Immune regulation following pediatric cardiac surgery

What goes up must come down

Alvin W.L. Schadenberg

Cover: Close-up photograph of dolerite rock in Aussenkehr, Namibia (photo & design A. Schadenberg). This rock originated 2 million years ago, only to be crumbling down again to dust today. Despite the loose structure it has some solid sections providing excellent climbing – what goes up must come down.

Layout: Legatron Electronic Publishing, Rotterdam
Printing: Ipskamp Drukkers, Enschede

Printing of this thesis was financially supported by Infection and Immunity Utrecht.

This thesis is printed on FSC certified paper. FSC certification ensures that products come from well-managed forests that provide environmental, social and economic benefits.

ISBN: 978-94-6191-962-5

Immune regulation following pediatric cardiac surgery

What goes up must come down

Immuunregulatie na kinderhartchirurgie

Herstel van de balans

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op woensdag 11 december 2013 des middags te 2.30 uur

door

Alvin Wietse Leslie Schadenberg

geboren op 14 januari 1977 te Noordoostpolder

Promotor: Prof.dr. B.J. Prakken

Co-promotor: Dr. N.J.G. Jansen

Voor mijn ouders

CONTENTS

Chapter 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

General introduction

The immune system is designed to respond rapidly to potential harmful stimuli. An acute stress signal induces systemic inflammation, and can be either infectious in origin such as sepsis; or sterile such as trauma, surgery and burns. Regardless of the etiology, the systemic inflammatory responses to these different stresses are remarkably similar.[1] To study this acute inflammatory response in more detail, research has predominantly relied on animal models. However, translating animal immunology to human disease has proven to be challenging, especially in this field.[1;2] Still, the systemic inflammatory response remains an important clinical challenge. For example, in the clinical setting of intensive care, cardiac surgery is well known to induce an acute systemic inflammatory response. Therefore, elective cardiac surgery also creates an opportunity to study the inflammatory response in human in detail, from its initiation through the peak of inflammation up to recovery.

Pediatric cardiac surgery profoundly affects the immune response. While surgical intervention is often life saving, it also brings along risks by triggering the immune system. Children needing repair of a congenital heart disease are usually young, which is an additional risk factor. Although at birth the human immune system is complete with all the components present, it still needs further development and expansion.[3] Not surprisingly, it has been shown that age is a considerable risk factor for developing adverse effects of the inflammatory response following cardiac surgery.[4;5] In the past decades much research has been performed to characterize this inflammatory response. In the immediate peri-operative period both local and systemic inflammation is initiated. Leukocytes responsible for this response increase in the circulation alongside a myriad of stress proteins such as complement, cytokines and chemokines (Table 1). Subsequently, therapies have been pursued to reduce these instigators of a harmful inflammatory response, with varying success[6]. Like every biological system, the inflammatory cascade includes an endogenous feedback mechanism. This endogenous inhibitory response is far less well understood. Understanding how the inflammatory response is kept in control after surgery could aid therapies for when inflammation runs out of control. While inflammation following cardiac surgery can be harmful, in itself it is an appropriate response to inflicted harm and essential for the healing process after surgery, if kept in control. Thus, understanding the feedback mechanisms of this inflammatory response can also shed light further afield. Lessons can be learnt for immunological disorders where the immune system responds inappropriately to harmless stimuli, such as allergy and autoimmunity.

The first part of this thesis investigates the acute inflammatory response in children following cardiac surgery. The second part of this thesis describes the long-term effects of cardiac surgery in a select group of patients requiring neonatal thymectomy.

Table 1. Indicators of inflammatory response following pediatric cardiac surgery.

	Increased	Decreased	References
Cellular factors			
Neutrophils	Elastase	CD11b, CD18	4;107-110
	Myeloperoxidase	CXCR1,2	
Monocytes	TLR2/4	HLA-DR	111-114
	CD11b, CD16, CD18	LPS-response	
Lymphocytes	Apoptosis	Response to mitogens	115-118
	Treg numbers	Cytokine production	
		Treg function	
Soluble factors			
Complement	C3a, C5a, C5b-9		4;119-123
Chemokines	MCP-1	RANTES, ICAM-1,	123;124
		sE-selectin	
Cytokines	IL-10, IL1-RA,		4;14;120;125-129
	IL-1b, TNFa		
	IL-5, IL-6, sIL6R		
	IL-8		

1. Pediatric cardiac surgery

Congenital heart disease (CHD) occurs in 6–13 of every 1000 livebirths.[7] Approximately one quarter of these children will require surgical or catheter intervention in the first year of life. CHD account for over 30% of birth defect related deaths and are responsible for more deaths than any other type of malformation.[8;9] CHD range from simple defects requiring no treatment, to heart defects necessitating multiple operations or ultimately heart transplantation. Over the past decades improvement in antenatal screening and treatment options has reduced mortality significantly. Recently published post-operative mortality figures range from 0.6% to 18.4% depending on the type of procedure.[10] Besides immediate post-operative risks, children with CHD also are at increased risk later in life including developmental disorder or delay.[11;12] Hence, these patients remain a group at risk for morbidity and mortality at clinical presentation and later in life. Surgical intervention being on the one hand essential and lifesaving, also contributes to this risk. Through combined efforts from anesthesia, surgery, cardiology, intensive care and other subspecialties, surgery is being performed on more complex defects in younger patients. These advances in surgical options also increase the challenges for the post-operative management. One of the endogenous factors complicating post-operative recovery is the inflammatory response. Although still not completely understood, this leads to morbidity due

to either hyper-inflammation resulting in increased vascular permeability and ultimately organ damage, or on the other hand of the spectrum temporary immune deficiency, with subsequent risk of infections. Finding an appropriate balance between the two without compromising the healing process is a great challenge for the post-operative management of these complex patients.

2. Clinical significance of post-operative inflammatory response

Patients requiring cardiac surgery will be exposed to multiple triggers to which their immune system responds. The net result of these responses is characterized as the post-operative inflammatory response. Clinical outcome depends on the severity of each trigger and the extent to which the immune system responds. Regardless of the type of operation, all patients will be affected by anesthesia, surgical tissue injury, mechanical ventilation and some degree of ischemia-reperfusion and lipopolysaccharide (LPS) translocation from the intestines (Figure 1). Most pediatric procedures furthermore depend on cardiopulmonary bypass (CPB) with the use of an extracorporeal system to enable systemic perfusion while the heart is operated on. Pre-operative conditions such as hypoxemia or heart failure trigger the immune system before any iatrogenic event occurs.[13-15] And finally, once the patient returns from the operating theatre to recover on the Pediatric Intensive Care Unit (PICU), ongoing triggers include mechanical ventilation and secondary infection[16]. As the immune system responds to each trigger, the culmination of these triggers can prevent recovery.

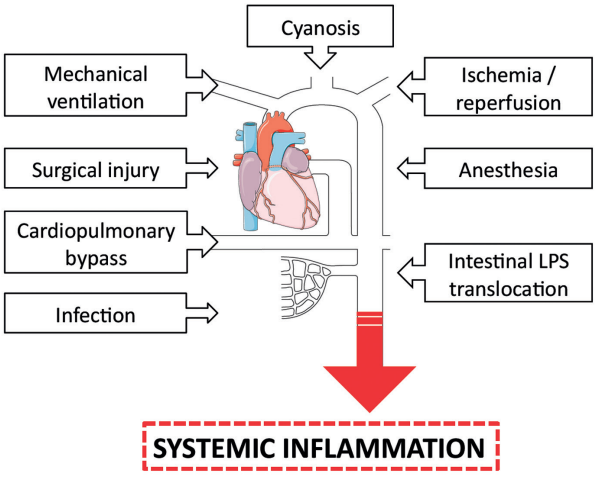

Figure 1. Peri-operative triggers of inflammation in pediatric cardiac surgery.
(LPS: lipopolysaccharide)

The inflammatory response can affect multiple organ systems. The basis to most morbidity is the effect on the endothelium, subsequent increase of permeability and leukocyte recruitment. Activated endothelium attracts leukocytes and loses its integrity resulting in increased vascular permeability. The subsequent vascular leak results in multi organ edema. The lungs are particularly prone to ischemia-reperfusion injury[17]. The resultant pulmonary edema results in reduced gas-exchange and subsequent morbidity. Following CPB leukocytes accumulate in the heart, contributing to a local inflammatory response and reduced function.[18] A reduced cardiac output after surgery, low cardiac output syndrome (LCOS), necessitates frequent use of inotropic support and fluid resuscitation.

Fluid retention post cardiac surgery, resulting from increased need for fluid resuscitation and increased vascular permeability, prolongs duration of ventilation and length of stay.[19] Acute kidney injury (AKI) and brain injury following cardiopulmonary bypass are well-recognized co-morbidities in the immediate post-operative period[20] especially in infants. Overall, multiple organs are affected by the inflammatory response due to cardiac surgery and contributes to post-operative morbidity and mortality.

3. Regulation of inflammation

It is obligatory for the immune system to respond rapidly to potential harmful stimuli, whether infectious, tissue injury or otherwise. Likewise, the same immune cells need to be tightly controlled and inhibited when no longer needed, to prevent collateral damage. Therefore, inflammation is a highly orchestrated response to tissue injury. Key to minimizing inflammatory damage is trafficking and localizing leukocytes to the injured tissue. At the required site, these leukocytes can switch on their pro-inflammatory gene pathways and produce inflammatory mediators. Uncontrolled production of inflammatory mediators, or production at the wrong site, can have detrimental effects. Therefore, endogenous mechanisms are available to keep the inflammatory response in check, and permit resolution of the inflammatory response. Understanding these control mechanisms has attracted much attention over the past decade. Insufficient control of inflammation is the hallmark of many diseases including autoimmunity, atherosclerosis, allergy, obesity and probably many more. The common characteristic of these clinical conditions is unresolved inflammatio.[21] Although the immune system is triggered through different mechanisms, the available points of control will be very similar. Thus, understanding endogenous resolution of the inflammatory response following surgery may also help understand human disease where resolution fails.

Control mechanisms are available at each level, from intracellular molecular feedback pathways, through anti-inflammatory proteins to highly specific regulatory leukocytes. Without giving a full overview of every known regulatory mechanism, some key mechanisms that influence the inflammatory response are described below. Section 3.1 describes the molecular pathways of importance in initiating inflammation through Toll like receptors (TLR) and

cytokine receptors. In addition their main negative feedback mechanisms are discussed. Next, in section 3.2 the function of the key anti-inflammatory cytokine IL-10 is discussed in order to highlight its importance for endogenous immune regulation. Finally, $FOXP3^+$ regulatory T cells (Treg) are introduced in section 3.3 as regulators of inflammation including phenotypical characteristics, function for immune homeostasis and development in humans.

3.1 Intracellular control of inflammation

Regulation of gene expression is an important control mechanism in inflammation. Exposure of cells to stress elicits a coordinated expression of stress-genes, which affect cell survival, apoptosis and cell-differentiation.[22] Subsequent post-transcriptional and finally translational regulation defines the outcome at protein level. Translation of specific mRNA is tightly controlled to prevent unwanted proteins and save energy expenditure.[23] Next, a summary is given of points of control in significant molecular pathways of importance for inflammation following cardiac surgery. To remain within the scope of this thesis, only those pathways are described briefly which are referred to in *Chapter 3* of this thesis. Primarily, the pro-inflammatory signaling cascade downstream of TLR4 resulting in NFkB activation is described alongside its endogenous feedback pathways. Then, the Jak family tyrosine kinases (JAK) / signal transducer and activator of transcription (STAT) pathway that is responsible for transmitting extracellular cytokine signals to the nucleus and the Suppressor of Cytokine Signaling (SOCS) is described. These signaling pathways with their main feedback pathways are illustrated in Figure 2.

TLR-MyD88 dependent pathway

The key transcription factor of inflammation is NFkB with a wide range of effects, both pro- and anti-inflammatory. As NFkB is present in the cell in an inactive form, it does not need new protein synthesis and therefore is a rapid first responder to harmful stimuli. NFkB is activated by many different stimuli.[24] The key activation step of the NFkB cascade is recognition of danger-associated molecular patterns (DAMPs) or pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRR). The best-described trans-membrane PRR is the family of Toll like receptors (TLR). In non-infectious inflammation so called DAMPs initiate the immune response following tissue damage. Examples of DAMPs include intracellular proteins (e.g. heat shock proteins, HMGB1), ATP and uric acid. Their infectious counterpart PAMPs, are small molecular motifs conserved within a class of microbes (LPS being the prototypic example). Recognition by TLR or other PRRs on the cellular surface leads to activation of NFkB through the myeloid differentiation factor 88 (MyD88) / interleukin-1 receptor-associated kinases (IRAK) dependent signaling pathway.[25] Following activation through the MyD88/IRAK pathway, IkB kinase (IKK) is released, which leads to degradation of IkB from the NFkB/IkB complex. Inactive NFkB is sequestered in the cytoplasm by its inhibitor IkB and degradation of IkB by IKK results in activation of NFkB. Once the NFkB complex is freed from IkB, it enters the nucleus where it can turn on the expression of genes with a binding site to NFkB,

including genes essential for inflammation (mediating synthesis of IL-1, IL-6, IL-8, TNFa, etc), cell survival and apoptosis. Simultaneously, NFkB activates its own inhibitor IkB, as a negative feedback. Additionally TLR activation leads to activation of responsive genes through the mitogen-activated protein kinase (MAPK) pathway. Multiple negative regulators exist of both NFkB and MAPK pathways including SOCS, IRAK-M and others (Figure 2).

JAK-STAT dependent pathway

The JAK-STAT pathway is used by many cytokine receptors to rapidly transmit extracellular signals to the nucleus.[26] The pleiotrophic cytokine IL-6 for instance is crucial in the acute-phase response to injury.[27] Signaling downstream of IL-6 results in activation of JAK, phosphorylates STAT3, which dimerizes and is translocated to the nucleus to activate transcription of genes containing STAT3 response elements. STAT3, which acts downstream of both IL-6 and IL-10 signaling, plays a crucial role in many cellular processes including cell survival and apoptosis.[28] STAT3 knockout mice exemplify the crucial role of STAT3 in systemic inflammation. Namely, such STAT3 knockout mice exhibit diminished recovery from endotoxic shock and hyperresponsiveness of certain LPS inducible genes.[29]

SOCS signaling

The family of SOCS proteins are important negative regulators of cytokine signaling. SOCS3 for example is strongly induced by IL-10, and suppresses the action of proinflammaotry cytokines such as IL-6 and TNFα.[30] In addition, proinflammatory cytokines induce SOCS3, thus acting as a negative feedback loop of these cytokines. The inhibitory function of SOCS results from binding to the activation loop of JAKs and suppression of their kinase activity.[31] Through inhibiting JAK-STAT signaling, SOCS acts as a negative regulator of a wide range of cytokines. In addition SOCS also inhibits JAK-independent pathways including IRAK-NFkB pathway.[32] Thus SOCS acts as an important negative feedback for several inflammatory signaling pathways (Figure 2).

The immune system needs to respond promptly to extracellular signals. Following activation of the specific receptors (e.g. through cytokines or DAMPs/PAMPs) an intracellular signaling pathway leads to expression of inflammatory genes. To turn off the inflammatory response in time and limit collateral damage, multiple intracellular negative feedback mechanisms exist which can interact at different sites in the signaling chain.

3.2 Anti-inflammatory cytokine IL-10

In controlling the degree and duration of the inflammatory response IL-10 plays a key role. It selectively blocks expression of pro-inflammatory genes, while simultaneously enhancing expression of anti-inflammatory molecules.[33] A wide range of cells (of both the innate and adaptive immune system) produce IL-10; including macrophages, monocytes, and all T cells.

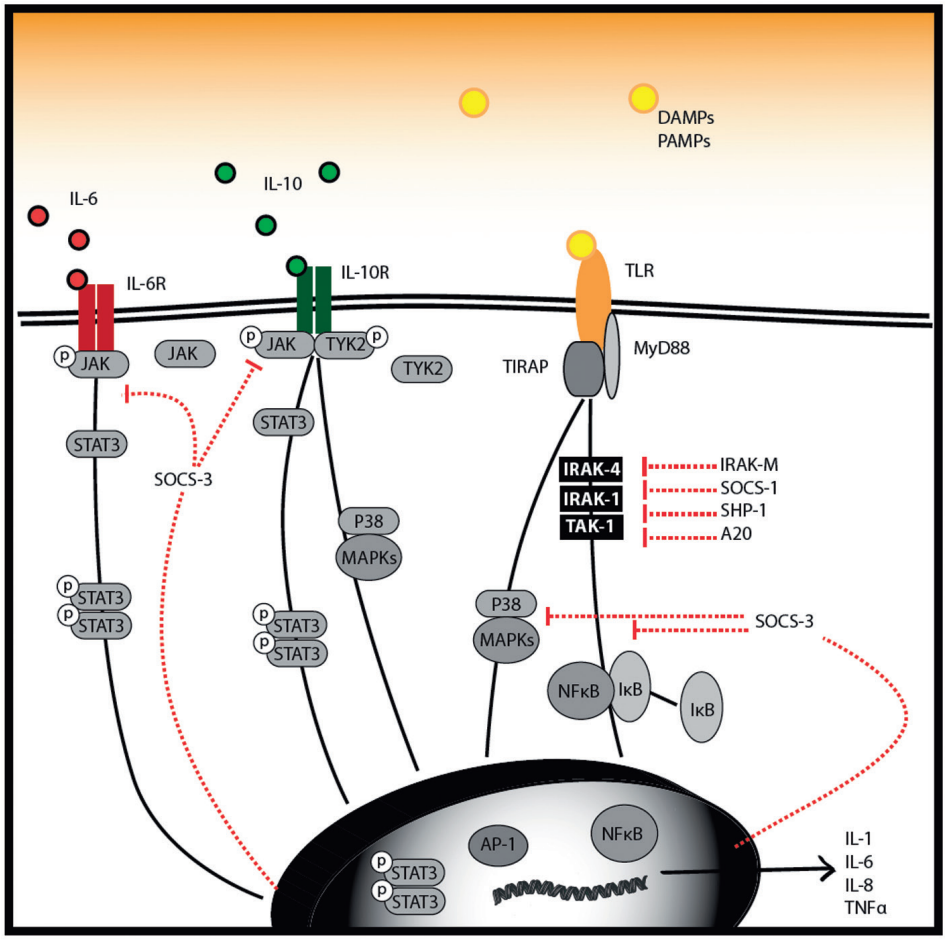

Figure 2. Feedback mechanisms of major signaling cascades of inflammation. The cell surface has multiple receptors available to engage in signals from outside the cell surface. Danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) mediate the induction of inflammation through recognition by Toll like receptors (TLR). MyD88 and Toll interleukin 1 receptor adaptor protein (TIRAP) couples TLR and Interleukin-1 receptor-associated kinases (IRAK1-4) that leads to activation of NFkB by dissociating and degradation of its inhibitory protein IkB. The activated NFkB is then translocated into the nucleus where it binds to specific sequences of DNA, ultimately leading to transcription of proteins responsible for inflammation (and cell survival (e.g. IL-1, IL-6, IL-8, TNFα). Stimulation through TLR also leads to activation of p38 / mitogen-activated protein kinases (MAPK) and subsequent activation of transcription factors (such as AP-1). IL-10 exerts its effect through its receptor IL-10R. The main signaling pathway downstream of this receptor is the JAK-STAT system. Engagement of the Jak family tyrosine kinases JAK1 and TYK2 with respectively IL10-R1 and IL10R2 induces tyrosine phosporylation and activation of signal transducer and activator of transcription 3 (STAT3). STAT3 dimerizes and translocates to the nucleus. In addition IL10R activation also leads to activation of MAPK pathway. The pleiotrophic cytokine IL-6 binds to its receptor which

also predominantly relies on JAK-STAT signaling to activate transcription of genes. At multiple sites in these signaling cascades inhibitory molecules interact, limiting further activation. The main negative feedback molecules include signaling of suppressor of cytokine signaling (SOCS), IRAK-M, Src homology region 2 domain-containing phosphatase-1 (SHP-1) and A20.

IL-10 exerts its function through binding to its receptor (IL-10R). IL-10R is composed of two subunits that are a member of the interferon receptor family.[34] While IL-10R2 is constitutively expressed on most tissues and cells, IL-10R1 needs upregulation to render cells responsive to IL-10.[35] Like many cytokines, JAK-STAT pathway plays an obligatory role in IL-10 signaling.[36] One of the main effects of activation of the IL-10 signaling pathway is transcription of SOCS3.[37;38] IL-10 induced SOCS3 provides a negative feedback regulation of IL-10 signaling itself but also contributes to IL-10 inhibition of inflammation by inhibiting pro-inflammatory cytokines (Figure 2). Another important anti-inflammatory property of IL-10 is the induction of IL-1 receptor antagonist (IL-1ra), which competes with IL-1 for binding to its receptor without activating its downstream signaling cascade. IL-10 enhances LPS-induced IL-1ra expression through binding of STAT3 to the IL-1ra promoter.[39] In this manner IL-10 directly abrogates the pro-inflammatory effects of IL-1. Beyond exerting its function through the JAK-STAT pathway, IL-10 inhibits other pro-inflammatory pathways. NFkB is inhibited on different levels, including inhibiting degradation of IkB and inhibiting NFkB DNA binding activity.[40]

The overall effect of IL-10 is inhibition of both myeloid and lymphoid cells. IL-10 potently inhibits monocyte / macrophage production of most proinflammatory cytokines (IL-6, TNFα, IL-1, IL-18 etc.), but also of IL-10 itself. In addition IL-10 inhibits production of chemokines (MCP-1, IL-8, MIP, etc) and prostaglandin E2.[33] IL-10 also downregulates macrophage expression of TLR, while increasing the expression of receptors required for scavenging such as CD16 (FcγRIIIa), CD64 (FcγRI) and CD163.[41] Likewise, IL-10 inhibits cytokine and chemokine production by neutrophils and suppresses microbicidal activity. $CD4^+$ T cell proliferation and cytokine production is strongly inhibited by IL-10. In contrast, IL-10 stimulates CD8+ T cells in recruitment, proliferation and cytotoxicity.[42]

As a result, collectively, IL-10 limits the duration of inflammation and contributes to clearance of harmful stimuli by enhanced phagocytosis. The protective effects of IL-10 have been illustrated in models of severe inflammation. Endogenous IL-10 confers significant protection from the harmful effects of LPS challenge in mice.[43] Likewise, IL-10 protects mice from different forms of lethal sepsis including streptococcal B infection and cecal ligation, and non-infectious systemic inflammation such as ischemia reperfusion and burns.[44-47]

In conclusion, the main action of IL-10 is to inhibit pro-inflammatory responses from both innate and adaptive immunity. By regulating and repressing the expression of pro-inflammatory cytokines during the resolution phase, IL-10 reduces tissue damage caused by these cytokines as illustrated in a model of cardiac ischemia-reperfusion injury.[48] Overall, IL-10 is a key mediator in controlling the degree and duration of the inflammatory response.

3.3 FOXP3$^+$ Regulatory T cells

FOXP3$^+$ Regulatory T cells (Treg) are a population of highly suppressive T cells which modulate the inflammatory response. Treg play an essential role in immune homeostasis, as disruption in their development or function results in autoimmunity and inflammatory diseases.[49] The most definitive marker for Treg is FOXP3, which is essential for its development and function.[50] FOXP3, a forkhead transcription factor, is considered a lineage specific transcription factor of Treg as genetic mutation of Foxp3 leads to deficiency in Treg resulting in severe autoimmunity in both humans and mice.[50;51] In addition, forced expression of FOXP3 converts naive T cells towards a Treg phenotype with similar suppressive function as true Treg.[52] However, the significance of FOXP3 expression has been complicated by the knowledge that CD4$^+$FOXP3$^-$, non-suppressive T cells can transiently express FOXP3 after activation without conferring a regulatory phenotype.[53;54]

Treg exert their suppressive function through different mechanisms, though their main effector function remains controversial. Different studies have shown their methods of action to be contact-dependent,[55;56] production of anti-inflammatory cytokines such as TGFb[57] and IL-10,[58] and competition for survival cytokines.[59;60] Treg inhibit effector T cell proliferation, cytokine production and cytotoxicity. Furthermore, Treg suppress antigen presenting cells, including monocytes and macrophages.[61] Treg not only suppress the adaptive immune system, but also have strong inhibitory effects on innate immune mechanisms.[62-64]

Over the past two decades ontogeny and mechanisms of suppression of Treg has been studied in great depth by means of experimental studies. Despite the obvious advantages of *in vitro* and animal models, there have been shown to be clear differences between experimental and human *in vivo* immunology.[65-67] Therefore, great care should be taken when translating experimental data to clinical human immunology and much work remains to be done to understand the full scope of Treg in human disease.

From the time that Treg were first discovered in mice, it was evident that they were produced by the thymus as a functionally distinct subpopulation of T cells.[68] Neonatal thymectomy in certain mouse strains resulted in severe autoimmune diseases due to removal of suppressive Treg, while transfer of these cells prevented autoimmune development.[69] These natural thymic derived Treg (nTreg) are generated in the thymus through major histocompatibility complex (MHC) class II-dependent T cell receptor (TCR) interaction, resulting in high avidity selection. Only those T cells that recognize self-ligands strongly (but below the apoptosis threshold) induce FOXP3 expression. However, FOXP3 expression alone is not sufficient for reliably delineating functional Treg cells. It has been shown that besides expressing FOXP3, nTreg also have specific demethylated DNA sites. These demethylated DNA sites (such as CNS2) are generally of importance for Treg function and contribute strongly to the stability of the Treg cell lineage.[70] The specific DNA demethylation patterns occur in the thymus and generally remain highly stable throughout the Treg cell's life, regardless of stimulation. Thus, Treg specific demethylated DNA sites, combined with Foxp3 expression, are reliable markers for nTreg.[71]

While beyond doubt FOXP3$^+$ Treg arise as a distinct lineage in the thymus, there is evidence that part of the peripherally circulating FOXP3$^+$ Treg in human developed from non-Treg cells. These induced Treg (iTreg) express similar Treg specific molecules such as FOXP3, cytotoxic T lymphocyte antigen-4 (CTLA-4), glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR) and IL-2 receptor (CD25). Evidence of their existence comes from adoptive transfer of CD25- naive T cells into lymphopenic mice. In subsequent homeostatic proliferation of the donor (non-Treg) T cells, some T cells become CD25$^+$CTLA-4$^+$GITR$^+$ T cells expressing FOXP3$^+$ and acquire suppressive activity.[72] Furthermore, numerous studies have reported on the induction of human FOXP3$^+$ Treg cells from non-Treg *in vitro* using different protocols.[73;74]

In human the peripheral FOXP3$^+$ T cell population can be divided into different subsets, with different proposed origin and function.[75] Based on the expression of FOXP3 and CD45RO (or CD45RA), FOXP3$^+$ cells can be separated into three subsets. The Treg subsets with functional suppressive capacity (CD45RA$^+$RO-FOXP3^{low}CD4$^+$ resting Treg and CD45RA-RO+FOXP3highCD4$^+$ active Treg) and a non-suppressive population (CD45RA-RO+FOXP3^{low}CD4$^+$ non-Treg). The active Treg population (CD45RA-FOXP3^{high}) is proposed to originate from the resting Treg population, although may also be induced from FOXP-conventional T cells.[76]

So far, limited studies on the dynamics of the FOXP3$^+$Treg population in human are available due to difficulty in distinguishing the separate populations from each other. The proposed origin of Treg in human is thus through thymopoiesis (natural thymic derived Treg) and peripheral induction (peripherally induced Treg). Their relative contribution in controlling immune homeostasis has not been completely elucidated. So far, it is assumed that both have a unique role in immune homeostasis.[77] Natural thymic derived Treg being essential for peripheral self-tolerance, while peripherally induced Treg are important in tolerance to non-pathogenic foreign antigens (such as intestinal microbiota).[78;79] Thus, an important question remains; how is the human Treg population maintained throughout life and during stimulation by different causes?

4. The thymus and thymectomy

T cell immunity depends on circulating T cell numbers and diversity of available TCR to respond adequately to encountered antigens. During maturation of the T-cell pool the number of T cells increases, paralleling the expanding body volume they have to patrol. T cells are produced through two mechanisms; thymopoiesis and proliferative expansion of post-thymic cells (Figure 3A). Thymic output generates diversity of the pool and proliferation achieves optimal clonal size of each specific TCR.[80] In the developing neonatal immune system, influx of new T cells from the thymus is essential to reassure that T cell diversity is not contracted due to increased clonal size.[81] TCR repertoire is generated in the thymus by ad random combinations of different gene segments leading to a diverse set of TCR-α and -β segments. Pairing of different

TCR-α and -β segments further enhances TCR diversity. Ultimately the TCR-αβ diversity of human naive T-cells is estimated at 2.5 x 10^7.[80]

The thymus is essential for the initial establishment of the peripheral T cell pool. In human it develops early in fetal life, being responsible for the outgrowth of the peripheral T cell population in both fetal and postnatal life. The thymus gradually atrophies after the age of approximately one year at the rate of 1–3% per year.[82] Regardless of involution, the thymus continues to serve as the site of T cell maturation and production throughout adulthood[83] while it largely degenerates into fatty tissue in the elderly.[84] Also, despite the decrease in thymus output during life, naive T cell numbers remain relatively stable during adult life.[85] In childhood, both thymopoiesis and peripheral proliferation contribute to the establishment and maintenance of the naive T cell compartment.[86] In adult life, in contrast to mice, the human naive T cell pool is predominantly maintained through peripheral proliferation and increased longevity of naive T cells.[65;87] However, in conditions of T cell depletion, the thymus is capable of re-establishing its role in contributing to reconstituting the T cell compartment. Thymic output is increased and rejuvenates the T cell repertoire after autologous stem cell transplant in adult MS patients[88] and patients treated for breast cancer.[89] Following antiretroviral treatment for HIV-1 infection the thymus contributes substantially in both young and adult patients to recover naive T cell count.[83;90]

Loss of thymopoiesis can result in a progressive decline in naive T cells (Figure 3). Insufficient circulating naive T cells may influence the capacity to mount immune responses to novel antigens. A study in aging primates showed that loss of naive T cells results in replacement with oligoclonal memory populations. The subsequent constricted TCR repertoire results in limited antigen responses.[91] Likewise, lymphopenic patients following bone marrow transplantation show a reduced vaccination response and the resistance to opportunistic infections correlates to naive T cell numbers.[92] Restoration of the TCR repertoire can only come from thymic rebound.[93]

Some genetic disorders, such as Di George anomaly, Down syndrome, and Wiskott-Aldrich syndrome, are known to present with thymic aplasia or hypoplasia. Di George anomaly is associated with a deletion of chromosome 22q11.2 in 80% of patients, and characterized by conotruncal cardiac defects, hypoparathyroidism, dysmorphic facial features and thymic hypoplasia or athymia.[94] A small proportion of Di George patients are born with a complete absence of thymus. The majority however display thymic hypoplasia, which presents with a relatively preserved T cell function, although reduced proportion of recent thymic emigrants, naive T cells and Treg.[95] The resultant lymphopenia is associated with an accelerated homeostatic proliferation resulting in reduced TCR diversity.[96] While complete Di George is fatal, partial Di George is associated with heterogeneous levels of immunodeficiency ranging from profound life-threatening immune deficiency to normal immunity. Interestingly, thymic transplantation seems a promising alternative for patients with limited thymic function.[97]

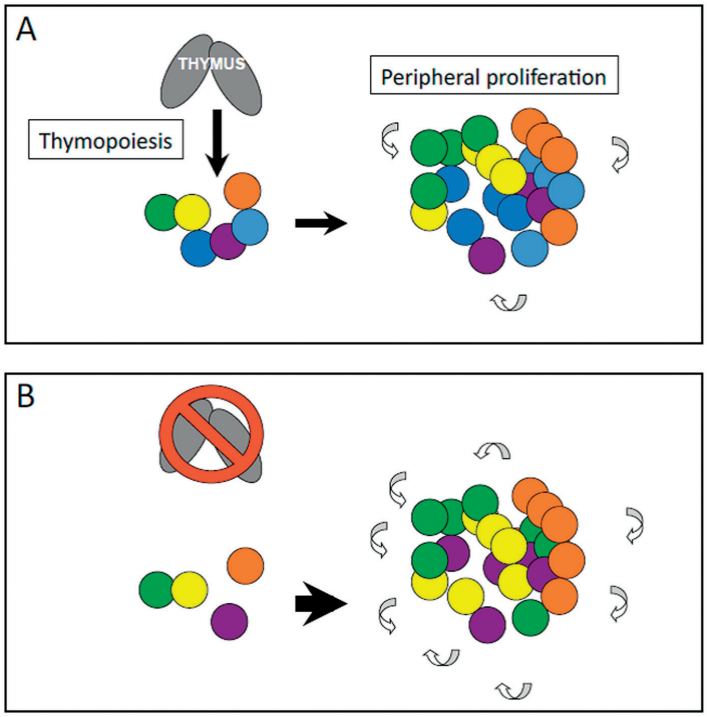

Figure 3. Maintenance of the T cell population.

