T cell Function in Patients with Dilated Cardiomyopathy

Erika Lindberg

UNIVERSITY OF GOTHENBURG

Department of Molecular and Clinical Medicine
The Wallenberg Laboratory for Cardiovascular Research
Institute of Medicine at Sahlgrenska Academy
2008

A doctoral thesis at a university in Sweden is produced as either a monograph or a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers. These have already been published or are in manuscript at various stages (i.e., in press, submitted, or in manuscript).

ABSTRACT

Dilated cardiomyopathy (DCM) is a heart muscle disease characterized by dilatation of one or both ventricles together with decreased systolic function. Its etiology is still largely unknown. However, immunological alterations such as the presence of autoantibodies, elevated cytokines in plasma, and viral genomes in the myocardium have been frequently reported. The aim of this thesis was to examine T cell function in patients with DCM.

First, cytokines in plasma were measured. In accordance with previous reports, plasma cytokines of TNF-α, IL-6, IL-10, and CRP were significantly elevated in patients with heart failure. Incorporating an etiology of DCM or ischemic heart disease together with clinical variables in a multivariate analysis, a diagnosis of DCM was found to be independently associated with lower IL-10 levels. Next, specific CD4$^+$ T cell response, accumulated cytokines in supernatant, and lymphocyte proliferation were measured using flow cytometry-based methods following culture of isolated peripheral blood mononuclear cells, and stimulation with Staphylococcus enterotoxin B or phytohaemagglutinin. The frequency of IFN-γ-producing CD4$^+$ (Th1) cells was significantly lower in patients than in healthy controls. In contrast, no difference was found in the number of IL-4-producing CD4$^+$ (Th2) cells. In addition, IL-10 production in the supernatant and lymphocyte proliferation were both significantly lower in patients. To conclude, these impairments of IFN-γ and IL-10 are both consistent with an increased susceptibility to chronic infections and autoimmunity.

Finally, we investigated the frequency of a single nucleotide polymorphism in the IFN-γ gene, which alter the transcription level. We found a significant association between the IFN-γ polymorphism and susceptibility to DCM. This previously unreported finding could be the first diagnostic marker of a DCM of autoimmune etiology.

In its entirety, this thesis supports the concept that DCM is a late sequela of myocarditis leading to a disease of autoimmune character.

LIST OF PUBLICATIONS

This thesis is based on the following individual works, referred to in the text by their Roman numerals.

Paper I   **Erika Lindberg**, Yvonne Magnusson, Kristjan Karason, Bert Andersson. (2008) Lower levels of the host protective IL-10 in DCM – a feature of autoimmune pathogenesis? Autoimmunity 41(6): 478-83.

Paper II   **Erika Lindberg**, Bert Andersson, Elisabeth Hultgren Hörnquist, Yvonne Magnusson. Impaired activity of the CD4$^+$IFN-γ^+ T lymphocyte subset in patients with dilated cardiomyopathy. Submitted.

Paper III   **Erika Lindberg**, Bert Andersson, Robert Eggertsen, Yvonne Magnusson. A functional polymorphism in the IFN-γ gene is associated with susceptibility to dilated cardiomyopathy. Manuscript.

ABBREVIATIONS

ACE	angiotensin-converting enzyme
CBA	cytokine bead array
CD	cluster of differentiation
CFSE	5,6-carboxylfluorescein diacetate succinimidyl ester
CRP	c-reactive protein
CTLA-4	cytotoxic T-lymphocyte antigen 4
CV	coefficient of variation
DC	dendritic cells
DCM	dilated cardiomyopathy
ECG	electrocardiogram
ELISA	enzyme-linked immunosorbent Assay
Foxp3	forkhead box p3
HLA	human leukocyte antigen
ICAM-1	intravascular adhesion molecule
IFN-γ	interferon-γ
Ig	immunoglobulin
IHD	ischemic heart disease
IL	interleukin
MHC	major histocompatibility complex
MHC2TA	major histocompatibility complex 2 transactivator
NF-κB	nuclear factor kappa beta
NYHA	New York heart association
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PHA	phytohemagglutinin
SEB	staphylococcal enterotoxin B
SNP	single nucleotide polymorphism
TCR	T cell receptor
Th	T helper
TNF	tumor necrosis factor
Treg	T regulatory
VCAM-1	vascular cell adhesion molecule

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"All our dreams can come true...
if we have the courage to pursue them."

Walt Disney

INTRODUCTION

THE HEART

Heart failure is defined as a pathophysiological condition in which the heart is unable to meet the blood supply demand of the body. Its prevalence has increased significantly during the last decades. About 2% of the population suffers from heart failure, which is a major cause of hospitalisation and death in the western world. Heart failure is considered a heterogeneous syndrome in which not one, but several different pathophysiological conditions may lead to clinical manifestations. However, one major cause of heart failure is cardiomyopathy.

The definition and classification of cardiomyopathy have varied over the years. Recently, the European Society of Cardiology defined cardiomyopathy as a 'myocardial disorder in which the heart muscle is structurally and functionally abnormal in the absence of coronary artery disease, hypertension, valvular disease and congenital heart disease'. It has been proposed to divide cardiomyopathy into five types, i.e., dilated, hypertrophic, arrhythmogenic right ventricular, restrictive, and unclassified cardiomyopathy [Elliott 2008].

Dilated cardiomyopathy

Dilated cardiomyopathy (DCM) is the most frequent form of heart failure in young adults, with an incidence of 5–8 cases per 100 000 individuals per year. It has a poor prognosis and is one of the major causes of heart transplantation in Sweden [Socialstyrelsen 2008]. DCM is characterized by dilatation of one or both ventricles, usually the left ventricle, together with decreased systolic function. Its etiology is largely unknown. Approximately 30–50% of patients have a familial form of DCM [Burkett 2005]. In fact, several different mechanisms might exist in DCM, such as viral-induced, autoimmune, and inflammatory mechanisms. Evidence for all three, i.e., the presence of viral genomes in the myocardium, autoantibodies against heart antigens, and increased immune activity in terms of elevated levels of plasma cytokines, is reported in the literature [Kuhl 2005; Magnusson 1994; Levine 1990; Mann 2002].

THE IMMUNE SYSTEM

The immune system is one of the most complex networks acting in the human body. It is divided into the fast non-specific innate immunity and the slower more specific adaptive immunity, although both types act together. Both are vital for protection against invading microbes such as viruses, bacteria, and protozoa, and for the maintenance of self-tolerance preventing autoimmune disorders.

T cells are of major importance in the adaptive immunity. During the development of T cells, interactions with stromal cells and the presence of various differentiating factors and cytokines are important. Initial stages of this development do not require the presence of antigens. However, once T cells express mature antigen receptors on the cell surface, they become antigen dependent in order to survive and differentiate. The adaptive immunity is time-consuming to mobilize, it take days to be fully activated due to the rearrangement of receptor genes needed to generate antigen specificity.

Central self-tolerance is established by the presentation of self-antigens to developing T cells in the thymus. T cells binding self-antigens with high affinity are subjected to apoptosis, while those not binding are released into the circulation. Occasionally, cells binding self-antigens escape the maturation site and are found circulating in the periphery. This dramatically increases the risk of autoimmune reactions. Autoimmune reactions, i.e., break of peripheral tolerance, however, is controlled by regulatory T cells.

Cytokines are the most important molecular components of the immune system during infections, autoimmunity, and self-tolerance. In the 1990s, cytokines were designated as either pro- or anti-inflammatory depending on whether they promoted or suppressed inflammation. Today the view is far more complicated, and many cytokines are now considered to have pleiotropic effects. Cytokines are produced by different cell subsets and their function depends on the cell from which they are produced, the target cell, and the surrounding milieu.

T CELL DEVELOPMENT

The T cell derived its letter designation from the site of maturation, the thymus. However, T cell development originates from bone marrow-derived progenitors. Notch signalling has been suggested to play an obligatory and selective role in the induction of the T cell lineage. In a Notch-deficient mouse model, progenitors failed to commit to the T cell lineage in the thymus while all other haematopoietic cell lineages developed normally [Radtke 1999].

Surface expression of various proteins reflects the stage of maturation. Maturation of T cells includes the rearrangement of the α- and β-chains of the T cell receptor (TCR) and the expression of the surface markers CD4 and CD8. Expression of the Th-POK transcription factor directs developing T cells to the CD4 lineage, while its absence seems to commit T cells to the default CD8 lineage [He 2005]. Once expressing a functional TCR, negative and positive selection takes place and results in T cells specific to foreign antigens (Figure 1) [Spits 2002].
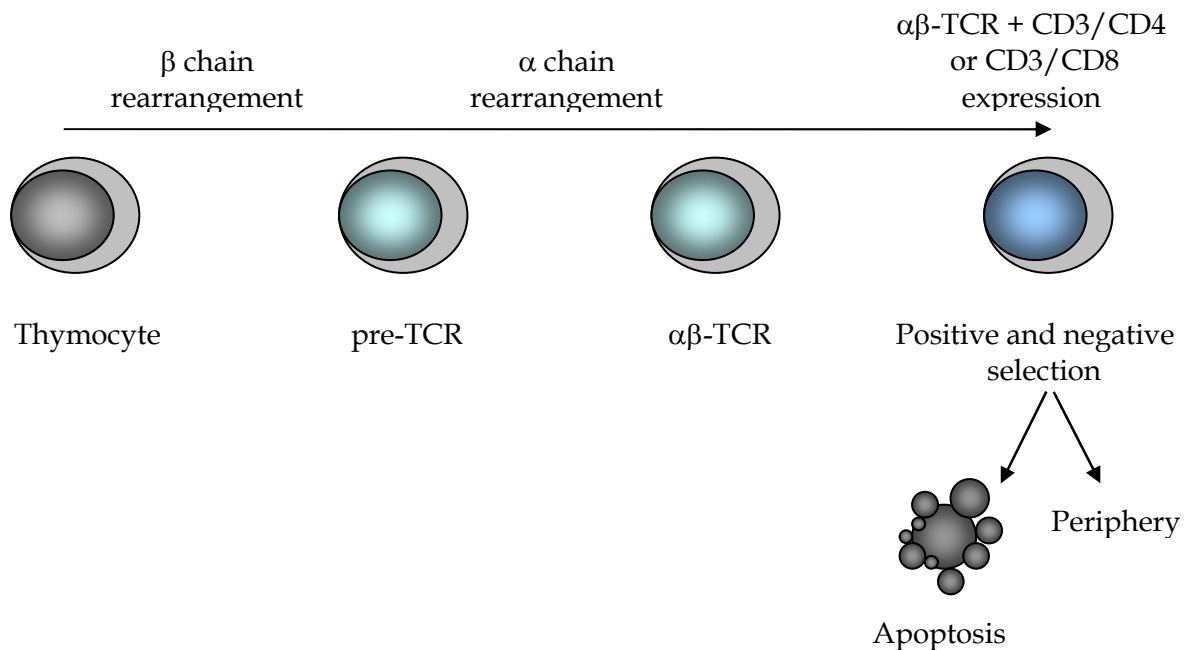

Figure 1. Overview of the T cell development in the thymus.

