 - 1.14)	0.15 (0.00 - 0.37)
		Cells/µl	13.30 (5.11 - 25.05)	13.64 (4.00 - 189.52)	14.61 (2.99 - 39.45)	15.78 (4.00 - 189.52)	14.32 (2.99 - 39.45)	11.42 (5.89 - 115.06)	14.71 (5.77 - 29.79)
Plasmacytoid Dendritic Cells		%	**0.10** [a, b] **(0.05 - 0.22)**	**0.07** [a, l] **(0.02 - 0.19)**	**0.08** [l] **(0.00 - 0.28)**	**0.05** [b] **(0.02 - 0.16)**	0.07 (0.00 - 0.18)	0.08 (0.03 - 0.19)	0.08 (0.03 - 0.28)
		Cells/µl	**9.12** [c] **(2.62 - 14.54)**	5.68 (1.77 - 16.74)	6.81 (0.35 - 16.74)	**4.67** [c] **(1.77 - 11.99)**	6.27 (0.35 - 16.74)	6.64 (2.48 - 16.74)	6.86 (2.27- 14.81)
	Plasmacytoid Dendritic Cells expressing CD86	%	**32.84** [g, h, i] **(16.36 - 53.62)**	**39.92** [o] **(21.08 - 85.16)**	**54.23** [g, o] **(21.63 - 89.80)**	41.70 (22.61 - 85.16)	**56.09** [h] **(21.63 - 76.82)**	**37.22** [p] **(21.08 - 75.00)**	**52.35** [i, p] **(32.57 - 89.80)**

* Relative to overall Monocytes

Results are expressed as median (range). *HG*: Healthy control Group; *HFP*: Heart Failure Patients; *HFP-To* (T0): Baseline assessment; *HFP-T6* (T6): Follow-up evaluation, 6 months after Cardiac Resynchronization Therapy (CRT); *WBC*: White Blood Cells. Statistically significant differences were considered when $P < 0.05$ (Mann-Whitney *U* test and Wilcoxon signed-rank)

HG *versus* HFP-To

[a] $P = 0.023$; [b] $P = 0.008$; [c] $P = 0.033$

HG *versus* HFP-T6

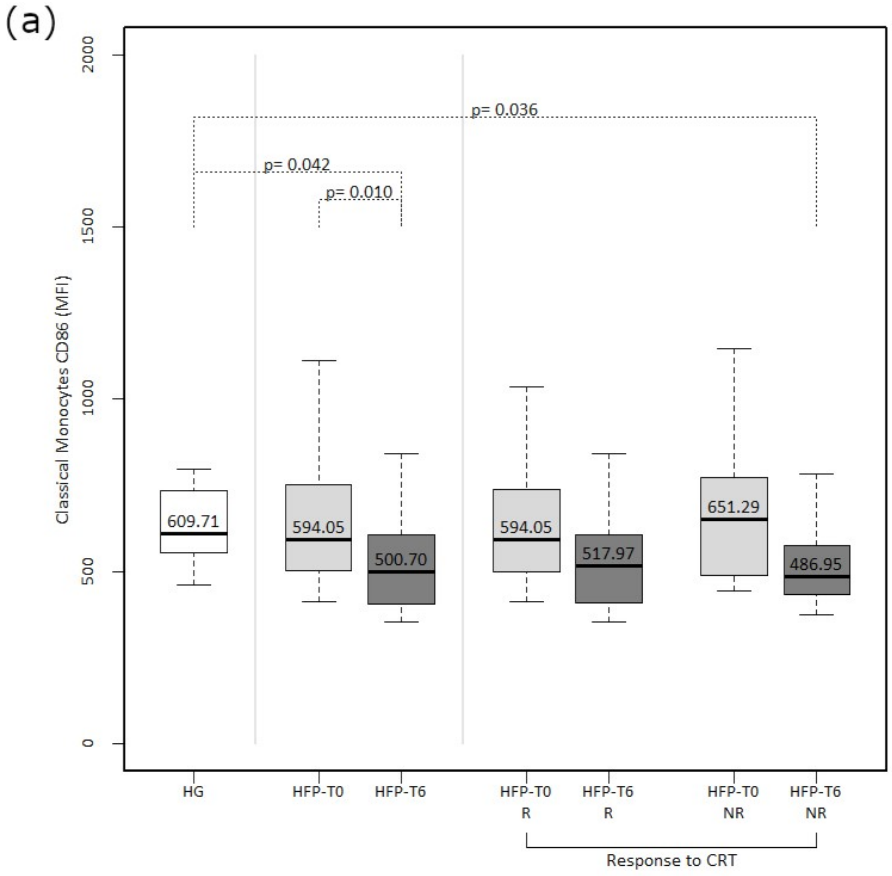
[d] $P = 0.006$; [e] $P = 0.004$; [f] $P = 0.012$, [g] $P = < 0{,}001$; [h] $P = 0{,}001$; [i] $P = 0.005$

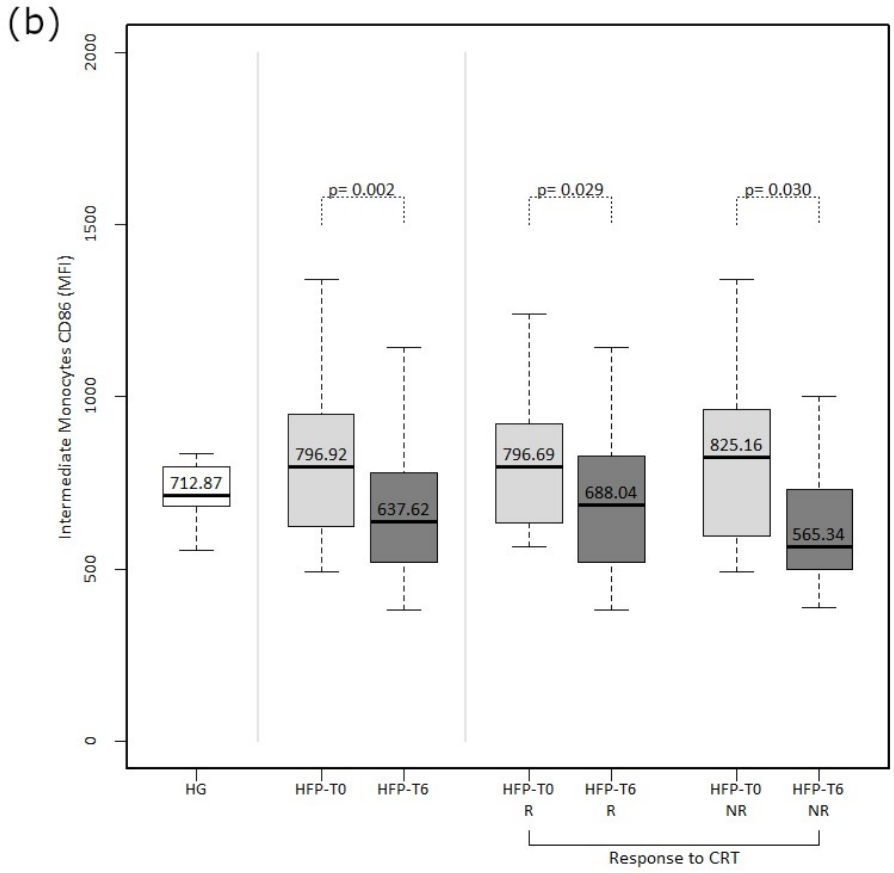
HFP-To *versus* HFP-T6

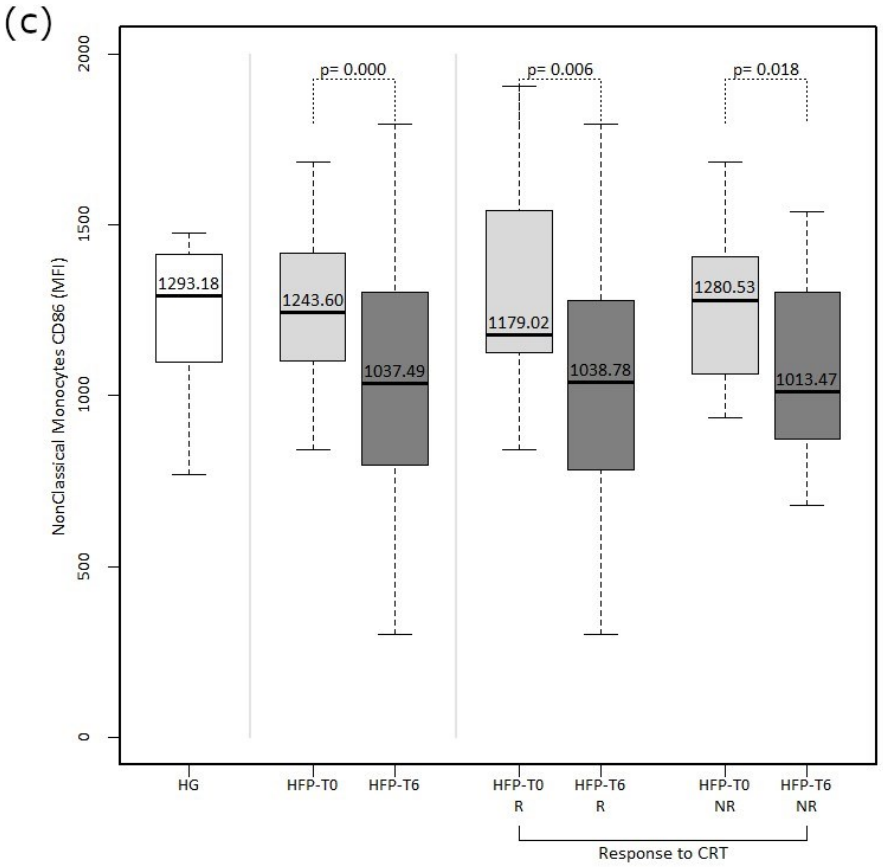
[j] $P = 0.007$; [k] $P = 0.006$; [l] $P = 0.046$; [m] $P = 0.002$; [n] $P = 0.006$; [o] $P = 0.007$; [p] $P = 0.021$

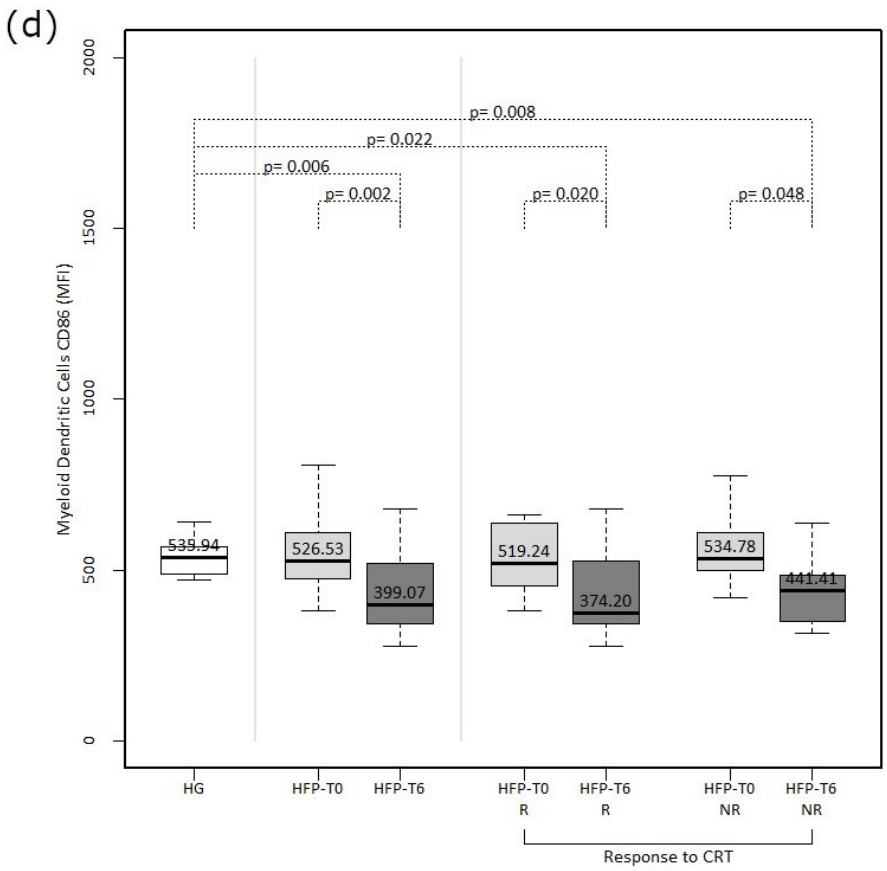
HFP-T6 (Responders) *versus* HFP-T6 (Non-responders)

[q] $P = 0.04$


(a)
Classical Monocytes CD86 (MFI)
0
500
1000
1500
2000
p= 0.036
p= 0.042
p= 0.010
609.71
594.05
500.70
594.05
517.97
651.29
486.95
HG
HFP-T0
HFP-T6
HFP-T0 R
HFP-T6 R
HFP-T0 NR
HFP-T6 NR
Response to CRT


(b)
Intermediate Monocytes CD86 (MFI)
0
500
1000
1500
2000
p= 0.002
p= 0.029
p= 0.030
712.87
796.92
637.62
796.69
688.04
825.16
565.34
HG
HFP-T0
HFP-T6
HFP-T0 R
HFP-T6 R
HFP-T0 NR
HFP-T6 NR
Response to CRT


(c)
NonClassical Monocytes CD86 (MFI)
0
500
1000
1500
2000
p= 0.000
p= 0.006
p= 0.018
1293.18
1243.60
1037.49
1179.02
1038.78
1280.53
1013.47
HG
HFP-T0
HFP-T6
HFP-T0 R
HFP-T6 R
HFP-T0 NR
HFP-T6 NR
Response to CRT


(d)
Myeloid Dendritic Cells CD86 (MFI)
0
500
1000
1500
2000
p= 0.008
p= 0.022
p= 0.006
p= 0.002
p= 0.020
p= 0.048
539.94
526.53
399.07
519.24
374.20
534.78
441.41
HG
HFP-T0
HFP-T6
HFP-T0 R
HFP-T6 R
HFP-T0 NR
HFP-T6 NR
Response to CRT

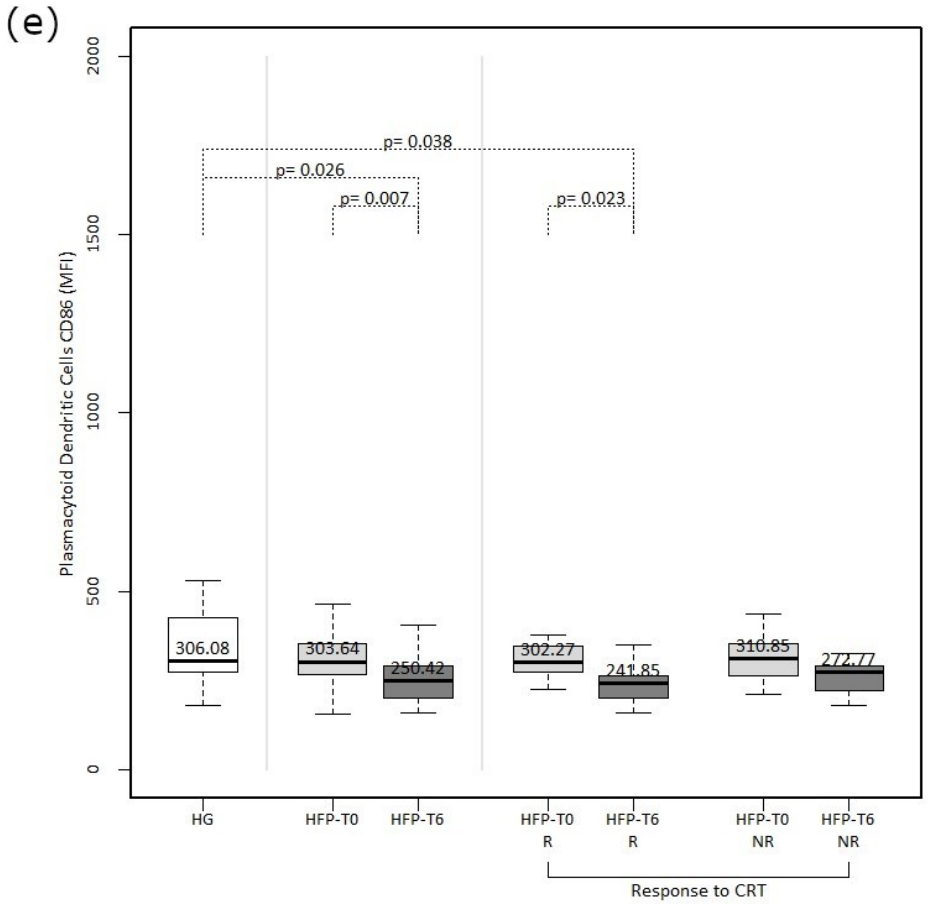


Figure 3.2 – Amount of CD86 per cell (based on the mean fluorescence intensity value) expressed by classical monocytes (cMo) (**a**), intermediate monocyte (iMo) (**b**), and non-classical monocytes (ncMo) (**c**), myeloid dendritic cells (mDC) (**d**) and plasmacytoid dendritic cells (pDC) (**e**) in healthy individuals (HG) and heart failure patients (HFP), at baseline assessment (HFP-T0) and 6 months after cardiac resynchronization therapy implantation (HFP-T6). Heart failure patients were distributed: according to response to cardiac resynchronization therapy: responders (R) and non-responders (NR). Statistically significant differences were considered when $P < 0.05$.

3.4.4. Impact of monocytes and dendritic cells subpopulations on CRT response

- **Frequency of monocytes and dendritic cells**

When we divide patients according to CRT response, only responders showed significantly lower levels of cMo after CRT than HG (P = 0.004, Table 3.1). Moreover, responders to CRT, but not non-responders, presented a significant reduction of cMo levels from baseline to post-CRT evaluation. The responder group also showed a significantly higher percentage of ncMo at follow-up compared to the HG (P = 0.012, Table 3.1).

On the other hand, after CRT, non-responders maintained similar levels of cMo and ncMo by comparison with the HG (cMo: HG = 81.00% versus non-responders (T6) = 76.24%, P = 0.056 and ncMo: HG = 8.55% versus non-responders (T6) = 8.68%, P = 0.902). Concerning the frequency of iMo, no differences were observed in this comparison with HG.

In relation to DC subpopulations, the significantly lower percentage of pDC compared to the HG was only observed in the responder group (pDC: HG = 0.10% versus responders(T0) = 0.05%, P = 0.008; and absolute values pDC: HG = 9.12 cell/µl versus responders(T0) = 4.67 cell/µl, P = 0.033, Table 3.1). No differences were found in the mDC subset.

In the analysis between the two moments of evaluation (T0 and T6), the overall HF population showed a significant decrease in the percentage of cMo at follow-up, and a significant increase in the frequency of iMo compared to baseline assessment (Table 3.1). Of note is that these significant differences were only observed in responders to CRT (Table 3.1); (cMo(non-responders): T0 = 77.50% versus T6 = 76.24%, P = 0.632; iMo(non-responders): T0 = 12.61% versus T6 = 14.82%, P = 0.298).

Regarding the ncMo subset, no differences were observed in the overall HF population between T0 and T6. However, responders to CRT presented higher levels of ncMo than non-responders at 6-month follow-up (P = 0.044, Table 3.1). Here, it was observed that, at 6-month follow-up, responders presented not only higher values of ncMo to the detriment of cMo, but also increased iMo frequency.

Considering DC, we only found one significant difference between baseline and follow-up evaluation: HF patients exhibited a higher frequency of pDC at follow-up (P = 0.046, Table 3.1).

- **Expression of CD86 by monocytes and dendritic cells**

As shown in Table 3.1, there was a remarkably higher frequency of CD86-expressing pDC after CRT in comparison with the HG, in both responders (P = 0.001) and non-responders (P = 0.005). However, as described previously, the MFI of CD86 decreased after CRT in HF patients compared with the HG, but only reaching significance in responders (Figure 3.2e) (pDC CD86 MFI: HG = 306.08 versus non-responders(T6) = 272.77, P = 0.069).