(**A**) The T cell population is maintained by two sources; thymopoiesis and peripheral proliferation. Diversity of the T cell repertoire depends on thymopoiesis, while peripheral proliferation maintains clonal size.

(**B**) Loss of thymopoiesis results in oligoclonality if the T cell numbers are to be maintained by increased peripheral proliferation

In human, removal of the thymus occurs for different reasons in both children and adults. Therapeutically, thymectomy is an accepted treatment for patients with myasthenia gravis. The precise mechanism of action of thymectomy is unknown, although possible explanations include the removal of the source of continued antigen stimulation, removal of Acetyl Choline Receptor antibody-secreting B-cells, and immunomodulation.[98] It has been shown that thymectomy at an adult age does not alter the composition of the T cell population while thymic output declines. Naive T cell numbers remain stable while thymic output decreases, suggesting that peripheral naive T cell proliferation and perhaps longevity are responsible for maintenance of the naive T cell compartment.[99;100] In pediatric cardiac surgery, thymectomy is performed to gain an unrestricted view of the operation site. Especially in neonates, where the thymus is comparatively large, surgical procedures involving the large vessels necessitate

complete removal of the thymus. Several studies have studied the effect of neonatal thymectomy on the developing immune system. Most studies evaluated the consequences of thymectomy in the first years after the procedure. Neonatal thymectomy results in reduced $CD4^+$ and $CD8^+$ T cells, already in the first year following thymectomy, but is sustained almost two decades thereafter.[101-104] As expected, especially the naive T cell population was affected.[102;105] This suggests that removal of the thymus early in life may affect the naive T cell compartment, and that the naive T cell compartment cannot be fully restored. In the first decade after neonatal thymectomy no obvious clinical immune dysfunction is observed. However, several studies do show signs of functional abnormalities due to reduced naive T cells. Neonatally thymectomized children show reduced response to a new antigen (tick-borne encephalitis vaccine).[105] Another study revealed marked immunological alterations in young adults who were thymectomized within the first two weeks of life. These alterations indicate premature signs of immune aging with reduced naive T cells, accumulation of oligoclonal memory T cell populations and increased markers of inflammation.[106]

Thus, while neonatal thymectomy is a necessary procedure in some cardiac procedures, the immunological consequences are not fully understood. Although these patients appear to have no clinical signs of immune deficiency in the first decades of life, there are clear observations of immunosenescence that may have consequences later in life. As cardiac procedures involving neonatal thymectomy are becoming more successful, and patients are surviving beyond the first decades of life, it is essential that the full consequences of thymectomy be better understood. In addition this patient group enables detailed human studies of the role of the thymus for maintaining different T cell subsets including naive T cells and Treg.

5. Outline of this thesis

This thesis describes the impact of pediatric cardiac surgery on the immune system. Cardiac surgery can affect the immune system in multiple ways. In the immediate post-operative period an inflammatory response is initiated. In some patients the immune system is directly affected because of the need to remove the thymus during the surgical procedure. Both effects of cardiac surgery tip the balance of the immune system, which requires regulating mechanisms to return to a healthy homeostasis.

In the first part of this thesis we focus on the acute systemic inflammatory response following pediatric cardiac surgery with an emphasis on activation and regulation of both monocytes and T cells. *Chapter two* gives an overview of the inflammatory response to pediatric cardiac surgery, the clinical implications and strategies employed to limit the debilitating effects of post-operative inflammation in children. *Chapter three* describes an endogenous negative feedback mechanism in monocytes during the inflammatory response after pediatric cardiac surgery. Here, the possible pathways are explored that could explain the hypo-responsiveness of monocytes to TLR-mediated activation. *Chapter four* scrutinizes Treg in the immediate

postoperative period following pediatric cardiac surgery. Treg dynamics during systemic inflammation are described alongside the role of soluble factors in plasma on their suppressive capacity.

The second part of this thesis focuses on the effect of neonatal thymectomy on the homeostasis of Tcell populations. *Chapter five* illustrates the plasticity of the human Treg population after neonatal thymectomy. In particular the homeostatic dynamics of two distinct subpopulations of Treg are studied. In *Chapter six* the short and long-term effects of neonatal thymectomy on the naive CD4 T cell compartment are illustrated up to 30 years after thymectomy.

In the summary and discussion section in *Chapter seven* the results of the previous chapters are discussed. The results of endogenous feedback during inflammation are interpreted with the current knowledge of systemic inflammation after not only cardiac surgery but also sepsis. Secondly, the effect of neonatal thymectomy for T cell homeostasis and potential clinical effects after thymectomy is discussed. Finally the potential of studying induced inflammation due to pediatric cardiac surgery is explored.

References

1. Seok J, Warren HS, Cuenca AG et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc.Natl.Acad.Sci.U.S.A 2013;110:3507-3512.
2. Marshall E. Drug trials. Violent reaction to monoclonal antibody therapy remains a mystery. Science 2006;311:1688-1689.
3. Ygberg S, Nilsson A. The developing immune system - from foetus to toddler. Acta Paediatr. 2012;101:120-127.
4. Ashraf SS, Tian Y, Zacharrias S et al. Effects of cardiopulmonary bypass on neonatal and paediatric inflammatory profiles. Eur.J.Cardiothorac.Surg. 1997;12:862-868.
5. Stiller B, Sonntag J, Dahnert I et al. Capillary leak syndrome in children who undergo cardiopulmonary bypass: clinical outcome in comparison with complement activation and C1 inhibitor. Intensive Care Med. 2001;27:193-200.
6. Allen M, Sundararajan S, Pathan N, Burmester M, Macrae D. Anti-inflammatory modalities: their current use in pediatric cardiac surgery in the United Kingdom and Ireland. Pediatr.Crit Care Med. 2009;10:341-345.
7. Botto LD, Correa A, Erickson JD. Racial and temporal variations in the prevalence of heart defects. Pediatrics 2001;107:E32.
8. Mahle WT, Newburger JW, Matherne GP et al. Role of pulse oximetry in examining newborns for congenital heart disease: a scientific statement from the American Heart Association and American Academy of Pediatrics. Circulation 2009;120:447-458.
9. Rosano A, Botto LD, Botting B, Mastroiacovo P. Infant mortality and congenital anomalies from 1950 to 1994: an international perspective. J.Epidemiol.Community Health 2000;54:660-666.
10. Jacobs JP, O'Brien SM, Pasquali SK et al. Variation in Outcomes for Risk-Stratified Pediatric Cardiac Surgical Operations: An Analysis of the STS Congenital Heart Surgery Database. Ann.Thorac.Surg. 2012
11. Gessler P, Schmitt B, Pretre R, Latal B. Inflammatory response and neurodevelopmental outcome after open-heart surgery in children. Pediatr.Cardiol. 2009;30:301-305.
12. Marino BS, Lipkin PH, Newburger JW et al. Neurodevelopmental outcomes in children with congenital heart disease: evaluation and management: a scientific statement from the American Heart Association. Circulation 2012;126:1143-1172.
13. Qing M, Schumacher K, Heise R et al. Intramyocardial synthesis of pro- and anti-inflammatory cytokines in infants with congenital cardiac defects. J.Am.Coll.Cardiol. 2003;41:2266-2274.
14. Hovels-Gurich HH, Schumacher K, Vazquez-Jimenez JF et al. Cytokine balance in infants undergoing cardiac operation. Ann.Thorac.Surg. 2002;73:601-608.
15. Ghorbel MT, Cherif M, Jenkins E et al. Transcriptomic analysis of patients with tetralogy of Fallot reveals the effect of chronic hypoxia on myocardial gene expression. J.Thorac.Cardiovasc.Surg. 2010;140:337-345.
16. Zupancich E, Paparella D, Turani F et al. Mechanical ventilation affects inflammatory mediators in patients undergoing cardiopulmonary bypass for cardiac surgery: a randomized clinical trial. J.Thorac.Cardiovasc. Surg. 2005;130:378-383.
17. Apostolakis E, Filos KS, Koletsis E, Dougenis D. Lung dysfunction following cardiopulmonary bypass. J.Card Surg. 2010;25:47-55.
18. Brix-Christensen V, Tonnesen E, Hjortdal VE et al. Neutrophils and platelets accumulate in the heart, lungs, and kidneys after cardiopulmonary bypass in neonatal pigs. Crit Care Med. 2002;30:670-676.
19. Arikan AA, Zappitelli M, Goldstein SL et al. Fluid overload is associated with impaired oxygenation and morbidity in critically ill children. Pediatr.Crit Care Med. 2012;13:253-258.
20. Dennen P, Altmann C, Kaufman J et al. Urine interleukin-6 is an early biomarker of acute kidney injury in children undergoing cardiac surgery. Crit Care 2010;14:R181.
21. Nathan C, Ding A. Nonresolving inflammation. Cell 2010;140:871-882.

22. Pearce AK, Humphrey TC. Integrating stress-response and cell-cycle checkpoint pathways. Trends Cell Biol. 2001;11:426-433.
23. Holcik M, Sonenberg N. Translational control in stress and apoptosis. Nat.Rev.Mol.Cell Biol. 2005;6:318-327.
24. Gordon JW, Shaw JA, Kirshenbaum LA. Multiple facets of NF-kappaB in the heart: to be or not to NF-kappaB. Circ.Res. 2011;108:1122-1132.
25. Kagan JC, Medzhitov R. Phosphoinositide-mediated adaptor recruitment controls Toll-like receptor signaling. Cell 2006;125:943-955.
26. Leonard WJ, Lin JX. Cytokine receptor signaling pathways. J.Allergy Clin.Immunol. 2000;105:877-888.
27. Brasier AR. The nuclear factor-kappaB-interleukin-6 signalling pathway mediating vascular inflammation. Cardiovasc.Res. 2010;86:211-218.
28. Levy DE, Lee CK. What does Stat3 do? J.Clin.Invest 2002;109:1143-1148.
29. Yoo JY, Huso DL, Nathans D, Desiderio S. Specific ablation of Stat3beta distorts the pattern of Stat3-responsive gene expression and impairs recovery from endotoxic shock. Cell 2002;108:331-344.
30. Berlato C, Cassatella MA, Kinjyo I et al. Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on lipopolysaccharide-induced macrophage activation. J.Immunol. 2002;168:6404-6411.
31. Naka T, Fujimoto M, Tsutsui H, Yoshimura A. Negative regulation of cytokine and TLR signalings by SOCS and others. Adv.Immunol. 2005;87:61-122.
32. Nakagawa R, Naka T, Tsutsui H et al. SOCS-1 participates in negative regulation of LPS responses. Immunity. 2002;17:677-687.
33. Moore KW, de Waal MR, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu. Rev.Immunol. 2001;19:683-765.
34. Mosser DM, Zhang X. Interleukin-10: new perspectives on an old cytokine. Immunol.Rev. 2008;226:205-218.
35. Crepaldi L, Gasperini S, Lapinet JA et al. Up-regulation of IL-10R1 expression is required to render human neutrophils fully responsive to IL-10. J.Immunol. 2001;167:2312-2322.
36. Davidson D, Zaytseva A, Miskolci V et al. Gene expression profile of endotoxin-stimulated leukocytes of the term new born: control of cytokine gene expression by interleukin-10. PLoS.One. 2013;8:e53641.
37. Qin H, Roberts KL, Niyongere SA et al. Molecular mechanism of lipopolysaccharide-induced SOCS-3 gene expression in macrophages and microglia. J.Immunol. 2007;179:5966-5976.
38. Cassatella MA, Gasperini S, Bovolenta C et al. Interleukin-10 (IL-10) selectively enhances CIS3/SOCS3 mRNA expression in human neutrophils: evidence for an IL-10-induced pathway that is independent of STAT protein activation. Blood 1999;94:2880-2889.
39. Tamassia N, Castellucci M, Rossato M et al. Uncovering an IL-10-dependent NF-kappaB recruitment to the IL-1ra promoter that is impaired in STAT3 functionally defective patients. FASEB J. 2010;24:1365-1375.
40. Schottelius AJ, Mayo MW, Sartor RB, Baldwin AS, Jr. Interleukin-10 signaling blocks inhibitor of kappaB kinase activity and nuclear factor kappaB DNA binding. J.Biol.Chem. 1999;274:31868-31874.
41. te Velde AA, de Waal MR, Huijbens RJ, de Vries JE, Figdor CG. IL-10 stimulates monocyte Fc gamma R surface expression and cytotoxic activity. Distinct regulation of antibody-dependent cellular cytotoxicity by IFN-gamma, IL-4, and IL-10. J.Immunol. 1992;149:4048-4052.
42. Mumm JB, Emmerich J, Zhang X et al. IL-10 elicits IFNgamma-dependent tumor immune surveillance. Cancer Cell 2011;20:781-796.
43. Standiford TJ, Strieter RM, Lukacs NW, Kunkel SL. Neutralization of IL-10 increases lethality in endotoxemia. Cooperative effects of macrophage inflammatory protein-2 and tumor necrosis factor. J.Immunol. 1995;155:2222-2229.
44. Cusumano V, Genovese F, Mancuso G et al. Interleukin-10 protects neonatal mice from lethal group B streptococcal infection. Infect.Immun. 1996;64:2850-2852.

45. Engles RE, Huber TS, Zander DS et al. Exogenous human recombinant interleukin-10 attenuates hindlimb ischemia-reperfusion injury. J.Surg.Res. 1997;69:425-428.
46. Kato T, Murata A, Ishida H et al. Interleukin 10 reduces mortality from severe peritonitis in mice. Antimicrob.Agents Chemother. 1995;39:1336-1340.
47. Florquin S, Amraoui Z, Abramowicz D, Goldman M. Systemic release and protective role of IL-10 in staphylococcal enterotoxin B-induced shock in mice. J.Immunol. 1994;153:2618-2623.
48. Frangogiannis NG, Mendoza LH, Lindsey ML et al. IL-10 is induced in the reperfused myocardium and may modulate the reaction to injury. J.Immunol. 2000;165:2798-2808.
49. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell 2008;133:775-787.
50. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of $CD4^+CD25^+$ regulatory T cells. Nat.Immunol. 2003;4:330-336.
51. Bennett CL, Christie J, Ramsdell F et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat.Genet. 2001;27:20-21.
52. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057-1061.
53. Tran DQ, Ramsey H, Shevach EM. Induction of FOXP3 expression in naive human $CD4^+$FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. Blood 2007;110:2983-2990.
54. Wang J, Ioan-Facsinay A, van der Voort E, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory $CD4^+$ T cells. Eur.J.Immunol. 2007;37:129-138.
55. von Boehmer H. Mechanisms of suppression by suppressor T cells. Nat.Immunol. 2005;6:338-344.
56. Zheng Y, Manzotti CN, Liu M et al. CD86 and CD80 differentially modulate the suppressive function of human regulatory T cells. J.Immunol. 2004;172:2778-2784.
57. Li MO, Wan YY, Flavell RA. T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. Immunity. 2007;26:579-591.
58. Rubtsov YP, Rasmussen JP, Chi EY et al. Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity. 2008;28:546-558.
59. Barthlott T, Moncrieffe H, Veldhoen M et al. $CD25^+$ $CD4^+$ T cells compete with naive $CD4^+$ T cells for IL-2 and exploit it for the induction of IL-10 production. Int.Immunol. 2005;17:279-288.
60. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. $CD4^+CD25^+Foxp3^+$ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector $CD4^+$ T cells. Nat.Immunol. 2007;8:1353-1362.
61. Taams LS, van Amelsfort JM, Tiemessen MM et al. Modulation of monocyte/macrophage function by human $CD4^+CD25^+$ regulatory T cells. Hum.Immunol. 2005;66:222-230.
62. Maloy KJ, Salaun L, Cahill R et al. $CD4^+CD25^+$ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. J.Exp.Med. 2003;197:111-119.
63. Murphy TJ, Choileain NN, Zang Y, Mannick JA, Lederer JA. $CD4^+CD25^+$ regulatory T cells control innate immune reactivity after injury. J Immunol. 2005;174:2957-2963.
64. Deane JA, Abeynaike LD, Norman MU et al. Endogenous regulatory T cells adhere in inflamed dermal vessels via ICAM-1: association with regulation of effector leukocyte adhesion. J.Immunol. 2012;188:2179-2188.
65. den Braber I, Mugwagwa T, Vrisekoop N et al. Maintenance of peripheral naive T cells is sustained by thymus output in mice but not humans. Immunity. 2012;36:288-297.
66. Wing K, Suri-Payer E, Rudin A. $CD4^+CD25^+$-regulatory T cells from mouse to man. Scand.J Immunol. 2005;62:1-15.
67. Shevach EM. Mechanisms of $FOXP3^+$ T regulatory cell-mediated suppression. Immunity. 2009;30:636-645.

68. Itoh M, Takahashi T, Sakaguchi N et al. Thymus and autoimmunity: production of $CD25^+CD4^+$ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. J.Immunol. 1999;162:5317-5326.

69. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. 1995;155:1151-1164.

70. Ohkura N, Hamaguchi M, Morikawa H et al. T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. Immunity. 2012;37:785-799.

71. Ohkura N, Kitagawa Y, Sakaguchi S. Development and maintenance of regulatory T cells. Immunity. 2013;38:414-423.

72. Curotto de Lafaille MA, Lino AC, Kutchukhidze N, Lafaille JJ. $CD25^-$ T cells generate $CD25^+Foxp3^+$ regulatory T cells by peripheral expansion. J.Immunol. 2004;173:7259-7268.

73. Allan SE, Song-Zhao GX, Abraham T, McMurchy AN, Levings MK. Inducible reprogramming of human T cells into Treg cells by a conditionally active form of FOXP3. Eur.J.Immunol. 2008;38:3282-3289.

74. Walker MR, Kasprowicz DJ, Gersuk VH et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human $CD4^+CD25^-$ T cells. J.Clin.Invest 2003;112:1437-1443.

75. Miyara M, Yoshioka Y, Kitoh A et al. Functional delineation and differentiation dynamics of human $CD4^+$ T cells expressing the FoxP3 transcription factor. Immunity. 2009;30:899-911.

76. Vukmanovic-Stejic M, Agius E, Booth N et al. The kinetics of $CD4^+Foxp3^+$ T cell accumulation during a human cutaneous antigen-specific memory response *in vivo*. J.Clin.Invest 2008;118:3639-3650.

77. Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. Nat.Rev.Immunol. 2003;3:253-257.

78. Kretschmer K, Apostolou I, Hawiger D et al. Inducing and expanding regulatory T cell populations by foreign antigen. Nat.Immunol. 2005;6:1219-1227.

79. Long SA, Rieck M, Tatum M et al. Low-dose antigen promotes induction of FOXP3 in human $CD4^+$ T cells. J.Immunol. 2011;187:3511-3520.

80. Nikolich-Zugich J, Slifka MK, Messaoudi I. The many important facets of T-cell repertoire diversity. Nat. Rev.Immunol. 2004;4:123-132.

81. Schonland SO, Zimmer JK, Lopez-Benitez CM et al. Homeostatic control of T-cell generation in neonates. Blood 2003;102:1428-1434.

82. Haynes BF, Markert ML, Sempowski GD, Patel DD, Hale LP. The role of the thymus in immune reconstitution in aging, bone marrow transplantation, and HIV-1 infection. Annu.Rev.Immunol. 2000;18:529-60.:529-560.

83. Douek DC, McFarland RD, Keiser PH et al. Changes in thymic function with age and during the treatment of HIV infection. Nature 1998;396:690-695.

84. Gruver AL, Hudson LL, Sempowski GD. Immunosenescence of ageing. J.Pathol. 2007;211:144-156.

85. Naylor K, Li G, Vallejo AN et al. The influence of age on T cell generation and TCR diversity. J.Immunol. 2005;174:7446-7452.

86. Hazenberg MD, Otto SA, van Rossum AM et al. Establishment of the $CD4^+$ T-cell pool in healthy children and untreated children infected with HIV-1. Blood 2004;104:3513-3519.

87. Freitas AA, Rocha B. Population biology of lymphocytes: the flight for survival. Annu.Rev.Immunol. 2000;18:83-111.

88. Muraro PA, Douek DC, Packer A et al. Thymic output generates a new and diverse TCR repertoire after autologous stem cell transplantation in multiple sclerosis patients. J Exp.Med. 2005;201:805-816.

89. Hakim FT, Memon SA, Cepeda R et al. Age-dependent incidence, time course, and consequences of thymic renewal in adults. J Clin Invest 2005;115:930-939.

90. Vrisekoop N, van Gent R, de Boer AB et al. Restoration of the CD4 T cell compartment after long-term highly active antiretroviral therapy without phenotypical signs of accelerated immunological aging. J.Immunol. 2008;181:1573-1581.

91. Cicin-Sain L, Smyk-Pearson S, Currier N et al. Loss of naive T cells and repertoire constriction predict poor response to vaccination in old primates. J.Immunol. 2010;184:6739-6745.
92. Lewin SR, Heller G, Zhang L et al. Direct evidence for new T-cell generation by patients after either T-cell-depleted or unmodified allogeneic hematopoietic stem cell transplantations. Blood 2002;100:2235-2242.
93. Roux E, Dumont-Girard F, Starobinski M et al. Recovery of immune reactivity after T-cell-depleted bone marrow transplantation depends on thymic activity. Blood 2000;96:2299-2303.
94. Barrett DJ, Ammann AJ, Wara DW et al. Clinical and immunologic spectrum of the DiGeorge syndrome. J.Clin.Lab Immunol. 1981;6:1-6.
95. Lean-Tooke A, Barge D, Spickett GP, Gennery AR. Immunologic defects in 22q11.2 deletion syndrome. J.Allergy Clin.Immunol. 2008;122:362-7, 367.
96. Piliero LM, Sanford AN, McDonald-McGinn DM, Zackai EH, Sullivan KE. T-cell homeostasis in humans with thymic hypoplasia due to chromosome 22q11.2 deletion syndrome. Blood 2004;103:1020-1025.
97. Markert ML, Alexieff MJ, Li J et al. Postnatal thymus transplantation with immunosuppression as treatment for DiGeorge syndrome. Blood 2004;104:2574-2581.
98. Garcia-Carrasco M, Escarcega RO, Fuentes-Alexandro S, Riebeling C, Cervera R. Therapeutic options in autoimmune myasthenia gravis. Autoimmun.Rev. 2007;6:373-378.
99. Sempowski G, Thomasch J, Gooding M et al. Effect of thymectomy on human peripheral blood T cell pools in myasthenia gravis. J.Immunol. 2001;166:2808-2817.
100. Storek J, Douek DC, Keesey JC et al. Low T cell receptor excision circle levels in patients thymectomized 25-54 years ago. Immunol.Lett. 2003;89:91-92.
101. Eysteinsdottir JH, Freysdottir J, Haraldsson A et al. The influence of partial or total thymectomy during open heart surgery in infants on the immune function later in life. Clin.Exp.Immunol. 2004;136:349-355.
102. Halnon NJ, Jamieson B, Plunkett M et al. Thymic function and impaired maintenance of peripheral T cell populations in children with congenital heart disease and surgical thymectomy. Pediatr.Res. 2005;57:42-48.
103. Prelog M, Keller M, Geiger R et al. Thymectomy in early childhood: significant alterations of the CD4(+) CD45RA(+)CD62L(+) T cell compartment in later life. Clin.Immunol. 2009;130:123-132.
104. Torfadottir H, Freysdottir J, Skaftadottir I et al. Evidence for extrathymic T cell maturation after thymectomy in infancy. Clin.Exp.Immunol. 2006;145:407-412.
105. Prelog M, Wilk C, Keller M et al. Diminished response to tick-borne encephalitis vaccination in thymectomized children. Vaccine 2008;26:595-600.
106. Sauce D, Larsen M, Fastenackels S et al. Evidence of premature immune aging in patients thymectomized during early childhood. J.Clin.Invest 2009;119:3070-3078.
107. Eggum R, Ueland T, Mollnes TE et al. Effect of perfusion temperature on the inflammatory response during pediatric cardiac surgery. Ann.Thorac.Surg. 2008;85:611-617.
108. Gessler P, Pfenninger J, Pfammatter JP, Carrel T, Dahinden C. Inflammatory response of neutrophil granulocytes and monocytes after cardiopulmonary bypass in pediatric cardiac surgery. Intensive Care Med 2002;28:1786-1791.
109. Gessler P, Pfenninger J, Pfammatter JP et al. Plasma levels of interleukin-8 and expression of interleukin-8 receptors on circulating neutrophils and monocytes after cardiopulmonary bypass in children. J.Thorac. Cardiovasc.Surg. 2003;126:718-725.
110. Gessler P, Pretre R, Hohl V et al. CXC-chemokine stimulation of neutrophils correlates with plasma levels of myeloperoxidase and lactoferrin and contributes to clinical outcome after pediatric cardiac surgery. Shock 2004;22:513-520.
111. Allen ML, Peters MJ, Goldman A et al. Early postoperative monocyte deactivation predicts systemic inflammation and prolonged stay in pediatric cardiac intensive care. Crit Care Med 2002;30:1140-1145.
112. de Jong PR, Schadenberg AW, van den Broek T et al. STAT3 regulates monocyte TNF-alpha production in systemic inflammation caused by cardiac surgery with cardiopulmonary bypass. PLoS.One. 2012;7:e35070.

113. Stocker CF, Shekerdemian LS, Horton SB et al. The influence of bypass temperature on the systemic inflammatory response and organ injury after pediatric open surgery: a randomized trial. J.Thorac. Cardiovasc.Surg. 2011;142:174-180.

114. Gessler P, Pretre R, Burki C et al. Monocyte function-associated antigen expression during and after pediatric cardiac surgery. J.Thorac.Cardiovasc.Surg. 2005;130:54-60.

115. Naldini A, Borrelli E, Carraro F, Giomarelli P, Toscano M. Interleukin 10 production in patients undergoing cardiopulmonary bypass: evidence of inhibition of Th-1-type responses. Cytokine 1999;11:74-79.

116. Riddle PR, Berenbaum MC. Postoperative depression of the lymphocyte response to phytohaemagglutinin. Lancet 1967;1:746-748.

117. Naldini A, Borrelli E, Cesari S, Giomarelli P, Toscano M. *In vitro* cytokine production and T-cell proliferation in patients undergoing cardiopulmonary by-pass. Cytokine 1995;7:165-170.

118. Schadenberg AW, Vastert SJ, Evens FC et al. FOXP3^{+} CD4^{+} Tregs lose suppressive potential but remain anergic during transient inflammation in human. Eur.J.Immunol. 2011;41:1132-1142.

119. Chenoweth DE, Cooper SW, Hugli TE et al. Complement activation during cardiopulmonary bypass: evidence for generation of C3a and C5a anaphylatoxins. N.Engl.J.Med. 1981;304:497-503.

120. Chew MS, Brandslund I, Brix-Christensen V et al. Tissue injury and the inflammatory response to pediatric cardiac surgery with cardiopulmonary bypass: a descriptive study. Anesthesiology 2001;94:745-753.

121. Jensen E, Andreasson S, Bengtsson A et al. Influence of two different perfusion systems on inflammatory response in pediatric heart surgery. Ann.Thorac.Surg. 2003;75:919-925.

122. Seghaye MC, Duchateau J, Grabitz RG et al. Complement activation during cardiopulmonary bypass in infants and children. Relation to postoperative multiple system organ failure. J.Thorac.Cardiovasc.Surg. 1993;106:978-987.

123. Tarnok A, Hambsch J, Emmrich F et al. Complement activation, cytokines, and adhesion molecules in children undergoing cardiac surgery with or without cardiopulmonary bypass. Pediatr.Cardiol. 1999;20:113-125.

124. Lotan D, Zilberman D, Dagan O et al. Beta-chemokine secretion patterns in relation to clinical course and outcome in children after cardiopulmonary bypass: continuing the search to abrogate systemic inflammatory response. Ann.Thorac.Surg. 2001;71:233-237.

125. Allen ML, Hoschtitzky JA, Peters MJ et al. Interleukin-10 and its role in clinical immunoparalysis following pediatric cardiac surgery. Crit Care Med. 2006;34:2658-2665.

126. Berdat PA, Eichenberger E, Ebell J et al. Elimination of proinflammatory cytokines in pediatric cardiac surgery: analysis of ultrafiltration method and filter type. J Thorac.Cardiovasc.Surg. 2004;127:1688-1696.

127. Brancaccio G, Villa E, Girolami E et al. Inflammatory cytokines in pediatric cardiac surgery and variable effect of the hemofiltration process. Perfusion 2005;20:263-268.

128. Franke A, Lante W, Fackeldey V et al. Proinflammatory and antiinflammatory cytokines after cardiac operation: different cellular sources at different times. Ann.Thorac.Surg. 2002;74:363-370.