The TCR recognizes both the major histocompatibility complex (MHC) molecule itself and a short peptide derived from the processed antigen presented by the MHC molecule. T cells undergo both positive and negative selection before entering the periphery. During positive selection, T cells binding with moderate affinity to self-MHC molecules presented by cortical epithelial cells will receive the survival signal, selecting them for the next selection phase, while those binding with low affinity are subjected to apoptosis. In negative selection, T cells binding the self-MHC molecules presenting a self-antigen will be eliminated, while those not binding will enter into the periphery. Approximately 95% of all T cells fail both selection steps and die within the thymus. Negative selection is an important mechanism for immunological tolerance, eliminating self-reactive T cells while allowing maturation only of those T cells specific to foreign antigens together with self-MHC molecules [von Boehmer 1990]. In the periphery, CD4$^+$ T cells recognize MHC class II molecules together with antigenic peptides, while CD8$^+$ T cells recognize MHC class I molecules binding peptides from foreign antigens.

The most common T cell population comprises those expressing the αβ-chain TCR. They are expressed on approximately 95% of the circulating T cells and are found in secondary lymphoid organs, such as the spleen, lymph nodes, and mucosal lymphoid tissues. They respond to infection by activating the humoral immune response and by lysis of infected target cells. They are dependent on antigen processing and presentation to initiate and maintain their effector function.

EFFECTOR CELLS

Triggering the TCR by antigen recognition induces naïve T cells to proliferate and differentiate into a variety of effector cells, such as T helper, T cytotoxic, and regulatory T cells. These subsets determine the type and magnitude of immune response, depending on the presented antigen and the surrounding cytokine milieu. In addition to T cells belonging to the adaptive arm of the immune system, NK and NKT cells are other important effector cells for a functional immune system.

Activation of effector cells following antigen presentation by dendritic cells

Crucial to the activation of effector T cells is the antigen presentation carried out by dendritic cells (DCs). DCs are a major cell type of the innate immune system known to link innate to adaptive immunity [Banchereau 1998]. The recognition of a specific antigen presented on the surface of an antigen-presenting cell is the first of two signals required for T cell activation. Immature DCs capture and process antigens, load the peptides onto the MHC II molecules, in the case of exogenous antigens, or onto the MHC I molecules if the antigens are, for example, viral proteins synthesized within the cell or released during apoptosis. Newly formed MHC II-antigen complexes have relatively long half-lives exceeding 100 h, in contrast to the MHC I-antigen complex which has a half-life of only 10 h [Cella 1997; Cella 1999].

Mature DCs up-regulate their expression of B7.1 (CD80) and B7.2 (CD86) molecules on the cell surface. These molecules bind the CD28 receptor expressed on T cells and mediate the second and final signal for T cell activation. CD28 triggers entry of the T cell into the G_1 phase of the cell cycle and induces transcription of the cytokine IL-2, leading to sustained activation and TCR signalling [Bretscher 1999]. In the absence of this second signal, T cells become anergic and apoptotic. In contrast to CD28, CTLA-4 (CD152) functions as an inhibitory receptor for B7.1 and B7.2, down-modulating the immune response by inhibition of cytokine production, down-regulating IL-2 receptor expression, and inducing cell cycle arrest. Unlike CD28, which is constitutively expressed and down-regulated following binding to

B7 molecules, CTLA-4 is up-regulated following CD28 engagement with B7 and induces anergy in activated T cells (Figure 2) [Walunas 1996].
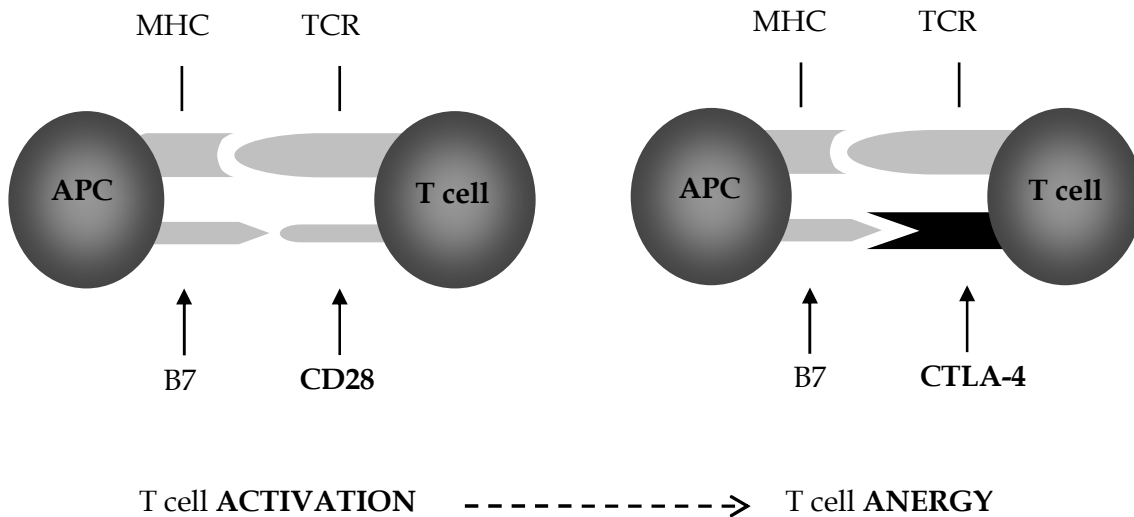

Figure 2. One way of inducing apoptosis and down-regulation of an active immune response. MHC, major histocompatibility complex; TCR, T cell receptor; APC, antigen-presenting cell.

T-helper cells

A central event in both the cellular and humoral immune responses is the activation of T helper (Th) cells. The Th cells are unusual in the sense that they do not have cytotoxic or phagocytic functions and are unable to kill infected host cells. In that sense, they could be considered useless to the immune system. That is not the case, however, since they have been shown to have important functions, such as activating both cytotoxic T cells and phagocytic cells, and initiating antibody class switching.

Following the initial activation steps, Th cells up-regulate numerous genes needed in the immune response. These are grouped according to how early they can be detected following activation, i.e., immediate, early, and late genes (Table 1) [Crabtree 1989]. IL-2 is the main Th cell-proliferative cytokine, the production of which is induced by B7-CD28 signaling as early as 45 min following antigen recognition, and it is responsible for massive Th cell proliferation.

Th cells are further divided into Th1 and Th2 cells, depending on their function and cytokine production. It has been shown that B7.2 is required for polarization toward the Th2 cell lineage, while B7.1 is considered a more general differentiative signal [Freeman 1995]. Th1 cells are responsible for activating a cell-mediated response including macrophages and cytotoxic T cells for phagocytosis and killing of intracellular pathogens. It is well known that IL-12 produced by mature DCs directs Th cells into a IFN-γ-producing Th1 cell subset, an effect mediated by the interaction of CD40 on the DC with CD40L on the Th cell [Heufler 1996; Cella 1996]. In contrast to CD40/CD40Ligand interaction, the OX40/OX40Ligand interaction and the presence of IL-4 in the surrounding milieu have been shown to influence the differentiation of Th2 cells, secreting IL-4, IL-5, IL-10, and IL-13 [Ito 2004]. Surface markers able to differentiate between the Th1 and the Th2 subsets are still lacking, and the most promising way to investigate these subsets is by studying cytokine production.

Two genes have been shown to regulate prototypic Th1 and Th2 cytokine production, IFN-γ and IL-4, respectively. The transcription factor Tbet initiates commitment to the Th1 subset from naïve T cells and controls the production of the prototypic IFN-γ cytokine, whereas GATA-3 has been shown to be selectively expressed in Th2 cells and to directly activate the IL-4 promoter. Reciprocal inhibition between these two subsets is known to occur both on the transcription levels and by means of the produced cytokines [Szabo 2000; Zheng 1997].

Cytotoxic T cells

The major role of cytotoxic T cells is to kill infected somatic cells and other damaged or dysfunctional cells, such as tumour cells. Cytotoxic T cells express both a TCR and a CD8 molecule and are activated when recognizing an antigen presented on the MHC class I molecule; to be fully activated, they are dependent on Th cells [Schoenberger 1998; Melief 2003]. When activated, they release the cytotoxic enzymes perforin and granzyme, which activate the FasLigand apoptotic pathway of the target cell. Cytotoxic T cells produce cytokines such as IFN-γ, TNF-α, IL-2, IL-4, and IL-10. In addition, some cytotoxic T cells also have a suppressive function [Mosmann 1997].

Table 1. Time course of gene expression by Th cells following antigen recognition. Adapted from Crabtree (1989).

Gene product	Function	Time mRNA expression begins	Location	Ratio A/NA
Immediate				
NF-AT	Th-specific transcription factor	20 min	Nucleus	50
NF-κB	Transcription factor	30 min	Nucleus	>10
Early				
IFN-γ	Cytokine	30 min	Secreted	>100
IL-2	Cytokine	45 min	Secreted	>1000
IL-3	Cytokine	1–2 h	Secreted	>100
TGF-β	Cytokine	<2 h	Secreted	>10
IL-2 receptor (p55)	Receptor	2 h	Cell membrane	>50
TNF-β	Cytokine	1–3 h	Secreted	>100
IL-4	Cytokine	<6 h	Secreted	>100
IL-5	Cytokine	<6 h	Secreted	>100
IL-6	Cytokine	<6 h	Secreted	>100
GM-CSF	Cytokine	20 h	Secreted	?
Late				
HLA-DR	MHC class II molecule	3–5 days	Cell membrane	10

NF-AF, nuclear factor of activated T cells; NT-κB, nuclear factor-kappa beta; IFN-γ, interferon gamma; IL-, interleukin; TNF-β, tumour necrosis factor-beta; GM-CSF, granulocyte monocyte colony-stimulating factor; HLA-DR, human leukocyte antigen-DR; A/NA, ratio of activated to non-activated cells.

Regulatory T cells

Another important T cell subset is the regulatory T (Treg) cells, which have received massive attention in recent years, although definitive answers as to how they develop, their phenotypic characterization, and exact mechanism(s) of

action are still lacking [Sakaguchi 2008]. However, it is known that they act by regulating the magnitude of the activated immune system in order to maintain homeostasis and tolerance to self-antigens. Immunological tolerance cannot be explained solely by clonal deletion during T cell development in the thymus, since self-reactive T cells are found in healthy individuals. In the mid 1990s, the CD4+ Th cells expressing the α-subunit of the IL-2 receptor (CD25) were identified as the naturally occurring Treg cells, which constitute approximately 10% of the CD4+ cells. In 1998 it was shown that the function of autoreactive T cells was blocked by CD4+CD25+ regulatory cells [Suri-Payer 1998]. However, since resting and activated human T cells also were shown to express CD25, this phenotypical characterization was not sufficient. The most specific marker so far reported for CD4+CD25+ naturally occurring Treg cells is the forkhead box transcription factor (Foxp3), which was also found to be essential for their development [Fontenot 2003]. The effect of naturally occurring Treg cells *in vitro* is well known, while the effect *in vivo* remains unidentified. Naturally occurring Treg cells are found to affect almost all cell types involved in the immune system, including by the following means: inhibiting the induction as well as the effector and memory functions of Th2 cells; inhibiting the proliferation, Ig production, and class switching of B cells; and inhibiting NK and NKT cytotoxicity as well as the function and maturation of DCs [Miyara 2007]. For naturally occurring Treg cells to become suppressive, they require antigenic TCR activation; however, once activated, their suppressive effects are completely antigen non-specific [Thornton 2000].