Compared with HG, the amount of CD86 per cell in cMo was significantly lower after CRT especially in non-responders (Figure 3.2a) (cMo CD86 MFI: HG = 609.71 versus responders(T6) = 517.97, P = 0.113). In the mDC subset this decrease was seen in both response groups (Figure 3.2d).

Comparing the baseline with follow-up (T0 versus T6) there was a significantly lower amount of CD86 per cell expressed in all monocytes and DC subpopulations after CRT. This pattern of decreased MFI of CD86 was seen independently of CRT response.

Considering the frequency of pDC expressing CD86, at 6-month follow-up the overall HF population presented a higher percentage of these cells compared to baseline assessment. However, this difference was mostly observed in non-responders (P = 0.021, Table 3.1).

- **Functional characterization of monocytes and dendritic cells**

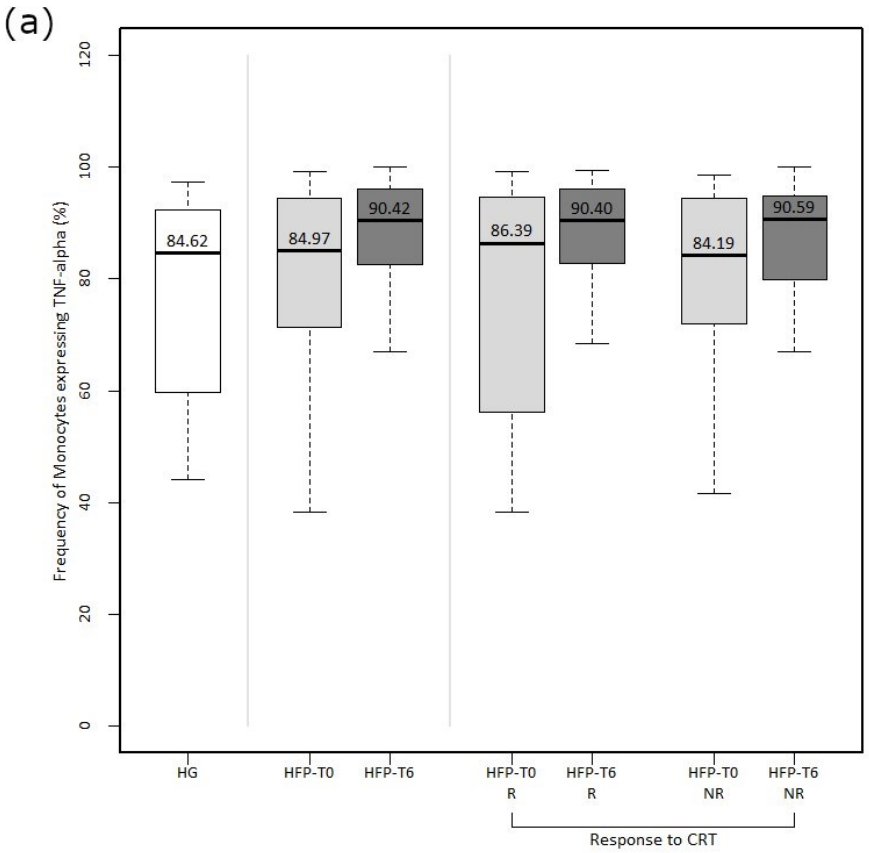
No significant differences were found between HF patients and the HG, nor between responders and non-responders to CRT, or between baseline and follow-up (T0 versus T6) regarding the frequency of TNF-α-producing monocytes (Figure 3.3a).

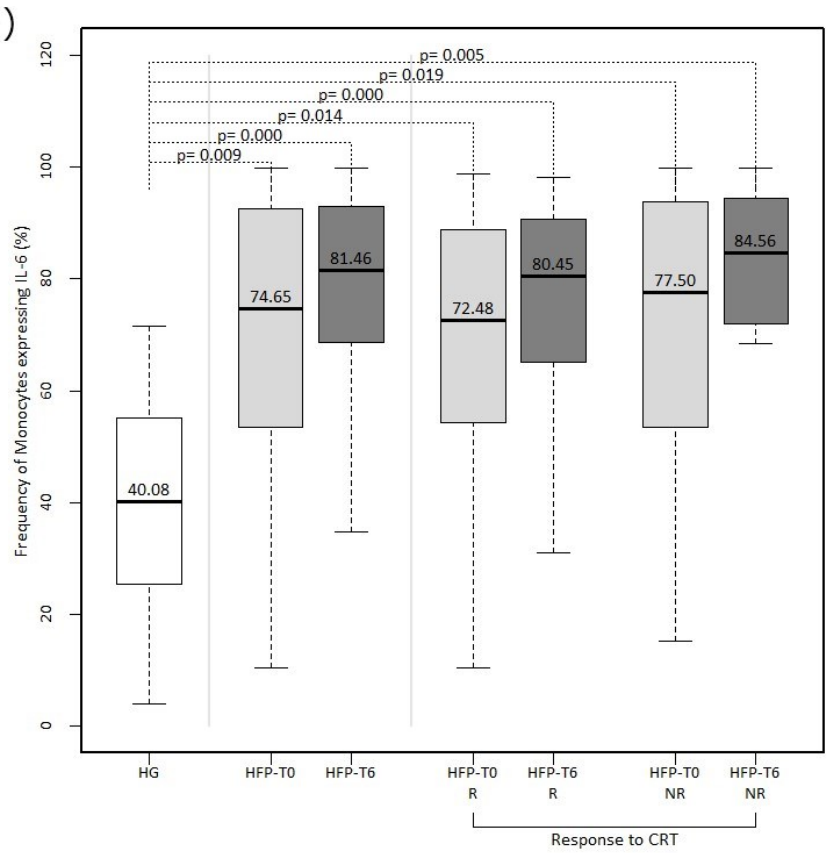
In the initial evaluation and even after CRT, both responders and non-responders presented a higher proportion of IL-6-producing monocytes, compared with the HG (Figure 3.3b). Likewise, responders also showed significantly more IL-1β-producing monocytes, at both times of evaluation, relative to the HG (Figure 3.3c).

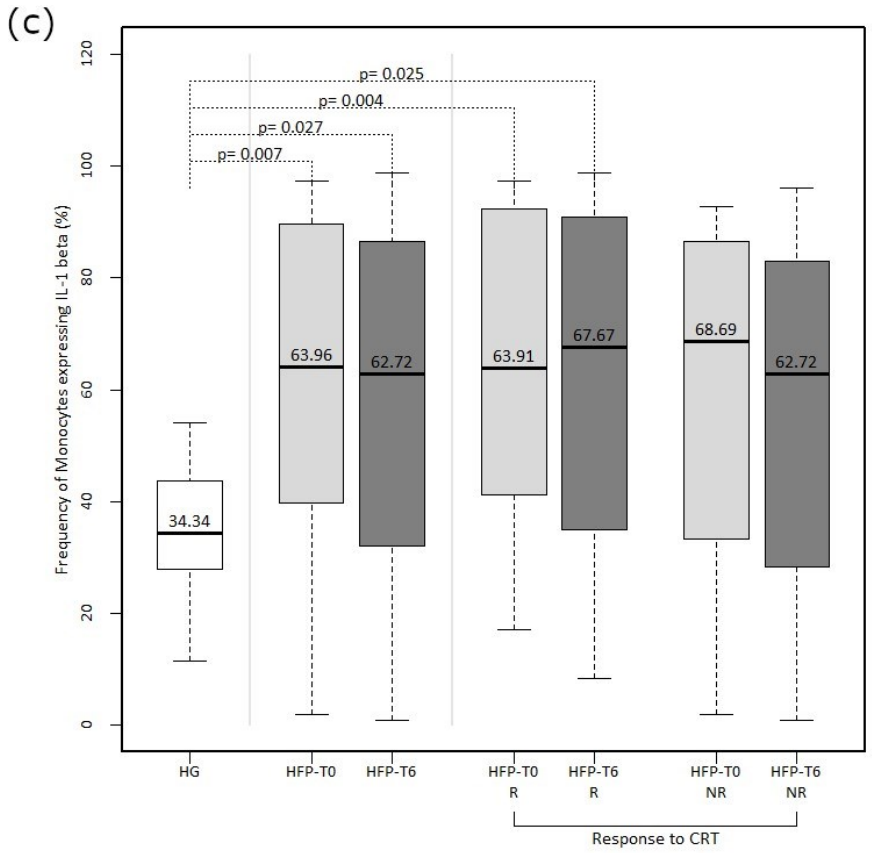
No significant differences were found regarding the frequency of IL6 and IL1β-producing monocytes, when comparing baseline with post-CRT (T0 versus T6) nor when comparing responders and non-responders (R versus NR) (Figure 3.3b, 3.3c).

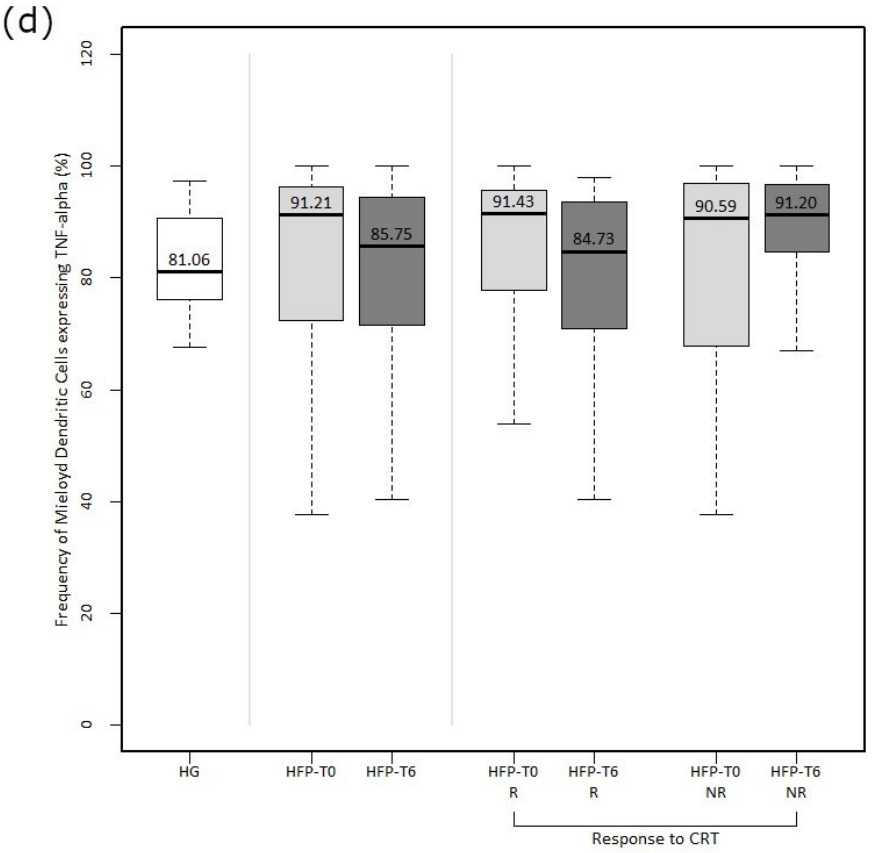
Regarding TNF-α-producing DC, we also found no significant differences between responder or non-responder patients and the HG (Figure 3.3d). However, both patient groups presented higher baseline levels of DC expressing IL-6 compared to the HG. Nonetheless, after CRT, this difference only persisted in the non-responder group (Figure 3.3e).

In addition, the proportion of IL-1β-producing DC was significantly increased in patients compared to the HG at both T0 and T6 evaluation times. Responders and non-responders presented a higher proportion of these cells compared to the HG. Curiously, the responder group showed a lower percentage of IL-1β-producing DC at follow-up compared to the baseline (Figure 3.3f).


(a)
Frequency of Monocytes expressing TNF-alpha (%)
0
20
40
60
80
100
120
84.62
84.97
90.42
86.39
90.40
84.19
90.59
HG
HFP-T0
HFP-T6
HFP-T0 R
HFP-T6 R
HFP-T0 NR
HFP-T6 NR
Response to CRT


(b)
Frequency of Monocytes expressing IL-6 (%)
0
20
40
60
80
100
120
p= 0.005
p= 0.019
p= 0.000
p= 0.014
p= 0.000
p= 0.009
40.08
74.65
81.46
72.48
80.45
77.50
84.56
HG
HFP-T0
HFP-T6
HFP-T0 R
HFP-T6 R
HFP-T0 NR
HFP-T6 NR
Response to CRT


(c)
Frequency of Monocytes expressing IL-1 beta (%)
p= 0.025
p= 0.004
p= 0.027
p= 0.007
34.34
63.96
62.72
63.91
67.67
68.69
62.72
HG
HFP-T0
HFP-T6
HFP-T0 R
HFP-T6 R
HFP-T0 NR
HFP-T6 NR
Response to CRT


(d)
Frequency of Mieloyd Dendritic Cells expressing TNF-alpha (%)
81.06
91.21
85.75
91.43
84.73
90.59
91.20
HG
HFP-T0
HFP-T6
HFP-T0 R
HFP-T6 R
HFP-T0 NR
HFP-T6 NR
Response to CRT

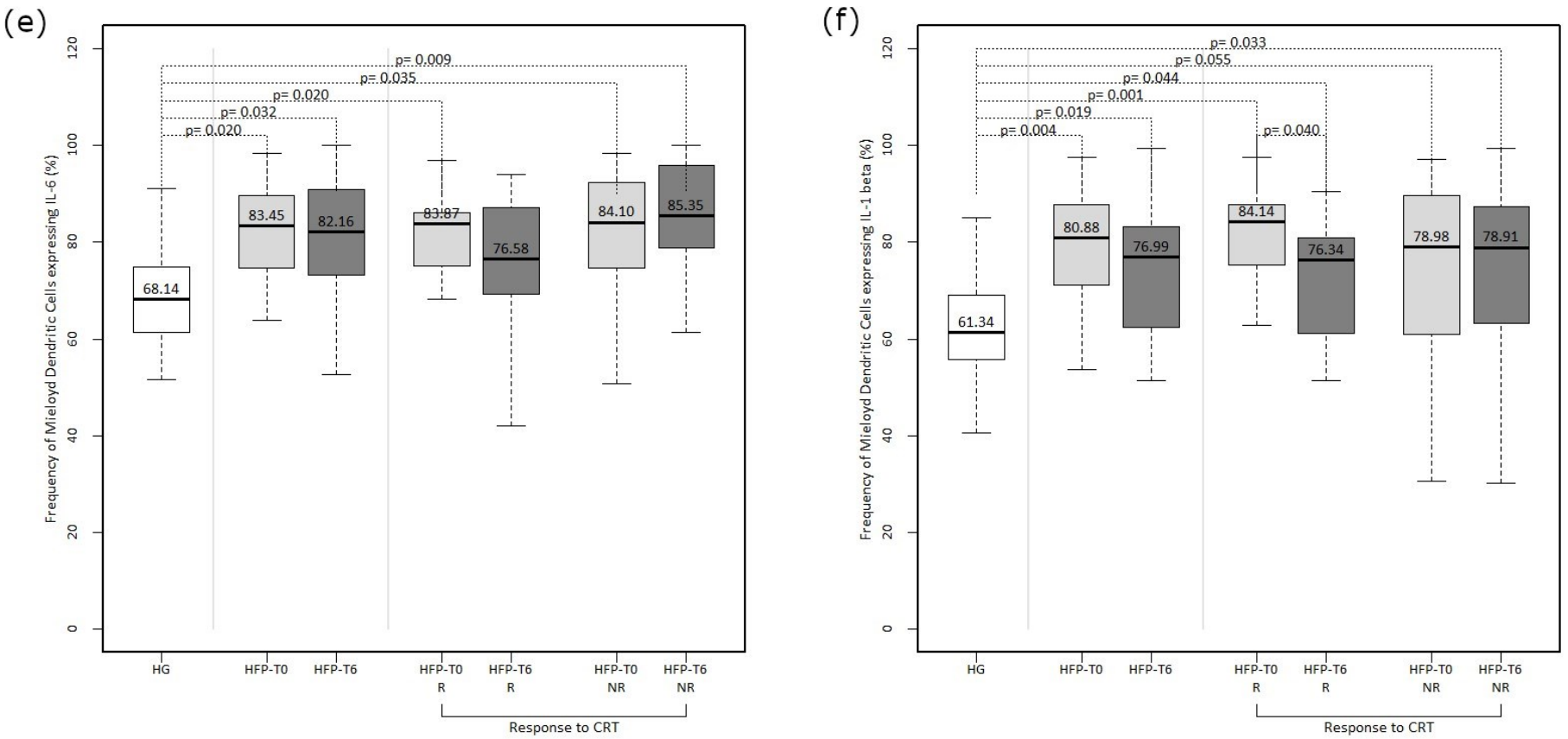


Figure 3.3 – Functional characterization of peripheral monocytes (identified by their characteristic FSC/ SSC light dispersion properties and concomitant expression of CD14, CD33 and HLA-DR) and myeloid dendritic cells (mDC) (Identified by their characteristic FSC/ SSC light dispersion properties, positive expression of CD33 and HLA-DR, and negative expression of all other lineage markers – CD3, CD19, CD56, and CD14 present in the mixture of Dendritic Cell Exclusion Kit). The percentage of monocytes producing TNF-α (**a**), IL-6 (**b**) and IL-1β (**c**) and mDC producing TNF-α (**d**), IL-6 (**e**) and IL-1β (**f**) were evaluated in healthy individuals (HG) and heart failure patients (HFP) at baseline assessment (HFP-T0) and 6 months after cardiac resynchronization therapy implantation (HFP-T6). Heart failure patients were divided according to response to cardiac resynchronization therapy: responders (R) and non-responders (NR). Statistically significant differences were considered when $P < 0.05$.

3.5. Discussion

Monocytes and DC have been implicated in the pathogenesis of HF as well as in its prognosis [19, 21, 28]. To the best of our knowledge, this is the first study to assess the potential role of DC subpopulations and monocyte subsets on cardiac reverse remodelling and CRT response.

In the first comparison between the HG and the HF population, the main findings are the lower frequency of pDC in HF patients before CRT and the lower frequency of cMo at the 6-month follow-up. Previous studies on whole blood DC and monocyte counts in HF patients have shown dissimilar results [6, 23, 25, 28, 29, 49, 50]. Some studies describe an increase in mDC subpopulation with unchanged levels of pDC [49] or comparable levels of both DC between HF patients and controls [23]. Nonetheless, a recent study performed by Pistulli R *et al.* (2016), reports lower counts of circulating mDC in patients with advanced HF [29]. Furthermore, Barisione C. *et al.* describe an increase in iMo and a decrease in ncMo subsets in HF patients compared to the control group [6] and in the latest study performed by Ptaszynska-Kopczynska K. *et al.* (2021) a decrease in circulating ncMo was only found in patients with advanced HF [25]. These distinct results may be due to the heterogeneity of the patient populations in these previous studies, linked to different inclusion and exclusion criteria, HF severity, HF aetiology and sample size. Of note is that our study has the advantage of enrolling a homogeneous population with advanced HF and class I indication for CRT.

Regarding the expression of CD86, HF patients showed similar levels of the amount of CD86 *per cell* at baseline compared to the HG, which suggests that the expression of this costimulatory molecule by the antigen-presenting cells under study is unaffected in HF. However, after CRT, patients presented a lower amount of CD86 *per cell* on cMo and mDC, which indicates an immunomodulation of the expression of this important costimulatory molecule. At the same time, although the frequency of pDC expressing CD86 increases after CRT (which could indicate that these cells may be peripherally activated, with a likely increased capacity to produce robust amounts of type I IFNs), the MFI of CD86 on pDC was also lower in patients than in healthy subjects. At this point, it appears that CRT can exert an impact on the amount of CD86 expressed by monocytes and DC.