129. Madhok AB, Ojamaa K, Haridas V et al. Cytokine response in children undergoing surgery for congenital heart disease. Pediatr.Cardiol. 2006;27:408-413.

Chapter

2

INFLAMMATORY RESPONSE TO PEDIATRIC HEART SURGERY

What goes up must come down

AWL Schadenberg[1,2], SO Algra[2], BJ Prakken[1], NJG Jansen[2]

[1] Dep. of Pediatric Immunology, University Medical Center Utrecht, The Netherlands
[2] Dep. of Pediatric Intensive Care / Children's Heart Center, University Medical Center Utrecht, The Netherlands

Submitted

Abstract

Many congenital heart defects require corrective surgery. Due to technical advances, cardiac surgery is increasingly being performed on younger children, involving more complex procedures. The sum of surgical damage, anesthesia, contact with foreign surfaces of the cardiopulmonary bypass circuit, ischemia-reperfusion and others, results in activation of the immune system. An activated immune system is essential for recovery; however, exaggeration can lead to either hyper-inflammation resulting in increased vascular permeability and organ damage or to hypo-inflammation and immune paralysis with ensuing risk of infection. Which children are prone to develop an unfavorable immunological response is not completely understood. This review discusses the possible activators of the inflammatory response due to cardiac surgery in children. Furthermore, the different immune components that initiate and regulate this response are highlighted. Finally, a summary is given of therapies previously examined or currently being used to control the immune response after surgery.

1. Introduction

Congenital heart disease (CHD) occurs in 9 of every 1000 livebirths,[1] which amounts to about 1250 new cases per year in the Netherlands. Approximately one quarter of these children will require surgical or catheter intervention in the first year of life. Survival of patients with CHD has improved significantly since surgical repair became available over 50 years ago. Instead of a mortality of 75% during infancy following the natural course, over 95% of children with a CHD now reach adulthood.[2] Due to improved antenatal screening and treatment options many patients follow a relatively uncomplicated recovery and can be discharged home within days of the surgical procedure. However, all patients remain at risk of serious morbidity and mortality. Recently published post-operative mortality figures range from 0.6% to 18.4% depending on the type of procedure.[3] Thus, these patients remain a group at risk for morbidity and mortality at clinical presentation and later in life. Through combined efforts from anesthesia, surgery, cardiology, intensive care and other subspecialties, surgery is being performed on more complex defects in younger patients. These advances in surgical options enhance the challenges in post-operative care. Surgical intervention being on the one hand essential and lifesaving, it also contributes to this risk. One of the endogenous factors complicating post-operative recovery is the inflammatory response. Although still not completely understood, this leads to morbidity due to either hyper-inflammation resulting in increased vascular permeability and ultimately organ damage, or on the other hand of the spectrum hypo-inflammation with subsequent risk of infections. Finding an appropriate balance between the two without compromising the healing process is a great challenge for the post-operative management of these complex patients.

2. Events leading to immune activation

Immunological sequelae of congenital heart surgery results from a cascade of events, which to some extent, all lead to immune activation. The extent of post-operative inflammation has been attributed to pre-operative clinical condition, intra-operative factors and post-operative events (Table 1). Over the past decades much effort has been put into improving peri-operative management to minimize immune activation, which will be discussed below.

Table 1. Events leading to immune activation after pediatric cardiac surgery.

Pre-operative	Intra-operative	Post-operative
Genetic predisposition	Anesthesia	Mechanical ventilation
Cyanosis	Cardiopulmonary bypass	Nosocomial infection
Clinical condition	Surgical injury	
	Ischemia / reperfusion injury	

2.1 Anesthesia

In the process of corrective heart surgery the first iatrogenic stimulant triggering the immune system will often be induction with anesthetic agents. Despite inducing inflammation, anesthesia can also have both direct and indirect protective effects on the immune system. Multiple efforts exist in employing agents necessary for inducing and maintaining anesthesia, which also have a protective effect during the surgical procedure. The best understood cardio-protective effect of anesthesia is based on diminishing oxygen demand by reducing contractility and metabolism and hereby protecting the myocardium and other organs during ischemia and reperfusion.[4;5] However, besides a direct effect in cardio-protection by diminishing metabolism, there is also increasing evidence of an indirect pre-conditioning effect.[6;7] Pre-conditioning, first described by Murry in 1986, is a technique in which mild stress (e.g. ischemia) leads to resistance of tissue to a subsequent ischemic insult[8]. Although pre-conditioning has been shown to improve basic indices (pulmonary and cardiac function) following pediatric cardiac surgery,[9;10] a recently performed large RCT by McCrindle, et al. showed no clinical benefit in length of stay or morbidity..

2.2 Cardiopulmonary bypass

Cardiopulmonary bypass (CPB) has increased surgical possibilities significantly by enabling to maintain perfusion while operating on a non-beating heart. Selective perfusion during some procedures (such as antegrade cerebral perfusion during aortic arch reconstruction) has further improved outcome.[12] However, contact of circulating blood components with foreign material has major effects on both coagulation and immune activation. The role of CPB in initiating an inflammatory response has been well studied in adults comparing on-pump with off-pump coronary artery bypass graft (CABG) surgery. The release of cytokines, but also organ dysfunction is clearly diminished in CABG surgery without the use of CPB (off-pump) compared to surgery with CPB (on-pump).[13] CPB induces a recognizable transcriptional response with pro-inflammatory elements being more or less balanced by protective elements.[14] We and others have described the inflammatory response following pediatric cardiac surgery with use of CPB.[15-18] Various improvements in extracorporeal perfusion have reduced activation of the inflammatory cascade (Table 2). Some advances benefitted clinical indices, such as pulsatile perfusion can improve organ perfusion[19;20] and heparin coated circuits can improve pulmonary function,[21] while others (eg leukocyte depletion) failed to show a significant clinical benefit.[22]

2.3 Surgical injury

Surgical tissue injury elicits an immune response both locally and systemically. While the inflammatory response due to tissue injury has potential side effects, the response in itself is essential to wound healing and starts as soon as damage occurs.[23] In damaged tissue necrotic cells will activate the NF-kB pathway leading to a local inflammatory response.[24] Furthermore,

injury leads to expression of chemokines resulting in recruitment of neutrophils.[25] Besides initiating a local response, surgical trauma also profoundly affects the systemic immune system, including both innate and adaptive immune responses.[26;27] While surgery has been shown to cause an innate host response with increased levels of circulating cytokines a more regulatory effect of the adaptive immune system also occurs. Circulating monocytes and T cells have been shown to be suppressed through a shift of Th1 to Th2 balance, which may explain why injured patients have an increased susceptibility to secondary infections.[28]

Table 2. Interventions to control inflammation due to cardiac surgery.

Intervention	Immunological effect	Clinical effect	References
Technical			
Pulsatile pump	Reduced inflammation	Increased organ perfusion (P)	19;20
Heparin coated circuit	Reduced inflammation	Improved pulmonary function (P)	127;128
Hypothermia	Minor / no cytokine differences	No effect (P)	129;130
Leukocyte depletion	Reduced neutrophils adhesion markers	No effect (P)	22;131
Modified ultrafiltration	Removal of cytokines, endotoxin	Increased postoperative perfusion and hematocrit, no effect on outcome (P)	132;133
Washed transfused blood products	Reduced IL-6:IL-10 ratio	Reduced number of transfusions (P), reduced infections (A)	134;135
Ischemic pre-conditioning	Pre-operatively increased, post-operatively attenuated cytokine release	Reduced inotrope score, improved pulmonary function, no improved clinical outcome (P)	9-11
Pharmacological			
Corticosteroids	Reduced pro-inflammatory cytokines, cardiac inflammation	No significant benefit for low-risk patients (P)	108;110; 113;136
Aprotinin	Reduced inflammation (factor XII, bradykinin, C5a, NO)	Reduced blood loss (P), severe adverse events (A)	137;138
Insuline (tight glycemic control)	Reduced pro-inflammatory cytokines	Conflicting results: no benefit and; reduced LOS and infections (P)	124-126
Sivelestat	Reduced circulating neutrophils and CRP	Improved pulmonary and cardiac indices (P)	122;123;139

A: adult, P: pediatric, NO: Nitric Oxide, LOS: length of stay, CRP: C-reactive protein

2.4 Ischemia / reperfusion injury

Despite the advantage of cardiopulmonary bypass, a majority of surgical procedures involve a period of myocardial ischemia. Reduced supply of oxygen and nutrients leads to tissue injury by intracellular accumulation of harmful metabolites and depletion of vital metabolites. Disruption of membrane ionic pumps results in further intracellular imbalance with fall in ATP and accumulation of calcium and lactate. While reperfusion on the one hand leads to restoration of oxygen and nutrient supply alongside excretion of harmful metabolites, it can also lead to additional injury. Reperfusion injury is the result of accumulation of intracellular calcium together with formation of reactive oxygen species (ROS). Following ischemia-reperfusion, injury can further accelerate due to attracted activated immune cells migrating across the vascular wall initiating an inflammatory response. Depending on the duration of cardiac hypoxia, damage may occur due to ischemia and subsequent reperfusion leading to ventricular fibrillation, reduced myocardial contractility (myocardial stunning and loss of intracellular proteins) and trigger inflammation.[29;30] Besides injury to the myocardium after cardioplegia, other tissues are also prone to ischemia-reperfusion injury, contributing to a systemic inflammatory response. Particularly the lungs are susceptible to ischemia-reperfusion injury resulting in post-operative pulmonary edema and reduced gas-exchange.[31] Post-ischemic reperfusion of the lungs causes upregulation of adhesion molecules in the pulmonary vasculature resulting in neutrophil sequestration and subsequent tissue injury through production of oxygen-derived free radicals. Continuing pulmonary perfusion during CPB can diminish post-operative lung injury.[32;33] Furthermore intestinal ischemia-reperfusion injury leads to reduced barrier function and subsequently transmigration of gut bacterial reagents like endotoxin.[34] Finally, ischemia-reperfusion probably contributes to post-operative acute kidney failure.[35] Overall, ischemia-reperfusion injury can lead to significant injury in tissues throughout the body. Some surgical interventions require a period of circulatory arrest and hypothermia with or without selective cerebral perfusion. While hypothermia is aimed to protect the tissue by minimizing metabolism, the ideal degree of hypothermia is still debated.[36] Also, the effect of hypothermia on the inflammatory response is not clear, with contrasting studies showing deep hypothermia resulting in both reduced and increased inflammation.[37-39]

3. The inflammatory cascade

The net result of above mentioned triggers is a potent inflammatory reaction. The inflammatory response is initiated both on a local and systemic level, summarized in figure 1a and b. Damaged cells release cell content, while stimulated cells activate specific intracellular cascades and release proteins (e.g. cytokines), which in turn activate other immune cells. The release of immune mediators can be either specific and controlled or uncontrolled, i.e. release of intracellular content (e.g. RNA) from lysed cells. Neutrophils are attracted to stressed tissues through locally released chemokines, such as IL-8. After adhering to the endothelium, neutrophils

migrate to the extravascular space and impair the endothelial barrier function, resulting in fluid extravasation.[40] Oxidative stress results in release of ROS by primarily neutrophils, which are activated by systemic factors and locally through damaged myocardium and endothelium. Following activation of neutrophils an augmented immune response is initiated through release of a multitude of cytokines and chemokines. These messenger proteins have a direct effect on all leukocytes besides other tissues including endothelium elsewhere in the body. Both pro- and anti-inflammatory cytokines are released following cardiac surgery in a more or less time dependent pattern.[41;42] The severity of tissue damage is to some extent correlated to circulating levels of cytokines.[43] However, systemically circulating inflammatory mediators may not reflect the ongoing inflammatory response at a local level.

Over the past decades different concepts of the ensuing inflammatory response have developed. Opposing effects of the same response have been divided into an early systemic inflammatory response syndrome (SIRS) with a hyper activated immune state (Figure 1a) versus a late compensatory anti-inflammatory response (Figure 1b). Although helpful in recognizing the opposing consequences of an overwhelming inflammatory response, some studies have questioned the time related aspect of this paradigm.[44] Furthermore, comparing a few major pro-inflammatory mediators versus anti-inflammatory mediators without taking into account the vast network of known and unknown mediators, interacting simultaneously, may be an oversimplification. More likely there is a multifaceted response with both pro-inflammatory and anti-inflammatory components acting at the same time (Figure 2).[45] The subsequent clinical effect probably depends more on localization, presence of pathogens or repetitive injury, than the sum of pro- and anti-inflammatory mediators.

3.1 Pediatric inflammatory response to injury

The inflammatory response to injury in children is functionally age-specific. Compared to adults, children may be relatively spared from multiple organ failure and are more at risk of developing infections due to debilitated immune function.[46;47] Although predisposing factors for developing organ failure in children are not well understood, young age and chronic illness have been recognized as important risk factors.[48] The distinct pattern of organ dysfunction in young children may well be due to major changes in developing organs with a different susceptibility to inflammatory damage.

At birth the innate immune system shows decreased complement, macrophage and phagocyte function compared to adulthood.[49] Besides clear shortcomings in innate immune components the adaptive immune system also needs further maturation.[50;51] Although mature function can be elicited under some circumstances, neonatal immune responses are often dampened or non-protective. This may explain the decreased hyper-inflammatory response and increased risk of developing secondary infections following major surgery. Infections after cardiac surgery in children remain a frequent complication occurring in up to 30% of cases.[52;53] Both exogenous and endogenous factors play a role in the increased risk of infection after cardiac

surgery. Important exogenous factors include hygiene, use of intravascular devices, duration and complexity of surgery, length of stay on PICU and delayed sternum closure. Endogenous factors determining outcome after major injury like cardiac surgery include age and immature immune system and genetic variables.

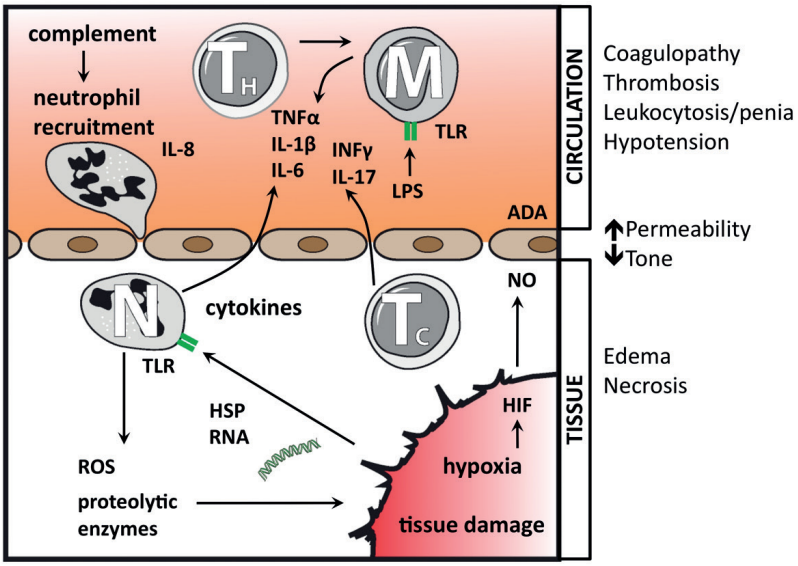

Figure 1a. Initiation of local and systemic inflammation. Local tissue damage initiates an inflammatory response through release of signals that attract and stimulate the immune system. Damaged cells release intracellular components such as ribonucleic acid (RNA) and heat shock proteins (HSP), which are strongly immunogenic through activation of toll like receptors (TLR). Chemokines released by eg stressed myocardium attract leukocytes to transmigrate from the circulation to the damaged tissue. Recruited neutrophils release reactive oxygen species (ROS) and proteolytic enzymes to digest damaged tissue. Local inflammatory cells stimulate the response by releasing pro-inflammatory cytokines. Systemically available endotoxin (translocated from the gut) activates monocytes through binding with TLR. Circulating cytokines such as TNFa, IL-1β and IL-6 further activate the systemic inflammatory response. Local hypoxemia also induces inflammation through activation of hypoxia-inducible transcription factor (HIF), which in turn results in production of nitric oxide (NO). NO is besides a strong vasodilator also important in regulation of the inflammatory response. Likewise endothelial adenosine deaminase (ADA) regulates vascular permeability by regulating the bioavailability of adenosine. The net result of above actions can have clinical relevance such as edema, hypotension, coagulopathy, and finally end organ failure. Cell types: Th = (CD4) Helper T-cell, Tc = (CD8) Cytotoxic T-cell, M = Monocyte, N = Neutrophil.

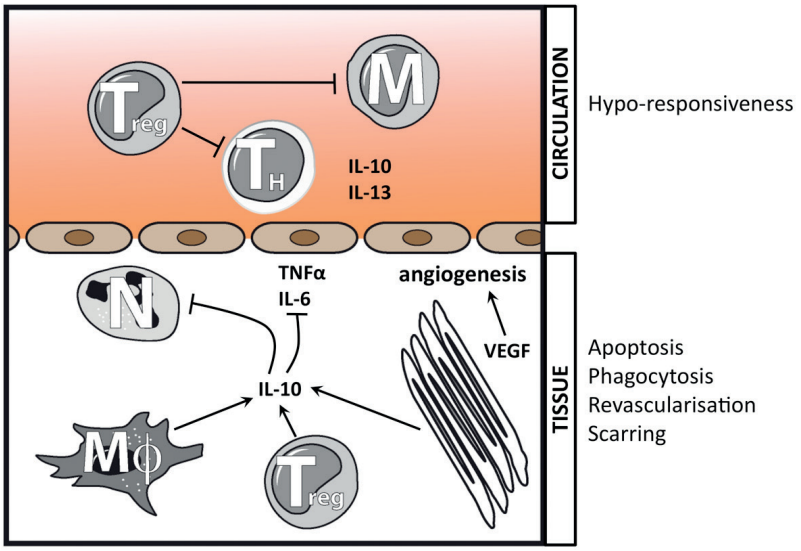

Figure 1b. Resolution of local and systemic inflammation. Inflammation is strictly controlled on both a local and a systemic level. Activated tissue produces the potent anti-inflammatory cytokine IL-10. Angiogenesis is stimulated by released vascular endothelial growth factor (VEGF) to promote wound healing and revascularisation. Tissue damage is removed effectively by macrophages, which in turn dampen further inflammatory damage through production of IL-10. Regulation of the inflammatory response is controlled by regulatory T cells, which act both through cell-cell contact and production of anti-inflammatory cytokines. Cell types: Th = (CD4) Helper T-cell, Treg = Regulatory T-cell, M = Monocyte, MΦ = Macrophage, N = Neutrophil.

3.2 Myocardium

Patients with congenital heart anomalies with associated mechanical stress show activation of inflammatory pathways in cardiomyocytes.[54] Tissue injury and oxidative stress can further cause initiation of an inflammatory response with subsequent postoperative complications.[55] Ischemia-reperfusion injury due to corrective surgery stimulates myocardium to produce ROS and cytokines such as IL-6 and IL-8.[56] Besides initiating a local inflammatory reaction, the heart also produces anti-inflammatory mediators such as IL-10.[57] Cytokines can have a local effect on the cardiomyocyte but also influence the inflammatory reaction systemically when released into the circulation. Pro-inflammatory cytokines generally have a negative inotropic effect on the heart through production of nitric oxide (NO) ultimately leading to low cardiac output syndrome.[58] While locally initiated inflammation correlates with myocardial damage and systemic augmented inflammatory response, the process in itself is necessary and essentially advantageous for wound healing of necrosis.[59]

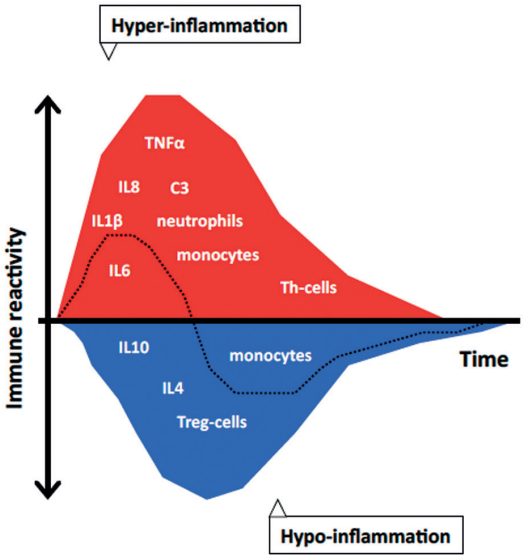

Figure 2. Inflammatory response following cardiac surgery characterized by circulating cytokines and leukocytes *(adapted with permission from de Jong et al, Cell Stress Chaperones 2009).* Multiple triggers during cardiac surgery result in a rapid release of pro-inflammatory cytokines activating multiple immune components. Simultaneously, anti-inflammatory cytokines are released that counterbalance the inflammatory response. Leukocytes are an important source of cytokines of both sides of the inflammatory balance. Depending on which side of the inflammatory balance dominates, the net result can be either a hyper-inflammatory or a hypo-inflammatory response with subsequent clinical signs.

3.3 Endothelium

Endothelium has a strategic position in the initiation and continuation of both local and systemic inflammation. At the interface between blood and tissues, endothelial cells control tissue perfusion besides guiding inflammatory cells to areas in need of defense or repair. Cardiac surgery with use of cardiopulmonary bypass initiates endothelial activation.[60] The endothelial response depends on specific tissue needs and adapts to local stress. Inflammation and oxidative stress, such as that induced by surgical trauma, disrupts endothelial homeostasis, thereby decreasing the bioavailability of NO.[61] This predisposes blood vessels to vasoconstriction, leukocyte adhesion and thrombosis. Coagulation is tightly controlled with an important role for endothelial cells that counteract coagulation by providing tissue factor and thrombin inhibitors and receptors for protein C activation. Activated protein C results in anti-thrombotic and anti-inflammatory effects.[62] Upon stress endothelial cells are activated through the NFκB pathway and initiate a local inflammatory response by recruiting leukocytes to the damaged tissue. Leukocyte recruitment by endothelium is a complex mechanism of rolling, adhesion

and transmigration.[6] Local hypoxia influences the inflammatory response strongly by having an effect on the integrity of the vessel wall and mobilizing leukocytes to the extravascular space. An important regulator of permeability is adenosine, which is under tight control of adenosine deaminase (ADA). During hypoxia ADA is induced, inhibiting extracellular levels of adenosine and hereby controlling vascular leak and inflammation.[64] Altered endothelial integrity due to inflammation can result in unfavorable clinical outcome including thrombosis, edema and hypotension.

3.4 Neutrophils

The archetypical scavengers of the immune response are polymorphic neutrophils, which actively seek and ingest damaged tissue and pathogens. Activation of neutrophils primarily occurs by components of both the complement and contact systems. Exposure to activated complement results in activation of neutrophils within seconds.[65] Activation during surgery involves increased expression of adhesion molecules of the integrin family such as CD11/CD18.[66] Upon activation neutrophils release granules, which contain cytotoxic enzymes, products for matrix degradation and mediators for attracting and activating other components of inflammation. The clinical consequence of activated neutrophils after CPB includes increased vascular permeability, interstitial edema, thrombosis, and local cell necrosis.[67] Impaired cardiovascular function after cardiac surgery correlates with neutrophil activation and is most prominent in children with a cyanotic heart lesion and those with a long CPB time.[68]

3.5 Monocytes

Monocytes play an important role in initiating and maintaining an inflammatory response. Upon activation of pattern recognition receptors monocytes produce pro- and anti-inflammatory cytokines besides stimulating T cells by presenting antigens through the MHC complex on their surface.[69] Furthermore, circulating monocytes enter the tissue and replenish the tissue macrophage populations and hereby contribute to local inflammation and tissue remodeling and repair.[70] Following surgery or major trauma peripheral monocytes show significant changes in surface biomarkers. Changes in HLA-DR expression and CX3CR1 have been observed during systemic inflammation.[71;72] HLA-DR is part of the MHC-II complex and is expressed on the surface of professional antigen presenting cells. Reduced monocytic HLA-DR expression is associated with post-traumatic immunoparalysis with reduced endotoxin induced cytokine production.[73] Despite some disagreement in its predictive value,[73;74] there is general consensus that during systemic inflammation HLA-DR expression on monocytes is reduced and is associated with clinical severity while a recovery of low monocyte HLA-DR expression parallels clinical improvement.[71;75;76] This anti-inflammatory phase is also characterized by a reduced toll-like receptor (TLR) responsiveness of monocytes.[77;78] Recently we found a crucial role for the STAT3 signaling pathway in the suppressed production of TNFa by monocytes in response to endotoxin after pediatric cardiac surgery.[79] These findings suggest a specific monocyte

reprogramming during the post-operative course, instead of a general immune suppression. Thus, monocytes are key players in the inflammatory response after cardiac surgery and are actively reprogrammed during the direct post-operative phase resulting in a suppressed responsiveness to TLR stimulation.

3.6 T- lymphocytes

T lymphocytes are central players in the cell mediated immunity. Different subsets exert either stimulatory or inhibitory functions in the inflammatory response. Following activation CD4 T helper cells facilitate the inflammatory response by secreting pro- and anti-inflammatory cytokines. The Treg subset expressing FOXP3 has been shown to be a potent inhibitor of various leukocytes. Since Riddle and Berenbaum in 1967 described that the lymphocyte responsiveness is diminished in the postoperative period,[80] it has been well established that injury impairs the adaptive immune system, including reduced T cell proliferation to polyclonal and antigen-specific stimulation.[81;82] In cardiac surgery T cells may become activated by sheer stress of the cardiopulmonary bypass,[83] effect of anesthesia[84] and TLR activation by both exogenous (endotoxin, peptidoglycan[85;86]) and endogenous (heat shock proteins[77;87]) ligands, which are released due to the procedure. In various inflammatory conditions $FOXP3^+$ Treg cells have been shown to be crucial regulators of the inflammatory response. We previously reported that during the inflammatory response after pediatric cardiac surgery Treg lose suppressive potential.[18] In pathological inflammatory responses to injury however it has been shown that Treg are an important contributor to post injury immune suppression.[88] Hence, reduced Treg suppression after surgery may well be a normal response, enabling active inflammation. In the event of prolonged Treg activation, inflammation is inhibited, resulting in immune paralysis, posing a risk of secondary infections.

3.7 Cytokines

As a derivative of systemic inflammation circulating cytokine levels are frequently studied. These proteins circulate after release by activated immune cells and tissue under stress, and represent the crosstalk between the various immune components. Roughly, cytokines are divided in pro- and anti-inflammatory, although the effect of the same cytokine can be opposite depending on the context. Prototypical pro-inflammatory cytokines include TNFα, IL-1β, IL-6 and IL-8 while IL-4, IL-10 and TGFβ are known inhibitors of inflammation. We and others have characterized the systemic inflammatory response ensuing pediatric surgery by measuring multiple circulating cytokines.[42;54;79;89] The immune response to cardiac surgery is a dynamic inflammatory balance, which is initially predominantly hyper-inflammatory in nature and is followed by a hypo-inflammatory phase (Figure 2). These phases however are not separate entities and vastly overlap in time with from the start rapid production of both pro- and anti-inflammatory mediators. The hypo-inflammatory phase renders innate and antigen specific immune cells hypo-responsive, hence this phase is also referred to as immunoparalysis.

Importantly however, the response varies from one organ to another; the inflammatory response is strictly compartmentalized.[90] Systemically circulating leukocytes could be hyporeactive, while at the same time leukocytes from inflamed tissue are activated and fully capable to respond to ex-vivo activation. While tissue inflammation can result in organ damage, immune paralysis puts a patient at risk of developing nosocomial infections.[91] A natural balance exists between pro- and anti-inflammatory mediators, both locally and systemically. Therefore, pharmacological therapies based on interpretation of circulating cytokines may well be contraproductive for other compartments.

3.8 Complement system

The complement system is an essential part of the early innate immune response. A multistep activation cascade through cleavage of different inactive precursors results in chemotaxis of leukocytes, opsonization and finally lysis of pathogens. Activation of the complement system during CPB was evident through the early studies by Chenoweth and Kirklin.[92] Activation occurs through both the alternative pathway (exposure of blood to the extra corporeal circuit, reperfusion of ischemic tissue, endotoxin) and the classic pathway (protamine).[93] Complement activation, predominantly through the alternative pathway,[94] has a role in postoperative organ dysfunction.[95] Especially in younger infants and prolonged bypass time complement activation contributes to morbidity.[96] The effects of complement-specific inhibitors have demonstrated the role of complement in the inflammatory response to cardiac surgery. TP10, a soluble complement receptor type 1, showed reduced complement activation in infants and adults, resulting in decreased vascular leak[97] and ischemia.[98] In an experimental model of neonatal CPB, Complement factor 1 esterase inhibitor (C1-inh) improved pulmonary and cardiac function.[99] In infants supplementation of C1-inh resulted in decreased activation of complement cascade and reduced capillary leak.[100] Complement activation occurs during CPB and contributes to morbidity in predominantly younger infants and prolonged bypass time.[96] However, thus far no intervention in inhibiting the complement cascade during CPB has improved clinical outcome in children significantly.

4. Efforts to control inflammation

Numerous attempts have been made to control the immediate inflammatory response following cardiac surgery.[101] A list of interventions to diminish the inflammatory response due to cardiac surgery in children is summarized in Table 2. Early on in the development of artificial surfaces for the CPB, heparin coated circuits were developed to inhibit thrombosis. Since then however, heparin coated circuits have also been recognized for the improved biocompatibility with the immune system. Heparin coated CPB circuits minimize immune activation by inhibiting direct contact, complement and neutrophil activation,[102;103] reduction of released cytokines[21] and less hemostatic activation.[104] Many studies have been published on the effect of removing leukocytes

from the circulation in order to inhibit the inflammatory response following cardiac surgery. Different leukocyte depleting filters within the CPB circuit have shown to effectively remove leukocytes from the circulation. Despite removal of leukocytes however, there is insufficient evidence that in a general patient population leukocyte depletion improves outcome.[105]

The longest relied on pharmacological agent to control inflammation in cardiac surgery must be corticosteroids. In both adult and pediatric cardiac surgery corticosteroids have been used for decades, despite limited data actually suggesting a positive effect on outcome. Many fundamental studies have shown a protective effect on myocardial and pulmonary cell integrity,[106] reduction of endothelial adhesion molecules and hereby reducing neutrophil mediated injury.[107] Also reduced levels of circulating complement and cytokine levels have been shown.[105;108;109] Although several studies could show a clear anti-inflammatory effect of corticosteroids given before cardiac surgery,[110;111] the value for diminishing clinical morbidity is less clear. Recently published meta-analyses focusing on both adult and pediatric corrective cardiac surgery separately concluded that there is insufficient evidence to propagate the universal use of corticosteroids in these patients.[112;113] Whether specific high-risk patient groups such as neonates and infants with complex congenital heart defects may benefit from the anti-inflammatory effects of corticosteroids remains a subject of debate. A retrospective study of high-risk cardiac surgery in neonates concluded a beneficial association between inta- plus pre-operative steroid and outcome (shorter duration of mechanical ventilation and hospital stay without increased risk of infection).[114]

The serine protease inhibitor aprotinin derives its anti-inflammatory effect from inhibiting the contact system. It inhibits trypsin, chymotrypsin, plasmin, kalikrein, elastase and thrombin,[115;116] and consequently decreased levels of pro-inflammatory cytokines circulate.[117] Besides these ex-vivo effects, several studies also suggest an overall decrease in mortality alongside reduced signs of reperfusion injury and improved bleeding due to platelet preservation.[118] However, a large well conducted study in adult patients by Mangano, et al. reports severe safety concerns (end-organ damage and long-term mortality) for the use of aprotinin and subsequently safer anti-fibrinolytic alternatives are preferred.[119]

Recently, clinical trials have been conducted with the neutrophil elastase inhibitor sivelestat. Sivelestat reduces neutrophil elastase, IL-6 and IL-8 and reduces acute lung injury in adult cardiac surgery with CPB.[120;121] Furthermore it has been shown to improve pulmonary and cardiac function in children following cardiac surgery.[122;123] These first studies show promising results, with reduced inflammatory mediators in some studies alongside improved clinical indices. However, due to the size of these trials and the uncomplicated post-operative course there was no difference in morbidity (such as length of stay or duration of mechanical ventilation) or mortality.