Another group of Treg cells has been demonstrated, namely, the inducible Treg cells. They were first recognized following antigenic stimulation of human and mouse CD4+ cells *in vitro* in the presence of IL-10. Stimulation resulted in an IL-10-producing, but not an IL-2- or IL-4-producing, CD4+ population able to inhibit antigen-specific immune response [Groux 1997]. As early as 1994, the importance of IL-10-producing cells was demonstrated. In patients with severe combined immunodeficiency, IL-10-producing cells suppressed the host-reactive T cells following HLA mismatch transplantation [Bacchetta 1994]. Inducible Treg cells seem to exert their suppressive effects mainly via cytokine production, unlike naturally occurring Treg cells, which use cell–cell contact and cytotoxicity. Inducible Treg cells mainly dampen the immunological response in an antigen-dependent manner,

while naturally occurring Treg cells act more directly on cell activity independent of antigen stimulation.

Natural killer cells

An important cell type in innate non-specific immunity is the natural killer (NK) cells. NK cells play an important role in the early response to invading pathogens while the adaptive immunity is being activated, as well as in fighting tumours. They possess cytotoxic activity following activation by cytokines and via their activating and inhibitory cell surface receptors, which distinguish between self and non-self cells. NK cells are phenotypically characterized by their cell surface expression of CD56 and CD16 and the lack of CD3 expression. Approximately 90% of the NK cell population has a low density of CD56 (CD56^{dim}) and a high density of CD16 (CD16^{bright}) and is the most cytotoxic NK population. The CD56^{bright}, CD16[dim/neg] subset constitutes approximately 10% and is the major cytokine-producing NK population. These cells produce large quantities of IFN-γ in particular, but also of other cytokines such as TNF-α and IL-10 [Lanier 1986; Stetson 2003]. Results from animal models report the order of NK cell frequency in the body to be lungs > liver > peripheral blood mononuclear cells > spleen > bone marrow > lymph node > thymus. They constitute approximately 9% of total lymphocytes in the lungs but slightly under 1% in the lymph nodes [Gregoire 2007].

Natural killer T cells

Another important cell population is natural killer T (NKT) cells. This population is defined as an αβTCR[+] T cell subset expressing NK cell-specific receptors. They are suggested to be the frontier between innate and adaptive immunity and to exert cytotoxic activity. Unlike T cells, NKT cells do not recognize antigens presented on MHC class I or II molecules. Instead, they are known to be activated by lipid antigens presented on the CD1d molecule, and are therefore also referred to as CD1-restricted T cells [Godfrey 2004]. Except for the expression of CD56 and CD16 observed on NK cells, NKT cells also express the T cell-specific

markers CD3 and TCR. The hallmark of mature NKT cells is the rapid production of tremendous amounts of IFN-γ and IL-4. They also produce other cytokines, such as IL-2, TNF-α, IL-5, IL-13, and GM-CSF. In animal models, NKT cells have been found in the thymus, lymph nodes, bone marrow, spleen, blood, liver, and lungs. They are most abundant in the lungs, constituting approximately 15% of the immune cells while they constitute less than 5% in other organs [Gregoire 2007].

It has been suggested that NKT cells play a role in both infection and autoimmunity. Defective NKT cells have been reported in patients with various autoimmune diseases, such as multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis. Their capacity to down-regulate T cell immunity has been shown *in vivo*, where CD1d-deficient mice failed to develop systemic tolerance [Ronchi 2008; van der Vliet 2001].

EFFECTOR MOLECULES – THE CYTOKINES

Cytokines are molecules needed for communication between cells in order to activate and inhibit immune responses. Many cytokines are now believed to have pleiotropic effects, and since some cytokines are of special interest in this thesis, a short description of each one and their characteristics will follow.

Interferons

Some of the most prominent proinflammatory cytokines are the interferons (IFNs), named for their main function of interfering with viral replication. The importance of IFNs goes beyond that of antiviral activity and includes an immunoregulatory function, which results in epigenetic changes of indeterminate naïve Th cells into either a Th1 or a Th2 phenotype. The IFNs are divided into type I (α,β) and type II (γ) according to their receptor complex and sequence homology. Production of type I IFNs is induced by double-stranded viral RNA and may be produced by, for example, macrophages, monocytes, and fibroblasts. IFN-γ is mainly produced by Th1 cells but also by cytotoxic T cells following TCR stimulation during adaptive immune responses, whereas NK and NKT cells are responsible for its production during innate immune activity in response to macrophage-derived cytokines, such as TNF-α and IL-12. In addition, IFN-γ may also be produced in response to IL-18, IFN-α and -β, and γδ$^+$ T cells (a T cell population constituting less than 5% of total lymphocytes); DCs and macrophages have also been shown to secrete IFN-γ [Boehm 1997].

The expression of IFN-γ is regulated on both the transcriptional and posttranscriptional levels. A variety of transcription factors, including nuclear factor (NF)-κB, T-bet, and GATA-3, have been shown to regulate IFN-γ transcription [Sica 1997; Szabo 2000; Zheng 1997]. NF-κB can induce IFN-γ expression indirectly by promoting the expansion of IFN-γ-producing cells or through the induction of IL-12, which in turn induces IFN-γ expression [Tato 2003]. Like many other cytokines, complex cross-talk exists between the IFNs; signalling by IFN-γ has been shown to depend on the IFN-α,β receptor components [Takaoka 2000].

Tumor necrosis factor-α

Tumor necrosis factor (TNF)-α, first discovered as an endotoxin-induced glycoprotein, exists in membrane-bound and soluble forms. The main source of TNF-α is macrophages and T cells. Other cell types, such as B and NK cells, mast cells, neutrophils, endothelial cells, smooth and cardiac muscle cells, have also been shown to produce minor amounts of TNF-α. TNF-α promotes inflammation by up-regulating various adhesion molecules on epithelial cells, which in turn attract inflammatory cells to the site of inflammation. Increased plasma levels were observed during inflammation and shown to correlate with the severity of infection [Kwiatkowski 1990].

TNF receptor-deficient mice were shown to be resistant to lethal dosages of both lipopolysaccharides (i.e., the induction of endotoxic shock) and *Staphylococcus aureus*, but in contrast displayed impaired clearance of the mycobacterium *Listeria monocytogenes* [Pfeffer 1993]. Although TNF-α is considered important for mounting an inflammatory response, excessive levels are harmful to the host. Major production of TNF-α from monocytes during endotoxic shock, together with the activation of neutrophils and coagulation factors, result in respiratory distress, multiple organ failure, and death [Morrison 1987]. Excessive levels of TNF-α are also believed to result in autoimmune reactions. TNF-α blocking treatment has been extensively used in rheumatoid arthritis, Crohn's disease, and psoriasis with promising results [Feldmann 2001]. However, TNF-α blocking treatment is not completely without adverse effects; for example, it has been shown to enhance the production of type I IFNs [Palucka 2005]. In fact, adverse effects were reported from clinical trials for heart failure, where treatment increased the risk of disease [Kwon 2003;].

Interleukin-2

One of the first cytokines characterized on a molecular level was interleukin-2 (IL-2). It was found to have growth-promoting activity on bone marrow-derived T cells. Following T cell activation, IL-2 is rapidly synthesized, which in turn up-regulates the IL-2 receptor expression in an autocrine manner, leading to the expansion of both CD4$^+$ and CD8$^+$ T cells. CD4$^+$ cells are the main source of IL-2.

However, minor amounts of IL-2 are also produced by B cells and DCs. The main function of IL-2 is to regulate the magnitude and duration of an activated immune response, inducing proliferation and differentiation, and to down-regulate an ongoing immune response to prevent autoimmune reactions. The latter is regulated by the transient production of IL-2, where the absence of antigenic stimulation leads to the death of activated T cells. IL-2 initiates pro-apoptotic pathways by inducing FasLigand expression on T cells [Van Parijs 1998]. It also has activating effects on NK cells, inducing the production of TNF-α and IFN-γ from NK cells, and acts synergistically with IL-12 to enhance NK cell cytotoxicity [Khatri 1998]. As for IFN-γ, IL-2 is also able to bind NF-κB, which increases cytokine gene expression [Kane 2002]. In contrast, the CTLA-4/B7 signalling induced by antigenic stimulation inhibits IL-2 production.

Interleukin-4

The major anti-inflammatory cytokine interleukin-4 (IL-4) was first discovered as a cytokine able to promote proliferation and differentiation of anti-IgM-treated B cells into IgG- and IgE-producing plasma cells. IL-4 is produced by Th2, NK and mast cells, eosinophils, and basophils. IL-4 function as inducer of the humoral B cell response by stimulating the proliferation and differentiation of B cells, inducing Ig class switching, up-regulating MHC class II expression on B cells and macrophages, and inducing Th2 polarization of T cells. IL-4 has an important immune regulatory function, characterized by down-regulation of macrophages and monocytes synergistically with other cytokines, such as IL-1, IL-6, IL-8, IL-12, and TNF-α. IL-4 has been used in animal models for treating Th1-driven autoimmune diseases, such as allergic encephalomyelitis and collagen-induced arthritis, with promising results [Schulze-Koops 2001; Horsfall 1997].

Interleukin-6

Interaction between T and B cells during antibody production was reported in 1968 without knowing the precise mechanism. Years later, interleukin-6 (IL-6) was

characterized as an important cytokine for terminal differentiation of plasma cells, antibody production, and the inflammatory response. In the early 1990s, it was shown that the antiviral activity in IL-6-deficient mice decreased by 90% following injection with stomatitis virus [Kopf 1994].

Today IL-6 is considered a pleiotropic cytokine, mainly produced by monocytes, macrophages, Th2 cells, and stroma cells. It is one of the most potent stimulators of acute phase inflammatory proteins such as C-reactive protein (CRP) and serum amyloid A [Gauldie 1987]. In addition, IL-6 induces T cell growth and cytotoxic T cell differentiation by augmenting IL-2 receptor expression and the potent proinflammatory cytokine inducer IL-12. IL-6 is also believed to play a role in T cell development in the thymus [Le 1988]. It induces differentiation of macrophages and up-regulates the expression of various adhesion molecules on endothelial cells. In conclusion, IL-6 is considered a key cytokine for the transition from acute to chronic inflammation, which is demonstrated by the transition from neutrophil to monocyte recruitment during inflammation [Kaplanski 2003]. IL-6 is thought to be involved in autoimmune reactions, where blocking its receptor has been shown to be a beneficial treatment for rheumatoid arthritis [Maini 2006].