As expected, at baseline, HF patients showed a higher frequency of Mo and mDC producing IL-6 and IL-1β compared to the HG. These higher levels of pro-inflammatory cytokine-producing cells reflect the inflammatory state of HF. This difference persisted

after CRT, suggesting that biventricular pacing is not able to decrease the frequency of these cells to normal values. Considering that the various monocyte subsets produce TNF-α, IL-6, IL-1β and that these inflammatory cytokines are important markers of active disease and HF prognosis [51], our study shows that CRT does not modulate the pro-inflammatory capacity of these cells, which may compromise the long-term response to CRT and even the overall survival of patients.

Regarding the comparison between responders and non-responders to CRT, the fact that responders showed significantly lower levels of cMo and higher levels of ncMo after CRT than the HG but that non-responders did not, suggests that the reduction of circulating cMo and the increase in ncMo play a role in CRT response. Some studies have shown that the inflammatory response induced by the innate immune system can be physiological and results in the upregulation of cytoprotective responses that allow the heart to adapt to stress [52, 53]. Therefore, it is tempting to speculate that the cMo reduction observed after CRT, exclusively in responders, indicates an increased recruitment of circulating monocytes to the injury site and a beneficial effect on reverse remodelling, or, on the other hand, it may be due to an increase in the ncMo subset, which after exerting their local function, emerge from the cardiac tissue into the peripheral blood circulation to die in the spleen [54].

Another important finding of the present study is the remarkable decrease in cMo and increase in iMo, from baseline to post-CRT, which was only due to responders. Furthermore, the responder group also presented higher ncMo values at follow-up compared to the non-responder group. Taken together, the increase in the frequency of ncMo and iMo and decrease in cMo in responders suggest a participation of these cells in cardiac reverse remodelling and CRT response. Our results are consistent with those of Ptaszynska-Kopczynska K. *et al.* (2021) [25], who describe an increase in ncMo and iMo subsets with a consequent reduction of cMo in HF patients after CRT. However, in that study the subdivision of patients according to CRT response was not carried out. Our study included an additional analysis based on responders and non-responders that distinguishes it from prior studies. Functionally iMo are involved in the induction of natural repair mechanisms such as regulation of immune response, pro-angiogenesis, and tissue regeneration [25, 26, 55, 56], while ncMo are known for their patrolling behaviour, surveying the endothelium for signs of inflammation or damage [26, 57]. In addition, despite being associated with inflammatory disease progression, ncMo are crucial for vascular homeostasis, removing damaged cells and debris from the endothelium, displaying an important role in wound-healing, collagen deposition, angiogenesis, and resolution of inflammation by linking innate to adaptive immune

response [57–60]. Moreover, a recent study by van de Bossche *et al.* [54] describes that, after completing their tissue-cleaning task, monocytes can migrate through the lymphatic system into the bloodstream, allowing phagolysosomal content to be evaluated and damage detection in this tissue to be used as a marker for therapeutic monitoring of several disease conditions. In this context, the differences in the frequency of peripheral ncMo observed between responders and non-responders after CRT can be explained by this behaviour of ncMo in the process of healing and cleaning of cardiac tissue and migration to peripheral blood.

Concerning cytokine production by monocytes, even responders showed an increased frequency of these cells expressing IL-6 and IL-1β at both times of evaluation, suggesting once again that CRT was not able to interfere with the functional inflammatory ability of monocytes. Interestingly, in the analysis of IL-6-producing mDC, although both responders and non-responders showed a higher frequency of cells at baseline, after CRT this difference disappears in responders. In this context, our results suggest that, after reverse remodelling, mDC might display lower functional inflammatory capacity. Furthermore, although CRT fails to reduce the inflammatory capacity of monocytes, the responder group showed a lower frequency of IL1β-producing mDC at follow-up compared to baseline, as well as lower values of IL-6 producing-mDC compared to non-responders. At this point, it can be concluded that there is also a tendency for CRT to suppress the inflammation produced by DC, but inflammatory values after stimulation remain higher than the HG.

Regarding the DC subpopulations, only responders presented lower frequency and absolute values of pDC at baseline compared to the HG. As producers of massive amounts of type-I IFNs when activated, pDC have been implicated in the development of autoimmune and inflammatory diseases [61–63]. In our work, the low frequency of pDC at baseline seems to be an indicator of positive response to CRT.

Another remarkable result of our study is the decrease in CD86 expression by monocytes and DC after CRT compared to the initial evaluation, suggesting an immune modulating role of CRT, whether in responder or non-responder patients, due to a lower ability of monocytes and DC to provide the second antigen-independent cosignal to T cells, which may compromise the adaptive immune response on the inflammatory process in HF. Interestingly, the increased frequency of pDC expressing CD86 observed in the general HF population at 6-month follow-up was primarily seen in non-responders.

Our group recently published a study on Treg cells in patients with HF. We showed that peripheral blood Treg cells were decreased in patients and remained reduced after CRT [64]. On the other hand, MI studies performed in animal models describe the recruitment of these tolerogenic cells to the heart in order to suppress the inflammatory response [65, 66]. Furthermore, it is described that Treg cells can not only improve healing after MI but also trigger monocyte differentiation [66]. In this sense, after CRT, the migration of Treg cells to the failing heart may continue to occur, which could, at least in part, explain the decrease in CD86 expression by monocytes and DC and the differentiation of monocytes into iMo and ncMo.

The present study has some limitations. Despite being a homogeneous population (with advanced HF submitted to CRT), one important limitation of our study is the small sample size, especially in comparisons between subgroups. Other studies with larger samples are needed to prove whether the reduction of monocytes and DC is in fact related to the positive response to CRT. Another limitation is the short follow-up period. We did not experience whether the possible anti-inflammatory effect of CRT is sustained over time.

In conclusion, our research suggests that the innate immune system participates in cardiac reverse remodelling and response to CRT. HF patients with less pDC appear to be more prone to respond to CRT, and the decrease in cMo values (which have proinflammatory effects) with the increase in iMo (with beneficial and anti-inflammatory properties) and ncMo (important in wound-healing) after CRT seem to be related to successful reverse cardiac remodelling. Furthermore, CRT is associated with a reduction in the amount of CD86 expressed by monocytes and DC subsets and in their potential to produce pro-inflammatory cytokines, which may influence the connection between the innate and adaptive immune response, contributing, at least in part, to the previously described anti-inflammatory effects of CRT.

3.6. References

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Chapter 4

Impact of cardiac resynchronization therapy on circulating IL-17-producing cells in patients with advanced heart failure

Martins S, Carvalheiro T, Laranjeira P, Martinho A, Elvas L, Gonçalves L, Tomaz C, António N, Paiva A.

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Chapter 4

4.1. Abstract

Purpose: IL-17-producing T cells have been implicated in the inflammatory milieu of chronic heart failure (CHF), which implies a dismal prognosis in affected patients. The aim of this study was to evaluate the impact of cardiac resynchronization therapy (CRT) on the frequency and functional activity of Th17 and Tc17 cells, as well as, on IL-17 mRNA expression in patients with CHF.

Methods: Twenty-eight patients with CHF, analysed before CRT (T0) and 6 months later (T6), and 15 healthy controls (HC) were enrolled in this study. Circulating Th17 and Tc17 cells were evaluated by flow cytometry. The quantification of IL-17A mRNA expression was performed by real-time PCR.

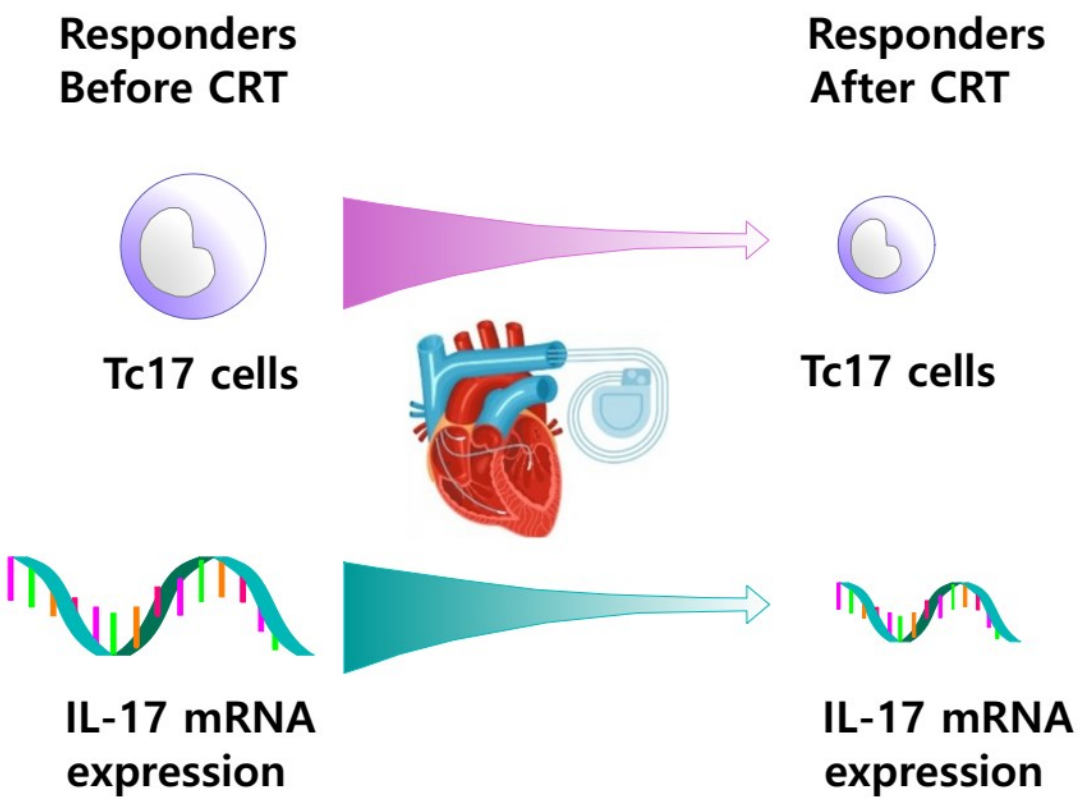
Results: Circulating Tc17 cells tended to be higher in CHF patients submitted to CRT than in HC (0.92% (0.24–3.32) versus 0.60% (0.09–3.68), although not reaching statistical significance. The frequency of Tc17 cells in CHF patients significantly decreases after CRT reaching levels similar to those of HC (0.92% (0.24–3.32) at T0 versus 0.56% (0.21–4.20) at T6, $P < 0.05$), mainly due to responders to CRT. Additionally, the expression of IL-17 mRNA was detected in a few number of responder patients at T0 (27%) and only detected in one responder at T6 (7%). Conversely, in non-responders, the proportion of patients exhibiting IL-17 mRNA expression increases from baseline (17%) to T6 (42%). No significant differences were observed in Th17 cells between HC, CHF patients in T0 and patients in T6.

Conclusion: The inflammatory response mediated by circulating IL-17-producing cells seems to be suppressed by CRT, particularly in responders.

Keywords

Chronic heart failure; cardiac resynchronization therapy; Th17 cell; Tc17 cell; cytokines

4.1.1. Graphical abstract



4.2. Introduction

Heart failure (HF) is a complex clinic pathophysiological syndrome with a large impact on modern societies due to its high mortality and morbidity [1, 2]. The histological features of HF include loss of myocardial cells and restructuring of the extracellular matrix. Myocardial fibrosis may be caused by humoral factors as cytokines, growth factors, and hormones, suggesting that immunologic and inflammatory responses play a significant role in the development and progression of HF [3–7]. Pro-inflammatory cytokines may have an adverse impact on left ventricular function, due to a negative inotropic effect and induction of ventricular remodelling [8].

Peripheral blood mononuclear cells (PBMC), like T cells and monocytes, have been suggested as a potential source for extended systemic cytokine production in CHF [7, 9]. Like T helper (Th) 1 cells, Th17 cells were recently implicated in the pathogenesis of chronic inflammatory and autoimmune disorders, including rheumatoid arthritis, multiple sclerosis, psoriasis, and inflammatory bowel disease [10]. Furthermore, Th17 cells seem to possess more potent abilities to induce inflammatory diseases, comparing with Th1 cells, being described that interleukin (IL)17 plays an important role in coordinating tissue inflammation and up-regulating a range of proinflammatory mediators, including tumor necrosis factor (TNF)-α, IL-1, IL-6, IL-8, and matrix metalloproteinases [10, 11].

The pathogenic role of the Th1/Th2 imbalance in the development of CHF is well accepted, but the role of Th17 and of other IL-17-producing cells, remains unclear and contradictory data have been reported [12–14]. A Th17/Treg imbalance, characterized by an increased frequency of Th17 cells and decreased frequency of Treg cells, was described in CHF, suggesting a potential role of these cells in the pathogenesis of the disease [13]. On the contrary, other study showed no differences in circulating Th17 cells among CHF patients and healthy individuals [15].

Similar to Th17 cells, a new subpopulation of $CD8^+$ T cells producing IL-17 (Tc17) cells was demonstrated to be increased in several chronic diseases, such as spontaneous urticaria, rheumatoid arthritis, and systemic lupus erythematosus [16–18]. In normal human peripheral blood, Tc17 cells are coregulated with Th17 cells during differentiation [19, 20] and it seems to exist a cooperative or synergistic function between Th17 and Tc17 cells in T cell-mediated immunity [19]. These observations support the hypothesis of the participation of Tc17 cells in the inflammatory environment of CHF; however, the role Tc17 cells in CHF is still unknown.

Cardiac resynchronization therapy (CRT) is a well-recognized, highly effective device-based treatment for advanced CHF patients aiming to restore mechanical synchrony [21, 22]. However, at least 30% of patients receiving CRT do not respond to this therapy [23–25]. Moreover, some studies have shown a reduction in inflammatory mediators in HF patients treated with CRT [26–28] and, in small and medium-sized trials, these observations were mainly observed in responder patients [29–31]. However, the impact of CRT in Th17 and Tc17 cells in CHF patients is still unknown. Therefore, we aimed to study the impact of CRT on IL-17-producing T cells, by evaluating the frequency and functional activity of Th17 and Tc17 cells in CHF patients submitted to CRT, as well as, IL-17 mRNA expression in circulating leukocytes.

4.3. Methods

4.3.1. Patient population

Twenty-eight patients with advanced heart failure scheduled for CRT, between 2010 and 2013, were prospectively enrolled in this study; their mean age was 60.3 ± 10.5 years, 18 patients were male and 10 were female (Table 4.1). Patients were assisted and followed up in the tertiary Cardiology Department, Centro Hospitalar e Universitário de Coimbra.

All patients were under stable, optimal pharmacological therapy for CHF at the time of inclusion, which includes an angiotensin-converting enzyme inhibitor or angiotensin receptor blocker, β-blocker, and aldosterone antagonist, unless contra-indicated or not tolerated. The inclusion criteria were as follows: class III or IV according to NYHA (New York Heart Association); left ventricular (LV) dysfunction with a LV ejection fraction (LVEF) ≤35%; QRS ≥120 ms with left bundle branch block; and normal sinus rhythm [24, 25, 32, 33]. The exclusion criteria included conditions that might influence the inflammatory response: clinical or biochemical manifestation of the presence of concomitant inflammatory disease; patients taking regular nonsteroidal anti-inflammatory drugs or patients on anticoagulants; active infections; known autoimmune or malignant diseases; severe valvular disease or congenital heart disease; cardiogenic shock; continuously or intermittently intravenous inotropic therapy; pregnancy; deep vein thrombosis or pulmonary embolism; severe peripheral arterial occlusive disease; severe and non-controlled arterial hypertension (systolic blood pressure > 180 mmHg or diastolic > 110 mmHg); comorbidities associated with a life expectancy less than 1 year; recent trauma or surgery (< 1 month); recent major

bleeding (< 6 months) requiring blood transfusion; renal insufficiency (creatinine > 2.0 mg/dl); anemia (hemoglobin < 8.5 g/dl) or thrombocytopenia (< 100,000/L); atrial fibrillation; prior arterial coronary bypass surgery; acute coronary syndrome, or percutaneous coronary intervention within 3 months; previously implanted CRT system; and excessive alcohol consumption or illicit drug abuse.

A baseline assessment of heart failure patients (HFP) scheduled for CRT (T0) performed before the device implantation ensured candidate eligibility.

Six months after CRT (T6), the patients were re-evaluated for the same variables.

4.3.1.1 Echocardiographic Evaluation

Each patient underwent echocardiographic assessment at T0 and T6. Standard echocardiography was performed using a Vivid 7 (GE Healthcare, Oslo, Norway) and 1.7-/3.4-MHz tissue harmonic transducer. Loops and three cardiac cycles were stored digitally and analysed offline using a customized software package (EchoPAC, GE Healthcare). The LV end-diastolic volume (LVEDV), LV end-systolic volume (LVESV), and LVEF were assessed by the biplane Simpson's equation in apical four-chamber and two-chamber views.

4.3.1.2. Definition of response to CRT

We classified responders to CRT as patients who were still alive and showed at least a 15% reduction in LVESV at the 6-month follow-up compared to baseline [34–37].

4.3.2. Healthy Control Group

The control group was composed by 15 sex- and age-matched healthy individuals (age 54 ± 12 years old; gender 7 females and 8 males). The inclusion criteria involved are the following: normal lipid profile, normal body mass, and normal cardiac evaluation. Exclusion criteria included the following: family history of heart disease and/or cardiomyopathy, active infections or inflammatory process, consumption of any drugs and/or alcohol, and inability to understand the informed consent.

4.3.3. Blood samples

Fasting blood samples were taken for chemistry assessment (including fasting glycaemia, creatinine, brain natriuretic peptide (BNP), high sensitivity C-reactive protein (hs-CRP), and hematological parameters) in all patients, at admission, just before the device implantation. Peripheral blood (PB) samples were collected from each individual in heparinized and Paxgene tubes, at T0 and T6, to analyse the inflammatory parameters and IL-17 mRNA expression.

4.3.3.1. Multiparameter flow-cytometry immunophenotypic studies of Th17 and Tc17 subsets

In vitro stimulation of PB T cells was performed as described by others [18].

Briefly, 500 μL of each PB sample were diluted 1:1 (vol/ vol), in RPMI-1640 medium (Gibco, Life Technologies, Paisley, Scotland, UK), supplemented with 2 mM L-glutamine. T cells were stimulated with 50 ng/mL of phorbol 12- myristate 13-acetate (PMA) (Sigma, Saint Louis, MO, USA) and 1 μg/mL of ionomycin (Sigma); after the addition of 10 μg/mL of brefeldin A (Sigma). The samples were incubated for 4h at 37 °C, in a humidified incubator with 5% CO_2 concentration.

Each cultured PB sample was aliquoted in three different tubes (200 mL/tube) and incubated with the following monoclonal antibodies: anti-CD3 peridinin-chlorophyll proteins-cyanine 5.5 (PerCP-Cy5.5) (clone SK7; Becton Dickinson Biosciences (BD), San Jose, CA, USA) and anti-CD8 allophycocyanin (APC) (clone B9.11; Beckman Coulter—Immunotech, Marseille, France). Then, a cell permeabilization protocol, with IntraPrep Permeabilization Reagent (Beckman Coulter, Brea, CA, USA), and an intracytoplasmic staining protocol was followed, according to manufacturer's instructions, in order to analyse the intracellular expression of IL-17 conjugated with phycoerythrin (PE) (clone 41802; R&D Systems, McKinley Place, MN, USA). Cell aliquots were also stained separately with IL-2 (clone MQ1-17H12; BD Pharmingen, San Diego, CA, USA), TNF-α (clone MAb11; BD Pharmingen), and interferon (IFN)-γ (clone 4S.B3; BD Pharmingen), all conjugated with fluorescein isothiocyanate (FITC). Finally, cells were resuspended in 0.5 mL of phosphate buffer saline (PBS) (Gibco BRL, Life Technologies, Vienna, Austria) and then acquired in a flow cytometer.

4.3.3.2. Flow cytometry data acquisition and analysis

Data acquisition was performed in a FACSCalibur flow cytometer (BD) equipped with an argon ion laser and a red diode laser.