Tight glycemic control in critically ill patients is an important post-operative management and may reduce the risk of secondary infection and general inflammation. A first large trial, with predominantly children following cardiac surgery showed reductions in mortality, length

of stay and rate of infection.[124] While a study from the same research group on tight glycemic control following neonatal cardiac surgery also shows cardio-protective effects together with reduced circulating pro-inflammatory cytokines, there were also concerns because of a high rate of hypoglycemia.[125] A recent study with infants after cardiac surgery showed, despite safely achieving normoglycemia, no improvement in infections, mortality or several organ specific end points.[126] Thus far, tight glycemic control in this patient population has generally not been implemented in routine protocols due to risk of hypoglycemia and insufficient proof of clinical benefit.

Overall, numerous attempts to control inflammation due to cardiac surgery have mainly focused on inhibiting the inflammatory response. Though reducing activation of the immune system (e.g. improved CPB circuits) has had a positive effect on outcome, actively inhibiting inflammation has thus far shown limited clinical benefit.

6. Conclusion

Inflammation following cardiac surgery is necessary for wound healing but also inflicts damage through systemic inflammation or by falling short when confronted with pathogens. As a rule, also in the case of post-operative inflammation; 'what goes up must come down'. In a natural course of inflammation the immune system is well equipped for regaining equilibrium. However, excessive responses, either hyper- or hypo-inflammatory, will have deleterious effect. Numerous therapeutic attempts to control inflammation have shown variable clinical success. Before initiating further trials in this field it is mandatory to identify which patient is most likely to benefit. Since the first attempts to control inflammation, peri-operative management has improved substantially resulting in less inflammation and lower post-operative morbidity. On the other hand, procedures have become more complex and are performed in younger patients. Bearing in mind that inflammation after major surgery is an essential part of recovery, outcome may be improved in those patients that are at risk to develop unbridled inflammation and subsequent damage. Ongoing knowledge in how to fine-tune the immune system is crucial in finding the appropriate therapy in this select patient group.

References

1. Botto LD, Correa A, Erickson JD. Racial and temporal variations in the prevalence of heart defects. Pediatrics 2001;107:E32.
2. Warnes CA. The adult with congenital heart disease: born to be bad? J.Am.Coll.Cardiol. 2005;46:1-8.
3. Jacobs JP, O'Brien SM, Pasquali SK et al. Variation in outcomes for risk-stratified pediatric cardiac surgical operations: an analysis of the STS Congenital Heart Surgery Database. Ann.Thorac.Surg. 2012;94:564-571.
4. De Hert SG, Preckel B, Schlack WS. Update on inhalational anaesthetics. Curr.Opin.Anaesthesiol. 2009;22:491-495.
5. Suleiman MS, Zacharowski K, Angelini GD. Inflammatory response and cardioprotection during open-heart surgery: the importance of anaesthetics. Br.J.Pharmacol. 2008;153:21-33.
6. Stadnicka A, Marinovic J, Ljubkovic M, Bienengraeber MW, Bosnjak ZJ. Volatile anesthetic-induced cardiac preconditioning. J.Anesth. 2007;21:212-219.
7. Frassdorf J, De Hert SG, Schlack W. Anaesthesia and myocardial ischaemia/reperfusion injury. Br.J.Anaesth. 2009;103:89-98.
8. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986;74:1124-1136.
9. Cheung MM, Kharbanda RK, Konstantinov IE et al. Randomized controlled trial of the effects of remote ischemic preconditioning on children undergoing cardiac surgery: first clinical application in humans. J.Am.Coll.Cardiol. 2006;47:2277-2282.
10. Zhou W, Zeng D, Chen R et al. Limb ischemic preconditioning reduces heart and lung injury after an open heart operation in infants. Pediatr.Cardiol. 2010;31:22-29.
11. McCrindle BW, Clarizia N, Khaikin S et al. Remote Ischemic Preconditioning in Children Undergoing Surgery with Cardiopulmonary Bypass: A Single Centre Double-Blinded Randomized Trial [abstract]. Circulation 2011;124:*A11013*.
12. Algra SO, Kornmann VN, van der Tweel I et al. Increasing duration of circulatory arrest, but not antegrade cerebral perfusion, prolongs postoperative recovery after neonatal cardiac surgery. J.Thorac.Cardiovasc. Surg. 2012;143:375-382.
13. Diegeler A, Doll N, Rauch T et al. Humoral immune response during coronary artery bypass grafting: A comparison of limited approach, "off-pump" technique, and conventional cardiopulmonary bypass. Circulation 2000;102:III95-100.
14. Liangos O, Domhan S, Schwager C et al. Whole blood transcriptomics in cardiac surgery identifies a gene regulatory network connecting ischemia reperfusion with systemic inflammation. PLoS.One. 2010;5:e13658.
15. Allan CK, Newburger JW, McGrath E et al. The relationship between inflammatory activation and clinical outcome after infant cardiopulmonary bypass. Anesth.Analg. 2010;111:1244-1251.
16. Gessler P, Pfenninger J, Pfammatter JP, Carrel T, Dahinden C. Inflammatory response of neutrophil granulocytes and monocytes after cardiopulmonary bypass in pediatric cardiac surgery. Intensive Care Med 2002;28:1786-1791.
17. Mou SS, Haudek SB, Lequier L et al. Myocardial inflammatory activation in children with congenital heart disease. Crit Care Med 2002;30:827-832.
18. Schadenberg AW, Vastert SJ, Evens FC et al. FOXP3+ CD4+ Tregs lose suppressive potential but remain anergic during transient inflammation in human. Eur.J.Immunol. 2011;41:1132-1142.
19. Rider AR, Schreiner RS, Undar A. Pulsatile perfusion during cardiopulmonary bypass procedures in neonates, infants, and small children. ASAIO J. 2007;53:706-709.
20. Sezai A, Shiono M, Nakata K et al. Effects of pulsatile CPB on interleukin-8 and endothelin-1 levels. Artif. Organs 2005;29:708-713.
21. Grossi EA, Kallenbach K, Chau S et al. Impact of heparin bonding on pediatric cardiopulmonary bypass: a prospective randomized study. Ann.Thorac.Surg. 2000;70:191-196.

22. Englander R, Cardarelli MG. Efficacy of leukocyte filters in the bypass circuit for infants undergoing cardiac operations. Ann.Thorac.Surg. 1995;60:S533-S535.
23. Cordeiro JV, Jacinto A. The role of transcription-independent damage signals in the initiation of epithelial wound healing. Nat.Rev.Mol.Cell Biol. 2013;14:249-262.
24. Li M, Carpio DF, Zheng Y et al. An essential role of the NF-kappa B/Toll-like receptor pathway in induction of inflammatory and tissue-repair gene expression by necrotic cells. J.Immunol. 2001;166:7128-7135.
25. Roupe KM, Nybo M, Sjobring U et al. Injury is a major inducer of epidermal innate immune responses during wound healing. J.Invest Dermatol. 2010;130:1167-1177.
26. Ni Choileain N, Redmond HP. Cell response to surgery. Arch.Surg. 2006;141:1132-1140.
27. Prondzinsky R, Knupfer A, Loppnow H et al. Surgical trauma affects the proinflammatory status after cardiac surgery to a higher degree than cardiopulmonary bypass. J.Thorac.Cardiovasc.Surg. 2005;129:760-766.
28. Murphy TJ, Paterson HM, Mannick JA, Lederer JA. Injury, sepsis, and the regulation of Toll-like receptor responses. J.Leukoc.Biol. 2004;75:400-407.
29. Imura H, Caputo M, Parry A et al. Age-dependent and hypoxia-related differences in myocardial protection during pediatric open heart surgery. Circulation 2001;103:1551-1556.
30. Caputo M, Mokhtari A, Rogers CA et al. The effects of normoxic versus hyperoxic cardiopulmonary bypass on oxidative stress and inflammatory response in cyanotic pediatric patients undergoing open cardiac surgery: a randomized controlled trial. J.Thorac.Cardiovasc.Surg. 2009;138:206-214.
31. Apostolakis E, Filos KS, Koletsis E, Dougenis D. Lung dysfunction following cardiopulmonary bypass. J.Card Surg. 2010;25:47-55.
32. Goebel U, Siepe M, Mecklenburg A et al. Reduced pulmonary inflammatory response during cardiopulmonary bypass: effects of combined pulmonary perfusion and carbon monoxide inhalation. Eur.J.Cardiothorac.Surg. 2008;34:1165-1172.
33. Suzuki T, Fukuda T, Ito T et al. Continuous pulmonary perfusion during cardiopulmonary bypass prevents lung injury in infants. Ann.Thorac.Surg. 2000;69:602-606.
34. Pathan N, Burmester M, Adamovic T et al. Intestinal injury and endotoxemia in children undergoing surgery for congenital heart disease. Am.J.Respir.Crit Care Med. 2011;184:1261-1269.
35. Li S, Krawczeski CD, Zappitelli M et al. Incidence, risk factors, and outcomes of acute kidney injury after pediatric cardiac surgery: a prospective multicenter study. Crit Care Med. 2011;39:1493-1499.
36. Luehr M, Bachet J, Mohr FW, Etz CD. Modern temperature management in aortic arch surgery: the dilemma of moderate hypothermia. Eur.J.Cardiothorac.Surg. 2013
37. Kamiya H, Hagl C, Kropivnitskaya I et al. The safety of moderate hypothermic lower body circulatory arrest with selective cerebral perfusion: a propensity score analysis. J.Thorac.Cardiovasc.Surg. 2007;133:501-509.
38. Le Deist F, Menasche P, Kucharski C et al. Hypothermia during cardiopulmonary bypass delays but does not prevent neutrophil-endothelial cell adhesion. A clinical study. Circulation 1995;92:II354-II358.
39. Tassani P, Barankay A, Haas F et al. Cardiac surgery with deep hypothermic circulatory arrest produces less systemic inflammatory response than low-flow cardiopulmonary bypass in newborns. J.Thorac. Cardiovasc.Surg. 2002;123:648-654.
40. Boyle EM, Jr., Pohlman TH, Johnson MC, Verrier ED. Endothelial cell injury in cardiovascular surgery: the systemic inflammatory response. Ann.Thorac.Surg. 1997;63:277-284.
41. Chew MS, Brandslund I, Brix-Christensen V et al. Tissue injury and the inflammatory response to pediatric cardiac surgery with cardiopulmonary bypass: a descriptive study. Anesthesiology 2001;94:745-753.
42. Franke A, Lante W, Fackeldey V et al. Proinflammatory and antiinflammatory cytokines after cardiac operation: different cellular sources at different times. Ann.Thorac.Surg. 2002;74:363-370.
43. Pasquale MD, Cipolle MD, Monaco J, Simon N. Early inflammatory response correlates with the severity of injury. Crit Care Med. 1996;24:1238-1242.
44. Osuchowski MF, Welch K, Siddiqui J, Remick DG. Circulating cytokine/inhibitor profiles reshape the understanding of the SIRS/CARS continuum in sepsis and predict mortality. J.Immunol. 2006;177:1967-1974.

45. Xiao W, Mindrinos MN, Seok J et al. A genomic storm in critically injured humans. J.Exp.Med. 2011;208:2581-2590.
46. Calkins CM, Bensard DD, Moore EE et al. The injured child is resistant to multiple organ failure: a different inflammatory response? J.Trauma 2002;53:1058-1063.
47. Wood JH, Partrick DA, Johnston RB, Jr. The inflammatory response to injury in children. Curr.Opin. Pediatr. 2010;22:315-320.
48. Proulx F, Joyal JS, Mariscalco MM et al. The pediatric multiple organ dysfunction syndrome. Pediatr.Crit Care Med. 2009;10:12-22.
49. Levy O. Innate immunity of the newborn: basic mechanisms and clinical correlates. Nat.Rev.Immunol. 2007;7:379-390.
50. Adkins B, Leclerc C, Marshall-Clarke S. Neonatal adaptive immunity comes of age. Nat.Rev.Immunol. 2004;4:553-564.
51. Chelvarajan RL, Collins SM, Doubinskaia IE et al. Defective macrophage function in neonates and its impact on unresponsiveness of neonates to polysaccharide antigens. J.Leukoc.Biol. 2004;75:982-994.
52. Barker GM, O'Brien SM, Welke KF et al. Major infection after pediatric cardiac surgery: a risk estimation model. Ann.Thorac.Surg. 2010;89:843-850.
53. Algra SO, Driessen MM, Schadenberg AW et al. Bedside prediction rule for infections after pediatric cardiac surgery. Intensive Care Med. 2012;38:474-481.
54. Qing M, Schumacher K, Heise R et al. Intramyocardial synthesis of pro- and anti-inflammatory cytokines in infants with congenital cardiac defects. J.Am.Coll.Cardiol. 2003;41:2266-2274.
55. Christen S, Finckh B, Lykkesfeldt J et al. Oxidative stress precedes peak systemic inflammatory response in pediatric patients undergoing cardiopulmonary bypass operation. Free Radic.Biol.Med. 2005;38:1323-1332.
56. Qing M, Woltje M, Schumacher K et al. The use of moderate hypothermia during cardiac surgery is associated with repression of tumour necrosis factor-alpha via inhibition of activating protein-1: an experimental study. Crit Care 2006;10:R57.
57. Heying R, Wehage E, Schumacher K et al. Dexamethasone pretreatment provides antiinflammatory and myocardial protection in neonatal arterial switch operation. Ann.Thorac.Surg. 2012;93:869-876.
58. Stangl V, Baumann G, Stangl K, Felix SB. Negative inotropic mediators released from the heart after myocardial ischaemia-reperfusion. Cardiovasc.Res. 2002;53:12-30.
59. Jiang B, Liao R. The paradoxical role of inflammation in cardiac repair and regeneration. J.Cardiovasc. Transl.Res. 2010;3:410-416.
60. Onorati F, Rubino AS, Nucera S et al. Off-pump coronary artery bypass surgery versus standard linear or pulsatile cardiopulmonary bypass: endothelial activation and inflammatory response. Eur.J.Cardiothorac. Surg. 2010;37:897-904.
61. Duran WN, Breslin JW, Sanchez FA. The NO cascade, eNOS location, and microvascular permeability. Cardiovasc.Res. 2010;87:254-261.
62. Joyce DE, Nelson DR, Grinnell BW. Leukocyte and endothelial cell interactions in sepsis: relevance of the protein C pathway. Crit Care Med. 2004;32:S280-S286.
63. Fernandez-Borja M, van Buul JD, Hordijk PL. The regulation of leucocyte transendothelial migration by endothelial signalling events. Cardiovasc.Res. 2010;86:202-210.
64. Eltzschig HK, Faigle M, Knapp S et al. Endothelial catabolism of extracellular adenosine during hypoxia: the role of surface adenosine deaminase and CD26. Blood 2006;108:1602-1610.
65. Craddock PR, Fehr J, Brigham KL, Kronenberg RS, Jacob HS. Complement and leukocyte-mediated pulmonary dysfunction in hemodialysis. N.Engl.J.Med. 1977;296:769-774.
66. Seely AJ, Pascual JL, Christou NV. Science review: Cell membrane expression (connectivity) regulates neutrophil delivery, function and clearance. Crit Care 2003;7:291-307.
67. Vinten-Johansen J. Involvement of neutrophils in the pathogenesis of lethal myocardial reperfusion injury. Cardiovasc.Res. 2004;61:481-497.

68. Gessler P, Pretre R, Hohl V et al. CXC-chemokine stimulation of neutrophils correlates with plasma levels of myeloperoxidase and lactoferrin and contributes to clinical outcome after pediatric cardiac surgery. Shock 2004;22:513-520.
69. Medzhitov R. Recognition of microorganisms and activation of the immune response. Nature 2007;449:819-826.
70. Gordon S, Taylor PR. Monocyte and macrophage heterogeneity. Nat.Rev.Immunol. 2005;5:953-964.
71. Allen ML, Peters MJ, Goldman A et al. Early postoperative monocyte deactivation predicts systemic inflammation and prolonged stay in pediatric cardiac intensive care. Crit Care Med 2002;30:1140-1145.
72. Pachot A, Cazalis MA, Venet F et al. Decreased expression of the fractalkine receptor CX3CR1 on circulating monocytes as new feature of sepsis-induced immunosuppression. J.Immunol. 2008;180:6421-6429.
73. Dehoux MS, Hernot S, Asehnoune K et al. Cardiopulmonary bypass decreases cytokine production in lipopolysaccharide-stimulated whole blood cells: roles of interleukin-10 and the extracorporeal circuit. Crit Care Med. 2000;28:1721-1727.
74. Perry SE, Mostafa SM, Wenstone R, Shenkin A, McLaughlin PJ. Is low monocyte HLA-DR expression helpful to predict outcome in severe sepsis? Intensive Care Med. 2003;29:1245-1252.
75. Docke WD, Randow F, Syrbe U et al. Monocyte deactivation in septic patients: restoration by IFN-gamma treatment. Nat.Med. 1997;3:678-681.
76. Tschaikowsky K, Hedwig-Geissing M, Schiele A et al. Coincidence of pro- and anti-inflammatory responses in the early phase of severe sepsis: Longitudinal study of mononuclear histocompatibility leukocyte antigen-DR expression, procalcitonin, C-reactive protein, and changes in T-cell subsets in septic and postoperative patients. Crit Care Med. 2002;30:1015-1023.
77. Dybdahl B, Wahba A, Lien E et al. Inflammatory response after open heart surgery: release of heat-shock protein 70 and signaling through toll-like receptor-4. Circulation 2002;105:685-690.
78. Hadley JS, Wang JE, Michaels LC et al. Alterations in inflammatory capacity and TLR expression on monocytes and neutrophils after cardiopulmonary bypass. Shock 2007;27:466-473.
79. de Jong PR, Schadenberg AW, van den Broek T et al. STAT3 regulates monocyte TNF-alpha production in systemic inflammation caused by cardiac surgery with cardiopulmonary bypass. PLoS.One. 2012;7:e35070.
80. Riddle PR, Berenbaum MC. Postoperative depression of the lymphocyte response to phytohaemagglutinin. Lancet 1967;1:746-748.
81. Angele MK, Faist E. Clinical review: immunodepression in the surgical patient and increased susceptibility to infection. Crit Care 2002;6:298-305.
82. Lederer JA, Rodrick ML, Mannick JA. The effects of injury on the adaptive immune response. Shock 1999;11:153-159.
83. Ijichi S, Mishima M, Matsuda T et al. Concentration of activated T lymphocytes in extracorporeal blood circulation for plasma separation. J.Clin.Apher. 1991;6:88-89.
84. Schneemilch CE, Hachenberg T, Ansorge S, Ittenson A, Bank U. Effects of different anaesthetic agents on immune cell function *in vitro*. Eur.J.Anaesthesiol. 2005;22:616-623.
85. Jansen NJ, van Oeveren W, Gu YJ et al. Endotoxin release and tumor necrosis factor formation during cardiopulmonary bypass. Ann.Thorac.Surg. 1992;54:744-747.
86. Tsunooka N, Maeyama K, Hamada Y et al. Bacterial translocation secondary to small intestinal mucosal ischemia during cardiopulmonary bypass. Measurement by diamine oxidase and peptidoglycan. Eur.J.Cardiothorac.Surg. 2004;25:275-280.
87. de Jong PR, Schadenberg AW, Jansen NJ, Prakken BJ. Hsp70 and cardiac surgery: molecular chaperone and inflammatory regulator with compartmentalized effects. Cell Stress.Chaperones. 2009;14:117-131.
88. Ni Choileain N, MacConmara M, Zang Y et al. Enhanced regulatory T cell activity is an element of the host response to injury. J.Immunol. 2006;176:225-236.
89. Hovels-Gurich HH, Schumacher K, Vazquez-Jimenez JF et al. Cytokine balance in infants undergoing cardiac operation. Ann.Thorac.Surg. 2002;73:601-608.

90. Cavaillon JM, Annane D. Compartmentalization of the inflammatory response in sepsis and SIRS. J.Endotoxin.Res. 2006;12:151-170.
91. Sarvikivi E, Lyytikainen O, Nieminen H, Sairanen H, Saxen H. Nosocomial infections after pediatric cardiac surgery. Am.J.Infect.Control 2008;36:564-569.
92. Chenoweth DE, Cooper SW, Hugli TE et al. Complement activation during cardiopulmonary bypass: evidence for generation of C3a and C5a anaphylatoxins. N.Engl.J.Med. 1981;304:497-503.
93. Kirklin JK, Chenoweth DE, Naftel DC et al. Effects of protamine administration after cardiopulmonary bypass on complement, blood elements, and the hemodynamic state. Ann.Thorac.Surg. 1986;41:193-199.
94. Seghaye MC, Duchateau J, Grabitz RG et al. Complement activation during cardiopulmonary bypass in infants and children. Relation to postoperative multiple system organ failure. J.Thorac.Cardiovasc.Surg. 1993;106:978-987.
95. Moat NE, Shore DF, Evans TW. Organ dysfunction and cardiopulmonary bypass: the role of complement and complement regulatory proteins. Eur.J.Cardiothorac.Surg. 1993;7:563-573.
96. Stiller B, Sonntag J, Dahnert I et al. Capillary leak syndrome in children who undergo cardiopulmonary bypass: clinical outcome in comparison with complement activation and C1 inhibitor. Intensive Care Med. 2001;27:193-200.
97. Li JS, Sanders SP, Perry AE et al. Pharmacokinetics and safety of TP10, soluble complement receptor 1, in infants undergoing cardiopulmonary bypass. Am.Heart J. 2004;147:173-180.
98. Lazar HL, Bokesch PM, van LF et al. Soluble human complement receptor 1 limits ischemic damage in cardiac surgery patients at high risk requiring cardiopulmonary bypass. Circulation 2004;110:II274-II279.
99. Baig K, Nassar R, Craig DM et al. Complement factor 1 inhibitor improves cardiopulmonary function in neonatal cardiopulmonary bypass. Ann.Thorac.Surg. 2007;83:1477-1482.
100. Tassani P, Kunkel R, Richter JA et al. Effect of C1-esterase-inhibitor on capillary leak and inflammatory response syndrome during arterial switch operations in neonates. J.Cardiothorac.Vasc.Anesth. 2001;15:469-473.
101. Allen M, Sundararajan S, Pathan N, Burmester M, Macrae D. Anti-inflammatory modalities: their current use in pediatric cardiac surgery in the United Kingdom and Ireland. Pediatr.Crit Care Med. 2009;10:341-345.
102. Mollnes TE, Videm V, Gotze O, Harboe M, Oppermann M. Formation of C5a during cardiopulmonary bypass: inhibition by precoating with heparin. Ann.Thorac.Surg. 1991;52:92-97.
103. Ovrum E, Fosse E, Mollnes TE et al. Complete heparin-coated cardiopulmonary bypass and low heparin dose reduce complement and granulocyte activation. Eur.J.Cardiothorac.Surg. 1996;10:54-60.
104. Jensen E, Andreasson S, Bengtsson A et al. Changes in hemostasis during pediatric heart surgery: impact of a biocompatible heparin-coated perfusion system. Ann.Thorac.Surg. 2004;77:962-967.
105. Warren O, Alexiou C, Massey R et al. The effects of various leukocyte filtration strategies in cardiac surgery. Eur.J.Cardiothorac.Surg. 2007;31:665-676.
106. von Spiegel T, Giannaris S, Wietasch GJ et al. Effects of dexamethasone on intravascular and extravascular fluid balance in patients undergoing coronary bypass surgery with cardiopulmonary bypass. Anesthesiology 2002;96:827-834.
107. Cronstein BN, Kimmel SC, Levin RI, Martiniuk F, Weissmann G. A mechanism for the antiinflammatory effects of corticosteroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial-leukocyte adhesion molecule 1 and intercellular adhesion molecule 1. Proc.Natl. Acad.Sci.U.S.A 1992;89:9991-9995.
108. Kawamura T, Inada K, Nara N, Wakusawa R, Endo S. Influence of methylprednisolone on cytokine balance during cardiac surgery. Crit Care Med. 1999;27:545-548.
109. Lerzo F, Peri G, Doni A et al. Dexamethasone prophylaxis in pediatric open heart surgery is associated with increased blood long pentraxin PTX3: potential clinical implications. Clin.Dev.Immunol. 2011;2011:730828.
110. El Azab Sr, Rosseel PM, de Lange JJ et al. Dexamethasone decreases the pro- to anti-inflammatory cytokine ratio during cardiac surgery. Br.J.Anaesth. 2002;88:496-501.

111. Jansen NJ, van Oeveren W, van den Broek L et al. Inhibition by dexamethasone of the reperfusion phenomena in cardiopulmonary bypass. J.Thorac.Cardiovasc.Surg. 1991;102:515-525.

112. Dieleman JM, van Paassen J, van Dijk D et al. Prophylactic corticosteroids for cardiopulmonary bypass in adults. Cochrane.Database.Syst.Rev. 2011CD005566.

113. Pasquali SK, Hall M, Li JS et al. Corticosteroids and outcome in children undergoing congenital heart surgery: analysis of the Pediatric Health Information Systems database. Circulation 2010;122:2123-2130.

114. Clarizia NA, Manlhiot C, Schwartz SM et al. Improved outcomes associated with intraoperative steroid use in high-risk pediatric cardiac surgery. Ann.Thorac.Surg. 2011;91:1222-1227.

115. Poullis M, Manning R, Laffan M et al. The antithrombotic effect of aprotinin: actions mediated via the proteaseactivated receptor 1. J.Thorac.Cardiovasc.Surg. 2000;120:370-378.

116. van Oeveren W, Jansen NJ, Bidstrup BP et al. Effects of aprotinin on hemostatic mechanisms during cardiopulmonary bypass. Ann.Thorac.Surg. 1987;44:640-645.

117. Greilich PE, Brouse CF, Whitten CW et al. Antifibrinolytic therapy during cardiopulmonary bypass reduces proinflammatory cytokine levels: a randomized, double-blind, placebo-controlled study of epsilon-aminocaproic acid and aprotinin. J.Thorac.Cardiovasc.Surg. 2003;126:1498-1503.

118. Goudeau JJ, Clermont G, Guillery O et al. In high-risk patients, combination of antiinflammatory procedures during cardiopulmonary bypass can reduce incidences of inflammation and oxidative stress. J.Cardiovasc.Pharmacol. 2007;49:39-45.

119. Mangano DT, Tudor IC, Dietzel C. The risk associated with aprotinin in cardiac surgery. N.Engl.J.Med. 2006;354:353-365.

120. Fujii M, Miyagi Y, Bessho R et al. Effect of a neutrophil elastase inhibitor on acute lung injury after cardiopulmonary bypass. Interact.Cardiovasc.Thorac.Surg. 2010;10:859-862.

121. Ryugo M, Sawa Y, Takano H et al. Effect of a polymorphonuclear elastase inhibitor (sivelestat sodium) on acute lung injury after cardiopulmonary bypass: findings of a double-blind randomized study. Surg.Today 2006;36:321-326.

122. Morimoto N, Morimoto K, Morimoto Y et al. Sivelestat attenuates postoperative pulmonary dysfunction after total arch replacement under deep hypothermia. Eur.J.Cardiothorac.Surg. 2008;34:798-804.

123. Toyama S, Hatori F, Shimizu A, Takagi T. A neutrophil elastase inhibitor, sivelestat, improved respiratory and cardiac function in pediatric cardiovascular surgery with cardiopulmonary bypass. J.Anesth. 2008;22:341-346.

124. Vlasselaers D, Milants I, Desmet L et al. Intensive insulin therapy for patients in paediatric intensive care: a prospective, randomised controlled study. Lancet 2009;373:547-556.

125. Vlasselaers D, Mesotten D, Langouche L et al. Tight glycemic control protects the myocardium and reduces inflammation in neonatal heart surgery. Ann.Thorac.Surg. 2010;90:22-29.

126. Agus MS, Steil GM, Wypij D et al. Tight glycemic control versus standard care after pediatric cardiac surgery. N.Engl.J.Med. 2012;367:1208-1219.

127. Jensen E, Andreasson S, Bengtsson A et al. Influence of two different perfusion systems on inflammatory response in pediatric heart surgery. Ann.Thorac.Surg. 2003;75:919-925.

128. Seeburger J, Hoffmann J, Wendel HP, Ziemer G, Aebert H. Gene expression changes in leukocytes during cardiopulmonary bypass are dependent on circuit coating. Circulation 2005;112:I224-I228.

129. Stocker CF, Shekerdemian LS, Horton SB et al. The influence of bypass temperature on the systemic inflammatory response and organ injury after pediatric open surgery: a randomized trial. J.Thorac. Cardiovasc.Surg. 2011;142:174-180.

130. Eggum R, Ueland T, Mollnes TE et al. Effect of perfusion temperature on the inflammatory response during pediatric cardiac surgery. Ann.Thorac.Surg. 2008;85:611-617.

131. Chen YF, Tsai WC, Lin CC et al. Leukocyte depletion attenuates expression of neutrophil adhesion molecules during cardiopulmonary bypass in human beings. J.Thorac.Cardiovasc.Surg. 2002;123:218-224.

132. Kuratani N, Bunsangjaroen P, Srimueang T et al. Modified versus conventional ultrafiltration in pediatric cardiac surgery: a meta-analysis of randomized controlled trials comparing clinical outcome parameters. J.Thorac.Cardiovasc.Surg. 2011;142:861-867.

133. Yndgaard S, Andersen LW, Andersen C, Petterson G, Baek L. The effect of modified ultrafiltration on the amount of circulating endotoxins in children undergoing cardiopulmonary bypass. J.Cardiothorac.Vasc. Anesth. 2000;14:399-401.