Interleukin-10

Interleukin-10 (IL-10) was first described as cytokine synthesis inhibitory factor produced by Th2 cells in mice and shown to inhibit cytokine production by Th1 cells [Fiorentino 1989]. Today, IL-10 is known to be produced by a variety of cell types, including B cells, mast cells, eosinophils, macrophages, DCs, cytotoxic T cells, and regulatory T cells. The exact mechanism by which IL-10 exerts its suppressive activity is still unclear. However, IL-10 is known to be involved in inhibiting macrophage and DC functions, leading to decreased production of proinflammatory cytokines [Kuhn 1993; Murphy 1994]. However, IL-10 also inhibits MHC class II expression, which in turn increases the risk of chronic infection due to impaired antigen presentation and clearance. In addition, IL-10 specifically decreases TNF-α production by macrophages, resulting in a limited ability to kill intracellular pathogens [Gazzinelli 1996]. Production of IL-10 has been shown to be important in reducing excessive IFN-γ. Interestingly, in mice infected with *Toxoplasma gondii*, the

IFN-γ-secreting Th1 cells were also shown to produce IL-10. The IL-10 production was induced more rapidly in recently activated than in resting cells, indicating that IL-10 has a regulatory function [Jankovic 2007].

The cytokines constitute a complex network of effector molecules; Table 2 summarizes the cellular source of the cytokines and their main function.

Table 2. Cytokines, their cellular sources, and main function.

Cytokine	Cellular source	Function
IL-2	Th1 cells, B cells, DC	Regulates the magnitude and duration of the immune response
		Induces proliferation and differentiation
IL-4	Th2 cells, mast cells, basophils and eosinophils	Induces B cell response, Ig class switching, Th2 polarization, up-regulates the MHC class II expression on B cell and DC
IL-6	Th2 cells, monocytes, MQ	Induces T cell growth and differentiation of cytotoxic T cells
		Induces differentiation of MQ and up-regulates the expression of adhesion molecules
		Stimulates the production of acute-phase proteins
IL-10	T and B cells, MQ, DC, mast cells, eosinophils	Regulatory functions, i.e., inhibiting MQ and DC functions and decreasing proinflammatory cytokine production
		Down-regulates the MHC class II expression
TNF-α	MQ, NK, mast cells, T and B cells	Promotes inflammation
IFN-α	Virus-infected cells	Induction of resistance to viral infection
IFN-β	Virus-infected cells	Induction of resistance to viral infection
IFN-γ	Th1 cells, NK and NKT cells	Inhibits Th2 cells, activates MQ, regulatory functions

IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; Th, T helper; DC, dendritic cells; MQ, macrophage; MHC, major histocompatibility complex; NK, natural killer; NKT cells, natural killer T.

IMMUNOLOGICAL ALTERATIONS IN DCM

The cause of DCM is still largely unknown. One hypothesis is that DCM is initiated by a viral myocarditis, which, due to immunological malfunction, does not fully resolve and therefore develops into a chronic disease of an autoimmune character [Kawai 1999; Mason 2003]. However, DCM is a heterogeneous disease and this hypothesis might explain disease development in a subset of patients.

Myocarditis and DCM are suggested to represent the acute and chronic states of an organ-specific autoimmune disease of the myocardium [Richardson 1996; Caforio 1996; MacLellan 2003]. As early as 1968, Orinius reported heart disease in humans' years after a coxsackie virus infection. Twenty years later, Bowles et al. reported coxsackie B virus-specific RNA in myocardial biopsies from patients with myocarditis and DCM [Orinius 1968; Bowles 1986].

In 1957, Witebsky and Rose postulated the criteria of autoimmune disease, criteria that are met in myocarditis and DCM [Witebsky 1957]. In the late 20th century, the specificity and functional activity of autoantibodies against the β_1-adrenergic receptor were defined [Magnusson 1990; Magnusson 1994]. Since then, the adaptive humoral response to various cardiac antigens has been well explored and several heart-directed autoantibodies have been found circulating in patients with DCM. In contrast, little research has been done examining the cellular response in these patients. However, numerous studies have examined patients with DCM and found various immunological characteristics associated with autoimmunity and infection.

Table 3 summarizes the immunological alterations found in DCM. These strongly indicate that DCM is a disease of autoimmune and infectious origin. Attention now needs to be directed toward the cellular response and its impact on the pathophysiology of DCM.

Table 3. Examples of infectious and autoimmune characteristics found in dilated cardiomyopathy.

Immunological characteristics		Reference
Viral genomes in the myocardium	CMV, EBV	Kühl 2005
Elevated plasma cytokines	TNF-α, IL-6, IL-10	Levine 1990, Torre-Amione 2005
Aberrant adhesion molecule expression in the myocardium	ICAM, VCAM	Devaux 1997, Noutsias 2003
Expression of HLA class II in the myocardium		Caforio 1990, 1994
Circulating autoantibodies	β_1-AR, myosin, mitochondrial	Magnusson 1994, Caforio 1992, Schultheiss 1985
Infiltrating T cells in the myocardium		Kühl 1996, Noutsias 2002

CMV, cytomegalovirus; EBV, Epstein–Barr virus; ICAM, intercellular adhesion molecule; VCAM, vascular cell adhesion molecule; TNF-α, tumor necrosis factor-α; IL, interleukin; β_1-AR, β_1-adrenergic receptors.

AIMS

The cause of DCM is still largely unknown. Autoantibody production, presence of viral genomes in the myocardium, and increased peripheral cytokine levels have been explored in recent decades. However, T cell function in DCM has received considerably less attention. The main focus of this thesis was therefore to investigate T cell function in patients with DCM. Our initial hypothesis was that patients with DCM would have an altered T cell function, most likely evident in increased IL-4 production. The specific aims of this thesis were therefore to:

ଓ Establish methods and experimental conditions for measuring specific T cell functions in patients with DCM

ଓ Study natural variances in relevant genes coding for immunological proteins for possible influence on the pathophysiology of DCM

METHODOLOGICAL CONSIDERATIONS

General descriptions of the study subjects and methods are given in each paper. In this section, they are described in detail and specific considerations are discussed.

Idiopathic dilated cardiomyopathy

Patients with idiopathic dilated cardiomyopathy (DCM) were recruited from a prospective multicenter study of DCM (papers I–III). Patients were recruited consecutively from seven Swedish hospitals starting in May 1997. The inclusion criteria were left ventricle dilatation of >32 mm/m^2 and an ejection fraction <0.45. The exclusion criteria were: ischemic heart disease diagnosed by coronary angiography or a history of myocardial infarction, hypertension with a blood pressure of >170/100 mm hg, significant valvular disease, significant systemic infection, excessive alcohol consumption, insulin-treated diabetes, endocrine disorders, cancer treatment including irradiation, and tachycardia-induced cardiomyopathy or other primary cardiomyopathy, such as hypertrophic cardiomyopathy [Magnusson 2005].

In paper III, n = 156 patients were also recruited from an epidemiological survey of idiopathic DCM performed in five counties of western Sweden from 1980 to 1987. All patients were investigated using echocardiography and had a clinical examination including ECGs, chest radiographs, and routine laboratory tests [Andersson 1995]. Patients with idiopathic DCM and patients with heart failure of unknown cause diagnosed with left ventricular dilatation and systolic dysfunction, i.e., ejection fraction <0.50 were included. Coronary angiography was retrospectively performed in a subset of theses patients, 15% of whom were found to have coronary disease [Andersson 1995]. This means that in paper III the DCM group may include <6% patients with ischemic heart disease.

Ischemic heart disease

Patients with ischemic heart disease (IHD) attending the Sahlgrenska University Hospital for investigation or treatment of chronic heart failure were included as a control group (paper I). These patients had been diagnosed with coronary disease by prior angiography. Patients with IHD were hospitalized for evaluation before heart transplantation, implantation of an internal cardioverter defibrillator, or implantation of a biventricular pacing system.

By using a control group with heart failure, it was possible to investigate etiological differences, which is not possible using a healthy control group.

Healthy controls

A healthy control group of adult subjects was randomly selected from the western Sweden county population, since the majority of patients with DCM (78%) also were recruited from the western regions of Sweden.

In paper II, a healthy control allows identification of altered T cell function in patients with DCM, a defect that could also be of mechanistic nature. In paper III the use of a large control group from the general population allows identification of susceptibility genes.

All studies complied with the Declaration of Helsinki and were approved by the Ethics Committee of the Medical Faculty, Göteborg University, Sweden. All study subjects gave informed written consent to participate.

Clinical parameters

The New York Heart Association (NYHA) functional class, which places the patients in one of four categories relating to limiting symptoms during physical activity, is determined by the treating physician. Ejection fraction is measured by two-dimensional echocardiography. A right heart catheterisation was perfomed at rest for hemodynamic evaluation, including intracardiac pressures and cardiac output obtained by thermodilution technique. Cardiac index is calculated by

cardiac output, which is the stroke volume x heart rate, divided by the body surface area; this relates heart performance to individual size.

Analysis of plasma cytokines

The usual method for quantifying plasma cytokines is the enzyme-linked immunosorbent assay (ELISA). ELISA is a reliable method that makes use of the highly specific and strong binding between an antibody and an antigen, in this case, the cytokine. However, cytokines are rapidly degraded following extreme changes in temperature and should therefore be analysed using samples that have not been previously thawed, or thawed as few times as possible. The coefficient of variation (CV) for TNF-α, IL-6, and IL-10 were reported from the manufacturer as 13.4%, 7.7%, and 11.3%, respectively. An investigation of the CV in our laboratory was similar for TNF-α and IL-6, 15.2% and 6.0%, respectively, while for IL-10 it was 38.8%. When the concentration differed greatly between duplicates or between two individual analyses, samples were re-analysed.

Isolation and stimulation of PBMC

Venous blood samples were collected in lithium–heparin tubes from which peripheral blood mononuclear cells (PBMC) were isolated using Ficoll Paque. Ficoll Paque yields a cell preparation with 60 ± 20% recovery of lymphocytes, 95 ± 5% recovery of mononuclear cells, 3 ± 2% recovery of granulocytes, 5 ± 2% recovery of erythrocytes, and <0.5% recovery of the total platelet content present in the blood. Between collection and isolation, the blood was kept at room temperature and isolation was performed within 4 h. Kaplan et al. reported that a delay in cell preparation exceeding 24 h affected lymphocyte markers and blastogenic responses [Kaplan 1982]. To minimize undesirable activation of lymphocytes during culture procedures, human AB sera were used. AB sera contain no antibodies against any blood group, i.e., A, B, or O, and are therefore the most suitable sera to use when culturing human blood cells.