Among positive CD3 cells, CD4 positive T cells were identified by the absence of CD8; and CD8 positive T cells were identified by the co-expression of CD3 and CD8. The cytokine production (IL-2, TNF-α, and IFN-γ) was evaluated in IL-17 positive cells, within $CD4^+$ and $CD8^+$ T cells, on an electronic $CD3^+$ gate with at least 20,000 events, after a first acquisition step of 20,000 of total events.

Results illustrate the percentage of positive cells within each cell subset or/and their mean fluorescence intensity (MFI).

Data were analysed using the Infinicyt™ software, V.1.5 (Cytognos SL, Salamanca, Spain).

4.3.3.3. Gene expression analysis

Analysis of IL-17A mRNA expression from whole blood was performed in blood collected in a PAXgene Blood RNATube (PreAnalytiX GmbH, Switzerland) with automated RNA purification in QIAcube (Qiagen, Hilden, Germany). One microgram of RNA was reverse transcribed with iScript TM Reverse Transcription Supermix for RTqPCR (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. Relative quantification of gene expression by real-time PCR was performed using a thermocycler (LightCycler 480 II; Roche, Basel, Switzerland). Normalization for gene expression quantification was performed with a geNorm Housekeeping Gene Selection Human Kit (Primer Design, Southampton, UK) and geNorm software (Center for Medical Genetics, Ghent University Hospital, Ghent, Belgium) to select optimal housekeeping genes for this study [38]. Real-time PCR reactions used specific QuantiTect Primer Assays (Qiagen) with optimized primers for IL-17A (QT00009233) and endogenous controls TOP1 (QT00068915) and SF3A1 (QT00061257), together with QuantiTect SYBR Green PCR Kit Gene expression (Qiagen), according to the manufacturer's instructions. Reactions were performed with the following thermal profile — 10 min at 95 °C plus 50 cycles of 10 s at 95 °C, 20 s at 55 °C, and 30 s at 72 °C. Quantitative real-time PCR results were analysed with LightCycler 480 software (Roche) and quantification was performed using the qBasePlus software package (Biogazelle, Zulte, Belgium).

4.3.4. Statistical analysis

Statistical analyses were performed using the non-parametric Mann-Whitney *U* test for independent variables. Wilcoxon signed-rank test was used to compare T0 vs. T6. Results were expressed as mean ± standard deviation or median (range). All statistical analyses were performed using Statistical Package for Social Sciences IBM SPSS 20 (IBM, Armonk, NY. USA) and Graphpad Prism version 5 (GraphPad Software, San Diego, CA, USA). Differences were considered to be statistically significant when the *P* value was < 0.05.

4.4. Results

4.4.1. Clinical evolution

The baseline characteristics of the studied population are described in Table 4.1. Before CRT, 14.3% of the patients (n = 4) were in NYHA class IV and 85.7% (n = 24) in class III. At the 6-month follow-up, the proportion of responders to CRT, according to the echocardiographic definition, was 55.6%.

As shown in Table 4.1, there were no statistically significant differences between responders and non-responders to CRT, regarding baseline characteristics. Despite the baseline longer QRS duration and the higher high sensitivity C-reactive protein (hs-CRP) and B-type natriuretic peptide (BNP) levels of non-responders by comparison to responders to CRT, these differences were not statistically significant at baseline assessment. After CRT, responders showed significantly lower BNP levels compared with non-responders. During the 6-month follow-up, one HFP died (due to HF) and none has been transplanted.

Table 4.1 – Characteristics of the heart failure patients enrolled in the study.

	Global Population Mean ± standard deviation (n = 28)	Responders Mean ± standard deviation (n = 15)	Non-Responders Mean ± standard deviation (n = 13)	*P* Value
Baseline assessment				
Age (years)	61.3 ± 10.5	65.2 ± 9.6	56.8± 9.8	0.011
Gender (Male/Female)	18 / 10	9 / 6	9 / 4	0.989
Aetiology (Non-Ischemic/Ischemic)	22 / 6	12 / 3	10/ 3	0.308
NYHA (III/IV)	24 / 4	14 / 1	10 /3	0.426
LVEF (%)	25.6 ± 7.0	24.9 ± 6.4	25.5 ± 7.6	0.274
LVESV (mL)	183.6 ± 95.8	178.4 ± 62.1	215.3 ± 124.6	0.465
LVEDV (mL)	235.5 ± 94.0	230.5 ± 64.0	264.1 ± 121.7	0.419
QRS	144.5 ± 31.0	138.8 ± 14.6	148.3 ± 38.6	0.447
hs-CRP (mg/dL)	5.8 ± 6.2	4.7 ± 4.3	7.0 ± 8.8	0.408
BNP (pg/mL)	262.9 ± 188.2	207.3 ±126.1	324.2 ±230.5	0.160
Cholesterol (mg/dL)	184.6 ± 58.9	191.1 ± 60.0	171.1 ± 45.3	0.338
Triglycerides (mg/dL)	134.5 ± 55.8	118.7 ± 51.0	143.1 ± 61.6	0.230
Uric Acid (mg/dL)	6.2 ± 1.7	5.7 ± 1.6	6.7 ± 1.9	0.149
After CRT				
hs-CRP (mg/dL)	4.0 ± 4.6	2.7 ± 1.8	5.9 ± 6.7	0.118
BNP (pg/mL)	189.9 ± 295.0	80.3 ±118.3	336.0 ± 395.3	0.033

NYHA, New York Heart Association; *LVEF*, left ventricular ejection fraction; *LVESV*, left ventricular end-systolic volume; *LVEDV*, left ventricular end-diastolic volume; *hs-CRP*, high sensitivity C-reactive protein; *BNP*, B-type natriuretic peptide.

4.4.2. Frequency of circulating Th17 and Tc17 cells in heart failure patients

As shown in Table 4.2, when considering all HF patients together, the frequency of Tc17 cells at baseline (HFP-T0) displayed a tendency to be increased in comparison

to the healthy group (HG) (P > 0.05). The percentage of Tc17 cells was also significantly higher at HFP-T0 compared to 6 months after CRT. Notably, at T6, the frequency of Tc17 decreased to the same levels observed in HG. In the same line, a slight increase of the amount of IL-17 produced at single cell level (MFI) in those cells was observed in HF patients, both at T0 and T6, compared to the control group. No significant differences were observed in Th17 cells among the three studied groups. The decrease in the frequency of Tc17 cells from baseline to T6 is even more significant in responders to CRT. Regarding the comparison between responders and non-responders to CRT, we found no statistically significant differences in the frequency of Tc17 or Th17 cells, neither for the baseline values nor for the 6-month follow-up frequencies.

4.4.3. Functional characterization of peripheral blood Th17 and Tc17 cells from heart failure patients

Considering all HF patients together, no differences were found in the frequency of Th17 and Tc17 cells producing IL-2, TNF-α, or IFN-γ, neither among HG and HFP, nor when comparing T0 and T6. However, responders to CRT presented at baseline an increased percentage of Tc cells IFN-γ^+/IL-17^+ compared to non-responders (P < 0.05) (Figure 4.1).

4.4.4. IL-17 mRNA expression in whole peripheral blood cells from heart failure patients

IL-17 mRNA was detected in a small proportion of patients (22%, 6 out 27) and almost undetectable in HG (7%, 1 out 15) (Figure 4.2A).

Interestingly, in responders to CRT, IL-17 mRNA expression was detected in a few number of patients at baseline (27%, 4 out 15) and only detected in one patient 6 months after CRT (7%, 1 out 15) (Figure 4.2A and B). Conversely, in non-responders, the proportion of patients exhibiting IL-17 mRNA expression increases from baseline (17%, 2 out 12) to T6 (42%, 5 out 12) (Figure 4.2A and B). Furthermore, this increase was associated to a higher IL-17 mRNA expression at T6 compared with baseline (P < 0.05) (Figure 4.2A).

Table 4.2 – Frequency of Th17 cells among $CD4^+$ T cells and of Tc17 among $CD8^+$ T cells, and amount of IL-17 per cell (MFI) in the different groups, after in vitro stimulation with PMA plus Ionomycin.

	% Th17	IL-17 MFI in Th17 cells	% Tc17	IL-17 MFI in Tc17 cells
HG (n = 15)	1.83 (0.28-3.70)	79.06 (44.72-364.33)	0.60 (0.09-3.68)	**57.45 (16.18-167.56)**
P values HG vs HFP-To	*0.339*	*0.126*	*0.268*	***0.007***
HFP– To (n = 28)	1.66 (0.52-6.22)	111.53 (55.32-245.52)	**0.92 (0.24-3.32)**	**87.20 (31.02-210.71)**
P values HFP-To vs. HFP-T6	*0.716*	*0.509*	***0.026***	*0.412*
HFP – T6 (n = 28)	1.80 (0.10-6.49)	92.70 (38.99-476.49)	**0.56 (0.21-4.20)**	**88.75 (24.26-416.66)**
P values HG vs HFP-T6	*0.665*	*0.593*	*0.959*	***0.050***
Responders (n=15)				
P values HG vs HFP-To	*0.709*	*0.178*	*0.384*	***0.004***
HFP – To	1.39 (0.52-6.15)	100.61 (55.32-229.20)	**0.91 (0.24-3.32)**	**117.82 (31.02-210.71)**
P values HFP-To vs. HFP-T6	*0.865*	*0.532*	***0.020***	*0.334*
HFP – T6	1.67 (0.26-4.26)	80.46 (51.11-236.35)	**0.37 (0.21-2.10)**	83.35 (24.26-194.03)
P values HG vs HFP-T6	*0.836*	*0.852*	*0.604*	*0.120*
Non-responders (n = 13)				
P values HG vs HFP-To	*0.189*	*0.205*	*0.300*	*0.102*
HFP – To	2.02 (0.63-6.22)	122.45 (58.93-245.52)	1.08 (0.28-2.18)	71.80 (32.23-179.08)
P values HFP-To vs. HFP-T6	*0.727*	*0.650*	*0.382*	*0.807*
HFP – T6	1.92 (0.10-6.49)	117.80 (38.99-476.49)	0.64 (0.30-4.20)	88.94 (43.35-416.66)
P values HG vs HFP-T6	*0.580*	*0.447*	*0.628*	*0.069*

Results expressed as median (minimum-maximum). Statistically significant differences were considered when $P < 0.05$. Mann–Whitney *U* test was used to compare HFP-To versus HG and HFP-T6 versus HG. Wilcoxon signed rank test was used to compare HFP-To versus HFP-T6. *HG*: Healthy control group; *HFP-To*: Heart failure patients at baseline assessment; *HFP-T6*: Heart failure patients 6 months after cardiac resynchronization therapy (CRT) implantation.

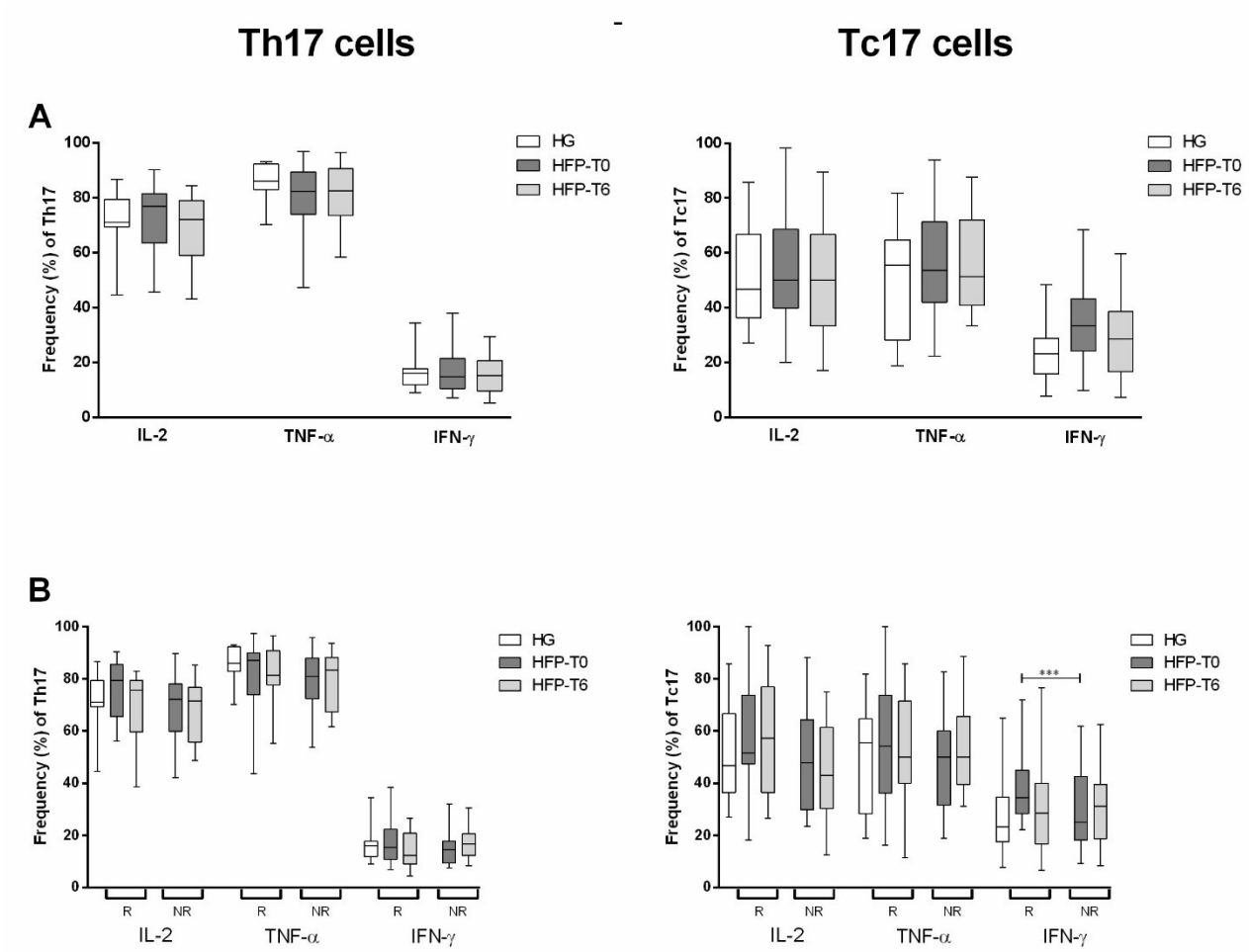


Figure 4.1 – Functional characterization of peripheral blood Th17 and Tc17 cells. The percentage of IL-2, TNF-α, and IFN-γ-producing Th17 and Tc17 cells was evaluated in healthy individuals (HG) and heart failure patients (HFP) distributed as follows: **A** total patients at baseline assessment (HFP-T0) and 6 months after cardiac resynchronization therapy implantation (HFP-T6); and **B** according to the response to cardiac resynchronization therapy: responders (R) and non-responders (NR) patients. ***Statistically significant differences were considered when $P < 0.05$

A

	% of cases expressing IL-17 mRNA (n)	Median (minimum-maximum)*
HG	7% (1/15)	1.74 (-)
HFP-T0	22% (6/27)	0.65 (0.28-0.75)
HFP-T6	22% (6/27)	1.99 (0.32-5.35)
According to response to cardiac resynchronization therapy		
HFP-T0	27% (4/15)	0.65 (0.28-0.75)
HFP-T6	7% (1/15)	0.77 (-)
HFP-T0	17% (2/12)	0.61 (0.51-0.71)***
HFP-T6	42% (5/12)	2.18 (0.32-5.35)

B

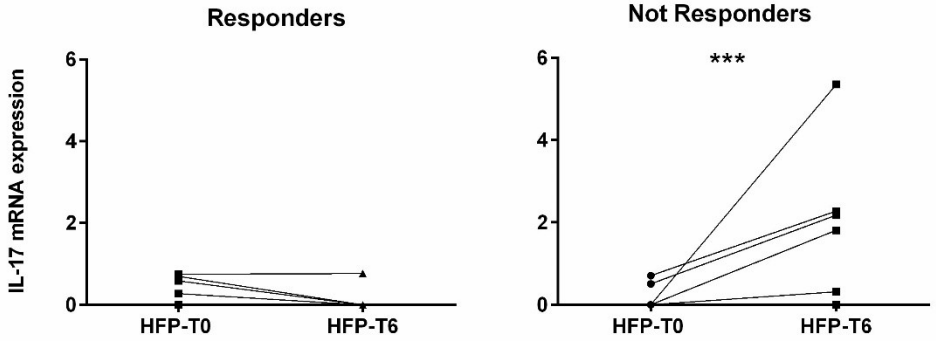


Figure 4.2 – **A** IL-17 mRNA expression in whole peripheral blood cells from healthy individuals (HG, n = 15) and heart failure patients (n = 27), distributed according to the response to cardiac resynchronization therapy. The results are expressed as percentage (number of cases) wherein mRNA expression was detected by RT-PCR. *Only considering the samples in which IL-17 mRNA was detected. **B** IL- 17 mRNA expression on whole PB cells from heart failure patients distributed according to the response to cardiac resynchronization therapy. *** Statistical significant differences were considered when $P < 0.05$.

4.5. Discussion

CHF is characterized by a chronic inflammatory status. T cells seem to be part of the inflammatory response during CHF, independently of the aetiology of the disorder [9]. To the best of our knowledge, this study is the first that has evaluated the impact of CRT on peripheral blood Th17 and Tc17 cells. Here, we demonstrate that responders to CRT exhibit a reduction in Tc17 cells reaching similar levels of healthy controls.

T cells are involved in the pathogenesis of cardiac disease, both by direct cytotoxicity and by enhancing the inflammatory functions of other cells [39]. Th17 cells and/or IL-17 seem to be involved in the maintenance of chronic inflammation in CHF [13, 40 44], although there are no studies describing the role of Tc17 cells in heart diseases. Based in recent reports made by our group, describing the involvement of Tc17 cells in other inflammatory diseases, we considered relevant to study the behaviour of both Th17 and Tc17 cells in patients with advanced HF submitted to CRT [16–18].

Multiple lines of evidence suggest crucial roles of IL-17 in cardiac pathology [40, 42, 43]. IL-17 can stimulate epithelial cells, endothelial cells, fibroblasts, and other cells to release massive cytokines such as granulocyte colony-stimulating factor (G-CSF), IL-6, and matrix metalloproteinase [40, 41]. Consequently, IL-17 can inhibit the reconstruction of the heart by myocardial fibrosis through dissolution, breakage, and reduced synthesis of intercellular collagen. Studies made in animal models raised the hypothesis that IL-17 can cause damage in the heart through several mechanisms: through direct toxic effects on myocardial cells, reduction in myocardial intracellular calcium levels, and enhancement of the activity of pro-inflammatory cytokines such IL-6 and IL-1β. These mechanisms may lead to cardiac hypertrophy, increased cellular necrosis, accelerated myocardial apoptosis, and extracellular matrix remodelling and, thus, accelerate heart failure progression [41].

CRT can reduce both morbidity and mortality in a subset of patients with HF; however, it is not well known how CRT affects the immune system. One study made in a small cohort of patients with HF reports a decrease of inflammatory markers, as IL-8, IL-6, monocyte chemoattractant protein 1 (MCP-1), and B-type natriuretic peptide (BNP), 6 months after CRT [45]. In our work, levels of Th17 cells in HFP were similar to the control group levels. This finding is in agreement with another report that describes comparable results in circulating Th17 cells frequencies, serum IL-17 levels, and ROR-γt expression between CHF patients and healthy individuals [15]. Considering Tc17 cells, we observed a trend to an augmented frequency among HFP, compared to healthy

controls, accompanied by an increased amount of IL-17 produced *per cell* (MFI) in HFP. When we evaluated the same HF patients 6 months after CRT, we found a significant decrease in Tc17 frequency. Notably, this decrease is mainly due to the responder group, which showed a significant decrease in the frequency of Tc17 cells from baseline (T0) to T6.

These achievements seemed to be in agreement with the lower number of responder patients in whom had been detected IL-17 mRNA expression and the maintenance/decrease of mRNA levels observed. On contrary, we found a higher number of cases with detectable IL-17 mRNA, as well as, an increase of IL-17 mRNA expression between T6 and T0, in non-responders patients.