134. Cholette JM, Henrichs KF, Alfieris GM et al. Washing red blood cells and platelets transfused in cardiac surgery reduces postoperative inflammation and number of transfusions: Results of a prospective, randomized, controlled clinical trial*. Pediatr.Crit Care Med. 2012;13:290-299.

135. Bilgin YM, van de Watering LM, Eijsman L et al. Double-blind, randomized controlled trial on the effect of leukocyte-depleted erythrocyte transfusions in cardiac valve surgery. Circulation 2004;109:2755-2760.

136. Schroeder VA, Pearl JM, Schwartz SM et al. Combined steroid treatment for congenital heart surgery improves oxygen delivery and reduces postbypass inflammatory mediator expression. Circulation 2003;107:2823-2828.

137. Schouten ES, van de Pol AC, Schouten AN et al. The effect of aprotinin, tranexamic acid, and aminocaproic acid on blood loss and use of blood products in major pediatric surgery: a meta-analysis. Pediatr.Crit Care Med. 2009;10:182-190.

138. Mojcik CF, Levy JH. Aprotinin and the systemic inflammatory response after cardiopulmonary bypass. Ann.Thorac.Surg. 2001;71:745-754.

139. Inoue N, Oka N, Kitamura T et al. Neutrophil elastase inhibitor sivelestat attenuates perioperative inflammatory response in pediatric heart surgery with cardiopulmonary bypass. Int.Heart J. 2013;54:149-153.

Chapter 3

STAT3 REGULATES MONOCYTE TNF-ALPHA PRODUCTION IN SYSTEMIC INFLAMMATION CAUSED BY CARDIAC SURGERY WITH CARDIOPULMONARY BYPASS

PR de Jong[1,2,*], AWL Schadenberg[1,2,*], T van den Broek[1,2], JM Beekman[3], F van Wijk[2], PJ Coffer[2,3], BJ Prakken[2,*], NJG Jansen[1,*]

[1] Dep. of Pediatric Intensive Care, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands
[2] Center for Molecular and Cellular Intervention, Dep.t of Pediatric Immunology, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands
[3] Dep. of Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

[*] These authors (P.R.J. & A.W.L.S. and B.J.P. & N.J.G.J.) contributed equally to this paper.

PLoS One 2012. 7: e35070

Abstract

Background: Cardiopulmonary bypass (CPB) surgery initiates a controlled systemic inflammatory response characterized by a cytokine storm, monocytosis and transient monocyte activation. However, the responsiveness of monocytes to Toll-like receptor (TLR)-mediated activation decreases throughout the postoperative course. The purpose of this study was to identify the major signaling pathway involved in plasma-mediated inhibition of LPS-induced tumor necrosis factor (TNF)-α production by monocytes.

Methodology/Principal findings: Pediatric patients that underwent CPB-assisted surgical correction of simple congenital heart defects were enrolled (n=38). Peripheral blood mononuclear cells (PBMC) and plasma samples were isolated at consecutive time points. Patient plasma samples were added back to monocytes obtained pre-operatively for ex vivo LPS stimulations and TNF-α and IL-6 production was measured by flow cytometry. LPS-induced p38 mitogen-activated protein kinase (MAPK) and nuclear factor (NF)-κB activation by patient plasma was assessed by Western blotting. A cell-permeable peptide inhibitor was used to block STAT3 signaling. We found that plasma samples obtained 4 h after surgery, regardless of pre-operative dexamethasone treatment, potently inhibited LPS-induced TNF-α but not IL-6 synthesis by monocytes. This was not associated with attenuation of p38 MAPK activation or IκB-α degradation. However, abrogation of the IL-10/STAT3 pathway restored LPS-induced TNF-α production in the presence of suppressive patient plasma.

Conclusions/Significance: Our findings suggest that STAT3 signaling plays a crucial role in the downregulation of TNF-α synthesis by human monocytes in the course of systemic inflammation *in vivo*. Thus, STAT3 might be a potential molecular target for pharmacological intervention in clinical syndromes characterized by systemic inflammation.

Introduction

Cardiopulmonary bypass-assisted surgery initiates a systemic inflammatory response associated by extrinsic (e.g. anesthesia, contact activation within the extracorporeal circuit, endotoxemia) and intrinsic (e.g. tissue damage, endothelial cell activation, ischemia-reperfusion injury of myocardium) factors.[1-3] Monocytes are important players in systemic inflammation and the main producers of pro- and anti-inflammatory cytokines upon activation of innate pattern recognition receptors.[4] Significant changes in surface biomarkers on circulating monocytes such as HLA-DR[5;6] and chemokine receptor CX3CR1[7] have been observed in critical illness. Moreover, monocytes activated by the extracorporeal circuit extravasate to peripheral tissues with upregulation of adhesion molecule CD11b.[8] During this dysregulation of inflammatory homeostasis, increased levels of pro-inflammatory plasma mediators such as TNF-α, IL-6 and IL-8 are joined by anti-inflammatory cytokines such as IL-10 and TGF-β.[9-12] Importantly, the net effect of these circulating inflammatory mediators appears to be biased towards inhibition of innate immune cells, thereby providing timely negative feedback. However, the molecular and cellular mechanisms responsible for suppression of the immune system after on-pump cardiac surgery remain unclear.[13]

The anti-inflammatory phase in systemic inflammation is associated with a reduced TLR responsiveness of monocytes.[14;15] Monocytes respond to LPS stimulation through association of LPS/LPS-binding protein (LBP) with CD14 and TLR4,[16;17] which results in NF-κB activation. Altered monocyte reactivity to LPS after on-pump cardiac surgery by plasma mediators may therefore be caused by a reduced availability of TLR ligands (i.e. free LPS), by upregulation of circulating LBP[18] or lipoproteins.[19] Alternative explanations include the downregulation of TLR4 and the resulting inhibition of downstream signaling cascades,[20;21] prevention of IκB-α degradation, the negative regulator of NF-κB[22;23] or finally, the effects of signaling cascades [e.g. Signal transducer and activator of transcription (STAT)3] activated by the prototypic anti-inflammatory cytokine IL-10.[15]

In the present study, we evaluated these possibilities in order to identify the molecular mechanism behind the diminished response of monocytes to LPS stimulation during human systemic inflammation *in vivo*. Set against (pre-)clinical sepsis models, CPB-assisted cardiac surgery allows serial sampling of cells and plasma from the incitement, expansion up to the resolution phase of human systemic inflammation, as previously shown.[24] Only patients with a favorable outcome were included in order to provide a controlled system of inflammatory evolution. We tested the capability of patient plasma isolated at different time points to inhibit LPS-induced TNF-α and IL-6 synthesis by monocytes. Subsequently we tested the requirement for IL-10/STAT3 signaling for the effects of anti-inflammatory plasma on monocytes ex vivo.

Results

Activation of the innate immune system after on-pump cardiac surgery

As expected, cardiac surgery led to *in vivo* activation of the innate immune system. Mean cell counts increased significantly 24 h after surgery for both the neutrophil (9.79 ± 2.74 vs. 3.10 ± 1.94·10^9/L, Figure 1A) and monocyte (1.87 ± 0.89 vs. 0.57 ± 0.25·10^9/L, Figure 1B) populations compared to baseline. Accordingly, the pro-inflammatory CD14+CD16+ monocyte subpopulation had expanded significantly 24 h after surgery (0.51 ± 0.34 vs. 0.044 ± 0.025·10^9/L; Figure 1C). These events were paralleled by elevated plasma levels of C-reactive protein 24-48 h after surgery (Figure 1D), whereas we observed a transient lymphopenia 4 h after surgery (Figure 1E). Analysis of plasma samples by multiplex immunoassay showed marked increases of biomarkers that have been associated with a deleterious course in human systemic inflammation,[25] including IL-6, IL-8, TNF-α, MIF (all pro-inflammatory) and IL-10 (anti-inflammatory, Figure 1F). Thus, on-pump cardiac surgery leads to a temporary, controlled activation of the innate immune system with both strong pro- and anti-inflammatory signals.

Inhibition of LPS-induced monocyte TNF-α synthesis by post-perfusion plasma

Next we assessed the functional consequences of the dramatic peri- and postoperative release of inflammatory mediators on TLR-mediated monocyte activation. To study this, we stimulated thawed PBMC from patients obtained at various time points with E. coli LPS for 4 h in standard culture medium. Monocytes were the major responders to LPS-stimulation in PBMC as determined by intracellular TNF-α synthesis measured by FACS. However, we found only a marginal decrease in TNF-α production by patient monocytes in the course of CPB surgery (Fiure 2A). Accordingly, TLR4 expression levels on monocytes did not significantly change during the study period (TLR4 MFI Pre-op, End-CPB, 24 h and 48 h after surgery was 2.4 ± 1.3, 2.3 ± 1.1, 2.6 ± 1.5 and 2.3 ± 1.6, respectively). We then stimulated fresh whole blood samples obtained from patients at consecutive time-points with LPS ex vivo. Importantly, we now found a marked decrease of monocyte TNF-α production, which was maximal 4 h after surgery compared to baseline (Figure 2B). These findings suggested that, although the intrinsic capacity of monocytes to respond to LPS did not change, plasma factors released in the course of on pump cardiac surgery might influence their capacity to synthesize TNF-α.

To test this, we next stimulated thawed patient PBMC isolated before surgery with LPS in the presence of autologous plasma obtained at different time points or with plasma from healthy donors (control). Importantly, by using the same monocyte population for all experimental conditions (see experimental setup in Figure 2C), we could specifically address the regulatory role of plasma components released in the course of human systemic inflammation on monocytes. As shown in Figure 2D, we found significantly reduced TNF-α production in the presence of plasma obtained before surgery, at end of CPB and maximal suppression mediated by 4 h post-surgery plasma (all $P<0.001$ vs. control). Importantly, the number of TNF-α positive

LPS-stimulated monocytes in the presence of 4 h post-surgery plasma was significantly lower compared to pre-operative and 24 h post-surgery plasma ($P<0.05$ and $P<0.001$, respectively). Surprisingly, we did not observe a similar inhibitory effect of 4 h post-surgery plasma on IL-6 synthesis (Figure 2E). Analysis of the mean fluorescence intensities of TNF-α and IL-6 in LPS-stimulated monocytes to compare their respective expression levels in the different plasma milieus reproduced the same results (Figure 2H). Thus, plasma mediators released in the circulation 4 h after open heart surgery strongly suppressed LPS-induced TNF-α but not IL-6 synthesis by monocytes.

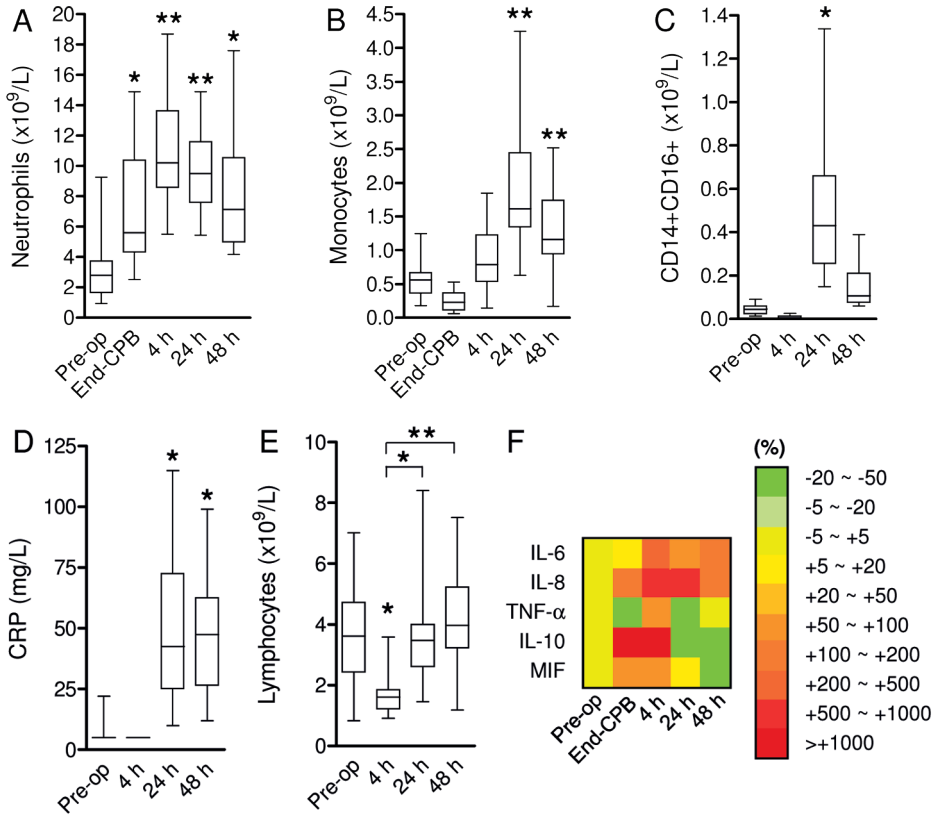

Figure 1. Inflammatory events induced by CPB surgery. Increased mean neutrophil (**A**) and monocyte (**B**) counts after on-pump cardiac surgery (n=21 and n=24, respectively). (**C**) Increased numbers of circulating CD14+CD16+ monocytes after CPB surgery (n=14). (**D**) Increased mean C-reactive protein (CRP) levels in patient blood samples post-surgery (n=22). (**E**) Lymphopenia was observed 4 h post-surgery (n=27). Box-and-whiskers plots. *$P<0.01$, **$P<0.001$ vs. pre-op (ANOVA). (**F**) Cyto- and chemokine color profiles of plasma samples (n=12) obtained at indicated time points, represented as % change compared to baseline. MIF: Macrophage migration inhibitory factor.

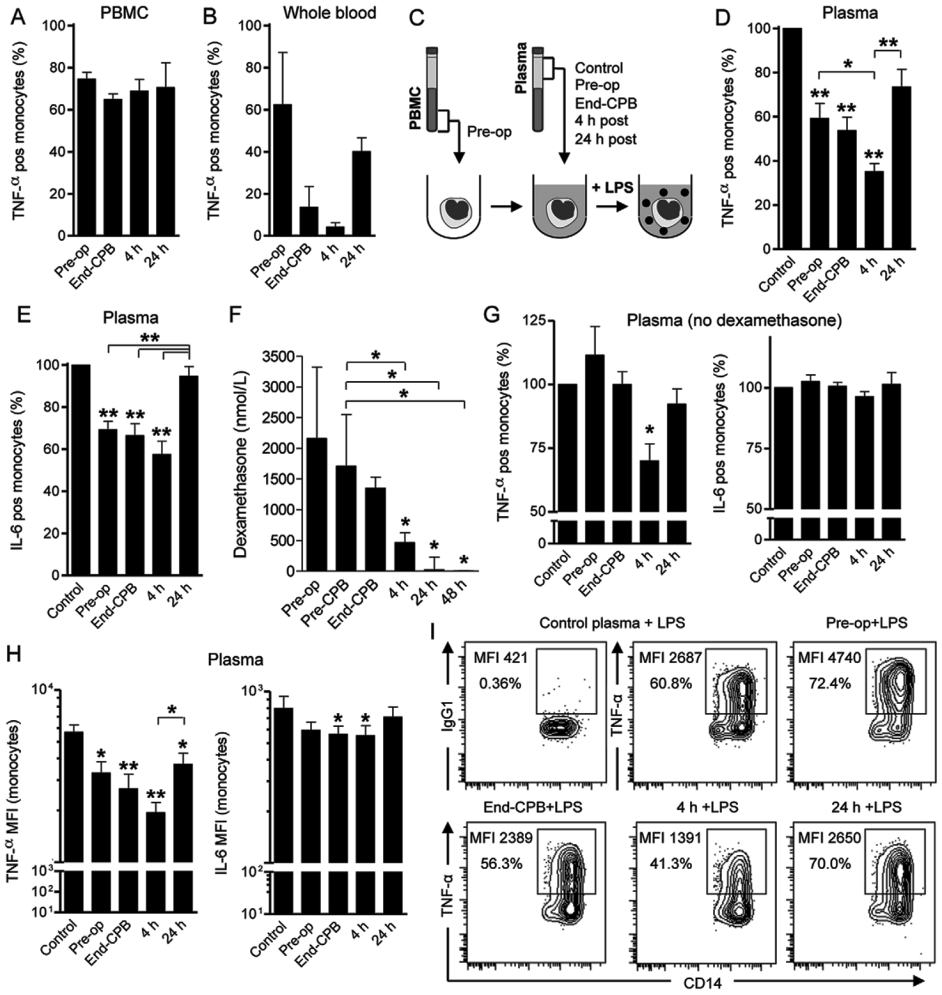

Figure 2. Post-perfusion plasma suppresses LPS-induced TNF-α production by monocytes. (**A**) Percentage of TNF-α producing cells in the monocyte population after ex vivo LPS stimulation (100 ng/mL) of patient PBMC isolated at various time points (n=4). (**B**) Reduced TNF-α synthesis by monocytes after LPS (10 ng/mL) stimulation in whole blood assays with patient samples obtained at the indicated time points (n=5). (**C**) Experimental setup for experiments shown in D,E,G-I. In short, patient PBMC obtained before surgery (Pre-op) were mixed with control (pooled AB plasma from healthy donors) or autologous patient plasma samples obtained at indicated time points, followed by LPS (100 ng/mL) stimulation for 4 h. Monocyte populations (CD14/SSC gate) were then analyzed for intracellular TNF-α and IL-6 synthesis. (**D**) Significantly reduced production of TNF-α by monocytes after LPS stimulation in the presence of plasma samples from different sources (n=13). Shown are percentages of TNF-α producing monocytes relative to control (100%) represented as mean ± SEM. **P*<0.05, ***P*<0.001 vs. control (ANOVA). (**E**) Percentages of IL-6 producing monocytes as in D. Mean ± SEM. ***P*<0.001 vs. control (ANOVA). (**F**) Dexamethasone levels in patient plasma samples as measured by radio-immunoassay (n=9). Median ± interquartile range. **P*<0.05 vs. pre-op (ANOVA). (**G**) Production

of TNF-α and IL-6 by monocytes after LPS stimulation in the presence of dexamethasone-free plasma samples (n=4). *P<0.05 vs. control (ANOVA). (**H**) Mean fluorescence intensities (MFI) of TNF-α and IL-6 in monocytes after LPS stimulation in different plasma milieus (n=7). *P<0.05, **P<0.001 vs. control (ANOVA). (**I**) Representative flow cytometry results (contour plots) of the LPS-induced TNF-α production by monocytes in the presence of control or patient plasma (Pre-op, End-CPB, 4 h or 24 h post-perfusion plasma from a No-dexamethasone patient). Isotype control: mouse IgG1. Data represented as mean ± SEM, unless otherwise indicated.

Since all patients analyzed had received dexamethasone pre-operatively, we had to exclude that this anti-inflammatory agent influenced our *ex vivo* monocyte assays. We therefore first measured dexamethasone levels in consecutive patient plasma samples and found that these were maximal in pre-operative samples, but already significantly reduced 4 h post-surgery (Figure 2F). To further exclude the potential influence of steroids on the effects of 4 h post-perfusion plasma, we enrolled a control group that did not receive dexamethasone before surgery. The clinical characteristics of these patients were comparable to the previously analyzed cohort of patients (Table 1). We repeated the ex vivo plasma assays as before and analyzed LPS-induced TNF-α and IL-6 production by monocytes. Again, we found a significant effect of 4 h post-perfusion plasma samples on TNF-α production by monocytes (Figure 2G). However, the inhibitory effects of Pre-op and End-CPB plasma samples on TNF-α synthesis were not found in the absence of dexamethasone. Moreover, there was no suppression of IL-6 in any of the steroid-free conditions tested (Figure 2G). Representative results of a patient from the No-dexamethasone group are shown in Figure 2I. We inferred from these data that 4 h post-perfusion plasma has a unique inhibitory effect on LPS-induced TNF-α but not IL-6 synthesis by monocytes.

Normal activation of p38 MAPK and NF-κB in the presence of post-perfusion plasma

Next, we sought to elucidate the molecular mechanisms that could account for the suppression of 4 h post-surgery plasma on LPS-induced TNF-α production by monocytes. To test whether this could be explained by either sequestration of LPS in post-surgery plasma or reduced TLR4 expression on monocytes, we evaluated for differences in activation of signaling pathways downstream of TLR4. To this end, we compared the effects of 4 h vs. 24 h post-perfusion plasma samples from the same patient, since the latter did not significantly reduce LPS-induced TNF-α synthesis (see Figure 2G). All three MAPK pathways i.e. p38, JNK/SAPK and ERK are activated by LPS in monocytes.[26] Since we found that the p38 MAPK pathway was most potently activated by LPS, we assessed the activation of p38 MAPK in purified monocytes isolated from healthy donors stimulated with LPS in the presence of patient plasma obtained 4 h or 24 h (control) post-surgery. As shown in Figure 3A, there was no attenuation of p38 activation in monocytes after LPS stimulation in the presence of 4 h post-surgery plasma compared to control plasma. In contrast, densitometric analysis of Western blots from 4 different patients showed even slightly

increased p38 MAPK phosphorylation in the presence of suppressive 4 h post-surgery plasma (Figure 3B). IκB-α negatively regulates NF-κB by sequestering this transcription factor in the cytosol.[27] LPS-mediated phosphorylation of IκB-α induces its ubiquitination and degradation, resulting in the release of NF-κB. Evaluation of IκB-α phosphorylation after LPS stimulation showed similar kinetics in the presence of either 4 h or 24 h post-surgery plasma (Figure 3A,C). Thus, we inferred from these results that suppression of LPS-induced TNF-α production by monocytes mediated by 4 h post-surgery plasma is not due to reduced TLR4 and subsequent p38 MAPK and NF-κB activation.

Table 1. Patient characteristics.

	Dexamethasone	No-Dexamethasone	P-value
Age (mo)	12 ± 34	7 ± 6	0.14
Male / female	19 / 15	1 / 3	
VSD	16	3	
ASD	12	1	
AVSD	2		
Aortic valvuloplasty	2		
Extracardiac conduit	1		
CoA	1		
Duration of CPB (min)	52 ± 28	49 ± 29	0.82
Duration of ACC (min)	32 ± 20	42 ± 17	0.26
PICU stay (days)	2 ± 1.2	1 ± 0	0.17

Age, CPB, ACC and PICU durations represented as median ± SD. ACC: aortic crossclamping, ASD: atrial septum defect, AVSD: atrioventricular septum defect, CoA: Coarctation aorta, CPB: cardiopulmonary bypass, Extracardiac conduit change due to stenosis after Fontan procedure, PICU: pediatric intensive care unit, VSD: ventricular septum defect. No significant differences were found between both patient groups (Mann-Whitney test).

A regulatory role of STAT3 signaling induced by inhibitory post-perfusion plasma

We next set out to assess the role of immunomodulatory cytokines in our system. As shown above, we identified high levels of the anti-inflammatory cytokine IL-10 in these plasma samples (Figure 1F). As monocytes/macrophages have been shown to be both the main producers[28] and target cells of IL-10,[29] we first evaluated the effect of IL-10 neutralization. Plasma samples obtained 4 h post-surgery were pre-treated with anti-hIL-10 mAb (10 or 100 μg/mL) or the appropriate isotype control (IgG2a, 100 μg/mL), before adding these samples back to PBMC in the presence of LPS. As shown in **Figure 4A**, we found that neutralization of IL-10 partially reversed the inhibitory effects of 4 h post-surgery plasma on TNF-α synthesis by monocytes.

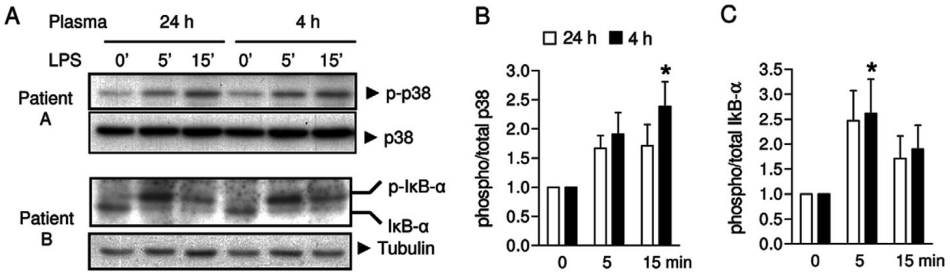

Figure 3. Post-perfusion plasma does not interfere with p38 MAPK or NF-κB activation.

Representative examples **(A)** and densitometric analyses **(B-C)** of LPS-induced p38 MAPK and IκB-α phosphorylation in monocytes in the presence of 24 h (control) or 4 h post-surgery plasma. Tubulin: loading control. Mean ± SEM (n=4). *P<0.05 vs. 0 min (ANOVA).

IL-10 activates the JAK1/STAT3 pathway by signaling through the IL-10 receptor (IL-10R) in mononuclear cells.[30;31] This IL-10R/STAT3 signaling axis results in the upregulation of various anti-inflammatory proteins that can inhibit pro-inflammatory cytokine synthesis.[32;33] Indeed, we found activation of STAT3 in monocytes by incubation with plasma isolated 4 h but not 24 h post-perfusion regardless of the presence of LPS (representative example in Figure 4B). Therefore, we next assessed the functional role of STAT3 signaling in monocytes with regard to the suppressive effects of post-perfusion plasma on cytokine production. We pre-treated patient PBMC with a cell-permeable STAT3 inhibitor peptide (phosphorylated peptide, pY-STAT3i) that contains a membrane translocating sequence that prevents nuclear translocation of STAT3 dimers.[34] After pre-treatment with pY-STAT3i or non-phosphorylated control peptide (STAT3i), the cells were again stimulated with LPS in the presence of 4 h post-perfusion plasma and the results were compared to those obtained with 24 h post-surgery (control) plasma. We found that STAT3 inhibition restored TNF-α production in the presence of suppressive patient plasma (Figure 4C, left panel), but it did not affect IL-6 synthesis (Figure 4C, right panel). STAT3 inhibition also restored levels of TNF-α, but not IL-6, in supernatants of LPS-stimulated monocytes incubated in the presence of 4 h post-perfusion plasma (Figure 4D). In all experiments, pre-treatment with control peptide had no effect on cytokine production (Figure 4C,D). Taken together, our findings suggest that STAT3 mediates the suppressive effects of plasma mediators (released shortly after CPB surgery) on TNF-α, but not IL-6, synthesis by monocytes.

Discussion

A suppressed immune system after cardiac surgery is extensively described in both adult and pediatric patients and is associated with an enhanced risk of nosocomial infections and prolonged hospital stay.[35;36] Previous studies identified both phenotypic cellular changes such as HLA-DR expressed on monocytes[5] and soluble factors including IL-10[15;37] to be associated with clinical outcome. Our data showed a transient suppression of monocyte function in the circulation after open heart surgery, which was mainly caused by plasma components (Figure 2). Previous dissections of signaling cascades responsible for suppression of the innate immune system in systemic inflammation have lead to the concept of 'endotoxin tolerance', particularly in human endotoxemia and sepsis. These conditions are associated with the upregulation of intracellular negative regulators of TLR4 signaling, including IL-1R-associated kinase (IRAK)-M,[38] MyD88s and single immunoglobulin interleukin-1 receptor-related molecule (SIGIRR).[39] However, we found that major signaling pathways downstream of TLR4 (i.e. p38 MAPK and NF-κB activation) were unimpaired in the presence of suppressive patient plasma (Figure 3). This suggests that the suppression of LPS-induced TNF-α production by monocytes in our model was not explained by endotoxin tolerance.

Transcriptional activity of STAT3 in macrophages and neutrophils has been shown to be essential for the orchestration of anti-inflammatory responses in experimental models of systemic inflammation.[40;41] Currently, there is limited information on the role of STAT3 in anti-inflammatory feedback on innate immune cells during human (sterile) systemic inflammation.[42;43] Here we demonstrate a crucial role for STAT3 in the suppression of TNF-α synthesis by monocytes in the course of systemic inflammation associated with on-pump cardiac surgery in a well described pediatric patient population (Figure 4). JAK1/STAT3 signaling has been studied broadly in primary mononuclear cells *in vitro* and both JAK1 and STAT3 are required for IL-10 mediated inhibition of LPS-induced TNF-α production.[44] On-pump cardiac surgery has been shown to induce the release of cytokines (IL-6, IL-10) associated with JAK1/STAT3 signaling,[9;15;45] as confirmed in the present study (Figure 1F). Neutralization of IL-10 in suppressive plasma samples partially reversed its inhibitory effects on LPS-induced TNF-α synthesis (Figure 4A), which suggested involvement of downstream JAK1/STAT3 signaling. We subsequently found that pre-treatment of monocytes with a specific STAT3 peptide inhibitor *ex vivo* indeed restored TNF-α (but not IL-6) synthesis by monocytes (Figure 4C,D). STAT3 is a critical signaling hub used by both pro- and anti-inflammatory signals mediated by IL-6 and IL-10, respectively.[46;47] These apparent paradoxical inputs are differentially regulated by suppressor of cytokine signaling (SOCS)3.[32] While the IL-6 receptor is susceptible to feedback inhibition by SOCS3, the IL-10R is not. The IL-10R-induced STAT3 pathway induces a transcriptional program of anti-inflammatory gene products resulting in the repression of pro-inflammatory transcripts.[48;49] Surprisingly, STAT3 inhibition in our study failed to reverse the suppression of IL-6 production by monocytes in the presence of post-perfusion plasma, in contrast to TNF-α (Figure 4C,D). This unexpected finding warrants further dissection of the exact molecular

mechanisms used by STAT3 to selectively regulate TNF-α synthesis in human monocytes. Besides an important regulator of inflammation, STAT3 plays a potential role in cytoprotection and regeneration. With regard to cardiac surgery, STAT3 contributes to cardioprotective mechanisms in ischemia-reperfusion injury,[50;51] with a major role for IL-6 in the induction of this pathway.[52] Thus, our results add another feature to the multifaceted properties of STAT3 signaling in different cell types to promote tissue homeostasis after cardiac surgery.