Optimization of antigen concentrations for lymphocyte stimulation was performed using the CFSE proliferation method following three days of culture. For PHA and SEB, 1 and 5 µg/mL, respectively, resulted in viable and proliferating cells with distinct forward and side scatter flow cytometry characteristics and were therefore chosen as optimal concentrations in our experimental model (Figure 3).
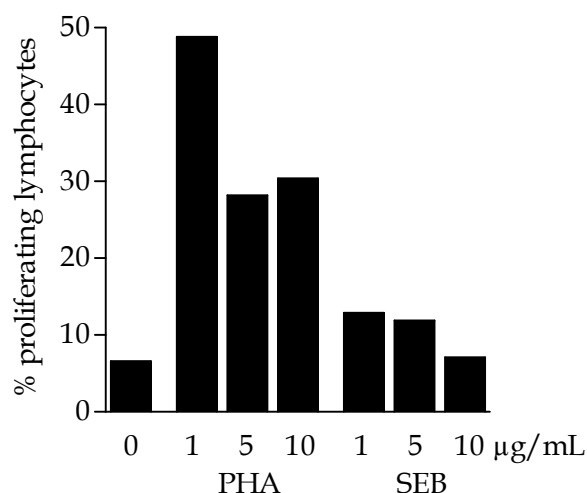

Figure 3. Optimization of PHA and SEB concentrations measured using the CFSE proliferation method following three days of culture.

Surface and intracellular cytokine staining of CD4+ T cells

Following antigen stimulation, lymphocytes rapidly secrete cytokines. This secretion could be measured in the supernatant. However, the amount of cytokines in the supernatant does not reflect the number or phenotype of the cytokine-producing cells. Surface and intracellular cytokine staining are probably two of the most specific methods allowing cytokine production by individual cells to be analysed. Both methods are based on flow cytometric detection, which allows the use of several fluorescent markers simultaneously, making it possible to couple cytokine production to one specific cell type, in our study, the CD4+ T cell.

Surface cytokine staining uses a bispecific antibody consisting of a conjugated pair of monoclonal antibodies, one directed against the cytokine and the other against CD45. CD45 is a leukocyte antigen present on the surface of all nucleated

cells. For the intracellular staining procedure, the cell membrane must be permeabilized to allow anti-cytokine antibodies to enter the cytoplasm. Following cytokine labelling, a fluorochrome-conjugated anti-CD4 antibody is used for selecting Th cells. In contrast to surface cytokine staining, intracellular staining reveals the number of cells that synthesize the cytokine and not the number that actively secrete the cytokine. In addition, intracellular staining results in non-viable cells, since the assay procedure includes blocking the cytokine secretion by brefaldin A, permeabilization of the plasma membrane, and cell fixation.

Surface cytokine staining is considered a more sensitive method than intracellular cytokine staining. In an experiment performed by Desombere et al., in which a defined number of IFN-γ-producing cells were added, the correlation coefficient for detected cells was 0.999. It was also shown that as few as 20 IFN-γ-producing cells could be reliably detected [Desombere 2004; Desombere 2005]. Higher sensitivity was also apparent in a control experiment performed in our laboratory in which surface cytokine staining was used to estimate cytokine production following three days of stimulation. The percentage of responding IFN-γ-producing CD4$^+$ T cells detected was approximately 40% for surface cytokine staining, but only 4% for the intracellular cytokine staining (data not shown). However, Oelke et al. reported good correlation, with similar degrees of detection, between surface and intracellular cytokine staining (r = 0.83) [Oelke 2000].

We have used both PBMC and whole blood in our analyses. Some reports have compared the use of whole blood and PBMC. Using intracellular cytokine staining, Suni et al. reported similar frequencies of activated T cells in PBMC and blood [Suni 1998]. In contrast, Hoffmeister et al. reported consistently higher response in PBMC than in whole blood, and concluded that the use of whole blood, could, if anything, underestimate the frequency of antigen-specific T cells [Hoffmeister 2003].

Proliferation

The ³H-thymidine incorporation proliferation assay has long been used to measure bulk cell division. We established a flow cytometry-based method in which cells are pre-labelled with the fluorescent dye 5,6-carboxylfluorescein diacetate succinimidyl ester (CFSE). CFSE consists of a fluorescent molecule

containing two acetate moieties and a succinimidyl ester functional group. In this form, CFSE is membrane permeant and non-fluorescent. After diffusion, endogenous esterases cleave the acetate group, making the non-fluorescent molecule fluorescent and non-permeant. Proliferation is detected as a decrease in fluorescence, as the amount of CFSE is diluted by each cell cycle. The CFSE method makes it possible to measure the number of cell divisions and to detect specific cell types.

CFSE concentration and days of culture were optimized. A concentration of 9 µM of CFSE and a culture time of three days were found to be optimal for our experimental model. The proportion of responding cells, i.e., the percentage of proliferating lymphocytes, was calculated by dividing the number of proliferating cells as detected in M2 by the total number of lymphocytes, M1 + M2 (Figure 4).
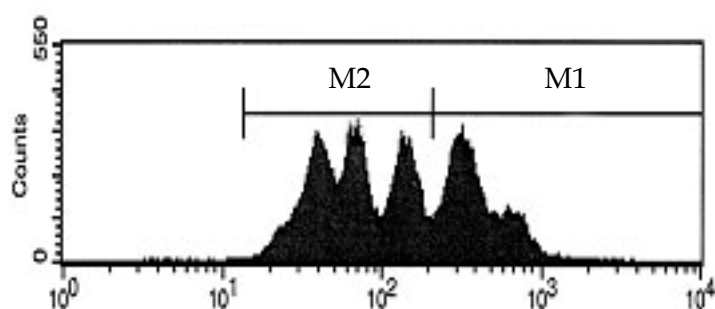

Figure 4. A typical histogram showing gate settings of CFSE-labelled lymphocytes.

Cytokines in supernatants from cultured PBMC

To quantify cytokines in supernatant from cultured PBMC, the flow cytometry-based cytokine bead array (CBA) method was used. CBA allows the simultaneous measurement of several different cytokines in as little as 25 µL of sample, compared with approximately 250 µL per well and cytokine in an ELISA. The method uses a mixture of beads coated with specific antibodies against each cytokine. The beads are uniform in size but contain different intensities of the fluorescence dye phycoerythrine. This is a solution-based method, which makes it more efficient in capturing antigens than a static surface like an ELISA plate [Tarnok 2003].

Allelic discrimination using TaqMan real-time PCR

Identifying single nucleotide polymorphisms (SNPs) has become a useful tool for investigating the genetic influence on various diseases. We determined the frequency of two known polymorphisms, one at position +874 in the first intron of the IFN-γ gene and one at position -168 of the MHC2TA gene, by using allelic discrimination with TaqMan real-time PCR. This method is highly accurate and reproducible for analyses of polymorphisms. Detection of the two alleles is achieved by differentiating between allele-specific probes. Bound probes are cleaved by 5′ nuclease activity, which in turn generate a permanent assay signal. All probes used were minor groove binding probes (MGB), which bind to and stabilize the DNA helix. All MGB probes include a non-fluorescent quencher to decrease background fluorescence, leading to increased sensitivity. We used control DNA of known genotypes, a kind gift from H. Nissbrandt and I. Kockum, previously verified by pyrosequencing and allelic discriminating TaqMan PCR, for each analysed SNP of a total of 12 individuals. Of a total of n = 470 patients and n = 425 controls, genotype was determined in all but one patient and one healthy control, IFN-γ and MHC2TA, respectively.

For IFN-γ (rs2430561), primer and probes earlier described by Rad et al. were used [Rad 2004] as follows: forward primer 5′ATT CAG ACA TTC ACA TTC ACA ATT GAT TTT ATT CTT AC 3′and reverse primer 5′ACT GTG CCT TCC TGT AGG GTA TTA TT 3′; probe 1, 5′MGB-AAT CAA ATC TCA CAC ACA C 3′-FAM, and probe 2, 5′MGB-ATC AAA TCA CAC ACA CAC 3′-VIC. The primer-to-probe concentration, reaction volume, and minimum concentration of DNA used in the PCR and allelic discrimination were optimized based on the cycle threshold values. Consequently, the reactions were carried out in a 96-well plate in a total volume of 15 µL containing approximately 60 ng of genomic DNA, 300 nM of each primer, and 50 nM of each probe.

For MHC2TA (rs3087456), pre-designed primers and probes were used (Applied Biosystems, New Jersey, US). A disadvantage of using pre-designed primers and probes is that the nucleotide sequences are unavailable to the customer, while the advantage is that they usually do not need further optimization and the total reaction volume is low. The PCR and end-point analysis for MHC2TA were carried

out in a 96-well plate in a total volume of 10 μL, with approximately 60 ng of genomic DNA.

Allelic discrimination analyses were performed on an ABI Prism 7900HT Sequence Detecting System using the SDS 2.1 software program.

RESULTS AND DISCUSSION

Inflammatory cytokines are today considered part of the heart failure syndrome. It is not clear, however, whether cytokines act as an initiating factor in the development of the disease or are a consequence of it. Immunological factors, such as the high frequency of autoantibodies, presence of viral genomes in myocardial biopsies, and elevated plasma cytokines, have been identified in patients with DCM. Considerably less is known about T cell function in DCM, which therefore has been the main focus in this thesis. The role of our findings will be interpreted in relation to infection and autoimmunity.

INCREASED PERIPHERAL IMMUNE ACTIVITY

Consistent with previous studies, we found increased levels of IFN-γ, TNF-α, IL-6, IL-10, and CRP in patients with DCM (papers I and II) [Levine 1990; Mann 2002; Ishikawa 2006]. This general increase of cytokine levels in plasma indicates chronic immune activation. Two hypotheses regarding enhanced immune activation can be found in the literature, namely, pathogen-driven and cardiac injury-driven activation, i.e., tissue antigens are exposed and capable of triggering an immune response [Torre-Amione 2005]. Pathogen-driven activation leading to myocarditis and its sequela, DCM, has been frequently reported following viral infections; initiation of DCM due to bacterial infections is less common [Kuhl 2005; Kuhl 2005; Davydova 2000]. A cardiac injury-driven activation can be seen following myocardial infarction where self-antigens are exposed and capable of triggering an immune response against the heart. In a rat model, self-reactive cytotoxic T cells have been detected and shown to exhibit direct cytotoxic effects on non-affected cardiomyocytes *in vitro* following myocardial infarction resulting in tissue damage [Varda-Bloom 2000]. In addition, increased bowel wall permeability accompanied by the translocation of endotoxins from the gut to the peripheral circulation has also been reported as one possible cause of the increased immune activity [Sandek 2007; Niebauer 1999].

The impact of cytokines on cardiac function

We found poor NYHA function class to be associated with increased plasma levels of IL-6 and CRP, but not with increased levels of TNF-α or IL-10 (paper I). We also found elevated IL-6, IL-10, and CRP levels to correlate with diastolic filling pressure (paper I). The association between NYHA function class and both IL-6 and TNF-α has been shown earlier [Torre-Amione 1996]. We do not have a clear explanation for the lack of association with NYHA and TNF-α in our study. It could depend on the composition of patients, as elevated TNF-α levels mostly has been found in severe heart failure only.