The decrease of IL-17-producing T cells in HFP after CRT implantation is possibly related to the reduction of the inflammatory process inherent to CHF and, probably, with the improvement of the cardiac function. The definition of response to CRT based on LV reverse remodelling has been used in the major clinical trials on CRT. However, the final objective of CRT is to prolong survival and/or to alleviate heart failure symptoms and a positive reverse remodelling response to CRT does not necessarily parallel a favourable outcome or symptomatic benefit [46]. Therefore, we may speculate that the anti-inflammatory effect of CRT could be a useful complementary marker to include in a composite definition of response to CRT.

From a functional point of view, Th17 cells are largely defined by the production of IL-17A and IL-17F, which have the ability to recruit neutrophils, possessing a proinflammatory function [47]. Th17 cells can also produce several other inflammatory cytokines such as IL-21, IL-22, IL-6, TNF-α, and IFN-γ [48]. In the failing heart, elevated left ventricular end-diastolic wall stress causes myocardial expression of cytokines, which directly or indirectly influence left ventricular contractile performance and remodelling. The proinflammatory cytokines, namely TNF-α, IL-1, and IL-6, lead to monocyte activation, while IL-2 leads to T-cell activation. Taken together, they promote monocyte-endothelial cell adhesive interaction, with subsequent cytokine production and free radical generation, giving rise to inflammation, tissue destruction, cardiovascular remodelling, and loss of function [49]. In an attempt to assess the impact of CRT on the functional inflammatory responses of Th17 and Tc17 cells in patients with CHF, we analysed the frequency of these cells producing not only IL-17 but also IL-2, TNF-α, and IFN-γ, after stimulation *in vitro*. We found no significant differences in the expression of IL-2, TNF-α, and IFN-γ by Th17 and Tc17 cells between healthy individuals and HFP, in both moments of evaluation, but responder patients

presented a significantly higher expression of IFN-γ by Tc17 cells. Tc17 cells possess a high plasticity and can convert to IL-17/IFN-γ-double producing cells (Tc17/IFN-γ cells), permitted by IL-12 signalling, with distinct properties from Tc1 lineage. In addition to their highly cytotoxic and antitumor activity, Tc17/ IFN-γ cells were found to be implicated in various inflammatory conditions in human and animal models [16, 17, 50].

In the case of T cell-mediated inflammation in the heart, IFN-γ has a complex combination of pro-inflammatory and anti-inflammatory effects, including a feedback inhibition of T cell activation and effector functions [39]. Since Tc17 cells display a greater plasticity of the cytokine-producing phenotype than their Th17 counterparts [51], we can speculate that this could translate into a different pathogenic role of Tc17 cells in CHF and could explain why only Tc17 cells are reduced by CRT. Taken together, our study raises novel insights on the impact of CRT over the immune behaviour of Th17 and Tc17 cells in advanced HF. The inflammatory response mediated by IL-17 producing cells seems to be effectively reduced by CRT, particularly Tc17, and this immune benefit may contribute to the positive response to CRT.

4.5.1. Limitations

The main limitation of our study is the small sample size. However, since the evaluation was performed in two different moments (before and after CRT), this study has the strength that each patient served as his own control. Another limitation is the short follow-up. As inflammation was not re-evaluated after the 6-month follow-up, we do not know whether the possible anti-inflammatory effect of CRT is sustained over time. Finally, we did not investigate whether changes in IL-17-producing cells were associated with clinical benefit after CRT and further studies are required to evaluate if the alleviation of inflammatory status translates into improved prognosis after CRT.

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Chapter 5

Reduced numbers of regulatory T cells in chronic heart failure seems not to be restored by cardiac resynchronization therapy

Martins S, António N, Carvalheiro T, Laranjeira P, Rodrigues R, Gonçalves L, Tomaz C, Paiva A.

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Chapter 5

5.1. Abstract

Background: T cells have been implicated in the development and progression of inflammatory processes in chronic heart failure (CHF). Cardiac resynchronization therapy (CRT) has beneficial effects on symptoms and cardiac remodelling in CHF. However, its impact on the inflammatory immune response remains controversial. We aimed to study the impact of CRT on T cells in heart failure (HF) patients.

Methods: Thirty-nine HF patients were evaluated before CRT (T0) and six months later (T6). Quantification of T cells, their subsets, and their functional characterization, after *in vitro* stimulation, were evaluated by flow cytometry.

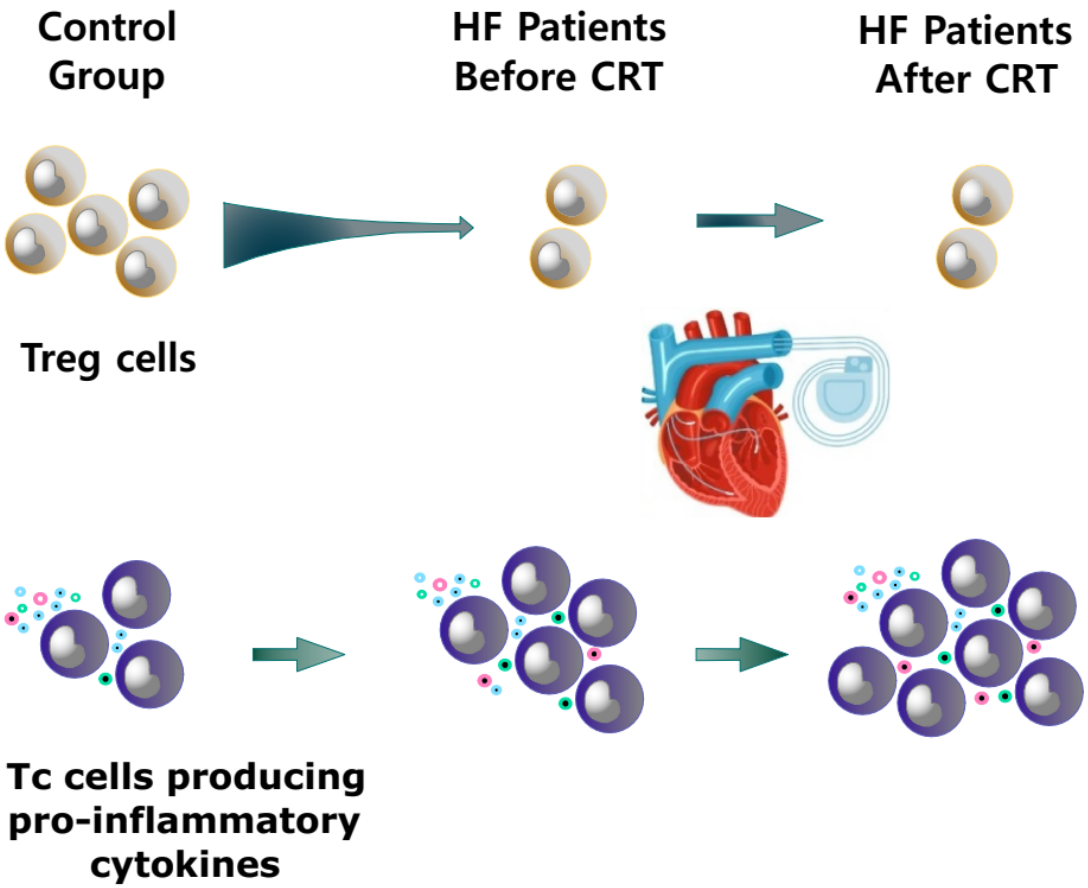
Results: T regulatory (Treg) cells were decreased in CHF patients (healthy group (HG): 1.08±0.50 *versus* (heart failure patients (HFP)-T0: 0.69±0.40, *P*=0.022) and remaining diminished after CRT (HFP-T6: 0.61±0.29, *P* = 0.003). Responders (R) to CRT presented a higher frequency of T cytotoxic (Tc) cells producing IL-2 at T0 compared with non-responders (NR) (R: 36.52±12.55 *versus* NR: 24.71±11.66, *P* = 0.006). After CRT, HF patients presented a higher percentage of Tc cells expressing TNF-α and IFN-γ (HG: 44.50±16.62 *versus* R: 61.47±20.54, *P*=0.014; and HG: 40.62±15.36 *versus* R: 52.39±18.66, *P* = 0.049, respectively).

Conclusion: The dynamic of different functional T cell subpopulations is significantly altered in CHF, which results in an exacerbated pro-inflammatory response. Even after CRT, it seems that the inflammatory condition underlying CHF continues to evolve with the progression of the disease. This could be due, at least in part, to the inability to restore Treg cells levels.

Trial registration: Observational and prospective study with no trial registration.

Keywords: chronic heart failure; cardiac resynchronization therapy; immune response; T cells, cytokines profile

5.1.1. Graphical abstract



5.2. Introduction

Chronic heart failure (CHF) is a common and debilitating disorder [1, 2] with significant rates of morbidity and mortality in modern societies [3]. The pathophysiology of CHF implicates progressive myocardial dysfunction associated with continuous ventricular remodelling, which is, by itself, a complex and multifactorial process [4, 5]. It is well established that multiple factors such as neurohumoral mediators, enzymes, oxidative stress and mechanical stress, as well as inflammation, are involved in pathological left ventricular (LV) remodelling and systolic dysfunction [4].

Multiple studies have demonstrated that the pro-inflammatory response contributes to the pathophysiology of CHF and that its up-regulation implies a dismal prognosis in affected patients [6-8].

T helper (Th) cells play a key role in several chronic inflammatory disorders and numerous studies propose their active participation in the pathogenesis of CHF [6-8]. The proinflammatory Th1 and Th17 cells are increased in CHF patients [9, 10]. Conversely, current evidence suggests that down-regulation or insufficient recruitment of regulatory T (Treg) cells results in worsened ventricular remodelling [11-13]. Moreover, it has been described that the Th1/Th2 imbalance [14] and the polarization of type 1 Th cells play a pathogenic role in CHF [10].

Treg cells seem to be reduced in different heart failure (HF) aetiologies and associated with a dismal prognosis. They can also participate in the pathophysiology of CHF by assuming an antiangiogenic and profibrotic profile Th1-like cells [15].

T cytotoxic (Tc) cells and their role in CHF have received less attention however, they seem to contribute to immune-mediated damage. Recent studies suggest that Tc cells contribute to cardiomyocyte apoptosis, adverse ventricular remodelling and deterioration of myocardial function [16]. Moreover, abundant Tc lymphocytes producing large amounts of IFN-γ were found in ischemic failing hearts [11].

Cardiac resynchronization therapy (CRT) is an effective treatment for patients with severe CHF. Based on biventricular pacing, CRT restores the electromechanical desynchrony of the heart, improving LV systolic function and reducing patient symptoms, re-hospitalizations and mortality [4, 17, 18]. Limited evidence suggests that CRT can reduce inflammatory mediators in HF patients [19], but the association between CRT response, T cells and cardiac remodelling in CHF is far from being

understood [4]. We performed an exploratory study to generate hypotheses based on the impact of CRT on the frequency and functional activity of T cell subpopulations in CHF patients submitted to CRT, by comparing baseline with post-CRT data.

5.3. Methods

5.3.1. Patient population

A total of thirty-nine consecutive and ambulatory patients with advanced heart failure, scheduled for CRT, were prospectively included in this study between 2010 and 2013; their mean age was 61.4 ± 10.5 years, 26 patients were male and 13 were female (Table 5.1). Patients were assisted and followed-up in a tertiary Cardiology Department (Centro Hospitalar e Universitário de Coimbra). To calculate the sample size, the software G*Power 3.1 was used [20]. Prior analysis was performed determining that 35 subjects would be needed for the study (Effect size dz:0.7, α error probability:0.05, power:0.80). Additionally, four elements were added to the sample as a matter of convenience.

At the time of inclusion, patients were under stable, optimal pharmacological therapy for CHF, including an angiotensin-converting enzyme (ACE) inhibitor or angiotensin receptor blocker, β-blocker, and aldosterone antagonist, unless contra-indicated or not tolerated. Inclusion criteria were restricted to patients with advanced heart failure, who met the criteria described in the guidelines, at that time, with class I recommendation for CRT: belonging to class II, III or IV according to NYHA (New York Heart Association); presenting LV ejection fraction (LVEF) ≤ 35%; QRS ≥ 120 ms with left bundle branch block morphology; and normal sinus rhythm [21, 22].

Exclusion criteria were defined, in an attempt to exclude any changes in patients that could interfere with our assessment of the immune response and bias the results.

As exclusion criteria we included several conditions that might influence the inflammatory response such as clinical or biochemical manifestation of the presence of concomitant inflammatory disease [23]; active infections (which could trigger an inflammatory immune response); autoimmune or malignant diseases (since T cells perform an important role in immune response in autoimmune diseases and cancer [24, 25]); severe valvular disease or congenital heart disease (because evidence indicates that an inflammatory state and immune alterations are present in patients

with valvular disease and congenital heart disease [26, 27]); cardiogenic shock (since implantation of CRT is not recommended at this stage); deep vein thrombosis or pulmonary embolism (because inflammation and coagulation are closely related, particularly in these diseases [28]); severe peripheral arterial occlusive disease (in which platelet activation and inflammation are usually abnormal [29]); severe and noncontrolled arterial hypertension (systolic blood pressure > 180 mmHg or diastolic > 110 mmHg) (it is described that the immune system, inflammation and hypertension are strongly related, and effector T and Treg cells play an important role in blood vessel constriction in hypertension [30]); recent trauma or surgery (< 1 month) (trauma can evoke a systemic reaction including a non-specific immune response which can result in multiple organ damage due to aggravated inflammation [31, 32]); recent major bleeding (< 6 months) requiring blood transfusion (considering that every blood component can promote inflammation [33]); renal insufficiency (creatinine > 2.0 mg/dl) (because inflammation is common in patients with chronic kidney disease) [34]); anaemia (haemoglobin < 8.5 g/dl) or thrombocytopenia (< 100000/L) (since alterations in haematological parameters may be linked to inflammatory processes described in infections, sepsis and anaemia of inflammation [35, 36]); pregnancy (considering that inflammation is essential for female reproduction and pregnancy is itself an inflammatory state [37] and radiation is also contraindicated during pregnancy); atrial fibrillation (because this arrhythmia is often associated with enhanced inflammatory response, which seems to be implicated in the pathophysiology of atrial fibrillation); prior arterial coronary bypass surgery (an inflammatory reaction occurs after arterial coronary bypass surgery and contributes to postoperative organ dysfunction and coagulation disorders [38]); acute coronary syndrome, or percutaneous coronary intervention within three months (since dysfunctional immune response and inflammation also have been implicated in the pathogenesis of acute coronary syndrome [39]); previously implanted electronic cardiac devices (in order to contribute to the study homogeneity, we only included patients with a class I recommendation for CRT); and comorbidities associated with a life expectancy less than one year (given the severe comorbid condition of these patients, they could exhibit some degree of inflammation).

Patients taking medication that could interfere with immune response were also excluded. Patients taking regular nonsteroidal anti-inflammatory drugs or patients on anticoagulants, or those on continuous or intermittent intravenous inotropic therapy and excessive alcohol consumption or illicit drug abuse.

Candidate eligibility was ensured by baseline assessment of heart failure (HF) patients scheduled for CRT (T0) before the implantation of device. Patients were followed-up and re-evaluated for the same variables at six months after CRT (T6).

5.3.2. Echocardiographic Evaluation

Each patient underwent echocardiographic assessment at T0 and T6. Standard echocardiography was performed using a Vivid 7 (GE Healthcare, Oslo, Norway) and 1.7/3.4-MHz tissue harmonic transducer. Loops and three cardiac cycles were stored digitally and analysed offline using a customized software package (EchoPAC, GE Healthcare). The LV end-diastolic volume (LVEDV), LV end-systolic volume (LVESV) and LVEF were assessed by the biplane Simpson equation in apical four-chamber and two-chamber views [40, 41].

5.3.3. Definition of response to CRT

We classified responders to CRT as patients who remained alive and showed at least a 15% reduction in LVESV at six months of follow-up compared to baseline.

5.3.4. Healthy Control Group

The healthy control group (HG) was constituted by 11 sex- and age-matched healthy individuals. Defined inclusion criteria were normal lipid profile (including cholesterol (< 240 mg/dL), high-density lipoprotein (HDL) cholesterol (> 60 mg/dL), low-density lipoprotein (LDL) cholesterol (between 130 to 159 mg/dL) and triglycerides (< 150 mg/dL)), normal body mass (body mass index (BMI) 18.5 to 24.9 range), and normal cardiac evaluation. Exclusion criteria were family history of heart disease and/or cardiomyopathy; active infections or inflammatory process; autoimmune, neoplastic, and allergic diseases; use of any drug within 30 days before inclusion and inability to understand informed consent.

5.3.5. Blood samples

At admission, just before the device implantation, peripheral blood (PB) samples were taken in all patients to determine haematological parameters and chemistry assessment (including glycemia, creatinine, high sensitivity C-reactive protein (hs-CRP), brain natriuretic peptide (BNP) and uric acid. In addition, to perform the analysis of inflammatory parameters, PB samples from each patient at T0 and T6 and from healthy individuals were collected into K_3-EDTA, heparin and serum tubes.

5.3.6. Quantification of T cells subpopulations

Quantification of total T cells ($CD3^+$), and Th ($CD4^+$) and Tc ($CD8^+$) subsets was performed using Lymphogram reagent (Cytognos, Salamanca, Spain), consisting of a mix of monoclonal antibodies (mAb): anti-CD19 and anti-CD8 conjugated with fluorescein isothiocyanate (FITC), anti-CD56 and anti-CD3 conjugated with phycoerythrin (PE), and anti-CD4 conjugated with Cyanine 5 tandem (PECy5). As described by others [42], Lymphogram reagent (Cytognos, Salamanca, Spain) was added to aliquots from PB sample, collected in K_3-EDTA, and incubated for 15 min at room temperature in darkness. After incubation period, lyse and wash protocol was followed: 2 ml of FACS Lysing Solution (BDB, San Jose, CA) (previously diluted 1:10 (vol/vol) in distilled water) were added to each sample and, after 10 minutes of incubation, cells were washed with 2 mL of phosphate buffer saline (PBS). In the end, cells were resuspended in 0.5 mL of PBS and acquired in FACSCalibur flow cytometer (BD).

5.3.7. Quantification of peripheral regulatory T cells

The immunofluorescent staining of Treg cells was performed according to a protocol established by others [42-44]. Aliquots for Treg evaluation were made from a PB sample, collected in K_3-EDTA, and anti-CD25-FITC (clone M-A251; Pharmingen BD, San Jose, CA, USA), anti-CD127-PE (clone hIL-7R-M21; Pharmingen BD, San Jose, CA, USA), and anti-CD4-peridinin-chlorophyll proteins-cyanine 5.5 (PerCP-Cy5.5) (clone SK3; Pharmingen BD, San Jose, CA, USA) were added. The aliquots were incubated for 15 minutes at room temperature in darkness, followed by the lyse and wash protocol described above.

5.3.8. Immunophenotypic and functional characterization of Th and Tc cells

PB T cells, collected in a heparin tube, were submitted to in vitro stimulation with PMA/ ionomycin, in the presence of brefeldin A, according to the immunofluorescence staining protocol described by others [42, 43]. Briefly, 500 μL of each PB sample were diluted 1:1 (vol/vol), in RPMI-1640 medium (Gibco, Life Technologies, Paisley, Scotland, UK), supplemented with 2 mM L-glutamine. T cells were stimulated with 50 ng/mL of phorbol 12-myristate 13-acetate (PMA) (Sigma, Saint Louis, MO, USA), 1 μg/mL of ionomycin (Sigma) and 10 μg/mL of Brefeldin A (Sigma). The samples were incubated for 4h at 37ºC, in a humidified incubator with 5% CO_2 concentration.