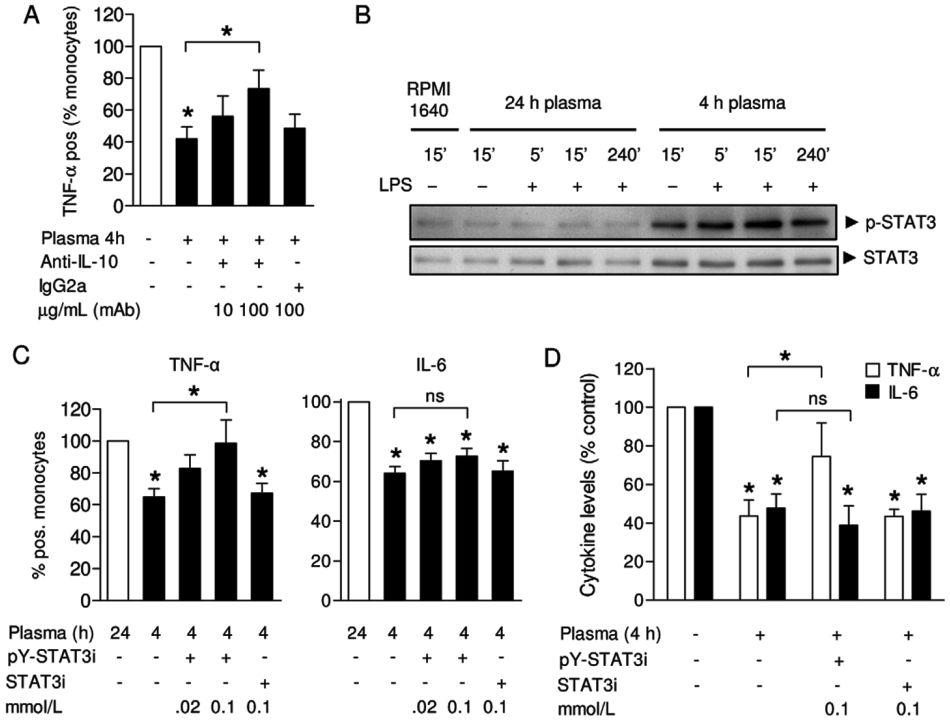

Figure 4. STAT3 signaling is required for the suppressive effects of post-perfusion plasma on TNF-α production. (**A**) Pre-treatment of 4 h post-surgery plasma samples with anti-IL-10 partially restored TNF-α production by patient monocytes in response to LPS (n=10). Control: plasma from healthy donors. (**B**)Activation of STAT3 in monocytes by incubation with suppressive (4 h post-perfusion) but not control (24 h post-perfusion) plasma. Cells were incubated in the absence or presence of LPS to match the experimental setup as in Figure 2. (**C**) Pre-treatment of patient PBMC with active STAT3 inhibitor (pY-STAT3i) but not control peptide (STAT3i) before LPS stimulation in the presence of post-surgery plasma restored TNF-α synthesis (left panel), in contrast to IL-6 (right panel). Shown are percentages of TNF-α and IL-6 producing monocytes normalized to control (24 h post-surgery) plasma (n=8). **D.** TNF-α and IL-6 levels measured in supernatants of LPS-stimulated monocytes after pre-treatment with STAT3 inhibitor or control peptide, in the presence of 4 h post-surgery plasma (n=8). Cytokine levels were normalized to LPS stimulation in control plasma from healthy donors due to interassay variability. All results are depicted as mean ± SEM. *P<0.05 vs. control condition (ANOVA), ns: not significant.

Pharmacological agents administered during and after the surgical and anesthesiological procedures could have affected our ex vivo plasma assays. In particular, the pre-operative administration of dexamethasone (standard practice for this type of surgery in our hospital and most other institutions[53]) may be of influence, as corticosteroids are known for their potent anti-inflammatory effects on innate immune cells. However, we found that the circulating levels of dexamethasone were already significantly reduced 4 h after surgery (Figure 2F). More importantly, we repeated the key experiments with plasma samples obtained from patients that did not receive steroids before the procedure (clinical characteristics in Table 1). Steroid-free plasma isolated shortly (4 h) after open heart surgery was still able to suppress LPS-induced TNF-α production by monocytes (Figure 2G). These results also suggest that the suppressive plasma components were not indirectly induced by steroids. By comparing the results obtained with plasma with and without dexamethasone (compare Figure 2D,E and Figure 2G, respectively), we inferred that only the suppressive effect of 4 h post-perfusion plasma on TNF-α synthesis was likely caused by endogenous plasma factors. No effect on IL-6 synthesis by monocytes was found with steroid-free plasma which is consistent with our observations that abrogation of IL10/STAT3 signaling did not affect IL-6 production in monocytes (Figure 4). Please note that due to limited availability of these steroid-free samples, we could however not perform additional experiments with IL-10 neutralizing antibodies and STAT3 inhibitor peptide.

We demonstrated a non-redundant role for STAT3 in mediating negative feedback on LPS-induced monocyte TNF-α (but not IL-6) production after on-pump cardiac surgery. This supports the concept of specific monocyte reprogramming in the course of human systemic inflammation, rather than general immune suppression. Our findings suggest that functional modulation of STAT3 activity offers a potential target for molecular intervention in suppressed states of the innate immune system in human disease.

Material and methods

Ethics Statement

Written informed consent was obtained from the parents of children participating in the study. A medical ethics committee (Medische Ethische Toetsings Commissie UMC Utrecht) approved this study (METC 03/049-K, 04/144-K UMC Utrecht, The Netherlands) and all procedures followed were in accordance with institutional guidelines.

Study population, surgical and anesthesiological procedures

Children admitted to our hospital for surgical repair of relatively simple congenital heart defects with an expected rapid recovery were enrolled. For this purpose we only included patients who underwent a surgical procedure from RACHS-1 (Risk Adjustment for Congenital Heart Surgery) score of 2 or less.[54] Patients that had signs of infection or a documented immunodeficiency were

excluded. A total of 38 children were enrolled in the study and all experienced an uneventful peri- and postoperative clinical course. Detailed clinical characteristics are depicted in Table 1. The surgical, anesthesiological and cardiopulmonary bypass procedures have been published previously.[24] Briefly, general anesthesia was always implemented using a standard technique involving sufentanil, midazolam, pancuronium, dopamine and milrinone. All patients received 48 hours perioperative antibiotic prophylaxis with Cefazolin. Patients receiving dexamethasone were given a single dose of dexamethasone (1 mg/kg) after induction of anesthesia. Four patients received no steroids before the procedure. Non-pulsatile cardiopulmonary bypass was used, the standard pump flow rate was 2.8 liter/m^2/min. Combined alpha and pH stat management of acid-base status was used during cardiopulmonary bypass. The cardioplegia procedure was standardized using St. Thomas' solution. After weaning from cardiopulmonary bypass all patients remained intubated and ventilated and were admitted to the pediatric intensive care for further management. At the pediatric intensive care patients were treated with milrinone, midazolam and morfine for maximally 24 hours. All patients were treated by the same surgical and anesthetic team.

Blood sampling and cell isolation

Blood samples were obtained at the following time points: immediately after insertion of a central venous catheter during anesthetic induction (Pre-op), at the end of cardiopulmonary bypass (End-CPB), 4 h, 24 h and 48 h after surgery. At these time points, total leucocyte, neutrophil, monocyte and lymphocyte counts and C-reactive protein (CRP) levels were determined. Fresh heparinized blood samples were used for full blood assays. For all other purposes, plasma samples were prepared by centrifugation and stored at -80°C, whereas PBMC were separated by density gradient centrifugation over Ficoll-Hypaque (Amersham Pharmacia Biotech) and stored in liquid nitrogen, as previously described.[55] In some assays, pooled human AB plasma from healthy volunteers (Sanquin Blood Bank, Utrecht, The Netherlands) was used as control plasma.

Antibodies

Fluorescently labelled or unconjugated monoclonal antibodies (mAb) directed against human CD14 (murine, clone MOP9, StemCell Technologies), mouse anti-FcγRIII/CD16 (3G8, BD Biosciences), mouse anti-CD284/TLR4 (HTA125, eBioscience), mouse anti-TNF-α (Mab11, eBioscience) and rat anti-IL-6 (MQ2-6A3, BD) were used for flow cytometry. MAbs directed against hIL-10 (JES3-19F1, rat IgG2a, BD) and rat IgG2a isotype (BD) were used for neutralization experiments. Antibodies directed against p-p38, p38, p-IκB-alpha and p-STAT3 (Cell Signaling), IκB-alpha and STAT3 (Santa Cruz) and Tubulin (Sigma) were used for Western blotting.

Cellular assays

Whole blood stimulation assays were performed in RPMI-1640 at 1:5 dilution. Cells were incubated with or without LPS (Escherichia coli O127:B8E, L4517, Sigma) at 10 ng/mL in a 96-wells plate (Costar®) for 4 h at 37°C, 5% CO_2 with 100% relative humidity. Cells were then washed and stained for surface markers followed by lysis of red blood cells (BD Lysing Solution) and intracellular cytokine staining. For ex vivo LPS stimulation assays, PBMC from various time points were plated in a 96-wells plate at 2 x 10^6 cells/mL in RPMI-1640 supplemented with 2 mmol/L glutamine, 100 U/mL penicillin/streptomycin (Gibco BRL, Invitrogen) and 10% (v/v) heat-inactivated human AB serum. LPS was added (100 ng/mL LPS) for 4 h, followed by intracellular cytokine staining. For plasma assays, patient PBMC isolated before surgery were adjusted to 2 x 10^6 cells/mL in supplemented RPMI-1640 (no serum). Pooled human AB plasma (control) and autologous patient plasma samples obtained at serial time-points were thawed and spinned (300 g, 10 min) and the supernatants were filtered (50 µm). Plasma samples mixed with LPS (100 ng/mL end concentration) were added to equal volumes of cell suspensions (50% v/v) and incubated for 4 h at 37°C, followed by intracellular cytokine staining. For IL-10 neutralization assays, patient plasma samples were pre-incubated with anti-hIL-10 mAb (10 – 100 µg/mL) or IgG2a isotype (100 µg/mL) for 1 h at 4°C on shaker. For STAT3 inhibition assays, PBMC obtained before surgery were pre-treated with 0.02 or 0.1 mM cell-permeable STAT3 Inhibitor Peptide (PpYLKTK-mts, Calbiochem) or 0.1 mM inactive control peptide (Ac-PpYLKTK-OH) for 1 h at 37°C in culture medium with 10% AB plasma. PBMC were then washed and mixed with plasma samples (4 h or 24 h post-surgery) and LPS (100 ng/mL) for a 4 h incubation period followed by intracellular cytokine staining.

Flow cytometry

Golgistop (2 µM, BD) was added during *ex vivo* incubations with LPS. Cells were then washed, blocked with normal mouse serum followed by extracellular staining, fixation in Cytofix/Cytoperm and washing in Perm/Wash solution (Cytofix/perm kit, BD). Finally, cells were incubated with mAbs for intracellular cytokine staining, as published.[24]

Multiplex immunoassay

Multiplex immunoassay with the Bio-Plex suspension array system (Bio-Rad Laboratories) was used to measure levels of TNF-α, IL-6, IL-8, IL-10 and MIF in patient plasma samples and culture supernatants, as previously described.[56]

Dexamethasone measurements

Dexamethasone in serum was measured after diethylether extraction using an in house competitive radio-immunoassay (RIA) employing a polyclonal anti-dexamethasone-antibody (IgG dex1 lot 1301; IgG Corporation). [1,2,4,6,7-3H]-dexamethasone (TRK645, Amersham) was used as a tracer following chromatographic verification of its purity. The lower limit of

detection was 20 pmol/L and intra-assay variation was <7%. All samples were included in one assay.

Western blot analysis

Purified monocytes from healthy donors were serum starved for at least 2 h, washed and resuspended in supplemented RPMI-1640. A total of $5x10^5$ cells per condition were stimulated in the absence or presence of LPS (100 ng/mL) for 0, 5, 15 or 240 min at 37°C in the presence of 50% (v/v) patient plasma. After *in vitro* stimulation, cells were washed with cold PBS and lysed in reducing Laemmli sample buffer. Proteins were separated with SDS-PAGE, transferred to PVDF membranes, blocked with 5% BSA followed by immunoprobing overnight at 4°C. Proteins were detected with HRP-conjugated secondary antibodies (Dako) and developed with Hyperfilm ECL (GE Healthcare). Densitometric analysis was performed with ImageQuant densitometric software (Molecular Dynamics).

Statistical analysis

Basic descriptive statistics were used to describe the patient population. Multiple data sets were analyzed by one-way ANOVA, as indicated. Significance was accepted at $*P<0.05$ and $**P<0.001$.

Acknowledgements

We thank J. Meerding and W. de Jager from the Dept. of Pediatric Immunology for their assistance in performing the multiplex cytokine immunoassays. We thank S.O. Algra from the Dept. of Pediatric Intensive Care and F. de Roo from the Dept. of Pediatric Cardiothoracic Surgery for their assistance in patient sampling. I. Maitimu-Smeele and E.G.W. Lentjes from the Dept. of Endocrinology, University Medical Center Utrecht, are acknowledged for the quantification of dexamethasone levels in patient plasma samples.

References

1. Chew MS, Brandslund I, Brix-Christensen V et al. Tissue injury and the inflammatory response to pediatric cardiac surgery with cardiopulmonary bypass: a descriptive study. Anesthesiology 2001;94:745-753.
2. Diegeler A, Doll N, Rauch T et al. Humoral immune response during coronary artery bypass grafting: A comparison of limited approach, "off-pump" technique, and conventional cardiopulmonary bypass. Circulation 2000;102:III95-100.
3. Tomic V, Russwurm S, Moller E et al. Transcriptomic and proteomic patterns of systemic inflammation in on-pump and off-pump coronary artery bypass grafting. Circulation 2005;112:2912-2920.
4. Xing L, Remick DG. Relative cytokine and cytokine inhibitor production by mononuclear cells and neutrophils. Shock 2003;20:10-16.
5. Allen ML, Peters MJ, Goldman A et al. Early postoperative monocyte deactivation predicts systemic inflammation and prolonged stay in pediatric cardiac intensive care. Crit Care Med 2002;30:1140-1145.
6. Peters M, Petros A, Dixon G, Inwald D, Klein N. Acquired immunoparalysis in paediatric intensive care: prospective observational study. BMJ 1999;319:609-610.
7. Pachot A, Cazalis MA, Venet F et al. Decreased expression of the fractalkine receptor CX3CR1 on circulating monocytes as new feature of sepsis-induced immunosuppression. J.Immunol. 2008;180:6421-6429.
8. Evans BJ, Haskard DO, Finch JR et al. The inflammatory effect of cardiopulmonary bypass on leukocyte extravasation *in vivo*. J.Thorac.Cardiovasc.Surg. 2008;135:999-1006.
9. Sablotzki A, Welters I, Lehmann N et al. Plasma levels of immunoinhibitory cytokines interleukin-10 and transforming growth factor-beta in patients undergoing coronary artery bypass grafting. Eur.J.Cardiothorac. Surg. 1997;11:763-768.
10. Seghaye M, Duchateau J, Bruniaux J et al. Interleukin-10 release related to cardiopulmonary bypass in infants undergoing cardiac operations. J.Thorac.Cardiovasc.Surg. 1996;111:545-553.
11. Tarnok A, Schneider P. Pediatric cardiac surgery with cardiopulmonary bypass: pathways contributing to transient systemic immune suppression. Shock 2001;16 Suppl 1:24-32.:24-32.
12. Franke A, Lante W, Fackeldey V et al. Proinflammatory and antiinflammatory cytokines after cardiac operation: different cellular sources at different times. Ann.Thorac.Surg. 2002;74:363-370.
13. Wilhelm W, Grundmann U, Rensing H et al. Monocyte deactivation in severe human sepsis or following cardiopulmonary bypass. Shock 2002;17:354-360.
14. Borgermann J, Friedrich I, Flohe S et al. Tumor necrosis factor-alpha production in whole blood after cardiopulmonary bypass: downregulation caused by circulating cytokine-inhibitory activities. J.Thorac. Cardiovasc.Surg. 2002;124:608-617.
15. Dehoux MS, Hernot S, Asehnoune K et al. Cardiopulmonary bypass decreases cytokine production in lipopolysaccharide-stimulated whole blood cells: roles of interleukin-10 and the extracorporeal circuit. Crit Care Med. 2000;28:1721-1727.
16. Haziot A, Tsuberi BZ, Goyert SM. Neutrophil CD14: biochemical properties and role in the secretion of tumor necrosis factor-alpha in response to lipopolysaccharide. J.Immunol. 1993;150:5556-5565.
17. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 1990;249:1431-1433.
18. Lequier LL, Nikaidoh H, Leonard SR et al. Preoperative and postoperative endotoxemia in children with congenital heart disease. Chest 2000;117:1706-1712.
19. Kitchens RL, Thompson PA, O'Keefe GE, Munford RS. Plasma constituents regulate LPS binding to, and release from, the monocyte cell surface. J.Endotoxin.Res. 2000;6:477-482.
20. Dybdahl B, Wahba A, Lien E et al. Inflammatory response after open heart surgery: release of heat-shock protein 70 and signaling through toll-like receptor-4. Circulation 2002;105:685-690.
21. Hadley JS, Wang JE, Michaels LC et al. Alterations in inflammatory capacity and TLR expression on monocytes and neutrophils after cardiopulmonary bypass. Shock 2007;27:466-473.

22. Shames BD, Selzman CH, Meldrum DR et al. Interleukin-10 stabilizes inhibitory kappaB-alpha in human monocytes. Shock 1998;10:389-394.
23. Takezako N, Hayakawa M, Hayakawa H et al. ST2 suppresses IL-6 production via the inhibition of IkappaB degradation induced by the LPS signal in THP-1 cells. Biochem.Biophys.Res.Commun. 2006;341:425-432.
24. Schadenberg AW, Vastert SJ, Evens FC et al. FOXP3^{+} CD4^{+} Tregs lose suppressive potential but remain anergic during transient inflammation in human. Eur.J.Immunol. 2011;41:1132-1142.
25. Pierrakos C, Vincent JL. Sepsis biomarkers: a review. Crit Care 2010;14:R15.
26. Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal. 2001;13:85-94.
27. Piao W, Song C, Chen H et al. Endotoxin tolerance dysregulates MyD88- and Toll/IL-1R domain-containing adapter inducing IFN-beta-dependent pathways and increases expression of negative regulators of TLR signaling. J.Leukoc.Biol. 2009;86:863-875.
28. Bazzoni F, Tamassia N, Rossato M, Cassatella MA. Understanding the molecular mechanisms of the multifaceted IL-10-mediated anti-inflammatory response: lessons from neutrophils. Eur.J.Immunol. 2010;40:2360-2368.
29. Pils MC, Pisano F, Fasnacht N et al. Monocytes/macrophages and/or neutrophils are the target of IL-10 in the LPS endotoxemia model. Eur.J.Immunol. 2010;40:443-448.
30. Williams L, Bradley L, Smith A, Foxwell B. Signal transducer and activator of transcription 3 is the dominant mediator of the anti-inflammatory effects of IL-10 in human macrophages. J.Immunol. 2004;172:567-576.
31. Williams LM, Sarma U, Willets K et al. Expression of constitutively active STAT3 can replicate the cytokine-suppressive activity of interleukin-10 in human primary macrophages. J.Biol.Chem. 2007;282:6965-6975.
32. Berlato C, Cassatella MA, Kinjyo I et al. Involvement of suppressor of cytokine signaling-3 as a mediator of the inhibitory effects of IL-10 on lipopolysaccharide-induced macrophage activation. J.Immunol. 2002;168:6404-6411.
33. Lee TS, Chau LY. Heme oxygenase-1 mediates the anti-inflammatory effect of interleukin-10 in mice. Nat. Med. 2002;8:240-246.
34. Turkson J, Ryan D, Kim JS et al. Phosphotyrosyl peptides block Stat3-mediated DNA binding activity, gene regulation, and cell transformation. J.Biol.Chem. 2001;276:45443-45455.
35. Michalopoulos A, Geroulanos S, Rosmarakis ES, Falagas ME. Frequency, characteristics, and predictors of microbiologically documented nosocomial infections after cardiac surgery. Eur.J.Cardiothorac.Surg. 2006;29:456-460.
36. Sarvikivi E, Lyytikainen O, Nieminen H, Sairanen H, Saxen H. Nosocomial infections after pediatric cardiac surgery. Am.J.Infect.Control 2008;36:564-569.
37. Allen ML, Hoschtitzky JA, Peters MJ et al. Interleukin-10 and its role in clinical immunoparalysis following pediatric cardiac surgery. Crit Care Med. 2006;34:2658-2665.
38. van 't Veer C, van den Pangaart PS, van Zoelen MA et al. Induction of IRAK-M is associated with lipopolysaccharide tolerance in a human endotoxemia model. J.Immunol. 2007;179:7110-7120.
39. Adib-Conquy M, Adrie C, Fitting C et al. Up-regulation of MyD88s and SIGIRR, molecules inhibiting Toll-like receptor signaling, in monocytes from septic patients. Crit Care Med. 2006;34:2377-2385.
40. Matsukawa A, Takeda K, Kudo S et al. Aberrant inflammation and lethality to septic peritonitis in mice lacking STAT3 in macrophages and neutrophils. J.Immunol. 2003;171:6198-6205.
41. Takeda K, Clausen BE, Kaisho T et al. Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. Immunity. 1999;10:39-49.
42. Oiva J, Mustonen H, Kylanpaa ML et al. Patients with acute pancreatitis complicated by organ failure show highly aberrant monocyte signaling profiles assessed by phospho-specific flow cytometry. Crit Care Med. 2010;38:1702-1708.
43. Tamassia N, Calzetti F, Menestrina N et al. Circulating neutrophils of septic patients constitutively express IL-10R1 and are promptly responsive to IL-10. Int.Immunol. 2008;20:535-541.
44. Riley JK, Takeda K, Akira S, Schreiber RD. Interleukin-10 receptor signaling through the JAK-STAT pathway. Requirement for two distinct receptor-derived signals for anti-inflammatory action. J.Biol.Chem. 1999;274:16513-16521.

45. Ogata M, Okamoto K, Kohriyama K et al. Role of interleukin-10 on hyporesponsiveness of endotoxin during surgery. Crit Care Med. 2000;28:3166-3170.

46. Niemand C, Nimmesgern A, Haan S et al. Activation of STAT3 by IL-6 and IL-10 in primary human macrophages is differentially modulated by suppressor of cytokine signaling 3. J.Immunol. 2003;170:3263-3272.

47. Yasukawa H, Ohishi M, Mori H et al. IL-6 induces an anti-inflammatory response in the absence of SOCS3 in macrophages. Nat.Immunol. 2003;4:551-556.

48. Murray PJ. The primary mechanism of the IL-10-regulated antiinflammatory response is to selectively inhibit transcription. Proc.Natl.Acad.Sci.U.S.A 2005;102:8686-8691.

49. Murray PJ. The JAK-STAT signaling pathway: input and output integration. J.Immunol. 2007;178:2623-2629.

50. Bolli R, Stein AB, Guo Y et al. A murine model of inducible, cardiac-specific deletion of STAT3: its use to determine the role of STAT3 in the upregulation of cardioprotective proteins by ischemic preconditioning. J.Mol.Cell Cardiol. 2011;50:589-597.

51. Heusch G, Musiolik J, Gedik N, Skyschally A. Mitochondrial STAT3 activation and cardioprotection by ischemic postconditioning in pigs with regional myocardial ischemia/reperfusion. Circ.Res. 2011;109:1302-1308.

52. Boengler K, Hilfiker-Kleiner D, Drexler H, Heusch G, Schulz R. The myocardial JAK/STAT pathway: from protection to failure. Pharmacol.Ther. 2008;120:172-185.

53. Checchia PA, Bronicki RA, Costello JM, Nelson DP. Steroid use before pediatric cardiac operations using cardiopulmonary bypass: an international survey of 36 centers. Pediatr.Crit Care Med. 2005;6:441-444.

54. Jenkins KJ, Gauvreau K, Newburger JW et al. Consensus-based method for risk adjustment for surgery for congenital heart disease. J.Thorac.Cardiovasc.Surg. 2002;123:110-118.

55. de Jong H, Lafeber FF, de JW et al. Pan-DR-binding Hsp60 self epitopes induce an interleukin-10-mediated immune response in rheumatoid arthritis. Arthritis Rheum. 2009;60:1966-1976.

56. de Jager W, Prakken BJ, Bijlsma JW, Kuis W, Rijkers GT. Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies. J.Immunol. Methods 2005;300:124-135.

Chapter

4

FOXP3^{+}CD4^{+} TREG CELLS LOSE SUPPRESSIVE POTENTIAL BUT REMAIN ANERGIC DURING TRANSIENT INFLAMMATION IN HUMAN

AWL Schadenberg[1,3], SJ Vastert[1], FCM Evens[2], W Kuis[1], AJ van Vught[3], NJG Jansen [3], BJ Prakken[1]

[1] Dep.t of Pediatric Immunology, Center for Molecular and Cellular Intervention (CMCI)
[2] Dep. of Pediatric Cardiothoracic Surgery
[3] Dep. of Pediatric Intensive Care Wilhelmina Children's Hospital, University Medical Center Utrecht, The Netherlands

European Journal of Immunology 2011. 41: 1132-1142

Abstract

Regulatory T cells are crucial in controlling inflammation. Although the transcription factor FOXP3 is the most applicable phenotype marker of Tregs, it does not indisputably characterize suppressive function during T cell activation *in vitro*. A question that remains is: what is the functionality of $FOXP3^+$ T cells during inflammation *in vivo*. We studied $FOXP3^+$ T cells in a human model of acute inflammation due to cardiac surgery. Twenty-five children who underwent cardiac surgery for correction of a septum defect were included. Following surgery we observed a transient systemic inflammatory response accompanied by an increased proportion of $CD25^{bright}$ T cells with sustained Treg phenotype. During this transient immune activation, both the percentage of $CD4^+FOXP3^+$ cells and the level of expression of FOXP3 in the $CD4^+CD25^{bright}CD127^{low}$ population increased. While Tregs remained present during systemic inflammation and continued to be anergic, the capacity to suppress effector T cells was reduced. The reduced suppressive state of Tregs could be induced *in vitro* by plasma obtained during the peak of inflammation after surgery. These data show that inflammation inhibits Treg function through soluble factors present in plasma. These results underscore the functional role of $FOXP3^+$ Tregs during inflammation *in vivo.*

Introduction

Regulatory T cells (Treg) have an important role in the maintenance of immune tolerance in both mice and humans. Besides a central role in autoimmunity and transplantation medicine, these cells have left their mark as regulators of inflammation such as in tumor immunology, allergy and infectious diseases. While the functionality of Tregs is indisputable in animal models, defining their *in vivo* role in human is problematic. For example, most markers associated with Tregs have been shown to be upregulated after *in vitro* T cell activation without necessarily qualifying the cells as suppressive Tregs. Therefore, measurement of Tregs in human disease is generally biased when conducted during inflammation.

In the following study we describe the functionality of $CD4^+CD25^+FOXP3^+$ T cells during the systemic inflammatory response in children undergoing cardiac surgery. Cardiac surgery with the use of cardiopulmonary bypass (CPB) induces a systemic inflammatory response.[1-4] Factors involved in triggering an immune response include anesthesia, surgical trauma and contact of immune competent cells with surface of extra-corporeal circuit. In uncomplicated cases, this is a temporary event. Depending on the preoperative clinical state and the extent of surgery, the pro-inflammatory response will be more or less effectively counteracted by an anti-inflammatory response. The balance between pro- and anti-inflammation is critical in determining clinical outcome.[5] Systemic inflammation after elective cardiac surgery therefore creates an opportunity to study in detail the activation of T cells directly ex vivo as the whole immune response can be scrutinized, from before triggering the immune system, through the peak of inflammation up to recovery. Moreover, samples can easily be obtained from the site of inflammation (systemic) in a human system. This study scrutinizes the induction of a human systemic inflammatory response and the subsequent functional ability of the $FOXP3^+$ T cell population.

Results

Patients and the ensuing systemic inflammatory response after cardiac surgery

Twenty-five patients who underwent surgical intervention for congenital VSD or ASD were included. Because these patients typically had a rapid recovery, with a short postoperative inflammatory response, we considered them ideal for monitoring the temporary systemic inflammatory response and subsequent restoration of immune homeostasis following cardiac surgery. Their median age was 40 weeks (range 7 weeks to 6 years). All patients recovered uneventfully following surgery and could be discharged from the pediatric intensive care unit within an average of 2 days. Patient characteristics are summarized in Table 1.

In response to the surgical insult, indeed all patients underwent a period of systemic inflammation. Clinically this could typically be observed with a rise in temperature after surgery alongside an increase of C-reactive protein. Furthermore both cellular and cytokine

characteristics of systemic inflammation were measured in obtained blood samples after surgery. Monocytes were released into the circulation soon after surgery, while the lymphocyte count decreased immediately after surgery with lowest numbers 4 hours post-operatively. Pro-inflammatory cytokines IL6 and IL8 were rapidly released systemically and returned back to baseline levels 48 hours after surgery (Table 2). TNFa and IL2, however, were less affected by the procedure. Thus, pediatric cardiac surgery is a suitable model for transient inflammation *in vivo*, characterized by clinical features which are accompanied by rapid and transient changes in immune activation parameters.

Table 1. Patient characteristics.

	Median (range)
Diagnosis (ASD/VSD)	15/10
Sex (male/female)	12/13
Age (weeks)	40 (7 – 349)
CPB duration (minutes)	48 (21 – 113)
ACC duration (minutes)	28 (7 – 80)
IC stay (days)	2 (1 – 3)

ASD: atrial septum defect, VSD: ventricular septum defect, CPB: cardiopulmonary bypass, ACC: aortic cross clamping,IC: intensive care

Table 2. Systemic inflammation in response to cardiac surgery in children.

	Pre-surgery	End CPB	4 h	24 h	48 h
Leukocytes (10^6/ml)	6.9 ± 1.8	6.9 ± 4.6	12 ± 3.3 **	15 ± 3.7 **	14 ± 4.0 **
Lymphocytes (10^6/ml)	4.0 ± 1.7	2.5 ± 1.3 *	1.4 ± 0.45 *	3.5 ± 1.6	4.5 ± 2.0
Monocytes (10^6/ml)	0.5 ± 0.2	0.2 ± 0.2 **	0.8 ± 0.4 *	2.0 ± 0.9 **	1.0 ± 0.5 **
IL6 (pg/ml)	92 ± 170	123 ± 202	240 ± 335 *	108 ± 175	111 ± 174
IL8 (pg/ml)	11 ± 14	27 ± 23 *	70 ± 38 *	13 ± 10	15 ± 11
TNFa (pg/ml)	25 ± 35	16 ± 29	46 ± 47	13 ± 26	25 ± 33
IL2 (pg/ml)	1.3 ± 1.2	2.1 ± 1.6	1.0 ± 0.5	0.8 ± 0.3	0.5 ± 0.1
CRP (mg/l)	5.0 ± 0	5.0 ± 0	5.0 ± 0	50 ± 28 **	46 ± 23 **

Cell count following cardiac surgery. Leukocyte, lymphocyte and monocyte count in million cells per ml. CPB: cardiopulmonary bypass, CRP: C-reactive protein. Data are represented as mean ± SD. Significance compared to before surgery is indicated by *P<0.05, **P<0.001.