The exact function and clinical significance of these cytokines in DCM and heart failure are poorly understood. The cytokine network is extremely complex, and one cytokine is enough to induce several others, affecting the outcome of an immune response. TNF-α is one of the most frequently studied cytokines in DCM, and quite apart from its inflammatory action, it is known to have direct negative inotropic effect on the myocardium by reducing the level of intracellular calcium during the systolic contraction phase [Yokoyama 1993]. Furthermore, TNF-α results in interstitial fibrosis, apoptosis, and ventricular remodelling, inducing left ventricular dilatation of the myocardium [Bradham 2002]. TNF-α induces the expression of IL-6, which is also known to have negative inotropic effects on cardiomyocytes [Finkel 1992]. IL-6, in turn, is further implicated in left ventricular remodelling and the development of ventricular hypertrophy through enhanced stimulation of the glycoprotein (gp) 130 receptor subunit expressed on cardiac myocytes [Kunisada 1996]. In fact, Aukrust et al. demonstrated increased expression of the soluble gp130 protein in heart failure [Aukrust 1999]. IL-6 is also known to rapidly induce CRP, which binds to a variety of microorganisms and induces a proinflammatory immune response. Although the matter has not been extensively studied, there are reports of elevated levels of CRP in heart failure and DCM [Ishikawa 2006]. CRP is known to have direct proinflammatory effects on human endothelial cells by increasing the expression of adhesion molecules such as ICAM-1, VCAM-1, and E-selectin [Pasceri 2000]. In fact, ICAM-1, VCAM-1, and soluble P-selectin have been associated with adverse clinical outcome in heart failure [Yin 2003]. In addition, endothelial dysfunction has been reported to influence the progression of heart failure, which indicates an important role for CRP in the pathophysiology [Chong 2004; Katz 2005].

The question of whether the heart itself is a source of cytokine production has been raised but the results are contradictory [Mann 1999; Munger 1996; Tsutamoto 2004]. We measured cytokine concentrations in the coronary sinus and systemic arterial blood without finding any difference between these two locations (Table 4).

Table 4. Cytokine gradient across the myocardium in patients with DCM.

	Transmyocardial gradient		
	Artery	Coronary sinus	p-value
TNF-α	2.41 ± 0.92	2.36 ± 0.91	0.74
IL-6	2.16 ± 0.41	2.15 ± 0.44	0.77
IL-10	0.88 ± 0.12	0.89 ± 0.18	0.73

IL-6, interleukin-6 (n = 19); IL-10, interleukin-10 (n = 9); TNF-α, tumor necrosis factor α (n = 16). Values are expressed as mean ± SEM pg/mL.

Increased lymphocyte activity

In addition to increased levels of cytokines in plasma, we also found elevated surface expression of both CD3 and the α-subunit (CD25) of the IL-2 receptor in patients with DCM (paper II). This indicates increased numbers of circulating T cells in the periphery together with increased immune activation. CD25 is considered an early activation marker expressed on T cells after the triggering of the TCR. The expression occurs within 2–24 h of stimulation and persists for only a few days if the stimulation is withdrawn [Poulton 1988]. Our findings are supported by Yndestad et al., who reported increased CD25 expression on both CD4^{+} and CD8^{+} T cells in chronic heart failure [Yndestad 2003].

Reduced production of IL-10

We found significantly and independently lower levels of IL-10 in patients with DCM than in patients with IHD even after incorporating of etiology, age, gender, and clinical variables in a multivariate analysis. To the best of our knowledge, this has not previously been demonstrated (paper I).

IL-10 is a major regulatory cytokine, down-regulating an enhanced immune response in order to limit collateral tissue damage and control peripheral autoreactivity [Murray 2005; O'Garra 2007; Gazzinelli 1996]. Knowing the function of IL-10, as well as the fact that autoantibodies against various heart antigens are frequently present in patients with DCM, in sharp contrast to IHD, the difference between DCM and IHD shown in this study is intriguing [Magnusson 1990; Magnusson 1994; Caforio 2008]. In addition, prevalence of viral genomes and viral persistence in the myocardial biopsies are detected in up to 50% of DCM [Why 1994; Kuhl 2005; Kuhl 2005; Magnusson 1990; Magnusson 1994]. Few reports have focused on comparing cytokine production in DCM and IHD. However, Fukunaga et al. studied *in vitro* activation of IFN-γ-producing CD4+ T cells and found significantly lower response in patients with DCM than in IHD [Fukunaga 2007].

We also found significantly lower production of IL-10 in the supernatant of cultured PBMC from patients with DCM than from healthy controls (paper II).

We speculate that these impaired IL-10 responses in patients with DCM could be insufficient in magnitude to fully control an infection, therefore increasing the likelihood of a disease of autoimmune character. The protective role of IL-10 is shown by its ability to reduce proinflammatory cytokines, such as, IFN-γ, TNF-α, and IL-2, in experimental autoimmune myocarditis [Gazzinelli 1996; Li 2005]. Notably, intravenous immunoglobulin treatment of patients with DCM has been shown to result in a marked increase of IL-10, which in turn correlated significantly with improved left ventricular ejection fraction [Gullestad 2001].

REDUCED T CELL ACTIVITY *IN VITRO*

DCM is a heterogeneous disease with several different etiologies and disease mechanisms. Measuring plasma cytokines only indicates whether there is ongoing immune activity without revealing anything about the cause of the altered production. It is therefore of great interest to use more specific methods to study T cell function.

Reduced response of IFN-γ-producing CD4^{+} T cells

In DCM, we observed a profound reduction in the number of responding IFN-γ-producing CD4^{+} T cells following stimulation with SEB and PHA, while there was no difference in the number of responding IL-4-producing CD4^{+} T cells (paper II). Decreased numbers of responding IFN-γ- but not of IL-4-producing CD4^{+} T cells have also been reported in patients with autoimmune type 1 diabetes [Kukreja 2002].

IFN-γ production measured in supernatant from stimulated PBMC has been shown to correlate well with the number of responding IFN-γ-producing CD4^{+} T cells [Desombere 2005]. We found further support for a defective IFN-γ response in DCM, seen by a significantly lower IFN-γ production from overnight non-stimulated PBMC, while no difference was found for IL-4. The reduced IFN-γ production in the supernatant was in the same range as the reduced mRNA expression of IFN-γ detected in the resting blood of newly diagnosed diabetic patients [Halminen 2001].

These findings were somewhat unexpected with regard to our hypothesis, according to which we expected elevated IL-4 production based on the presence of autoantibodies in these patients. Since the IL-4 production was found to be normal, the impaired IFN-γ was most likely not a result of any suppressive effects of IL-4. The balance of the Th1 (IFN-γ) and Th2 (IL-4) response was first proposed as a major regulatory mechanism of autoimmunity. Organ-specific autoimmune diseases have been suggested to have an increased Th1 response [Charlton 1995]. However, this simple classification has today been reassessed and may no longer be strictly applicable.

The importance of CD4^{+} T cells in activating a sufficiently strong CD8+ cytotoxic T cell response during infection is well known [Battegay 1994; Matloubian 1994]. This was shown in both CD4^{+} and CD8^{+} T cell-deficient mice immunized with the cardiac myosin peptide to induce organ-specific autoimmunity. Immunization of CD4^{+}-deficient mice resulted in the development of autoimmune myocarditis with autoantibody production, whereas immunization of CD8^{+}-deficient mice resulted in a more aggravated disease [Penninger 1993].

Taken together, our results indicate a defect in the IFN-γ response. This is reinforced by the lack of differences in the IL-4 response, in both the number of responding CD4^{+} T cells and IL-4 levels in the supernatant.

Reduced lymphocyte proliferation

Following three days of stimulation with SEB, there was a significant proportion of lymphocytes undergoing proliferation compared with non-stimulated cells. However, this proliferative response was significantly lower in patients with DCM than in controls (paper II). An impaired proliferative response to *Mycobacterium bovis* has been described in patients with DCM and has been found to correlate with disease progression [Bromelow 1997]. We did not observe any difference when stimulating with PHA (data not shown). This could be because PHA is dependent on accessory cells for T cell stimulation and would be expected to need a longer time to exert the same effect as seen with SEB. T cells need to be stimulated and to have initiated cytokine production before they can proliferate.

Even though we did not specifically study CD4^{+} T cell proliferation, we surmise that the decreased proliferation most likely reflects reduced Th cell activity. In the peripheral blood of our patients, approximately 60% of the lymphocytes were CD4^{+}, the remaining population consisting of 30% cytotoxic T cells and 10% B cells (data not shown). Despite impaired proliferation in DCM, we observed no significant differences between DCM and controls in the IL-2 levels measured in supernatants following either overnight or three days of SEB stimulation (data not shown). Since IL-2 is a major proliferative cytokine, one would have expected the decreased proliferation to be visible in the IL-2 levels. Therefore, it seems reasonable to believe that there is some sort of T cell defect present in DCM, possibly a defect in the TCR

signalling. SEB stimulation has been shown to result in phosphorylation of the TCR ζ-subunits. Such a connection has been described in haemodialysis patients, who displayed lower proliferation following SEB stimulation, caused by impaired phosphorylation of the ζ-chain [Niedergang 1998; Eleftheriadis 2004].

GENETICS OF DCM

Approximately 30–50% of cases of DCM are believed to have a genetic background, autosomal dominant inheritance being the usual form. Although genetic factors seem to be important, only a minority of known mutations have been associated with familial DCM. The first familial DCM was reported as early as 1949, whereas the first DCM-associated mutation, detected in the dystrophin gene, was characterized in 1993 [Towbin 1993]. Linkage analysis is difficult to perform due to the limited size of families and difficulties in the diagnosis of DCM. Today, over 20 mutations have been characterized in various cardiac-specific proteins leading to DCM. Considerable less is known about mutations in immunological proteins and their possible association to DCM. Two of the most frequently reported polymorphisms associated with various autoimmune diseases are found within the human leukocyte antigen (HLA) and the CTLA-4 surface molecule genes. The HLA is the antigen-presenting molecule in humans corresponding to the MHC molecule in animals [Bilbao 2003; Li 2008]. The IFN-γ polymorphism at position +874 has been of interest in inflammatory and autoimmune disorders such as hepatitis B, Wegener's granulomatosis, and Hashimoto's disease [Spriewald 2005; Ito 2006; Ben-Ari 2003].

We investigated the frequency of the polymorphism at position +874 in the IFN-γ gene and at position –168 in the MHC2TA gene in patients with heart failure. Both polymorphisms have been associated with differential transcription levels [Pravica 1999; Pravica 2000; Swanberg 2005]. To the best of our knowledge, we are the first to report a significant association between the high producing IFN-γ genotype and susceptibility to DCM. In contrast to IFN-γ, there was no difference in the MHC2TA polymorphism between patients and controls. There were no associations between either of these polymorphisms and 10-year mortality (paper III).