Each cultured PB sample was aliquoted in three different tubes (200 mL/tube) and incubated with the following monoclonal antibodies: anti-CD4-PerCP (clone SK3; Becton Dickinson Biosciences (BD), San Jose, CA, USA) and anti-CD8-allophycocyanin (APC) (clone B9.11; Beckman Coulter – Immunotech, Marseille, France). In order to analyse the intracellular expression of interleukin (IL)-2, tumour necrosis factor (TNF)-α, and interferon (IFN)-γ by Th and Tc cells, cell permeabilization protocol, using IntraPrep Permeabilization Reagent (Beckman Coulter, Brea, CA, USA), and intracytoplasmatic staining protocol were followed, according to manufacturer's instructions. All cell aliquots were stained separately with IL-2 (clone MQ1-17H12; BD Pharmingen, San Diego, CA, USA), TNF-α (clone MAb11; BD Pharmingen), and interferon (IFN)-γ (clone 4S.B3; BD Pharmingen), all conjugated with FITC. Finally, cells were resuspended in 0.5 mL of PBS (Gibco BRL, Life Technologies, Vienna, Austria) and then acquired in a FACSCalibur flow cytometer (BD).

5.3.9. Flow cytometry data acquisition and analysis

FACS analysis was performed blinded for patient's clinical information.

FACSCalibur flow cytometer (BD) was used to perform flow cytometry data acquisition.

The percentage of positive cells within each cell subset and/or their mean fluorescent intensity (MFI) were measured.

The identification and quantification of Treg were made according to the expression of the following phenotype: $CD4^+/CD25^{bright}/CD127^{-/low}$, after a first acquisition of 20 000 total events, followed by an acquisition on an electronic $CD4^+$ gate. The strategy used

for quantification of peripheral Treg cells is represented in Figure 5.1. For immunophenotypic and functional characterization of Th and Tc cells, T lymphocytes were identified according to their typical light scatter. Cytokine production was evaluated in both Th ($CD4^+$) and Tc ($CD8^+$) cells on an electronic gate with at least 20.000 events, after a first acquisition step of 20 000 total events. Data were analysed using the Infinicyt™ software, V.1.5 (Cytognos SL, Salamanca, Spain) and absolute counts were determined using two different instrumentation platforms (flow cytometer and haematological cell analyser).

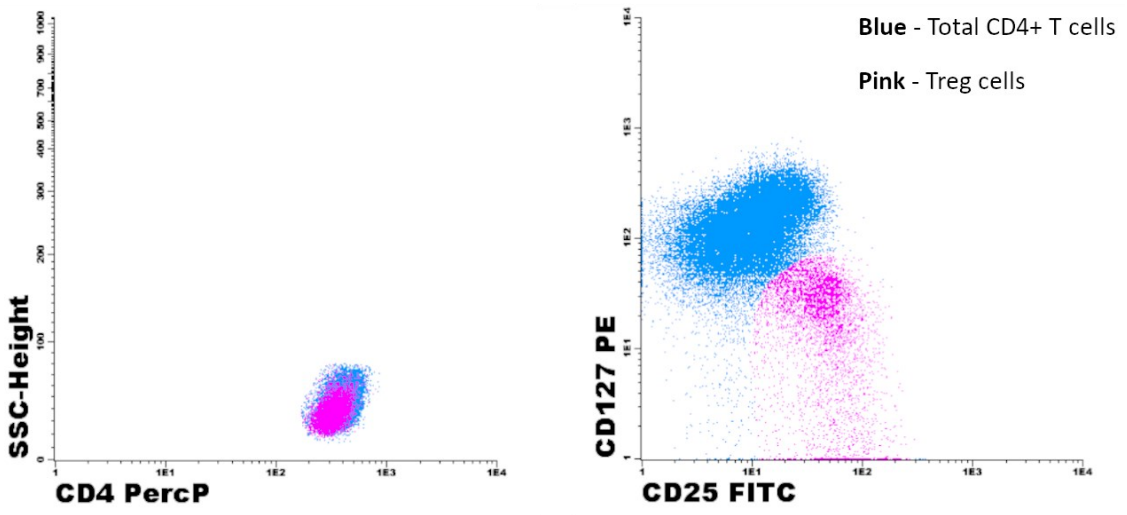


Figure 5.1 – Representative dot plots illustrating the identification of Treg cells in peripheral blood samples using a combination of anti-CD25-FITC, anti-CD127-PE and anti-CD4 PerCP-Cy5.5: $CD4^+/CD127^{Low}/CD25^{High}$.

5.3.10. Statistical analysis

Statistical analysis was performed using the non-parametric Mann-Whitney *U* test for independent variables. The Wilcoxon signed-rank test was used to compare T0 vs. T6 [45]. Results were expressed as mean ± standard deviation or median (range). The values used to establish the effect size were 0.20; small, 0.60; moderate, 1.20; large and 2.00; very large [46]. All statistical analyses were performed using R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www.R-project.org/), (version 3.4.1). Differences were considered to be statistically significant when *P* value was < 0.05.

Prespecified analysis plan is detailed in Supplementary Table 5.1.

Supplementary Table 5.1 – Prespecified analysis plan.

Main hypothesis		
CRT can have an impact on the T cell-mediated inflammatory response.		
Prespecified hypotheses	**Group comparison**	**Evaluated parameters**
Patients have impaired numbers and functional activity of T cells.	**HF patients** *versus* **Control group**	Frequency and absolute number of T $CD3^+$, $TCD4^+$ (Th), $TCD8^+$ (Tc) and Treg cells, and frequency of Th and Tc cells expressing pro-inflammatory cytokines (IL-2, TNF-α, IFN-γ)
CRT can reduce the functional activity of T cells and restore Treg cell numbers.	**HF patients at To** (before CRT) *versus* **HF patients at T6** (6 months follow-up post-CRT)	
Responders to CRT present, at baseline, lower inflammatory values compared to than non-responders.	**Responders** *versus* **Non-responders**	

5.4. Results

5.4.1. Baseline characteristics of healthy control group

HG was constituted by eight males and three females with an average age of 43.4 ± 10.8 years old. Considering the lipid profile, HG showed the following mean values: cholesterol: 184.3 ± 16.1 mg/dL; HDL cholesterol: 64 ± 8.6 mg/dL; LDL cholesterol: 96.5 ± 11.4 mg/ dL and triglycerides: 119.1 ± 24.0 mg/dL. Comparing to HF patients, HG presented a significantly higher value of HDL Cholesterol ($P < 0.001$). BMI average of HG was 22.8±1.3.

5.4.2. Clinical characteristics of responders and non-responders to cardiac resynchronization therapy

The characteristics of the global HF population are described in Table 5.1.

Regarding chronic medication, 72.2% of the patients were under ACE inhibitors, 19.4% under angiotensin II type 1-receptor blockers, 94.4% under beta-adrenergic blockers, 66.7% under spironolactone, 97.2% under furosemide, 27.8% under digoxin, 50% under statins, and 13.9% under ivabradine, before CRT.

Before CRT, the majority of patients were in NYHA class III or IV (79.5%). At the six-month follow-up, the proportion of responders to CRT was 54%, according to the echocardiographic definition. There were no changes in medication between baseline and the six-month re-evaluation.

Regarding baseline characteristics, responders to CRT were significantly older than non-responders and presented higher levels of HDL cholesterol. We found no other statistically significant differences in clinical characteristics. As expected, after CRT, responders presented significantly lower BNP levels compared to non-responders to CRT and significantly better LV geometry and systolic function (Table 5.1).

5.4.3. Evaluation of T cells subpopulations in heart failure patients by comparison with healthy group

Considering the subpopulations of T cells, no significant differences were found in the absolute numbers of Th and Tc cells between HG and HF patients (Table 5.2).

However, regarding Treg cells, HF patients showed significantly lower frequency and absolute values of these cells at baseline and even six months after CRT compared with healthy individuals (Figure 5.2a, 5.2b).

Table 5.1 – Clinical characterization of responders and non-responders to CRT.

	Global Population Mean ± standard deviation (n=39)	Responders Mean ± standard deviation (n=21)	Non-Responders Mean ± standard deviation (n=18)	*P* Value Responders vs Non-responders
Baseline assessment				
Gender (Male/Female)	26 / 13	14 / 7	12 / 6	1
Aetiology (Non-Ischemic/Ischemic)	29 / 10	17 / 4	12 / 6	0.418
NYHA (II/III/IV)	8/ 27/ 4	4/ 16/ 1	4/ 11 /3	0.465
LV lead position (L/PL/A/AL)	17/11/3/8	12/4/2/3	5/7/1/5	0.228
Age (years)	61.4 ± 10.5	**65.2 ± 9.6**	**56.9 ± 9.8**	**0.015**
LVEF (%)	24.9 ± 6.9	23.8 ± 6.5	26.3 ± 7.4	0.309
LVESV (mL)	190.2 ± 84.9	180.1 ± 55.3	202.1 ± 111.0	0.903
LVEDV (mL)	244.2 ± 83.9	233.3 ± 58.9	257.1 ± 106.8	0.583
QRS	148.4 ± 30.6	144.5 ± 22.1	151.4 ± 36.6	0.866
Total Leukocytes (x10^3 µl)	8.4 ± 1.7	8.1 ± 1.6	8.7 ± 1.8	0.242
hs-CRP (mg/L)	5.6 ± 6.0	5.2 ± 5.1	5.9 ± 6.9	0.934
BNP (pg/mL)	362.8 ± 358.7	264.3 ± 214.8	461.3 ± 448.1	0.362
Uric Acid (mg/dL)	6.0 ± 1.7	5.6 ± 1.5	6.5 ± 1.9	0.231
Cholesterol (mg/dL)	185.9 ± 56.5	194.47 ± 61.85	175,81 ± 49.55	0.466
HDL Cholesterol (mg/dL)	44.4 ± 11.7	**48.53 ± 11.04**	**39,56 ± 10.84**	**0.031**
LDL Cholesterol (mg/dL)	117.4 ± 46.3	121.47 ± 49.98	111,41 ± 42.33	0.499
Triglycerides (mg/dL)	132.8 ± 55.2	122.37 ± 50.20	146.00 ± 59.68	0.252
After CRT				
Total Leukocytes (x10^3 µl)	8.3 ± 1.8	8.1 ± 1.5	8.6 ± 2.1	0.220
LVEF (%)	33.9 ± 10.8	**39.1 ± 9.8**	**27.6 ± 8.4**	**0.001**
LVESV (mL)	151.4 ± 96.0	**100.4 ± 36.7**	**215.1 ± 109.6**	**<0.001**
LVEDV (mL)	220.2 ± 108.5	**168.9 ± 50.3**	**284.4 ± 127.9**	**0.001**
hs-CRP (mg/L)	4.1 ± 4.6	2.6 ± 1.8	6.2 ± 6.4	0.288
BNP (pg/mL)	245.3 ± 334.6	**139.6 ± 164.1**	**403.9 ± 456.0**	**0.043**

Bold: Statistically significant differences ($P < 0.05$).

NYHA New York Heart Association, *L* Lateral, *PL* posterolateral, *A* anterior, *AL* anterolateral, *LVEF* left ventricular ejection fraction, *LVESV* left ventricular end-systolic volume, *LVEDV* left ventricular end-diastolic volume, *hs-CRP* high sensitivity C-reactive protein, *BNP* B-type natriuretic peptide, *HDL Cholesterol* high-density lipoprotein cholesterol, *LDL Cholesterol* low-density lipoprotein cholesterol.

Table 5.2 – Comparative analysis of overall T cells and their subsets in healthy individuals and patient groups.

		HG (n=11)	HG vs HFP-To *P* values	HFP (n=39)		HG vs HFP-T6 *P* values
				HFP-To	HFP-T6	
WBC	**10^3/ µl**	7.52 ± 2.07	0.170	8.36 ± 1.71	8.34 ± 1.76	0.202
T cells						
	%	76.10 ± 9.50	0.513	77.39 ± 9.95	77.07 ± 10.73	0.699
	Cells/µl	1510.45 ± 381.77	0.717	1467.14 ± 638.21	1481.90 ± 588.58	0.885
Th cells						
	%	66.22 ± 7.87	0.545	63.63 ± 10.85	61.99 ± 12.91	0.326
	Cells/µl	994.93 ± 258.20	0.331	919.55 ± 426.25	918.63 ± 383.68	0.449
Tc cells						
	%	28.78 ± 7.35	0.578	31.33 ± 10.84	33.67 ± 12.27	0.302
	Cells/µl	439.63 ± 174.87	1.000	474.22 ± 274.04	519.76 ± 293.35	0.647

Results are expressed as mean±standard deviation.

Statistically significant differences were considered when $P < 0.05$ (Mann–Whitney *U* test and Wilcoxon signed-rank).

Th T helper ($CD4^+$) cells, *Tc* T cytotoxic ($CD8^+$) cells, *HG* healthy control group, *HFP* heart failure patients, *HFP-To (To)* baseline assessment, *HFP-T6 (T6)* follow-up evaluation, 6 months after Cardiac Resynchronization Therapy (CRT), *WBC* white blood cells.

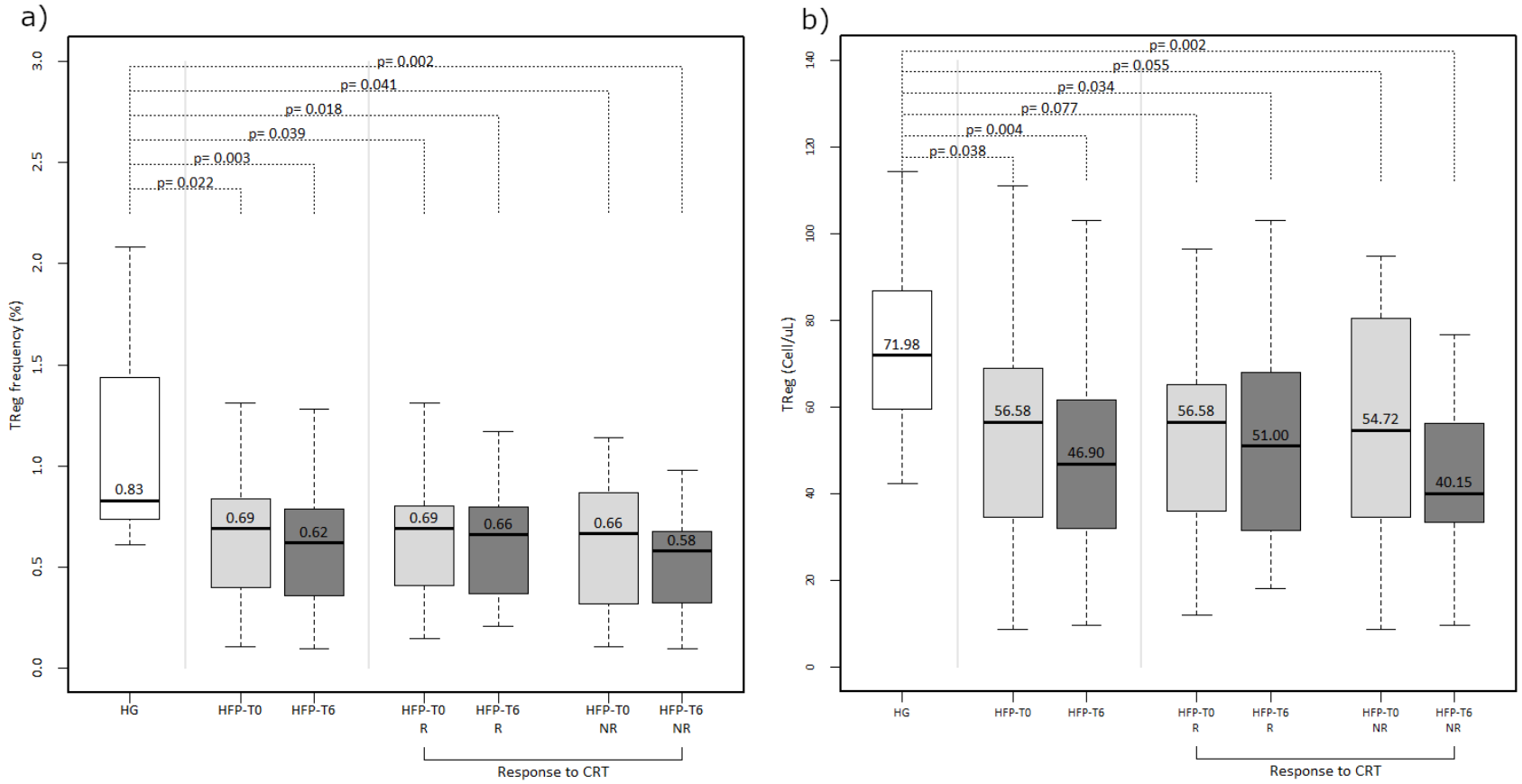


Figure 5.2 – Frequency (%) (a) and absolute number (Cell/µl) (b) of peripheral regulatory $CD25^{bright}/CD127^{low}$ $CD4^{+}$ T cells in total leukocytes, from healthy individuals (HG) and heart failure patients, at baseline assessment (HFP-T0) and 6 months after cardiac resynchronization therapy implantation (HFP-T6).
Heart failure patients were distributed: according to response to cardiac resynchronization therapy: responders (R) and non-responders (NR).
Statistically significant differences were considered when $P < 0.05$.

5.4.4. Impact of CRT on T cells subpopulations and differences according to CRT response

As shown in Table 5.3, comparing responders to non-responders to CRT, we found that non-responders presented a significantly higher frequency of total T cells at baseline and this difference remained after CRT. However, regarding the subpopulations Th and Tc cells, no significant differences were observed between responders and non-responders to CRT, neither for the baseline values, nor for the six-month follow-up quantification (Table 5.3). Likewise, when comparing baseline (T0) and follow-up (T6) assessments, no significant differences were also found in HF patients.

Analysing the impact of CRT in Tregs frequency, we found that both responder and non-responder patients displayed lower frequency and absolute values of Treg compared to HG, in both moments of the evaluation (Figure 5.2a, 5.2b).

5.4.5. Functional characterization of peripheral blood Th and Tc cells of heart failure patients

Regarding the frequency of Th cells producing IL-2 (Figure 5.3a), TNF-α (Figure 5.3c) and IFN-γ (Figure 5.3e), there were no significant differences between the overall HF patient's population and the HG. Moreover, no significant differences were observed when comparing HF patients at baseline with post-CRT (T0 and T6). According to CRT response, we also found no significant differences in the frequency of these T cells between responders and non-responders.

Considering Tc cells producing IL-2, no significant differences were found between HF patients and healthy individuals (HG Vs HFP-T0: $P = 0.903$ and HG Vs HFP-T6: $P = 0.429$) (Figure 5.3b). However, responders to CRT presented, a significantly higher frequency of IL-2 producing Tc cells at baseline than non-responders (Figure 5.3b).