Activation of T lymphocytes with sustained Treg characteristics

With the observation of a rapid decrease in circulating lymphocytes, we considered how this reflected the composition of lymphocyte subsets in particular with regard to Tregs. After surgery

$CD4^+$ T helper cells temporarily decreased (median $CD4^+$ lymphocyte count was before, 24 hours and 48 hours after surgery 2.19, 1.53 and 1.88 x10*9/L respectively, Figure 1A and supporting information Figure 1). The $CD4^+$ T cell population became activated as is typified by increased expression of CD25 (Figure 1B, $P<0.001$). Percentage of $CD69^+CD4^+$ T cells remained low (supporting information Figure 2). While total $CD4^+$ T cells decreased, $CD4^+CD25^+$ numbers remained stable (median $CD4^+CD25^+$ T cells before, 24 hours and 48 hours after surgery 0.14, 0.19 and 0.19 x10*9/L respectively, n.s.). A comparable increase was observed in $CD4^+$ T cells with high expression of CD25 ($CD4^+CD25^{bright}$) (Figure 1C). $CD4^+CD25^{bright}$ T cells contain $FOXP3^+$ Tregs; therefore we characterized the FOXP3 content in this population during the inflammatory response. $CD4^+$ cells were sorted by FACS based on low, intermediate and bright CD25 surface expression, after which FOXP3 mRNA expression was determined (Figure 1D). Twenty-four hours after surgery, FOXP3 mRNA expression per cell showed a moderate though not significant increase in both CD25 expressing cell populations, indicating that the increased percentage of $CD25^+$ cells during the activated immune state contain at least similar levels of FOXP3 mRNA compared with before surgery. Besides a stable FOXP3 mRNA expression, these cells also continued to express high levels of both GITR and CTLA-4, proteins associated with Treg function (Figure 1E and F). Twenty-four hours after surgery, $CD4^+$ T cells with the brightest expression of CD25, moderately upregulated GITR compared with before surgery.

Taken together, these results indicate activation of T cells during the transient inflammatory response ensuing cardiac surgery. Furthermore, the relative proportion of $CD4^+CD25^{bright}$ T cells also increased, which continued to have phenotypic characteristics of Tregs.

Induction of $CD4^+FOXP3^+$ T cells 24 hours after cardiac surgery

Subsequently, we determined if the systemic inflammatory response indeed influenced the composition of $FOXP3^+$ Tregs in the circulation. To quantify $CD4^+FOXP3^+$ cell kinetics we analyzed this cell population during the observation period by flowcytometry. The proportion $CD25^+FOXP3^+$ cells within $CD4^+$ population increased from 4.48% before surgery to 6.74% 24 hours after surgery ($P<0.01$), and returned back to 4.70% on the second day postoperatively (Figure 2A). Besides an increase in proportion of $FOXP3^+$ cells, mean intensity of FOXP3 expression increased significantly in $CD4^+CD25^+CD127^{low}$ population 24 hours after surgery, $P<0.01$ (FOXP3 MFI of $CD4^+CD25^+CD127^{low}$ population was before surgery, 24 hours and 48 hours after surgery; 10.8, 14.2 and 12.5 respectively, Fiure 2C). Furthermore, as localization of FOXP3 protein could influence activity of Tregs we examined FOXP3 localization by confocal microscopy 24 hours after surgery in the same $CD4^+CD25$ populations (Figure 2D). FOXP3 was typically localized in the nucleus, as expected. $CD4^+CD25^{bright}$ population showed predominantly FOXP3 positive cells, while $CD4^+CD25^-$ population lacked $FOXP3^+$ cells. Circulating $CD4^+FOXP3^+$ cell numbers remained statistically stable after surgery, while the total $CD4^+$ T cell population decreased in numbers ($CD4^+FOXP3^+$ cells before surgery,

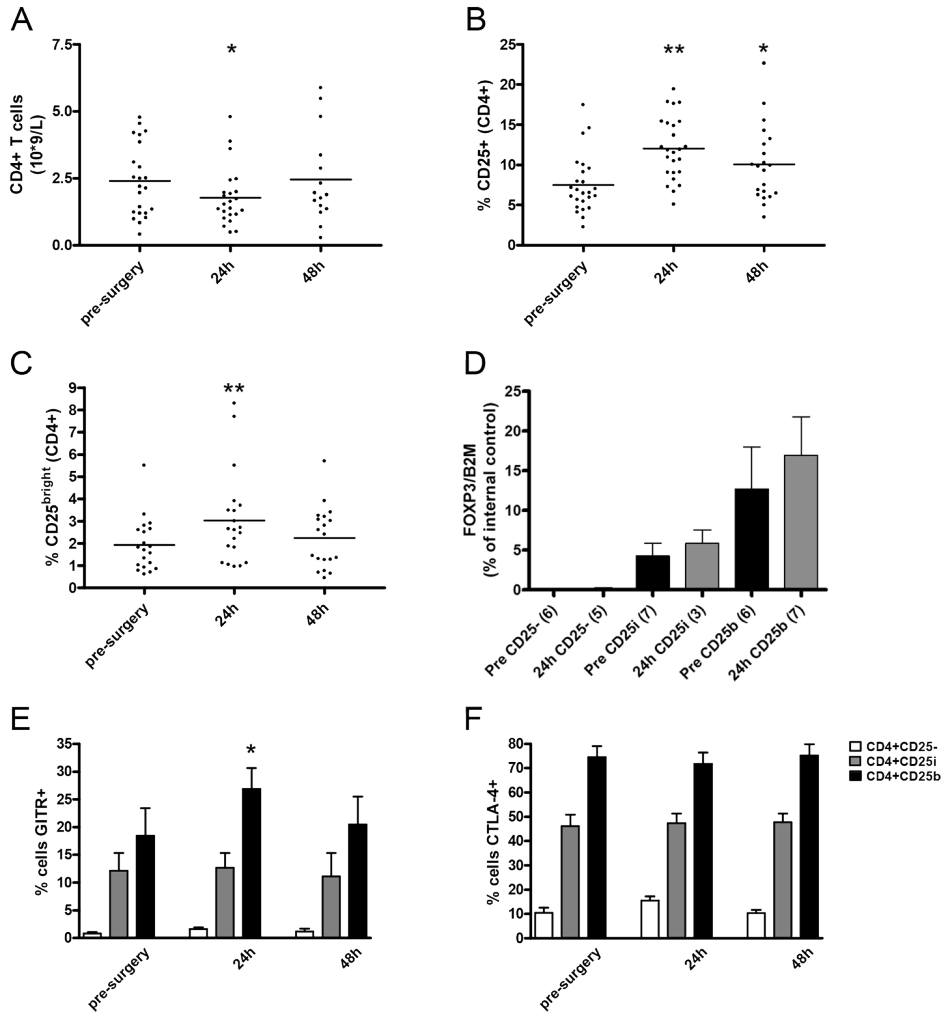

Figure 1. $CD4^+$ T cell phenotype before 24 and 48 hours after surgery. (**A**) $CD4^+$ T cell count; (**B**) percentage $CD25^+$ and; (**C**) $CD25^{bright}$ T cells within $CD4^+$ population. Data are represented as dot plots with median (n=25). (**D**) FOXP3 mRNA expression pre-surgery (black bars) and 24 hours after surgery (grey bars) in $CD4CD25^-$, CD4CD25intermediate (CD25i) and $CD4CD25^{bright}$ (CD25b) populations. The number between brackets ( ) shows the sample size per population. Mean values represent expression of FOXP3 compared to B2M as percentage of tetanus stimulated control. (**E**) Expression of glucocorticoid-induced tumor necrosis factor receptor (GITR) (n=13) and; (**F**) Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) (n=13) in $CD25^-$ (white bars), $CD25^{intermediate}$ (grey bars) and $CD25^{bright}$ (black bars) $CD4^+$ T cells. Significance compared to before surgery calculated by Wilcoxon test is indicated by *=P<0.05, **=P<0.001.

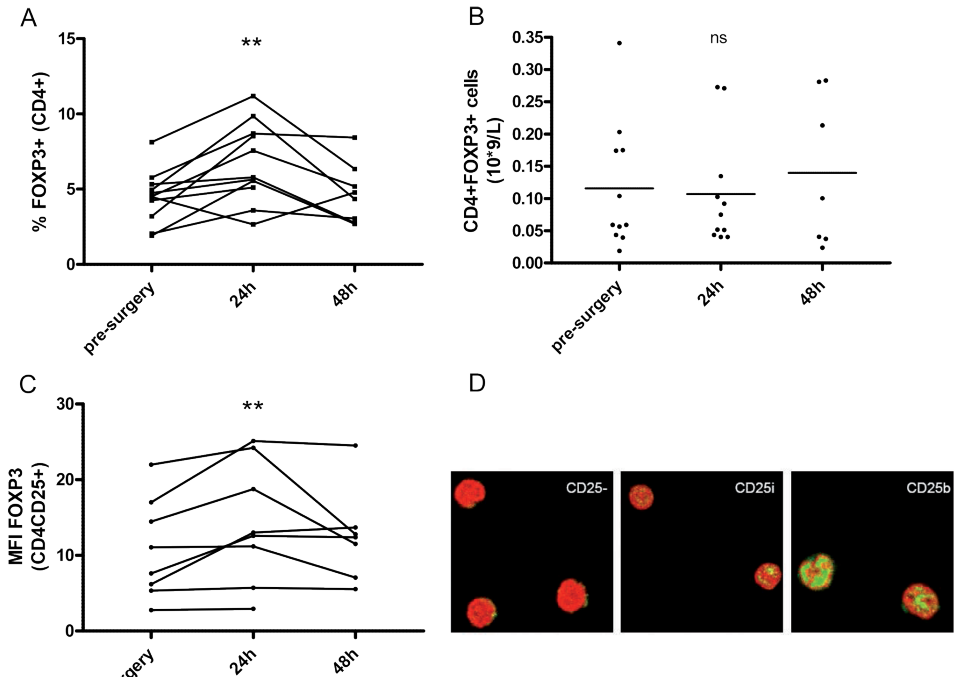

Figure 2. Kinetics of FOXP3 expression during systemic inflammation. (**A**) Percentage of FOXP3$^+$ cells before surgery compared to 24 h and 48 h after surgery. (**B**) Number of circulating CD4$^+$FOXP3$^+$ cells in million cells per mL. (**C**) Mean fluorescent intensity of FOXP3 expression of CD4$^+$CD25$^+$ population. (**D**) Representative FOXP3 expression pattern in sorted CD25 populations of one patient, 24 h after surgery (CD25$^-$, CD25^{int}, CD25^{bright}). FOXP3-Alexa488 and TO-PRO-3 iodide as nuclear staining were pseudo-colored in green and red, respectively. Images were recorded with a 100x objective. Significance compared to before surgery calculated by the Wilcoxon test is indicated **=P<0.001, ns = not significant.

24 hours and 48 hours after surgery; 0.12, 0.11 and 0.14 x 10*9 cells per L respectively, n.s., Figure 2B). Thus overall, within 24 hours after cardiac surgery the composition of the CD4 T cell population changed transiently in favor of FOXP3$^+$ cells. Not only did the ratio FOXP3$^+$ cells within the CD4$^+$ T cell population change, on a per cell basis FOXP3 expression increased in the population which typically contain Tregs.

Highest expression of proliferation marker Ki67 in CD4$^+$FOXP3$^+$ T cells

The question arose as to which mechanisms could explain the different kinetics between CD4$^+$ cells and CD4$^+$FOXP3$^+$ cells. While the first decreased rapidly from the circulation during the inflammatory response following surgery, the Tregs remained stable in numbers and increased

significantly in percentage of $CD4^+$ T cells (Figure 2A and B). For this purpose, we analyzed Ki67 expression in both total $CD4^+$ and $CD4^+FOXP3^+$ population. Ki67 is a protein important for cell division and is only expressed in proliferating cells. The percentage of $Ki67^+$ cells was substantially higher in $CD4^+FOXP3^+$ cells compared to total $CD4^+$ cell population at all time points. In all patients, $CD4^+$ T cells showed a higher division rate 24 hours after surgery ($CD4^+Ki67^+$ median before surgery and post-operative day one: 2.7% versus 7.8%, Figure 3A, $P<0.001$). The same pattern could be seen in $CD4^+FOXP3^+$ cells ($CD4^+FOXP3^+Ki67^+$ median before surgery and post-operative day one: 16% versus 40%, Figure 3B, $P<0.001$). Notably, the $FOXP3^+$ ratio in proliferating $CD4^+$ T cells remained constant during the inflammatory response (median ± SD before surgery, 24 hours and 48 hours after surgery 18.2 ± 4.2, 21.4 ± 6.3 and 21.3 ± 7.5, respectively). These findings indicate that proliferation increased in all $CD4^+$ T cells 24 hours after cardiac surgery, with highest proliferative activity in the $CD4^+FOXP3^+$ cells.

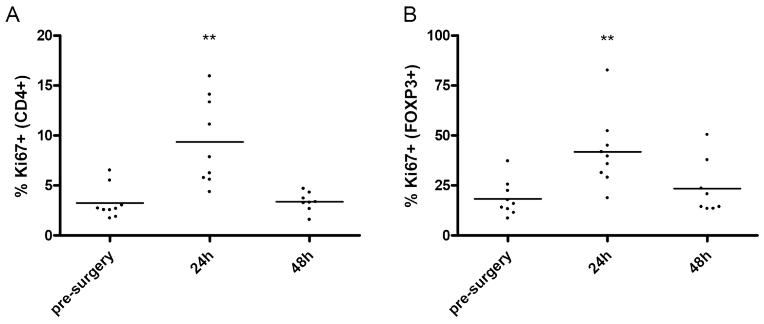

Figure 3. Kinetics of $Ki67^+$ cells representing proportion of proliferating cells of (**A**) $CD4^+$ and (**B**) $FOXP3^+$ population before, 24h and 48h after surgery. Each dot represents one patient; horizontal bar shows the median value. Significance compared to before surgery calculated by the Wilcoxon test is indicated **=$P<0.001$.

$FOXP3^+$ Tregs after surgery are anergic but less suppressive

In human, FOXP3 expression does not always indicate regulatory capacity. True FOXP3 Tregs are anergic *in vitro* to TCR stimulation and suppress effector T cell (Teff) proliferation. We determined the proliferative capacity of 5 x 10^3 Teffs ($CD4^+CD25^-$) and 5 x 10^3 Tregs ($CD4^+CD25^+CD127^{low}$) after TCR stimulation with anti-CD3 and compared these before and 24 hours after surgery. The determined $FOXP3^+$ Treg population was equally anergic 24 h after surgery as before surgery with approximately 3% proliferation compared to Teffs at the same time point (Figure 4A). Next, we determined suppressive potential of the $FOXP3^+$ Tregs at both time points, before and after surgery. Five thousand Teffs were co-cultured with or without equal numbers of Tregs from before and 24 h after surgery in the presence of plate bound

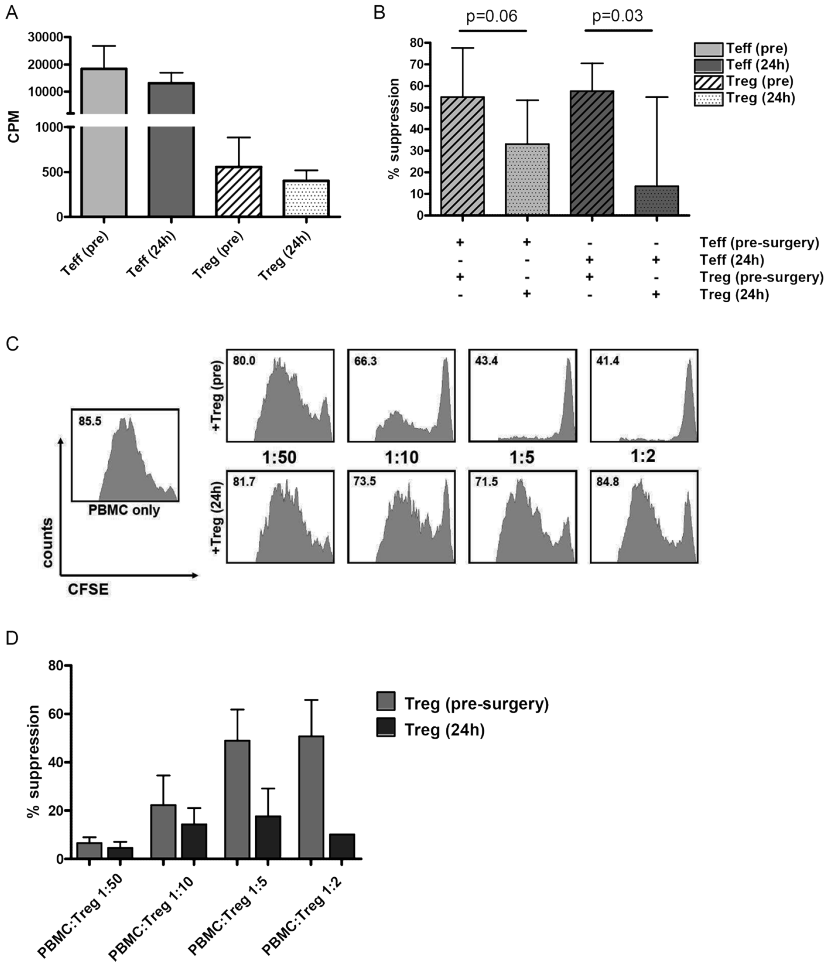

Figure 4. Functional characteristics after TCR stimulation of Teffs ($CD4^+CD25^-$) and Tregs ($CD4^+CD25^+CD127^{low}$) before and after surgery. Isolated PBMCs obtained immediately before cardiac surgery (pre) and 24 h after surgery (post) were sorted by flowcytometry. In total, 5 x 10^3 sorted cells were stimulated for 96 h with platebound anti-CD3 in the presence of 25 x 10^3 irradiated T cell-depleted cells (APC) from one time point. The final 16 h ^{3}H-TdR was added and proliferation was measured by ^{3}H incorporation. (**A**) Proliferation of Teffs and Tregs from before or 24 h after surgery in counts per minute (CPM), mean ± SEM of 7 representative patients. (**B**) Suppressive capacity of Tregs from before compared to 24 h after surgery. In total, 5 x 10^3 Teffs were co-cultured with 5 x 10^3 Tregs. The results shown represent the mean ± SEM of 7 representative patients with proliferation of the Teffs alone (either pre or 24 h after surgery) set at 100%. Difference between Tregs before and 24 h after surgery was compared using the Wilcoxon matched pair test. (**C**/**D**) Proliferation of 5 x 10^4 PBMCs alone and in co-culture with increasing ratio of Tregs (1:50-1:2). (**C**) Upper and lower panels illustrate CFSE dilution histogram of PBMCs in co-culture with Tregs from before and 24 h after surgery respectively. The mean percentage of divided cells of three experiments is shown in the left upper corner of each histogram. (**D**) Bar graph representing mean ± SEM of percentage suppression by Tregs from before (light grey) and 24 h after surgery (dark grey), n=3.

anti-CD3 and 25.000 irradiated antigen-presenting cells from before surgery. Tregs from before surgery could clearly suppress proliferation of Teffs (55% and 54% suppression of Teffs obtained before and 24 hours after surgery, respectively), while Tregs from 24 h after surgery showed diminished potential to suppress both T effector populations (28% and 17% suppression of Teffs obtained before and 24 h after surgery, respectively, Figure 4B and supporting information Figure 3). To further substantiate the functionality of Tregs before and after surgery, CFSE dilution assays were performed on PBMCs in co-culture with increasing ratio of Tregs. Consistent with the 3H-thymidine assays, Tregs from before surgery were potent suppressors of TCR-induced division of T cells in a dose-dependent manner. Tregs obtained 24 h after surgery, however, were less suppressive (Figure 4C and D). In conclusion, the increased population of $FOXP3^+$ T cells due to cardiac surgery had a diminished capacity to suppress T effector cell proliferation, whereas these $FOXP3^+$ T cells were intrinsically unable to proliferate upon TCR stimulation *in vitro*, and thus remained anergic.

Role of inflammatory milieu on Treg activity

As Tregs were inhibited in their suppressive activity due to cardiac surgery, we sought the mechanism behind the diminished effectiveness of Tregs. Cardiac surgery clearly evoked an inflammatory response with the release of multiple cytokines. As a putative mechanism of inhibiting the Tregs, we investigated the role of serum as inflammatory milieu after cardiac surgery. Therefore, we studied the effect of adding serum obtained from patients after cardiac surgery on the suppressive activity of Tregs from healthy subjects in a suppression assay. Co-culture with 20% serum obtained 4 h after surgery inhibited the suppressive activity of Tregs (76% and 33% suppression when comparing AB serum versus serum obtained 4 h post surgery). Twenty-four hours after surgery, when cytokine levels had returned to baseline values, suppression was equal or increased compared to healthy control serum (Figure 5A). As IL-6 showed the clearest increase 4 h after surgery and it has been described that this pro-inflammatory cytokine can inhibit Tregs, we subsequently investigated the role of IL-6. Adding plasma 4 h after surgery again clearly inhibited suppression, while adding IL-6 blocking antibodies showed no reversal of this plasma effect (Figure 5B), indicating no prominent role for IL-6 in the inhibiting effect of plasma.

The above-described observations clearly illustrate that Tregs are strongly influenced by the milieu in which these cells are to conduct their suppressive effect.

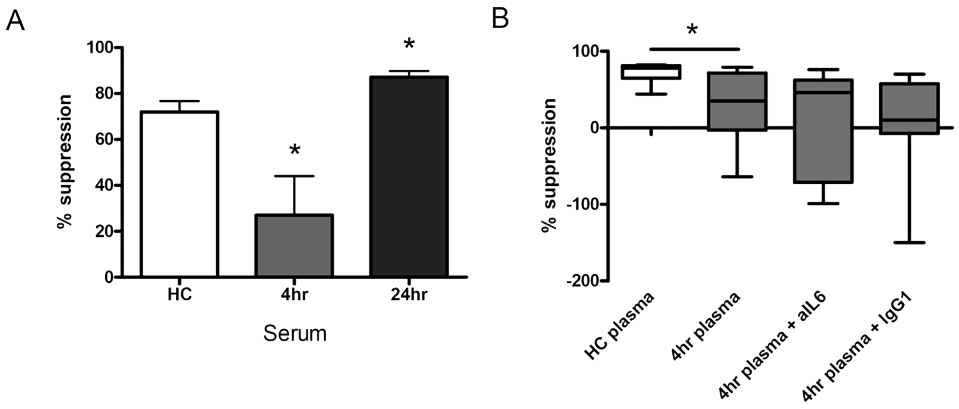

Figure 5. (A) Effect of postoperative plasma on suppressive capacity of Tregs. Sorted $CD4^+CD25^+CD127^{low}$ T cells (Tregs) from healthy subjects were co-cultured with 20% healthy AB serum (HC) or 20% serum obtained 4 h and 24 h after surgery. After 96 h culture with platebound anti-CD3 in the presence of 25 x 10^3 irradiated T cell-depleted cells (APCs), proliferation was estimated by ^3H incorporation. Depicted is the percentage of suppression by 10 x 10^3 Tregs added to 10 x 10^3 Teffs compared to Teffs alone. The results shown represent the mean ± SEM of eight plasma co-culture experiments. Difference between HC serum and 4 h or 24 h serum was compared using the Mann-Whitney U test, $*P<0.05$. (**B**) The role of IL-6 on the inhibitory effect of post-surgery plasma on the suppressive capacity of healthy Tregs. This experiment was performed as above with or without the addition of blocking antibodies for IL-6 or the appropriate isotype. The boxplot represents eight experiments. Significant difference compared to 4 h plasma alone was calculated by Wilcoxon matched pair test, $*P<0.05$.

Discussion

This study scrutinized the functionality of $FOXP3^+$ Tregs during transient inflammation in children who underwent cardiac surgery. While on the one hand $CD4^+$ cells became activated, alongside a release of pro-inflammatory cytokines IL-6 and IL-8 and changes in cell count of all leukocytes in the circulation, the frequency of $CD4^+FOXP3^+$ cells increased significantly. Just like true Tregs, the $FOXP3^+$ Treg population after surgery remained anergic. However, these cells were less capable of suppressing $CD4^+CD25^-$ T effector cells after TCR stimulation *in vitro*. Inflammatory serum obtained after cardiac surgery strongly inhibits the suppressive effectiveness of healthy Tregs.

Numerous studies have reported on the induction of $FOXP3^+$ cells from non-Tregs *in vitro*.[6;7] Furthermore, mechanisms important for induction of Treg *in vivo* have been demonstrated in rodents.[8;9] While observational studies of $FOXP3^+$ T cells in various human clinical states abound, the relevance of these cells is not always easy to ascertain. There are, however, strong indications that Tregs play a role in most inflammatory states. Numerous studies have clearly shown associations in autoimmune disease,[10-14] chronic infection,[15-19] cancer[20;21]

and transplantation.[22;23] Although some studies have been able to show correlations between numbers of Tregs and clinical outcome, it proves to be hard to show a direct link between the appearance or function of Tregs and disease.[24-27] One of the problems is that in the presence of inflammation, Tregs always appear with a diverse set of other inflammatory cells. Above all, these are not easily distinguished from each other, while different populations may have opposing functions. To distinguish true Tregs from activated T cells functional assays *in vitro* are mandatory.

We used pediatric cardiac surgery as a model of healthy, transient inflammation. Pediatric cardiac surgery has been described to provoke a systemic inflammation with consequences for various immunological cascades including monocytes and cytokines.[28;29] This model enabled us to collect samples from the site of inflammation and study the activation and regulation of the $CD4^+$ T cell compartment. Furthermore, the immune system could be monitored in a single patient over time, from before initiation to subsidence of the inflammatory response.

Although patients differed in both pre-surgery and post-operative cell numbers and expression of various proteins, virtually all patients followed the same trend during the systemic inflammatory response after surgery. Therefore, the observations during the aftermath of the surgical procedure are likely a general phenomenon during a systemic inflammatory response. The observed 'cytokine storm' will drive the systemic nature of the inflammation and hereby contribute in activating T cells. Furthermore, T cells may become activated by sheer stress of the CPB,[30] effect of anesthesia[31] and toll-like receptor activation by both exogenous (lipopolysaccharide, peptidoglycan[32;33]) and endogenous (heat shock proteins[34-36]) ligands which are released due to the procedure.

The observed loss of suppressive capacity of the Treg population may be explained through various mechanisms. First, the increase of $FOXP3^+$ T cells could be the result of a differential distribution of $FOXP3^+$ and $FOXP3^-$ T cells. Either effector FOXP3- T cells are more prone to migrate into the tissues or $FOXP3^+$ T cells are more rapidly mobilized into the circulation during an inflammatory response. Several migratory characteristics have been identified to be specific for Tregs.[37;38] However, this phenomenon can not explain the increased expression of FOXP3 per cell. Second, the increase of $FOXP3^+$ T cells could be due to preferential proliferation. While our data confirms that the $FOXP3^+$ T cell population has the highest percentage of proliferating $Ki67^+$ cells, the time period of 24 h would seem too short to explain any substantial increase in cell numbers. Finally, the observed altered composition of $FOXP3^+$ Tregs in the circulation could be due to *de novo* induction of FOXP3 in formerly $FOXP3^-$ T cells. This would also explain the observed diminished suppressive capacity of the Treg population as a whole. It has been shown *in vitro* that proliferating T cells temporarily upregulate FOXP3 without acquiring suppressive function.[39]

While we observed a unanimous increase in frequency of Tregs, total cell numbers remained stable during the inflammatory response. Therefore, the observed functional changes could also be attributed to suppressed Treg population as a whole. The inflammatory milieu

could influence the Treg suppressive capacity. Indeed, this has recently been demonstrated for IL-6, which is abundantly available after surgery, which prevents suppression by Tregs.[40] We were, however, not able to show a role for IL-6 in this setting, as blocking antibodies to IL-6 showed no concluding effect. Although it seems likely that cytokines are contributing factors in regulating Tregs, we cannot exclude other soluble factors. For example, medication could play a role, although there is little difference between prescribed medication 4 h and 24 h after surgery.

Interestingly, the $FOXP3^+$ cells remained anergic *in vitro*, like true Tregs. This implies that the induced FOXP3 is functional on the level of the cell itself, without acquiring additional characteristics of a true Tregs with suppressive capacity. Although Tregs are thought to be anergic *in vitro*, their anergic state can be overcome in the presence of pro-inflammatory cytokines IL-1 and IL-6, cytokines that are increased in plasma after surgery. More so, it was recently shown that Tregs are actually the first T cells to respond to IL-2 in an immune response.[41] Within 6-12 h, Tregs are activated and proliferate. In our patients, we were not able to measure significant levels of IL-2 in plasma; however, it is likely that IL-2 does play an important role on the local cell level. In a healthy situation, *in vivo*, Tregs have been shown to be the population with the fastest turnover rate.[42] Indeed, we found expression of proliferation marker Ki67 to be highest in the $CD4^+FOXP3^+$ population, both before and during the inflammatory response.

Consequently, modulation of Tregs in various inflammatory diseases is of interest. However, without a proper understanding of how these cells can be induced and subsequently function during an inflammatory response *in vivo*, proposed interventions in human can have deleterious consequences.[43] Up to date, several *in vitro* protocols have been developed to induce FOXP3 in human cells *in vitro*. TCR activation of $CD4^+CD25^-$ T cells induces $FOXP3^+$ T cells with regulatory activity.[7;44;45] Several studies have found that increased expression of FOXP3 after *in vitro* stimulation corresponds to increased suppressive potential.[6;46] We found that although FOXP3 levels increased in $CD4^+CD25^+CD127^{low}$ population during the systemic inflammatory response, this did not render these cells more potent suppressors, but rather less. This observation underlines the need to be cautious to extrapolate *in vitro* studies with Tregs to *in vivo* situations. Besides the issue of level of FOXP3 expression, duration of expression may be an important facet determining the function of induced Tregs. The reduced effectiveness of the induced FOXP3 T cells may be time-dependent as earlier *in vitro* studies report that continuous levels of FOXP3 are required to convert naive T cells into Tregs with full effectiveness.[6] In this setting of systemic inflammation, 24 hours seems to be too short to procure the full molecular and transcriptional changes necessary for suppression. On the other hand, it does seem to be sufficient to inhibit the cell from dividing after TCR stimulation *in vitro*. Accordingly, FOXP3 may act as an intrinsic regulator during inflammation, preventing collateral damage by temporarily silencing activated T cells.

In conclusion, during systemic inflammation due to cardiac surgery in children $FOXP3^+$ T cells loose suppressive capacity. While these cells are anergic to TCR stimulation, the transiently increased expressed FOXP3 is not capable of taking on a suppressive function. Furthermore,

the inflammatory milieu in which Tregs exert their action after cardiac surgery inhibits their suppressive activity. This study illustrates the functionality of FOXP3$^+$ T cells in a human model of inflammation and underlines the requirement of more human *in vivo* systems to understand the properties and potential of induced FOXP3$^+$ Tregs in human disease.