Interestingly, Rheka et al. reported an association between the high-producing IFN-γ genotype TT and Hashimoto's thyroiditis, whereas the low-producing IFN-γ genotype was associated with Graves' disease; the former disease is caused by autoreactive T cells and the latter by antibody-mediated immune reactions [Rekha 2006]. The direct anti-inflammatory effect of intravenous immunoglobulin treatment, which has been used successfully in DCM and shown to lead to improved cardiac function, has been linked to blockade of IFN-γ gene expression

and signalling [Park-Min 2007]. The role of IFN-γ polymorphism in heart failure has been sparsely investigated. It has been studied in regard to the progression to end-stage heart failure in children with cardiomyopathy or congenital heart disease, without any association being found [Webber 2002]. As shown in Figure 5, we found no association between the IFN-γ genotype and the number of IFN-γ responding CD4$^+$ T cells in either patients or controls (Figure 5).
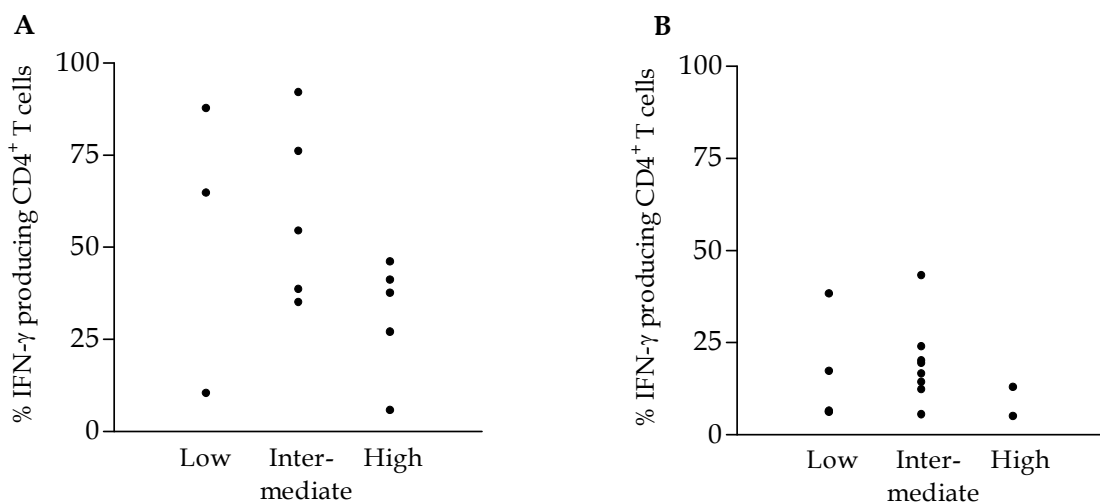

Figure 5. The percentage of IFN-γ-producing CD4$^+$ T cells in relation to IFN-γ transcription phenotype (low, intermediate, and high) in (A) healthy controls and (B) patients with DCM.

IFN-γ is a strong inducer of MHC expression, which in turn is controlled by MHC2TA. Aberrant HLA expression, especially HLA-DR4, has been reported in DCM. In addition, increased numbers of T cells expressing HLA-DR are found in the periphery of patients with DCM [Carlquist 1991; Ueno 2007]. The allele and genotype frequencies in our study were in the same range as reported by Swanberg et al.; they also reported an association between the G-allele and rheumatoid arthritis, multiple sclerosis, and myocardial infarction [Swanberg 2005]. Several studies have failed to confirm these results, however. Neither did we found any significant associations between the MHC2TA gene polymorphism and DCM or mortality. This is in contrast to the observed increased mortality in patients with type 1 diabetes and previous myocardial infarction having the low expressing variants of MHC2TA [Lindholm 2006].

IS THERE AN ACTUAL T CELL DEFECT IN DCM?

We have demonstrated impaired *in vitro* production of IFN-γ and IL-10 in CD4⁺ T cells and PBMC, respectively. This is in contrast to the increased peripheral levels of these cytokines and to the higher genetic ability to produce IFN-γ. It is important to discuss the experimental conditions used and to rule out factors that could have influenced the *in vitro* results, such as SEB specificity, anergy, and apoptosis.

SEB is a potent superantigen produced by the *Staphylococcus aureus* bacterium. Superantigens stimulate a large fraction of T cells by cross-linking the MHC class I or II on antigen-presenting cells and the corresponding TCR on CD4⁺ and CD8⁺ cells. SEB is therefore considered a strong physiological stimulator of T cells. This bridge bypasses the antigen processing and presenting step needed during normal antigen activation. SEB is known to bind specifically to certain T cell subsets expressing the TCR Vβ-3, -12, -14, or -17 chains in humans. This specificity could skew the results in an *in vitro* model such as ours [Choi 1989]. However, in our study, the reduced response of IFN-γ-producing CD4⁺ T cells was also apparent in the case of PHA stimulation. PHA is a non-specific mitogen-binding extracellular carbohydrate structure and is dependent on accessory cells for activation of T cells [Ijichi 1996].

What about anergy and apoptosis? Indeed, it is possible that PBMC isolated from patients with DCM displaying increased peripheral immune activation could already be in an anergic state of unresponsiveness or enter apoptosis when stimulated *in vitro*, i.e., activation-induced cell death [Song 2004; Kishimoto 1999; Redmond 2005]. An important consideration in this regard is the significantly reduced levels of IFN-γ found in the supernatant following overnight incubation without stimulation, despite the increased plasma levels of IFN-γ. If the reduced IFN-γ levels were due to increased anergy and apoptosis *in vivo*, this would then also be evident in peripheral cytokine levels. Although, to the best of our knowledge, lymphocyte apoptosis has not been reported for DCM, increased apoptosis in cardiomyocytes has been reported [Kuethe 2007].

In experiments measuring both the CD4⁺ T cell response and the PBMC production of cytokines, the anti-CD28 co-stimulatory molecule was added. Co-stimulation via CD28 is known to support T cell proliferation as well as survival.

CD28 counteracts the activation-induced cell death pathway by up-regulating pro-apoptotic molecules such as BCL-x$_L$ [Boise 1995; Kerstan 2004].

DCM is a progressive disease and may display different pathophysiology depending on disease duration. However, we did not find any correlation between disease duration and the number of responding IFN-γ-producing CD4$^+$ T cells (Figure 6). This lack of correlation means that the reduced number of IFN-γ-producing CD4$^+$ T cells in DCM reflects a mechanistic defect rather than a variable dependent on degree of heart failure.
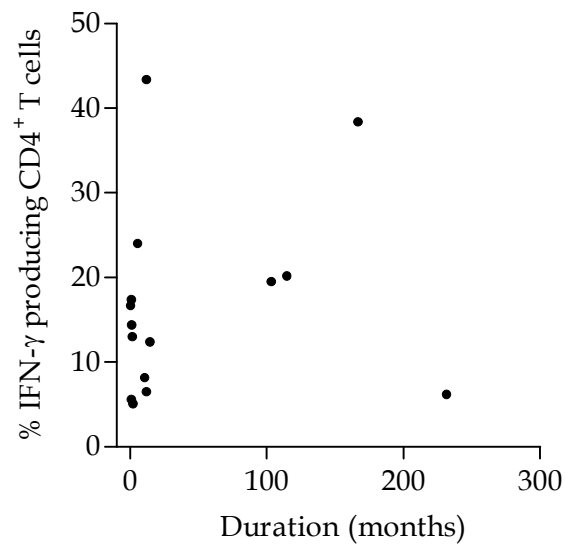
Figure 6. The percentage of IFN-γ-producing CD4$^+$ T cells in relation to heart failure duration.

The consistently normal IL-4 responses together with the lack of correlation between disease duration and the IFN-γ response further support the conclusion that the altered IFN-γ is a true defect in the immune response of DCM. Having ruled out possible influencing factors, the next question is, what impact would aberrant IFN-γ and IL-10 production have on infection and autoimmunity?

DUAL ROLE OF IL-10 AND IFN-γ IN THE REGULATION OF INFECTION AND AUTOIMMUNITY

> Some patients seem to die not of their infection
> but of the body's response to it.
>
> William Osler, 1849–1919

Shortly after autoimmune diseases were first recognized, they were associated with viral infections. Today, however, no autoimmune diseases have been consistently linked to a specific microorganism. Instead, several viruses have been associated with one disease. It is possible that viruses play a contributory rather than a causative role. Following infection with the cytomegalovirus in mice, Fairweather et al. demonstrated the development of myocarditis with a late, chronic phase of the disease. This phase was similar to the infection observed following immunization with the cardiac myosin antigen [Fairweather 2001]. In fact, this late, chronic phase of cytomegalovirus infection meets the criteria of an autoimmune disease by (1) being reproduced by immunization with a relevant organ-specific antigen, (2) causing production of autoantibodies, and (3) the ability to adoptively transfer with T cells from affected to non-infected mice [Rose 1993; Witebsky 1957].

It is therefore of interest to understand the adjuvant effects, such as cytokine production and the regulation of immune response during infection, instead of focusing on the pathogen itself.

IL-10

IL-10 is beneficial to both the host and the virus: to the host by limiting the immunopathology defined as damage caused to the host by the immune response as a result of infection, and to the virus by allowing transformation from an acute to a chronic phase of infection. Beneficial host effects have been shown in mice infected with encephalomyocarditis virus where treatment with recombinant human IL-10 resulted in significantly improved survival and attenuated myocardial lesions [Nishio 1999]. Strategies for augmenting pathogen clearance in order to prevent chronic infections have resulted in exacerbated tissue damage. IL-10-

deficient mice inoculated with *Toxoplasma gondii* and *Trypanosoma cruzi* were found to succumb to lethal infections due to overproduction of IFN-γ. The mortality seen following *T. cruzi* infection could be reversed by administration of recombinant IL-10 or neutralizing antibodies specific to IL-12 [Gazzinelli 1996; Hunter 1997]. Similar aggravating effects of an IL-10 deficiency have been shown in hepatitis, herpes simplex virus-1, and HIV, to mention a few [Couper 2008]. This indicates that maximal pathogen clearance does not necessarily lead to reduced disease prevalence. Instead, it indicates the importance of a properly balanced immune response. A strong regulatory response by IL-10 at the beginning of an infection may result in inadequate pathogen clearance due to its suppressive effects on proinflammatory cytokines, while an insufficient response may result in severe immunopathology due to the over-activation of proinflammatory cytokines.