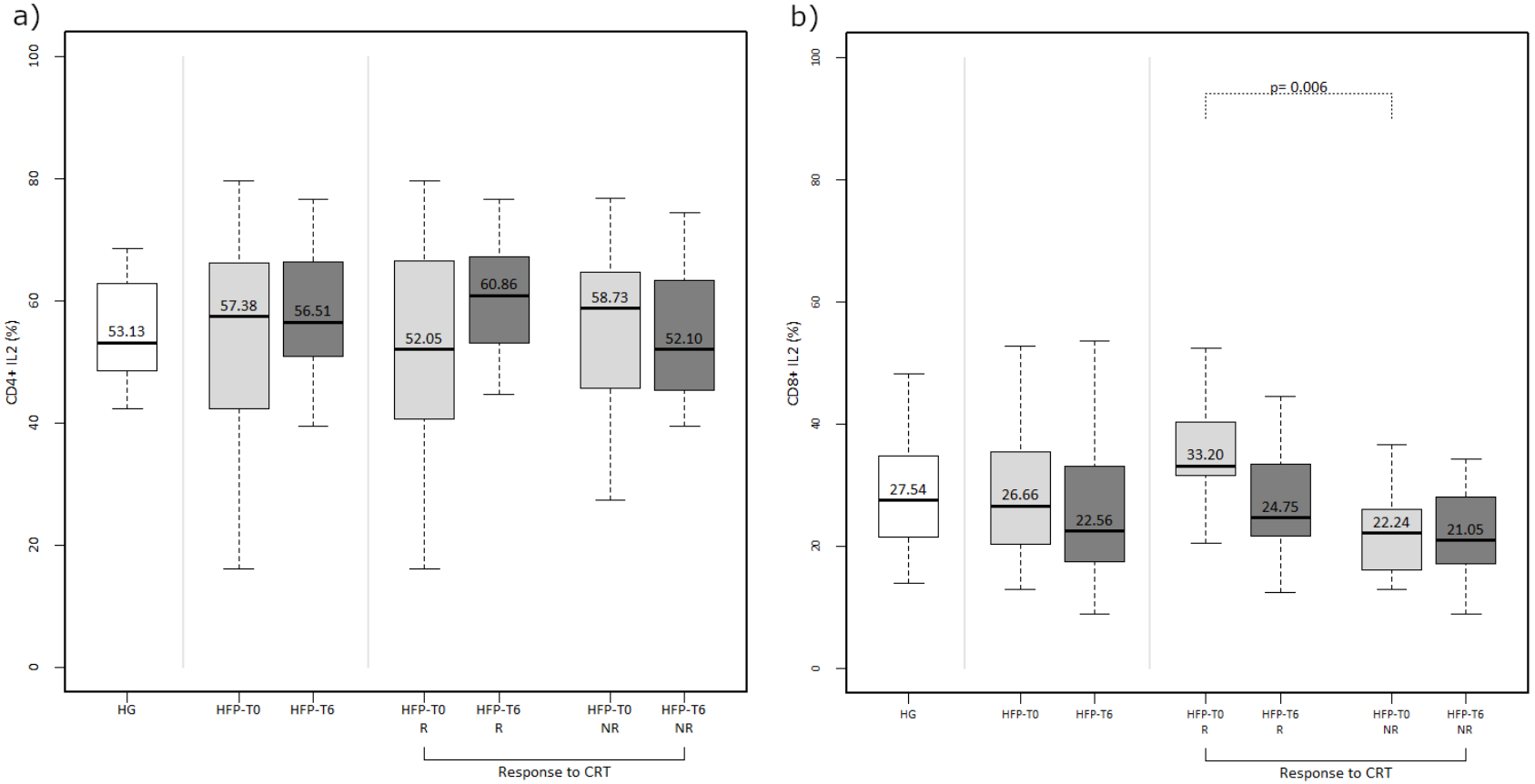
Another difference was observed in the frequency of TNF-α producing Tc cells, which was significantly higher in HF patients six-months post-CRT compared with healthy individuals (HG Vs HFP-T0: $P = 0.336$ and HG Vs HFP-T6: $P = 0.021$) (Figure 5.3d). This difference was even more evident in responders to CRT (Figure 5.3d).

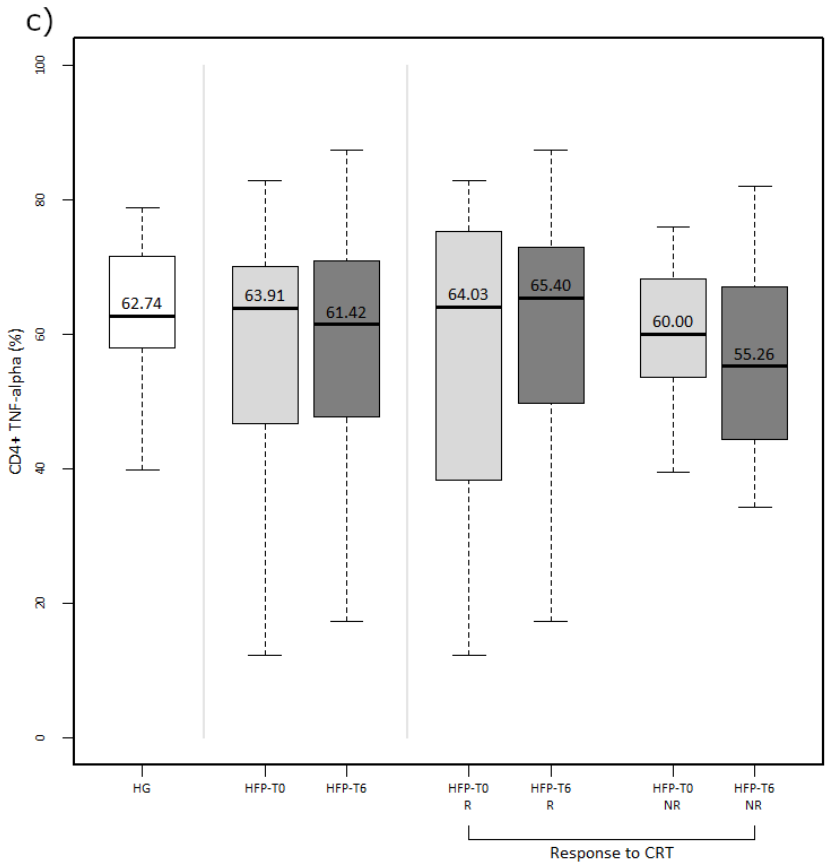
Table 5.3 – Comparative analysis of overall T cells and their subsets in patient groups, according to response to CRT.

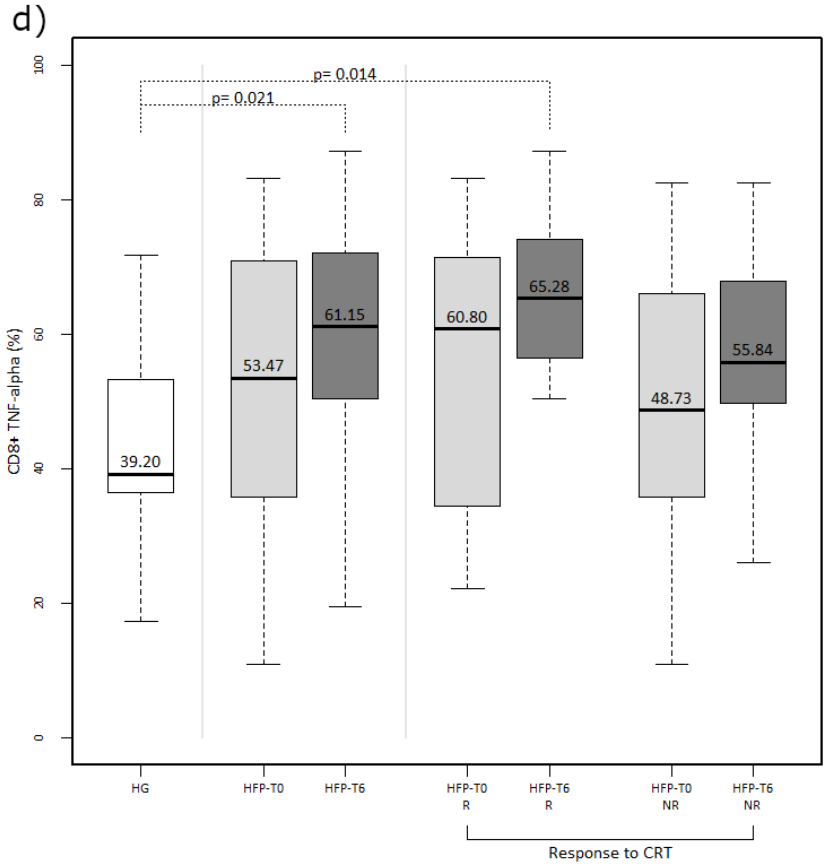
		HFP (n=39)		HFP-To vs HFP-T6 *P* values	According to response to CRT	Responders (n=21)		HFP-To vs HFP-T6 (R) *P* values	Non-Responders (n=18)		HFP-To vs HFP-T6 (NR) *P* values	R vs NR (HFP-To) *P* values	R vs NR (HFP-T6) *P* values
		HFP-To	**HFP-T6**			**HFP-To**	**HFP-T6**		**HFP-To**	**HFP-T6**			
WBC	**10^3/ µl**	8.36 ± 1.71	8.34 ± 1.76	0.726		8.10 ± 1.58	8.10 ± 1.50	0.672	8.66 ± 1.84	8.64 ± 2.06	0.842	0.242	0.220
T cells													
	%	77.39 ± 9.95	77.07 ± 10.73	0.633		**73.53 ± 11.44**	**73.33 ± 10.97**	0.225	**81.47 ± 6.04**	**81.99 ± 8.38**	0.433	**0.026**	**0.015**
	Cells/µl	1467.14 ± 638.21	1481.90 ± 588.58	0.326		1412.74 ± 573.74	1470.27 ± 611.70	0.768	1524.56 ± 712.17	1497.15 ± 576.25	0.404	0.518	0.892
Th cells													
	%	63.63 ± 10.85	61.99 ± 12.91	0.736		65.71 ± 10.77	62.76 ± 12.50	0.523	61.43 ± 10.78	61.02 ± 13.75	0.900	0.159	0.404
	Cells/µl	919.55 ± 426.25	918.63 ± 383.68	0.589		900.76 ± 324.14	930.13 ± 389.04	1.000	939.37 ± 522.19	904.26 ± 389.07	0.495	0.940	0.718
Tc cells													
	%	31.33 ± 10.84	33.67 ± 12.27	0.270		29.92 ± 10.21	33.11 ± 12.54	0.580	32.82 ± 11.57	34.37 ± 12.28	0.501	0.391	0.560
	Cells/µl	474.22 ± 274.04	519.76 ± 293.35	0.191		449.96 ± 284.46	515.26 ± 301.82	0.832	499.83 ± 268.33	525.40 ± 292.15	0.211	0.425	0.888

Results are expressed as mean ± standard deviation.

Th: T helper ($CD4^+$) cells; Tc: T cytotoxic ($CD8^+$) cells; *HFP*: Heart Failure Patients; *HFP-To* (To): Baseline assessment; *HFP-T6* (T6): Follow-up evaluation, 6 months after Cardiac Resynchronization Therapy (CRT); *R:* Responders; *NR*: Non-responders; *WBC*: White Blood Cells. Statistically significant differences were considered when $p<0.05$ (Mann-Whitney *U* test and Wilcoxon signed-rank).


a)
CD4+ IL2 (%)
0
20
40
60
80
100
53.13
57.38
56.51
52.05
60.86
58.73
52.10
HG
HFP-T0
HFP-T6
HFP-T0 R
HFP-T6 R
HFP-T0 NR
HFP-T6 NR
Response to CRT
b)
CD8+ IL2 (%)
p= 0.006
27.54
26.66
22.56
33.20
24.75
22.24
21.05
HG
HFP-T0
HFP-T6
HFP-T0 R
HFP-T6 R
HFP-T0 NR
HFP-T6 NR
Response to CRT


c)
CD4+ TNF-alpha (%)
0
20
40
60
80
100
62.74
63.91
61.42
64.03
65.40
60.00
55.26
HG
HFP-T0
HFP-T6
HFP-T0 R
HFP-T6 R
HFP-T0 NR
HFP-T6 NR
Response to CRT


d)
CD8+ TNF-alpha (%)
p= 0.014
p= 0.021
0
20
40
60
80
100
39.20
53.47
61.15
60.80
65.28
48.73
55.84
HG
HFP-T0
HFP-T6
HFP-T0 R
HFP-T6 R
HFP-T0 NR
HFP-T6 NR
Response to CRT

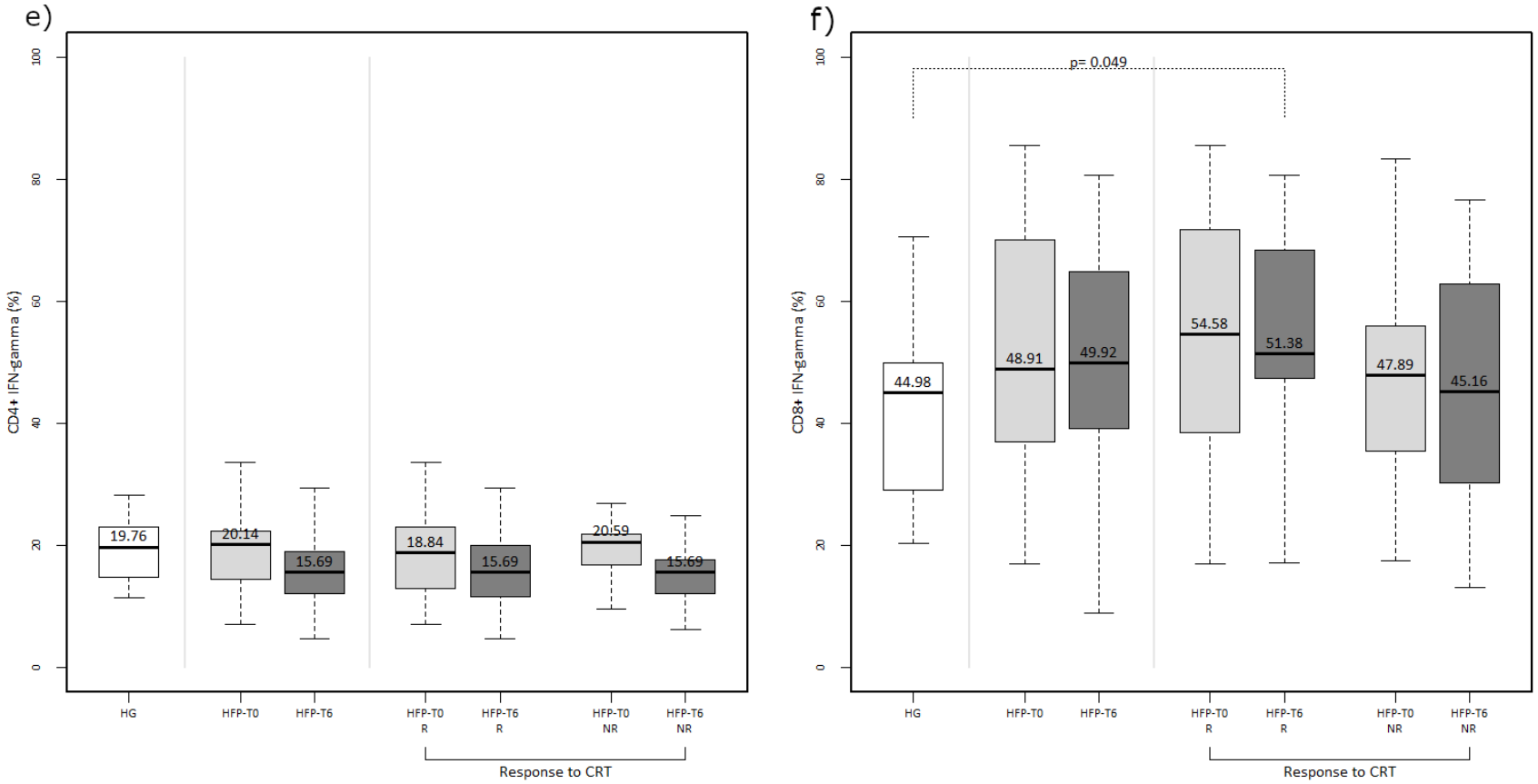


Figure 5.3 – Functional characterization of peripheral T helper ($CD4^+$) cells and T cytotoxic ($CD8^+$) cells.

The percentage of T helper cells producing IL-2 (a), TNF-α (c) and IFN-γ (e) and T cytotoxic cells producing IL-2 (b), TNF-α (d) and IFN-γ (f) were evaluated in healthy individuals (HG) and heart failure patients at baseline assessment (HFP-T0) and 6 months after cardiac resynchronization therapy implantation (HFP-T6). Heart failure patients were divided according to response to cardiac resynchronization therapy: responders (R) and non-responders (NR).

Statistically significant differences were considered when $P < 0.05$.

Moreover, responders to CRT also presented a significantly higher percentage of Tc cells expressing IFN-γ cells after CRT, compared to the control group (Figure 5.3f).
No other differences were seen between T0 and T6 nor between responders and non-responders concerning TNF-α and IFN-γ producing Tc cells (Figure 5.3d and 5.3f).

5.5. Discussion

The main findings of the present work can be summarized as follows: (1) Treg cells are decreased in CHF patients and CRT seems not to be able to restore their normal levels; (2) responders to CRT presented a higher frequency of Tc cells producing IL-2 at baseline than non-responders and a higher percentage of Tc cells expressing TNF-α and IFN-γ after CRT than healthy individuals. (3) CHF patients showed similar levels of overall T cells and Th and Tc subsets to those observed in healthy controls.

Treg cells are major mediators of immune tolerance [46] and homeostasis, preventing auto immune diseases and controlling inflammation [46, 47]. They can act suppressively in innate and adaptive immune response in a direct way: triggering a direct cellular action with the secretion of cytokines such as IL-10, TGF-β and IL-35; and indirectly: expressing high levels of CD25, competing with effector T cells for IL-2, and thus limiting their proliferation [48].

In the failing heart, Treg cells play a cardioprotective role, regardless of HF aetiology [49]. In animal myocardial ischaemia/reperfusion injury models, Treg cells are presented as responsible for attenuating cardiomyocyte apoptosis and activating a pro-survival pathway [50]. In virus-induced myocarditis cases, Tregs cells also suppress immunopathology and prevent tissue damage, avoiding the progression of the disease [51]. Another example is found in myocardial infarction mice studies, where therapeutic Tregs activation increases de novo collagen expression within the scar [52, 53]. Beyond that, Treg activation attenuates interstitial fibrosis, myocardial matrix metalloproteinase activity and cardiac apoptosis, and decreases neutrophils, macrophages, and lymphocytes infiltration as well as TNF-α and IL-1β production [5]. Conversely, Tregs depletion in infarcted mice accelerates ventricular dilation and accentuates apical remodelling [54].

In clinical studies, the frequency of circulating Treg cells is decreased in patients with CHF which may contribute to disturbed immune regulation, chronic inflammation [9, 55] and progression of HF [13, 41]. A study performed by Tang TT *et al.* (2010)

demonstrated that patients with CHF presented not only a decrease in Treg cells numbers but also a loss of suppressive capacity on proliferation and production of cytokines. They also described an inverse correlation between the suppressive function of Treg cells and the severity of the disease, suggesting that reduced levels of these cells may be responsible for uncontrolled T cell activation and consequently for myocardial injury and aggravation of cardiac function [55].

According to these studies, our results confirm that circulating Treg cells are decreased in CHF patients. On the other hand, Tc cells producing TNF-α are significantly increased, indicating that there is an alteration in T-cell homeostasis in CHF patients. We can assume that the increased frequency of pro-inflammatory cytokines-producing T cells may be related to the decreased frequency of Treg cells.

According to their cytokine secretion, effector T cells can be divided into several subpopulations. As known, Th type 1 cells express IFN-γ (its signature cytokine) [12, 56–58], TNF-α and IL-2 [12, 57] whereas Th2 produce high amounts of IL-4 and IL-5 [56, 58]. More recently, the existence of IL-17 producing Th cells, designated Th17, were also described as the third subset of $CD4^+$ effector T cells [56, 57, 59].

Th1 and Tc1 cells are potent effector cells through the secretion of IFN-γ and TNF-α. However, type 1 immunity might also play a pathogenic role in several pathologies, including autoimmune disorders, and chronic inflammatory disorders [57]. In CHF, immune activation can be initiated by direct antigenic stimulation or secondary to cardiac injury, with exposure of "new antigens" that consequently trigger an immune response against the heart. In either case, the immune pathways that follows are similar and implicate the development of T cell–specific responses [6, 60], as well as antibody responses and complement activation [60].

Prior studies in CHF patients established a shift in Th1/ Th2 balance towards Th1 and a shift in Th17/Treg balance towards Th17 [9, 12, 55]. Furthermore, the increased Th1 response in HF is proportional to the severity of the disease [10]. However, little is known about the role of human Tc cells in CHF.

In the present study, we found a higher baseline percentage of Tc cells expressing IL-2 in responders compared to non-responders. Similarly, patients with HF, especially responders, presented an increased frequency of TNF-α and IFN-γ producing Tc cells compared to the control group. These results suggest that the type 1 Tc cells phenotype may be important to reverse remodelling and CRT response. No differences in the percentage of Tc cells expressing TNF-α and IFN-γ were found between healthy

controls and CHF patients at baseline, as well as between responders and non-responders.