IL-10 is known to control the proinflammatory arm of the immune system, such as TNF-α release. Exogenous administration of IL-10 has been reported to protect against tissue injury induced by TNF-α-mediated oxidative stress [Mulligan 1993]. In addition, IL-10 has also been shown to shift the protease–antiprotease balance favouring matrix preservation and tissue healing [Lacraz 1995]. Uncontrolled proinflammatory cytokine production can induce undesirable outcomes, such as autoimmune reactions due to activation of macrophages and cytotoxic T cells. These cells cause collateral damage to normal tissue by the release and leakage of proteases and reactive oxygen, which in turn causes exposure of self-antigens and activates an immune response (Figure 7). This could be one plausible hypothesis as to what is observed in DCM, a chronic immune activity with autoimmune characteristics possibly caused by insufficient levels of IL-10 to control the immune activity.

The complexity of the IL-10 response and its regulatory mechanisms has made it difficult to sort out the role of specific IL-10-producing cells during infection. However, in virulent infections, naturally occurring Treg cells are insufficient to control the magnitude of the immune response. Instead, the important inducible Treg cells producing IL-10, generated in the periphery, take over and act in an antigen-dependent manner [Shevach 2006]. In our study, we were unable to identify the type of cell responsible for the impaired production of IL-10. However,

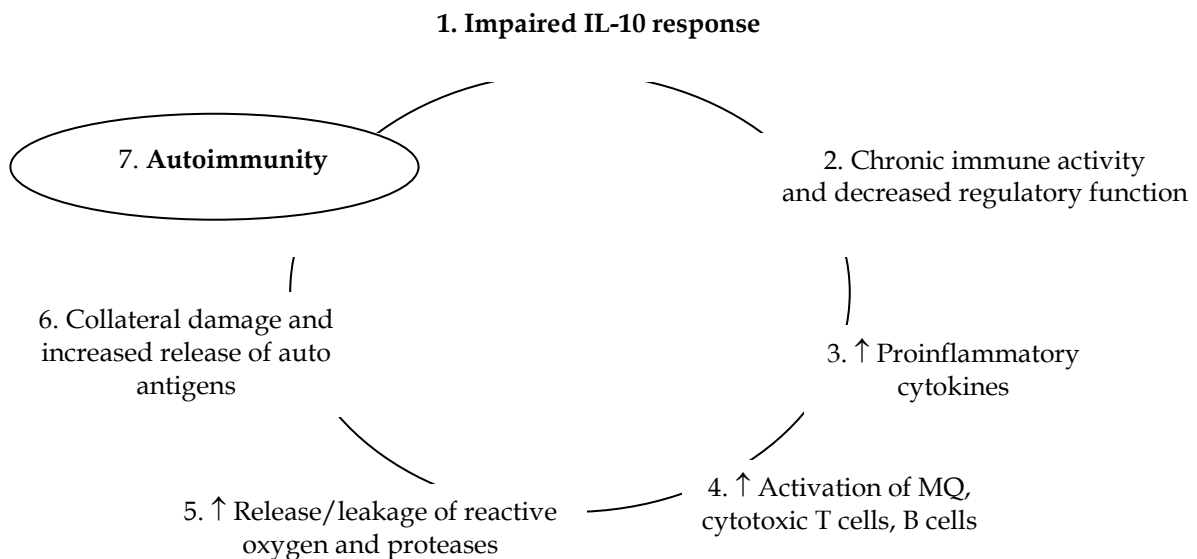irrespective of the source, IL-10 seems to have similar effects on various infections [Couper 2008].

1. Impaired IL-10 response

7. **Autoimmunity**

2. Chronic immune activity
and decreased regulatory function

6. Collateral damage and
increased release of auto
antigens

3. ↑ Proinflammatory
cytokines

5. ↑ Release/leakage of reactive
oxygen and proteases

4. ↑ Activation of MQ,
cytotoxic T cells, B cells

Figure 7. The vicious circle of an over-activated immune response. ↑, increased; MQ, macrophages.

IFN-γ

Over the past few years, the Th1 cytokine IFN-γ has been shown to have regulatory functions and to influence autoimmune reactions. Depending on the disease, it has been shown to function as both a disease-promoting and -inhibiting component. In animal models in which either IFN-γ or the IFN-γ receptor were genetically deleted, most autoimmune diseases, for example, experimental autoimmune encephalomyelitis, collagen-induced arthritis, and uveitis, resulted in increased disease progression and severity [Rosloniec 2002]. In experimental autoimmune myocarditis, IFN-γ deficiency resulted in exacerbated myocarditis and the development of fatal autoimmune disease [Afanasyeva 2001; Eriksson 2001]. There are conflicting findings as to the precise role of IFN-γ in autoimmunity. However, Wang et al. reported IFN-γ to be a strong inducer of Foxp3 expression in experimental autoimmune encephalomyelitis and important for the conversion of

CD4$^+$CD25$^-$ T cells into CD4$^+$ Treg cells, in both mice and humans [Wang 2006]. This is in line with the association found by Afanasyeva et al. between the impaired up-regulation of CD25 expression on CD4$^+$ T cells in IFN-γ-deficient mice and the progression of myocarditis to DCM. [Afanasyeva 2005]. Yet another control mechanism exerted by IFN-γ-producing Th1 cells is the co-production of IL-10 in a self-controlling manner following infection with *Toxoplasma gondii* [Jankovic 2007].

Taken together, not only IL-10, but also IFN-γ is important for the induction and regulation of Treg cells and the development of autoimmune diseases (Figure 8). This strengthens the importance of our findings of impaired IFN-γ as well as IL-10 production in DCM.
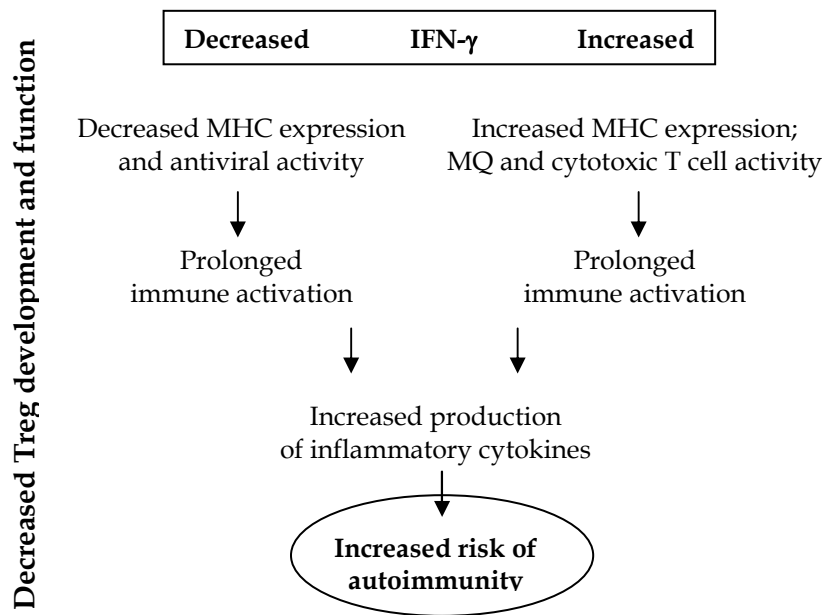
Figure 8. Effects of altered IFN-γ production on the immune response.

THE IMPACT OF HEART FAILURE TREATMENT ON
THE IMMUNE SYSTEM

It is well documented that the sympathetic nervous system plays a major role in cardiovascular disease. Patients with heart failure have profoundly elevated levels of catecholamines; these in turn have been shown to have deleterious effects in experimental studies of mammalian cardiomyocytes, in which higher levels lead to decreased myocyte viability [Mann 1992]. In addition, norepinephrine, in particular, has been shown to be independently related to the risk of heart failure progression and mortality [Cohn 1984]. In turn, norepinephrine is known to alter the activity of the immune system through the signalling of β-adrenergic receptors, which are known to be expressed on T and B cells [Sanders 2002]. Norepinephrine is believed to increase sympathetic drive which leads to increased immune activity, most often seen as increased cytokine levels. It is therefore of importance to discuss the impact of heart failure treatment on immunological studies.

A major breakthrough in the treatment of heart failure came in mid 1970 when Waagstein et al. reported improved cardiac function following β-blocker treatment of patients with heart failure. Today β-blockers and angiotensin-converting enzyme (ACE) inhibitors are mandatory in the treatment of heart failure [Waagstein 1975]. This treatment reduces sympathetic activity by lowering plasma norepinephrine, and thereby also immune activity. Most significant is the influence of ACE inhibitors by lowering plasma IL-6 and of β-blockers by lowering plasma TNF-α. However, even following heart failure therapy, a high degree of immune activation persists [Gullestad 1999; Ohtsuka 2001].

Of patients with DCM in whom we measured plasma cytokines, 92% and 99% received β-blockers and ACE inhibitors, respectively, in paper I, whereas 88% and 100% of patients were treated in paper II. All patients received $β_1$-selective β-blockers. This would not have any direct blocking effect on immune cells, since they lack the $β_1$-AR [Kohm 2001; Sanders 2002]. Levels of IL-6 and TNF-α in plasma are elevated in patients with DCM and heart failure despite treatment with β-blockers and ACE inhibitors (papers I and II). It could be speculated that the results observed in paper II, in which decreased responses of both IFN-γ and IL-10 were observed, were partly due to treatment effects. This is not particularly likely, since we found

no difference between DCM and controls in either IL-6 or TNF-α production measured in the supernatant from cultured PBMC (Table 5).

Table 5. TNF-α and IL-6 measured in the supernatant from PBMC cultured overnight. Values are expressed as mean ± SEM.

	TNF-α			IL-6		
	Controls	DCM	p-value	Controls	DCM	p-value
Basal	2460 ± 330	2270 ± 440	0.45	14 400 ± 1500	18 600 ± 3000	0.66
SEB	2430 ± 220	2950 ± 330	0.35	12 600 ± 1100	17 800 ± 2400	0.09
PHA	2340 ± 240	2880 ± 360	0.32	14 000 ± 1400	19 100 ± 3600	0.56

In a study designed to evaluate heart failure therapy, Gage et al. reported that the TNF-α production and IFN-γ/IL-10 ratio of stimulated PBMC was significantly lower in treated than untreated patients [Gage 2004].

Finally, the immune system and sympathetic nervous system are two complex networks in the human body. How and the extent to which these two systems regulate one another is still controversial, so it is difficult to interpret the possible effects of conventional heart failure therapy on T cell function.

CONCLUSION

This thesis aimed to study T cell function in DCM. Our results are consistent with the general belief that DCM is a disease with immunological pathophysiology. The major immunological alterations reported in DCM, i.e., presence of autoantibodies and elevated cytokines in plasma, indicate chronic peripheral immune activity in these patients. We suggest that this chronic immune activity is a consequence of a reduced capacity to regulate the immune response, seen by decreased ability to produce INF-γ and IL-10. Dysfunctional immune regulation dramatically increases the risk of both chronic infections and autoimmune reactions.

However, we have only studied a minor part of the complex area of immunology, and there is much more to explore in order to define what impact the immune system has on DCM. Here, we offer one piece of the major puzzle that is DCM and encourage others to continue this line of research, by defining T cell function in DCM.

ACKNOWLEDGMENTS

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