Patients responding to CRT have shown a reduction of inflammatory status in small and medium sized clinical studies [61–64]. In fact, Michelucci *et al.* (2007), observed a decrease in IL-6 and high sensitivity C-reactive protein (hs-CRP) in 140 HF patients who underwent an evident reverse remodelling with CRT [62]. Moreover, Lappegård *et al.* (2006), in a small clinical study with 9 HF patients, also found a reduction of inflammatory parameters such IL-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) after CRT [63]. Additionally, a recent study carried out by Gambardella J *et al.* (2021) showed that baseline level of glycation of type 1 ryanodine receptor in circulating lymphocytes can be used as a novel independent biomarker of CRT response [65].

However, no changes in serum markers or inflammatory mediators were reported in other studies [64, 66, 67]. Boriani *et al.* [66] and Tarquini *et al.* [67] showed that CRT had no effect on inflammatory markers such as IL-6, TNF, and soluble TNF receptors. Within these unclear settings, doubts about the impact of CRT on inflammatory mediators' neutralization may arise, despite its beneficial effects on symptoms and cardiac remodelling.

After myocardial injury, T effector cells promote apoptosis of cardiomyocytes [49]. In animal models, it is also well established that Th1 and Th17 cells can induce cardiac fibrosis and adverse cardiac remodelling [11, 68, 69]. On the other hand, Tregs protect LV remodelling and induce an anti-inflammatory milieu by inhibition of neutrophils, monocytes and T cells accumulation and consequent inflammatory cytokine production (such as TNF-α, IL-1β, IFN-γ) [49]. However, little is known about the impact of CRT on the inflammatory response mediated by T cells. To the best of our knowledge this is the first study that has evaluated the impact of CRT on circulating Th1/Tc1 cells and Treg cells.

Here we suggest that CRT had no impact on the frequency and absolute values of Treg cells in patients with HF. Despite the decreased Treg cells values, CRT does not appear to have a therapeutic ability to correct Treg cells levels to normal, even in responders. In the same way, regarding pro-inflammatory cytokine producing Tc1 cells, no differences were found in CHF patients from baseline assessment to follow-up. Moreover, it was after CRT that we found the greatest difference in TNF-α and IFN-γ producing Tc cells between control and patient groups. These observations suggest that CRT does not decrease the frequency of pro-inflammatory cytokine producing Tc1 cells

even in responder patients. Nevertheless, the frequency of IL2-producing Tc cells in responders seems to stabilize to similar values to those of the control group, after CRT.

Taken together, considering the pattern of chronicity and natural course of the CHF, our data supports the idea that inflammation mediated by T cells continues to expand despite CRT even in responders (regardless of reverse remodelling).

5.6. Conclusion

T cell subpopulations are altered in patients with HF, which may result in an exacerbated pro-inflammatory pathway (Treg cells decline *plus* predominance of a Tc1 cells phenotype). Our results suggest that CRT cannot restore Treg cells or inhibit T cell-mediated proinflammatory pathway. It seems that the inflammatory condition underlying HF continues to evolve with the progression of the disease despite CRT.

5.7. Limitations

Healthy controls were selected according to clinical history, considering available and recent analytical results and cardiac exams. But the inclusion and exclusion criteria were not extensively evaluated as for the patient group. The control group was constituted by healthy and active people who apparently did not have comorbidities such as those presented in the exclusion criteria for HF patients. However, we did not parentally exclude these same morbidities.

Another important limitation of our study was a small sample size. Especially when comparing patients with the control group or comparing responders and non-responders, statistical power could be partially lost. Our work included multiple comparisons between groups with a smaller sample size that may not be representative of the population under study due to random sampling error. Further studies with larger number of samples are required to evaluate whether the alleviation or worsening of inflammatory status mediated by T cells translates into different prognosis after CRT.

5.7. References

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Chapter 6

General discussion and conclusion

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HF is a highly prevalent syndrome, associated with significant mortality and morbidity. Systemic inflammation has been recognized as a common pathobiological feature of CHF [1]. Patients with HF present increased levels of pro-inflammatory cytokines regardless of systolic or diastolic dysfunction [2]. Importantly, inflammation is related to the development, progression, and complications of the disease, and can also predict adverse clinical outcomes independent of traditional metrics, such as LVEF or NYHA class [1-3].

Inflammation contributes to the pathogenesis and progression of HF across the spectrum of HFrEF, HFmrEF, and HFpEF subtypes. However, recent research suggests that the various HF stages have distinct inflammatory features [1, 3-5]. Inflammatory biomarkers measured in patients with ischaemic and non-ischaemic aetiologies were mostly associated with HFpEF, whereas biomarkers of cardiac stretch were mainly associated with HFrEF [1, 3-5]. Accordingly, it has been proposed that HFpEF results from a chronic pro-inflammatory state induced by comorbid conditions such as obesity, hypertension, and diabetes, which triggers microvascular endothelial cell inflammation and stress oxidation [6]. On the other hand, HFrEF is usually promoted by a direct cardiac insult, as occurs in myocarditis or ischaemia. Nonetheless, in patients with HFrEF, the release of DAMPs and PAMPs, following cardiac insults, can also promote inflammatory processes. These processes have been extensively studied and found to cause activation of inflammatory innate and adaptive cells, production of pro-inflammatory cytokines, and release of autoantibodies against cardiac antigens [3, 7, 8]. All these factors are associated with an increased severity, a poor prognosis, and a higher likelihood of rehospitalization for patients with HFrEF.

Recent research has found a relationship between EF and levels of several inflammatory biomarkers [9, 10]. Patients with lower LVEF have a distinct inflammatory biomarker profile and are at a higher risk of adverse clinical outcomes compared to those with higher LVEF [10]. In addition, inflammation-related biomarkers have been associated with clinical outcomes in multi-etiologic HF populations. For instance, in patients with CHF, an elevated level of plasma hs-CRP is linked to a worse prognosis. High concentrations of interleukin-6, TNF-α, and

adiponectin also indicate disease severity and can identify high-risk HF patients [11, 12]. Therefore, in our work, we sought to broadly evaluate the role of peripheral immune cells on inflammation in patients with end-stage HFrEF undergoing CRT. Our study plan included the investigation of APC innate immune cells like monocytes and DC, as well as cells responsible for adaptive immunity, namely different functional subsets of T lymphocytes.

Additionally, despite the confirmed efficacy of CRT as a medical treatment for patients with HFrEF [13], the alterations in the inflammatory process observed after CRT have not been sufficiently investigated. To the best of our knowledge, there is currently no extensive data evaluating the frequency of peripheral immune cells in CHF patients or studying their functional characterization post-CRT, hence the importance of studying these cells before and after treatment.

In this investigation, the comparative study performed between the group of HF patients and the control group allowed us to characterize peripheral immune cells in HF patients, determining the differences in their frequency and functional activity. Moreover, the comparison between the two evaluation moments (before CRT and at follow-up) of HF patients enabled us to verify the influence of CRT on these cells and their functionality. Here, it is important to mention that our study population was strictly defined as patients with a class I recommendation for CRT, according to the ESC guidelines, providing the advantage of composing a homogeneous group of patients. In addition, the evaluation of patients at two different moments permitted each patient to serve as their own control. Finally, considering that inflammation can compromise the response to CRT, the comparison between responders and non-responders to CRT allowed us to establish the differences between them and to provide new information on favourable response values to CRT. Additionally, it should be considered that flow cytometry is a technique that can be completely standardized, enhancing its ability to identify peripheral biomarkers of response to CRT.

The role of APC under CHF condition depends on the different cell subset number and may involve inflammatory response, resulting in tissue damage or repair. Research has demonstrated that circulating cMo constitute the predominant monocyte subset in HF, followed by iMo and ncMo in the bloodstream [14]. Accordingly, our results revealed the same consistent pattern in monocyte subtypes. However, we found no differences in monocyte total frequency or absolute count compared to healthy individuals, which contradicts the study by Van Craenenbroeck *et al.* [15]. Nonetheless, in alignment with

our study, this research group also found no differences in the monocyte subset ratio between healthy group and patients.

Functionally, monocytes and macrophages are crucial for wound healing and tissue repair through angiogenesis, phagocytosis, and favourable remodelling of the extracellular matrix. However, prolonged inflammation leads to detrimental remodelling when cells synthesize too much fibroblast and collagen content, promoting cardiomyocyte apoptosis. In the case of ischaemic HF, monocyte activation is involved in systemic and cardiac inflammation induced by early cardiac muscle necrosis. The production of pro-inflammatory cytokines and chemokines by monocytes increases inflammation of cardiac tissue even further. Cytokine production by monocytes for instance, triggers uncontrolled oxidative stress, cardiomyocyte apoptosis, and even tissue necrosis. The loss of cardiomyocytes consequently contributes to the deterioration of the contractile function of the heart muscle and, therefore, to the development of cardiac remodelling [14].

Cardiac remodelling involves not only the infarcted myocardium but also occurs in non-ischaemic cardiomyopathy. During cardiac remodelling, the unaffected myocardium tries to compensate for the function of the impaired cardiac area, and at the same time, the damaged myocardium is replaced by a collagen scar which expands into the healthy area of the heart. Taken together, excessive infiltration of monocytes and macrophages into the damaged myocardium provokes cardiac fibrosis and adverse myocardial remodelling when LVEF becomes reduced and insufficient to provide the necessary supplements and oxygen to the tissues [14].

In our research, HF patients presented a higher frequency of monocytes producing intracellular inflammatory cytokines, such as IL-6 and IL-1β, when compared to the control group. Considering that patients with HFrEF may present circulating levels of IL-1β, IL-6, and TNF-α two to six times higher than in control individuals [16-18], our findings support that inflammation mediated by monocytes and macrophages could participate in the development of HFrEF [14].

Recognized as the most potent APC among all inflammatory cells, DC cells have also been the subject of heart diseases research [19-24]. However, there have been conflicting results regarding the frequency of circulating mDC and pDC in HF [25-27]. In our study, HF patients presented a lower frequency of pDC compared to healthy control, with a comparable percentage of mDC. In a study performed by Jing Y *et al.* [28], ageing was associated with the decline in function and numbers of pDC, whereas mDC numbers and function are relatively unchanged in healthy elderly people. There

are several similarities between the pathophysiology of ageing and HF, including endothelial dysfunction, oxidative stress and chronic inflammation, with a large overlap between age-related inflammatory markers and the inflammatory mediators involved in HF [29]. Therefore, the reduced levels of pDC in our HF population seem to be in line with these findings.

Regarding their functional activity, like monocytes, the frequency of mDC-producing intracellular IL-6 and IL-1β in patients was higher than in healthy individuals, which shows an activation of these cells and their possible involvement in the chronic inflammatory state [30] documented in HF. In addition, IL-6, IL1-β produced by APC have been proposed as being involved in human Th17 cell polarization [31] through the induction of naive $CD4^+$ T cells by the selective secretion of these Th17 cell-polarizing cytokines.

Nowadays it is recognized that the Th1/Th2 imbalance occurs in HF with a shift towards Th1, as well as the Th17/Treg with a shift towards Th17 [26, 32-34]. In the study of adaptive immune cells, we found no differences in total T cells and within its effector Th1 and Tc1 subpopulations, as well as in the frequency of these cells producing intracellular IL-2, IFN-γ or TNF-α. However, we observed a trend for increased Tc17 cells numbers and a significant decrease in Treg cells in HF patients compared to the control group. Similarly to ours, the study published by Li *et al.* [32] demonstrated that circulating IL-17-producing T cells increased and Treg cells significantly decreased in patients with HFrEF when compared to healthy individuals. They also report that IL-17-producing cells can hypothetically be correlated with left ventricular dilatation and remodelling like MMPs due to their increase in patients with HFrEF compared to HFpEF. To continue, the decreased value of Treg cells appears to result in a blockade of immunosuppression and imbalance of the immune system, which further promotes the release of inflammatory cytokines and eventual myocardial injury [32].

All things considered, our work showed that there are alterations in the homeostasis of innate and adaptive immune cells in HF, supporting the acknowledged activation of inflammatory pathways, which may explain its contribution to progression of the disease and adverse outcomes.

Regarding the impact of CRT on inflammatory immune response, we found significant differences in the values of monocyte subsets from baseline to post-CRT. Specifically, we observed a decrease in the percentage of cMo and an increase in iMo after CRT compared to baseline. Our findings are consistent with the research conducted by Ptaszyńska-Kopczyńska *et al.* in HF patients submitted to CRT [35]. Both studies

suggest a possible shift in the role of monocytes in HF after CRT. Moreover, we found that these differences were mainly due to responder patients. Interestingly, a significantly higher frequency of ncMo was also observed in responders when compared to non-responders, suggesting the involvement of different monocyte subsets in reverse cardiac remodelling after CRT.

The precise role of different monocyte subtypes in HF reverse remodelling remains unclear. Most studies have been performed on MI specific mouse models [36-39]. They report that circulating monocytes are recruited to the heart and then differentiate into macrophages to mediate the onset and resolution of inflammation. Within the first 30 minutes following a MI, there is a significant mobilization and influx of monocytes into the myocardial tissue. These monocytes are first recruited from the patrolling pool in the peripheral blood and subsequently from the splenic reservoir through signalling via the angiotensin II-Type 1a receptor [40].

Distinctly, cMo can differentiate into macrophages and dendritic cells in the heart, and produce pro-inflammatory cytokines, labelled as inflammatory monocytes [36, 41]. The blockage of C-C chemokine receptor type 2 (CCR2), highly expressed by cMo, was associated with an increase of LVEF and a decrease of LVEDV in MI mice models [37, 38]. In addition, elevated levels of cMo were found to be related with impaired LV functions at 6-months follow-up after an acute MI [42]. These reports suggest a possible adverse involvement of cMo in reverse cardiac remodelling. On the other hand, the ncMo has been defined as a patrolling population, being found close to the vessel wall in the circulation and scavenging oxidized lipids, cellular debris, and pathogens [40]. Their role is believed to be beneficial in reducing chamber dilation volume and improving systolic and diastolic function. Furthermore, ncMo has been considered essential for the resolution of inflammation in MI, regulating scar formation, angiogenesis, and myocardial healing, and by secreting vascular endothelial growth factor (VEGF) and TGF-β [43]. The absence of an increase in the ncMo subset was also considered an unfavourable prediction linked to the reduction of LVEF [39]. Based on these researches and on the normal behaviour of these cells [44], we consider that, after completing their beneficial role of reversing cardiac remodelling, ncMo can migrate from the heart to the bloodstream to subsequently be destroyed in the spleen. This phenomenon could be the reason behind the higher percentage of ncMo that was observed in the peripheral blood of HF patients following CRT. Relatively to iMo, they probably represent a transitional step between cMo and ncMo, and their role in favourable cardiac remodelling includes natural reparatory mechanisms through regulation of immune response, angiogenesis, and tissue regeneration [35].

In summary, the shift in monocyte subtypes found in responder patients after CRT seems to be related to successful cardiac reverse remodelling.

Analysing their functionality after CRT, the frequency of monocytes producing inflammatory cytokines remained increased after treatment, even in responder patients. The persistence of higher levels of pro-inflammatory cytokine-producing cells after CRT raises questions about CRT's ability to modify the pro-inflammatory capacity of these cells. However, the amount of CD86 *per cell* decreased in all its subpopulations in both responder and non-responder patients. Likewise, the amount of CD86 *per cell* decreased in both mDC and pDC, demonstrating that after CRT, APC cells present a reduced ability to provide the second antigen-independent co-stimulatory signal to T cells. These remarkable observations may indicate an immune-modulating role of CRT and an amendment in cellular immune mechanisms between the innate and adaptive systems in HF post-CRT. By reducing the amount of CD86 expressed by monocytes and DCs, T cell activation can decrease and, consequently, the inflammatory condition of HF may be improved. Furthermore, considering DCs intracellular production of IL-6 and IL-1β, their frequency decreased after CRT in responder patients, becoming probably lesser inducers of inflammation.

At the same time, concerning adaptive immune cells, CRT seems to exert a significant impact on T cell subpopulations in HF patients, particularly on Tc17 cells. Our findings demonstrate a notable reduction in Tc17 cells in responders to CRT, achieving levels comparable to healthy controls. This diminished presence of IL-17-producing T cells post-CRT is particularly interesting, suggesting a remarkable reduction in the inflammatory process inherent to CHF. The expression of IL-17 mRNA was also maintained or decreased in those patients during follow-up.

IL-17 has been implicated in adverse cardiac remodelling after myocarditis and its progression to DCM in mouse models [45]. This cytokine contributes to cardiac fibrosis, activation of MMPs and increased cardiac cell death, as it increases the release of pro-inflammatory cytokines, such as IL-1β and IL-6 [46] and decreases intracellular calcium levels [47]. The downregulated expression of IL-17 suppresses the inflammatory response and contributes to improving cardiac function [48-50]. Thus, the reduction of Tc17 cells after CRT may be associated with the improvement in inflammation and subsequent amelioration of cardiac activity post-resynchronization. This effect could be pivotal in the improvement of cardiac function following CRT.

Even so, we found no differences in the frequency of Th1 and Tc1 cells or producing TNF-α, IFN-γ and IL-2 after CRT. This evidence reveals that the type-I inflammatory

immune response was not inhibited by CRT, indicating a selective immunomodulatory effect of this therapy. On the other hand, type-I T cells may also be somehow involved in reverse cardiac remodelling. Furthermore, Treg cells, known for their role in mediating immune tolerance and controlling inflammation, were found to be decreased in HF patients after CRT, even in responders. Intriguingly, CRT did not appear to restore Treg cells to their normal levels. It is possible that the lower levels of Treg cells in the bloodstream after treatment might be due to their migration to the heart during the response process to CRT. Once in the heart, these cells could play a vital role in suppressing inflammation and promoting tolerance during the reverse remodelling process. This could explain the decrease in CD86 expression by monocytes and DC, as well as the differentiation of monocytes into iMo and ncMo, as previously described. On the other hand, this finding may also suggest a persistent imbalance in the immune homeostasis of CHF patients, even post-CRT. The maintenance of the increased frequency of TNF-α and IFN-γ-producing Tc1 cells after CRT may be related to the reduced value of Treg. These cells may be insufficient to inhibit type I cytokine-producing cells. Either way, further studies are needed to prove both arguments.

Taken together, our findings indicate that following CRT there is a change in the inflammatory immune response linked to the inherent process of reverse cardiac remodelling. One important challenge is to determine whether modifications in peripheral inflammation biomarkers result from their role in the remodelling process or from phenomena induced by CRT.

Lastly, upon analysing the cellular values for response to CRT, we found that responder patients presented a lower pDC percentage at baseline assessment than non-responders. It seems that HF patients with lower levels of pDC are more likely to benefit from CRT treatment; however, further studies, enrolling higher number of patients, are necessary to confirm and consider the value of pDC as a predictor of CRT response.

In conclusion, our research provides compelling evidence of the interaction between CRT and the immune system in patients with heart failure. The reduction in inflammatory markers post-CRT, especially in responders, suggests a beneficial immunomodulatory effect that may be fundamental in the more effective treatment of HF. However, the persistent imbalance in certain immune cell populations highlights the need for a more cautious approach to treating HF when considering cardiac function and immune regulation.

The mechanisms behind the observed changes in immune cell populations after CRT remain speculative. However, these changes can contribute significantly to clinical outcomes, such as reverse remodelling and symptomatic improvement in HF. It is important to recognize the limitations of our studies, including the small sample size and relatively short follow-up periods. Future research should aim to explore the long-term effects of CRT on immune modulation, potentially revealing sustained anti-inflammatory effects. Furthermore, larger studies are needed to confirm these preliminary results and better understand how these immune changes translate into clinical benefits in patients with HF undergoing CRT.

Nonetheless, understanding immune cell dynamics in HF and the potential role of CRT in modulating the immune response offers new insights into the complex interplay between immune regulation and cardiac therapy outcomes. Recognizing and knowing the interaction between CRT and the immune system can provide valuable information on patient stratification, predicting CRT response and potentially identifying targets for adjuvant therapies to improve CRT outcomes.

6.1. References

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