Materials and Methods

Patient selection

Children admitted to our hospital for surgical repair of either a ventricular septum defect (VSD) or an atrial septum defect (ASD) were enrolled in this study. Patients were excluded from the study if at the time of admission they had received steroids within two weeks before surgery, had signs of infection or had a documented immunodeficiency. Informed consent was obtained from the parents of children participating in the study. The medical ethics committee approved this study (METC 03/049-K, UMC Utrecht, The Netherlands).

Surgical and anesthesiological procedures

General anesthesia was always implemented using a standard technique involving high dose sufentanil, midazolam, pancuronium, dopamine and milrinone. All patients were given a single dose of dexamethason 1 mg/kg after induction of anesthesia. Non-pulsatile cardiopulmonary bypass was used, the standard pump flow rate was 2.8 liter/m^2/min. Combined alpha and pH stat management of acid-base status was used during cardiopulmonary bypass. The cardioplegia procedure was standardized using St. Thomas' solution. After weaning from cardiopulmonary bypass all patients remained intubated and ventilated and were admitted to the pediatric intensive care for further management. All patients were treated by the same surgical team.

Blood sampling

Blood samples were obtained from a central venous catheter at the following time points; immediately after insertion during anesthetic induction (T1), at the end of the cardiopulmonary bypass (T2) and at 4 hours (T3), 24 hours (T4) and 48 hours after surgery (T5). At these time points, total white blood cell count, lymphocyte count, and monocyte count were determined. Sample volumes were adjusted to patient's body weight with a maximum for all samples combined of 10% of circulating volume. Due to limited amount of blood volume obtainable from the often young patients, not all assays could be performed on all 25 patients.

Cell isolation and flowcytometry

Mononuclear cells were isolated from heparinized blood samples (T1, T4 and T5) using Ficoll Isopaque density gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden). Peripheral blood mononuclear cells were washed in FACS buffer (PBS containing 2% FCS and 0.1% sodium azide), adjusted to 4.0 x 10^6 cells/ml in FACS buffer and blocked with normal

mouse serum. The cells were incubated in 50 µl FACS buffer containing the appropriately diluted Fitc, PE, PercP or APC labeled antibodies against human CD3, CD4, CD25, CD69, CD127, or glucocorticoid induced tumor-necrosis-factor receptor (GITR). For cytoplasmatic staining of cytotoxic T lymphocyte antigen 4 (CTLA-4) and Ki-67 the cells were first surface stained, then fixed in Cytofix/Cytoperm (20 min., 4°C) and washed twice in Perm/Wash solution (Cytofix/perm kit, BD Biosciences, San Jose, CA, USA), followed by incubation with the appropriate antibody. Intranuclear staining of FOXP3 was performed after fixation and permeabilisation according to the manufacturer's protocol and subsequently incubated with the appropriate antibody. Antibodies against CD4 (clone SK3), CD25 (2A3), CD69 (L78), CD127 (hIL-7R-M21) and CTLA-4 (BN13) were obtained from BD Bioscience, GITR (110416) from R&D (Minneapolis, MN, USA) and Ki67 (MIB-1) from Immunotech (Marseilles, France), FOXP3 (PCH101) from eBioscience (San Diego, CA, USA). Finally, stained mononuclear cells were washed twice in FACS buffer and run on a FACS Calibur (BD Biosciences). CellQuestPro software (BD Biosciences) was used for analyses. The gates for the different populations were set for the sample prior to surgery and kept identical for the following samples (supporting information Figure I A).

Multiplex immunoassay

From plasma obtained at five timepoints (immediately before and after surgery, 4 hours, 24 hours and 48 hours after surgery), IL6 and IL8 levels were determined by multiplex immunoassay as previously described.[47;48]

mRNA analysis by quantitative PCR

According to the intensity of CD25 expression, $CD4^+CD25^{bright}$, $CD4^+CD25^{intermediate}$ and $CD4^+CD25^-$ T cells were isolated from samples before surgery and 24 hours after surgery. The gates for these three populations were kept identical at both time points. Isolation of total RNA and quantification of FOXP3 mRNA was performed as previously described.[11]

Confocal fluorescent imaging

Forty million isolated peripheral blood mononuclear cells were stained for CD4 and CD25 as described above. Cells were fixated and stained for FOXP3 Alexa-488 (PCH101) according to manufacturer's instructions (eBioscience). The cell sample was sorted by FACS in the three appropriate populations according to the intensity of CD25 expression. Sorted cells were applied on a Poly-L-lysine pre-coated coverslip and incubated with the biochemical nuclear marker TO-PRO-3 iodide (Invitrogen, Carlsbad, CA, USA). After washing the coverslips twice in FACS buffer, they were applied onto slides and left to dry overnight. Fluorescence was imaged with Leica TCS SP confocal microscope equipped with an Argon / HeNe laser for double fluorescence at 488 nm and 633 nm. Confocal images were recorded with a 100x objective and processed with Leica Confocal software. Higher magnification images were composed digitally.

Alexa 488 and TO-PRO-3 iodide were pseudo-colored in green and red, respectively. Gain and offset settings were identical for the three sorted slides.

Functional Treg Assay

Suppression assays were performed to ascertain the functional ability of the identified regulatory T cells. Isolated mononuclear cells were divided by magnetic separation (MACS) into CD3 positive and negative populations (>90% purity). Per well 25.000 irradiated (3500 Rad) CD3 negative cells were used as antigen presenting cells (APC). CD3 positive cells were sorted on a FACS Aria (BD bioscience) according to expression of CD4, CD25 and CD127 in to effector (Teff) and Treg populations (supporting information Figure 1B). Teffs were identified by positive expression of CD4 and negative expression of CD25. Treg cells were sorted by positive expression of CD4 and CD25 and low expression of CD127. Cells were incubated for 96 hours in 37 C, 5% CO2 and stimulated with platebound anti-CD3 (OKT03, 1 ug/ml, eBioscience). For the final 16 hours tritium thymidine (^{3}H) was added. The proliferation of Teffs and Tregs alone was determined by ^{3}H incorporation. Suppressive capacity of Tregs was assessed in co-culture conditions with equal amounts of Teffs and Tregs. The subsequent proliferation of Teffs in the presence of Tregs was related to the proliferation of Teffs alone. An average value from triplicate wells per condition was set off against medium value.

To further substantiate the functionality of Tregs before and after surgery CFSE dilution assay was performed on three patients using different ratio of Tregs to PBMC. 5 x 10^4 PBMC from before surgery were labeled with CFSE according to protocol.[49] Cells were cultured as described before with platebound anti-CD3 with different ratios of sorted Tregs from both before and 24 hours after surgery. Division of PBMC was determined after 96 hours by analyzing CFSE dilution by means of FACS analysis.

Treg suppression assay in co-culture with inflammatory plasma

In order to evaluate the role of soluble factors present during the inflammatory response on Treg functionality a standardized suppression assay was performed in the presence of patient plasma. Teffs (10.000 cells) and Tregs (10.000 cells) were sorted from healthy subjects and co-cultured with 20% heat inactivated AB serum (Sanquin Blood Bank, Utrecht, The Netherlands) and 20% plasma obtained from patients 4 hours and 24 hours after surgery. Cells were incubated for 96 hours in 37 C, 5% CO2 and stimulated with platebound anti-CD3 (OKT03, 1 ug/ml, eBioscience). For the final 16 hours tritium thymidine (^{3}H) was added. The proliferation of Teffs and Tregs alone was determined by ^{3}H incorporation. To investigate the effect of IL6 we added IL6 neutralizing antibodies (MQ2-13A5, BD Biosciences) and the appropriate rat IgG1 isotype control (50ng/ml).

Statistical analysis

Basic descriptive statistics were used to describe the patient population. Data involving 2 time points within one population were compared using the Wilcoxon matched pair test. For differences in median between two independent groups the Mann-Whitney U test was used to test for significance. Significance was accepted at $p < 0.05$ indicated in the graphs by * or $P<0.001$ indicated by **.

Acknowledgements

The authors would like to thank W. de Jager from the Center for Molecular and Cellular Intervention for his assistance with the Luminex analysis, M. Klein for technical assistance with FACS sorting and J. Meerding for performing the CFSE assays.

Grant support

This study was supported by the Wilhelmina Children's Hospital Research Fund. B.J. Prakken was supported by grants from the Netherlands Organisation for Scientific Research (NWO Vidi innovation grant) and the Dutch Arthritis Foundation.

References

1. Chew MS, Brandslund I, Brix-Christensen V et al. Tissue injury and the inflammatory response to pediatric cardiac surgery with cardiopulmonary bypass: a descriptive study. Anesthesiology 2001;94:745-753.
2. Diegeler A, Doll N, Rauch T et al. Humoral immune response during coronary artery bypass grafting: A comparison of limited approach, "off-pump" technique, and conventional cardiopulmonary bypass. Circulation 2000;102:III95-100.
3. Tomic V, Russwurm S, Moller E et al. Transcriptomic and proteomic patterns of systemic inflammation in on-pump and off-pump coronary artery bypass grafting. Circulation 2005;112:2912-2920.
4. Wan S, LeClerc JL, Vincent JL. Inflammatory response to cardiopulmonary bypass: mechanisms involved and possible therapeutic strategies. Chest 1997;112:676-692.
5. Zimmerman JJ. Congenital heart disease, cardiopulmonary bypass, systemic inflammatory response syndrome, compensatory anti-inflammatory response syndrome, and outcome: evolving understanding of critical care inflammation immunology. Crit Care Med 2002;30:1178-1179.
6. Allan SE, Song-Zhao GX, Abraham T, McMurchy AN, Levings MK. Inducible reprogramming of human T cells into Treg cells by a conditionally active form of FOXP3. Eur.J.Immunol. 2008;38:3282-3289.
7. Walker MR, Kasprowicz DJ, Gersuk VH et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human $CD4^+CD25^-$ T cells. J.Clin.Invest 2003;112:1437-1443.
8. Gabrysova L, Wraith DC. Antigenic strength controls the generation of antigen-specific IL-10-secreting T regulatory cells. Eur.J.Immunol. 2010;40:1386-1395.
9. Visekruna A, Huber M, Hellhund A et al. c-Rel is crucial for the induction of Foxp3(+) regulatory CD4(+) T cells but not T(H)17 cells. Eur.J.Immunol. 2010;40:671-676.
10. Brusko T, Wasserfall C, McGrail K et al. No alterations in the frequency of $FOXP3^+$ regulatory T-cells in type 1 diabetes. Diabetes 2007;56:604-612.
11. de Kleer IM, Wedderburn LR, Taams LS et al. $CD4^+CD25^{bright}$ regulatory T cells actively regulate inflammation in the joints of patients with the remitting form of juvenile idiopathic arthritis. J Immunol. 2004;172:6435-6443.
12. Han GM, O'Neil-Andersen NJ, Zurier RB, Lawrence DA. $CD4^+CD25^{high}$ T cell numbers are enriched in the peripheral blood of patients with rheumatoid arthritis. Cell Immunol. 2008;253:92-101.
13. Saresella M, Marventano I, Longhi R et al. $CD4^+CD25^+FoxP3^+PD1$- regulatory T cells in acute and stable relapsing-remitting multiple sclerosis and their modulation by therapy. FASEB J. 2008;22:3500-3508.
14. Valencia X, Yarboro C, Illei G, Lipsky PE. Deficient $CD4^+CD25^{high}$ T regulatory cell function in patients with active systemic lupus erythematosus. J.Immunol. 2007;178:2579-2588.
15. Cao Y, Zhao J, Lei Z et al. Local accumulation of $FOXP3^+$ regulatory T cells: evidence for an immune evasion mechanism in patients with large condylomata acuminata. J.Immunol. 2008;180:7681-7686.
16. Ebinuma H, Nakamoto N, Li Y et al. Identification and *in vitro* expansion of functional antigen-specific $CD25^+$ $FoxP3^+$ regulatory T cells in hepatitis C virus infection. J.Virol. 2008;82:5043-5053.
17. Harris PR, Wright SW, Serrano C et al. Helicobacter pylori gastritis in children is associated with a regulatory T-cell response. Gastroenterology 2008;134:491-499.
18. Miyara M, Amoura Z, Parizot C et al. The immune paradox of sarcoidosis and regulatory T cells. J Exp. Med. 2006;203:359-370.
19. Toulza F, Heaps A, Tanaka Y, Taylor GP, Bangham CR. High frequency of $CD4^+FoxP3^+$ cells in HTLV-1 infection: inverse correlation with HTLV-1-specific CTL response. Blood 2008;111:5047-5053.
20. Clark RA, Huang SJ, Murphy GF et al. Human squamous cell carcinomas evade the immune response by down-regulation of vascular E-selectin and recruitment of regulatory T cells. J.Exp.Med. 2008;205:2221-2234.
21. Perrone G, Ruffini PA, Catalano V et al. Intratumoural FOXP3-positive regulatory T cells are associated with adverse prognosis in radically resected gastric cancer. Eur.J.Cancer 2008;44:1875-1882.

22. Bunnag S, Allanach K, Jhangri GS et al. FOXP3 expression in human kidney transplant biopsies is associated with rejection and time post transplant but not with favorable outcomes. Am.J.Transplant. 2008;8:1423-1433.

23. Veronese F, Rotman S, Smith RN et al. Pathological and clinical correlates of FOXP3$^+$ cells in renal allografts during acute rejection. Am.J.Transplant. 2007;7:914-922.

24. Costantino CM, Baecher-Allan C, Hafler DA. Multiple sclerosis and regulatory T cells. J.Clin.Immunol. 2008;28:697-706.

25. Miyara M, Amoura Z, Parizot C et al. Global natural regulatory T cell depletion in active systemic lupus erythematosus. J.Immunol. 2005;175:8392-8400.

26. Lee JH, Wang LC, Lin YT et al. Inverse correlation between CD4$^+$ regulatory T-cell population and autoantibody levels in paediatric patients with systemic lupus erythematosus. Immunology 2006;117:280-286.

27. Piccirillo CA, d'Hennezel E, Sgouroudis E, Yurchenko E. CD4$^+$Foxp3$^+$ regulatory T cells in the control of autoimmunity: *in vivo* veritas. Curr.Opin.Immunol. 2008;20:655-662.

28. Allen ML, Peters MJ, Goldman A et al. Early postoperative monocyte deactivation predicts systemic inflammation and prolonged stay in pediatric cardiac intensive care. Crit Care Med 2002;30:1140-1145.

29. Gessler P, Pfenninger J, Pfammatter JP et al. Plasma levels of interleukin-8 and expression of interleukin-8 receptors on circulating neutrophils and monocytes after cardiopulmonary bypass in children. J.Thorac. Cardiovasc.Surg. 2003;126:718-725.

30. Ijichi S, Mishima M, Matsuda T et al. Concentration of activated T lymphocytes in extracorporeal blood circulation for plasma separation. J.Clin.Apher. 1991;6:88-89.

31. Schneemilch CE, Hachenberg T, Ansorge S, Ittenson A, Bank U. Effects of different anaesthetic agents on immune cell function *in vitro*. Eur.J.Anaesthesiol. 2005;22:616-623.

32. Jansen NJ, van Oeveren W, Gu YJ et al. Endotoxin release and tumor necrosis factor formation during cardiopulmonary bypass. Ann.Thorac.Surg. 1992;54:744-747.

33. Tsunooka N, Maeyama K, Hamada Y et al. Bacterial translocation secondary to small intestinal mucosal ischemia during cardiopulmonary bypass. Measurement by diamine oxidase and peptidoglycan. Eur.J.Cardiothorac.Surg. 2004;25:275-280.

34. de Jong PR, Schadenberg AW, Jansen NJ, Prakken BJ. Hsp70 and cardiac surgery: molecular chaperone and inflammatory regulator with compartmentalized effects. Cell Stress.Chaperones. 2008

35. Dybdahl B, Wahba A, Lien E et al. Inflammatory response after open heart surgery: release of heat-shock protein 70 and signaling through toll-like receptor-4. Circulation 2002;105:685-690.

36. Schett G, Metzler B, Kleindienst R et al. Myocardial injury leads to a release of heat shock protein (hsp) 60 and a suppression of the anti-hsp65 immune response. Cardiovasc.Res. 1999;42:685-695.

37. McFadden C, Morgan R, Rahangdale S et al. Preferential migration of T regulatory cells induced by IL-16. J.Immunol. 2007;179:6439-6445.

38. Zhang N, Schroppel B, Lal G et al. Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response. Immunity. 2009;30:458-469.

39. Wang J, Ioan-Facsinay A, van der Voort E, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4$^+$ T cells. Eur.J.Immunol. 2007;37:129-138.

40. Goodman WA, Levine AD, Massari JV et al. IL-6 signaling in psoriasis prevents immune suppression by regulatory T cells. J.Immunol. 2009;183:3170-3176.

41. O'Gorman WE, Dooms H, Thorne SH et al. The initial phase of an immune response functions to activate regulatory T cells. J.Immunol. 2009;183:332-339.

42. Akbar AN, Vukmanovic-Stejic M, Taams LS, Macallan DC. The dynamic co-evolution of memory and regulatory CD4$^+$ T cells in the periphery. Nat.Rev.Immunol. 2007;7:231-237.

43. Marshall E. Drug trials. Violent reaction to monoclonal antibody therapy remains a mystery. Science 2006;311:1688-1689.

44. Bisikirska B, Colgan J, Luban J, Bluestone JA, Herold KC. TCR stimulation with modified anti-CD3 mAb expands CD8$^+$ T cell population and induces CD8$^+$CD25$^+$ Tregs. J.Clin.Invest 2005;115:2904-2913.

45. Kretschmer K, Apostolou I, Hawiger D et al. Inducing and expanding regulatory T cell populations by foreign antigen. Nat.Immunol. 2005;6:1219-1227.

46. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. Nat.Immunol. 2007;8:277-284.

47. de Jager W, Prakken BJ, Bijlsma JW, Kuis W, Rijkers GT. Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies. J.Immunol. Methods 2005;300:124-135.

48. de Jager W, te VH, Prakken BJ, Kuis W, Rijkers GT. Simultaneous detection of 15 human cytokines in a single sample of stimulated peripheral blood mononuclear cells. Clin.Diagn.Lab Immunol. 2003;10:133-139.

49. Vercoulen Y, Wehrens EJ, van Teijlingen NH et al. Human regulatory T cell suppressive function is independent of apoptosis induction in activated effector T cells. PLoS.One. 2009;4:e7183.

Supporting information

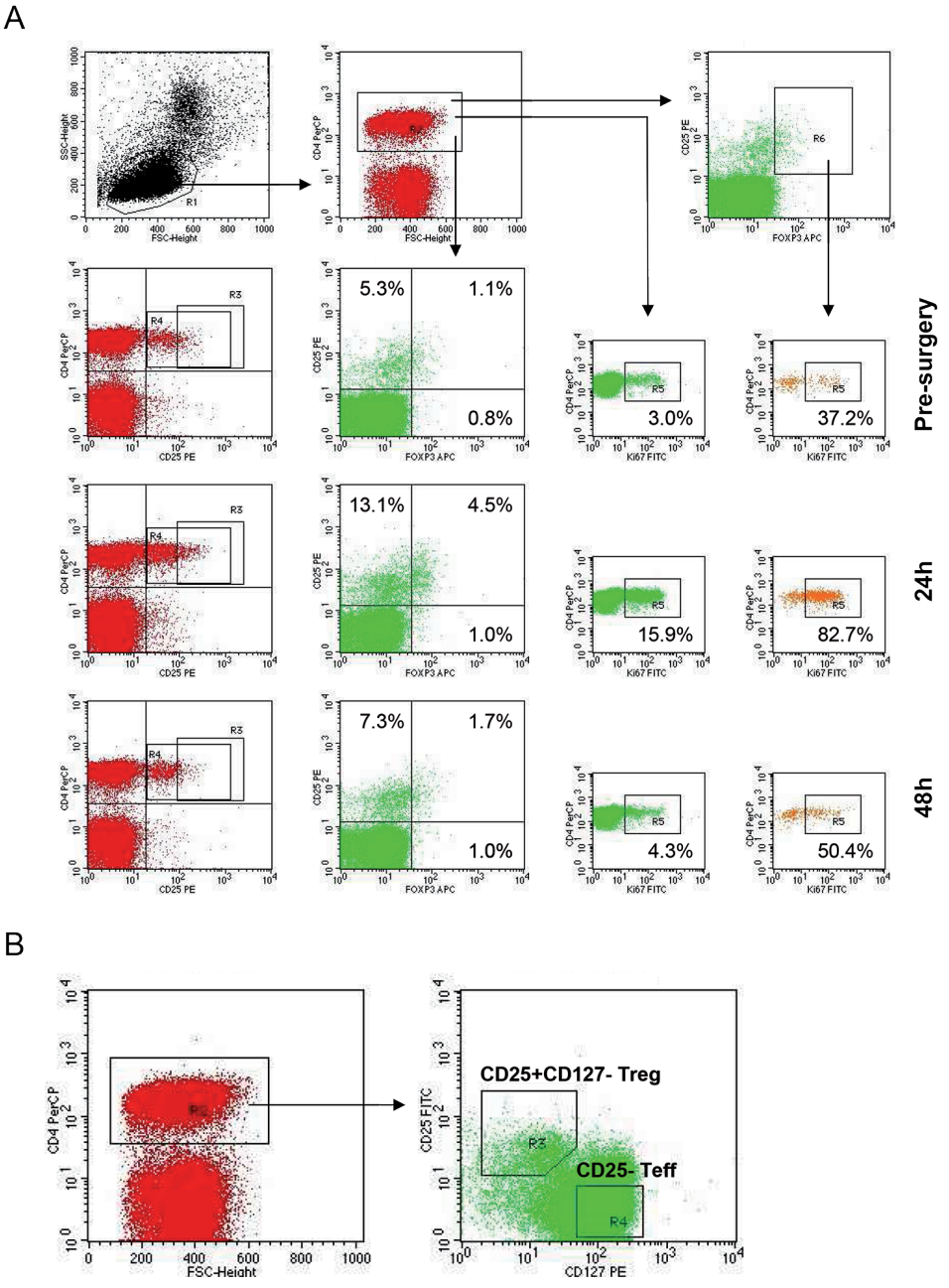

Figure 1. Gating strategies for FACS analysis and sorting. For the sake of comparison gating regions are copied between the different samples of the same patient.

(**A**) Gating strategies for figure 1 A, B, C, Figure 2 A, Figure 3 A, B.

(**B**) Gating strategy for sorting Treg ($CD4^+CD25^+CD127^-$) and Teff ($CD4^+CD25^-$) for suppression assays with 3H-thymidine incorporation (Figure 4 A, B and 5) and CFSE dilution assay (Figure 4 C).

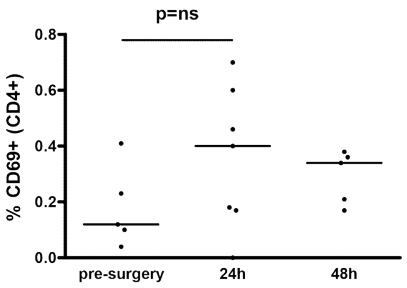

Figure 2. CD69 expression of $CD4^+$ lymphocytes before and after surgery. Data are presented as dot plots with median. Significance was calculated by the Wilcoxon matched pair test.

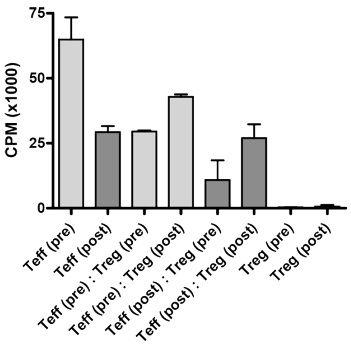

Figure 3. Functional characteristics after TCR stimulation of Teff ($CD4^+CD25^-$) and Treg ($CD4^+CD25^+CD127^{low}$) before and after surgery. Isolated PBMC obtained immediately before cardiac surgery (pre) and 24 h hours after surgery (post) were sorted by flowcytometry. 5 x 10^3 sorted cells were stimulated for 96 h with platebound anti-CD3 in the presence of 25 x 10^3 irradiated T cell depleted cells (APC) from one timepoint. The final 16 hours ^{3}H-TdR was added and proliferation was measured by ^{3}H incorporation. 3H-thymidine suppression assay illustrating absolute counts per minute per condition. The results depicted represent the mean ± SEM from triplicate conditions of 1 representative patient.

Chapter 5

DIFFERENTIAL HOMEOSTATIC DYNAMICS OF HUMAN TREG CELL SUBSETS FOLLOWING NEONATAL THYMECTOMY

AWL Schadenberg[1,2], T van den Broek[1,2], MA Siemelink[3], SO Algra[2], PR de Jong[1,2], NJG Jansen[2], BJ Prakken[1,*], F van Wijk[1,*]

[1] Center for Molecular and Cellular Intervention (CMCI), Department of Pediatric Immunology and Laboratory of Translational Immunology, Wilhelmina Children's Hospital, University Medical Centre Utrecht, Utrecht, The Netherlands
[2] Dep. of Pediatric Intensive Care / Pediatric Cardiothoracic Surgery, University Medical Center Utrecht, The Netherlands
[3] Dep. of Experimental Cardiology, University Medical Center Utrecht, The Netherlands

[*] These authors contributed equally to this paper

Journal of Allergy and Clinical Immunology 2013, accepted

Capsule Summary

Neonatal thymectomy during cardiac surgery results in an increased percentage of circulating FOXP3$^+$ Treg through compensatory peripheral proliferation. However, the composition of FOXP3$^+$ Treg subsets is altered, which may affect immune regulation later in life.

Keywords: Regulatory T cells, FOXP3, thymectomy, homeostasis, children

Introduction, Results and Discussion

FOXP3 expressing CD4$^+$ regulatory T-cells (Treg) are important in the maintenance of self-tolerance and immune homeostasis. There are two possible origins for Treg[1]: 1) thymus-derived natural Treg (nTreg) and 2) peripherally induced Treg (iTreg). Although both Treg populations express similar phenotypic proteins, it has been proposed that they exert different functions in maintaining immune homeostasis. While nTreg have been shown to be essential in self-tolerance, iTreg may be important in tolerance to non-pathogenic foreign antigens.[2] Disturbing the production of either Treg population may affect immune regulation later in life. Recently it has been shown for example that alterations at neonatal age in thymic Treg maturation affect clinical outcome.[3] It was demonstrated that in atopic children, thymic Treg function is significantly delayed early on in life. However, further data on human Treg development early in life are scarce.

The functional Treg population contains two distinct populations, a naive CD45RA+RO-FOXP3low fraction and an activated/memory CD45RA-RO+FOXP3high fraction, both equally capable of suppressive activity.[4] Though both subpopulations are true Treg, they have distinct differentiation dynamics. We hypothesized that Treg population dynamics would be affected in patients that undergo neonatal thymectomy during cardiac surgery. Previously we showed the effect of neonatal thymectomy on long-term restoration of the naive T-cell compartment.[5] In the present study we evaluated the dynamics of distinct Treg subpopulations in the first 3 years following neonatal thymectomy.

Twenty-six children with a median age of 11.4 months (range 2.5 – 34.7 months) were included who were previously thymectomized during the correction of a cardiac defect (table E1, see Online Repository (OR)). The study was approved by the medical ethical committee of the University Medical Centre Utrecht (METC 05-041 and 06-149) and written informed consent was obtained. Thymectomy was performed within the first month of life (10.0 ± 9.0 days) in all participants. At the time of blood sampling all children showed no sign of infection or immune dysregulation. For full information on the study population and flow cytometry staining protocols, see method section in OR.

First, we determined the impact of thymectomy on the total, peripheral CD4$^+$ T-cell population. Following thymectomy, absolute CD4 counts dropped significantly compared to healthy infants (Figure 1A & E1A). Similarly, total FOXP3$^+$CD4$^+$ T-cell numbers were significantly lower in thymectomized patients compared to healthy age-matched controls (Figure 1B & E1B). At a young age, CD31$^+$ (PECAM-1) T-cells represent recent thymic emigrants.[6] Compatible with a loss of thymic production of Treg, thymectomized patients showed a significantly lower percentage of CD31$^+$FOXP3$^+$ T cells compared to age-matched controls (Figure 1C). Taken together, neonatal thymectomy results in a loss of thymus-derived Treg and a reduced number of circulating Treg.

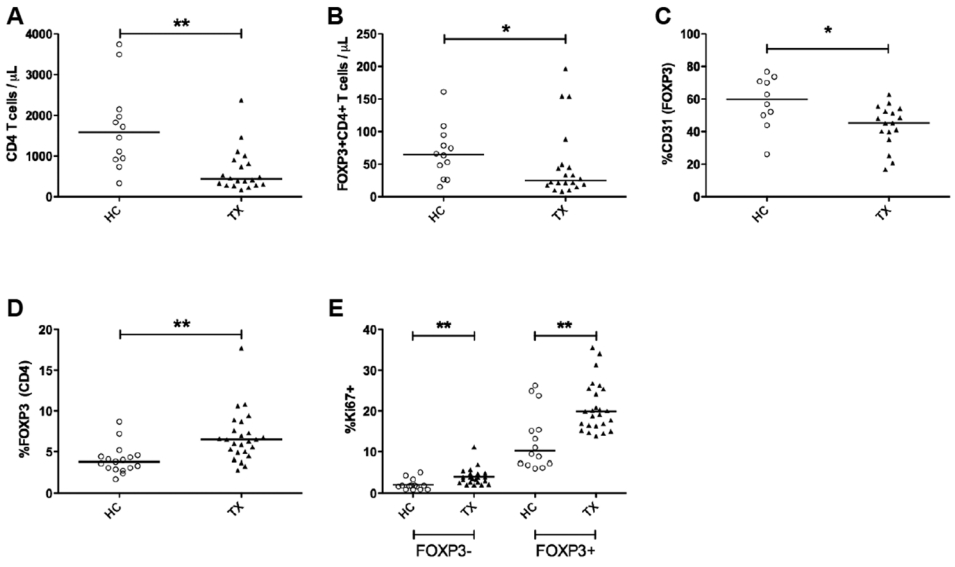

Figure 1. CD4⁺ and FOXP3⁺ T cell dynamics and expression of CD31 and Ki67 after thymectomy. (**A**) CD4⁺ T cell count; (**B**) FOXP3⁺CD4⁺ Treg cell count; (**C**) Percentage of CD31⁺ (FOXP3⁺) Treg cells; (**D**) Percentage FOXP3⁺ (CD4⁺) cells; (**E**) Percentage of FOXP3⁻ and FOXP3⁺ T cells in cell cycle (Ki67⁺) in thymectomized subjects (TX) (▲) and age matched controls (HC) (○). Horizontal line represents median value per group, **P*<0.05, ***P*<0.001.

Interestingly, after thymectomy the percentage of FOXP3⁺CD4⁺ T-cells was increased compared to controls (Figure 1D & E1C). Therefore we investigated if there was an increase in peripheral proliferation to compensate for the loss of thymic output. Compared to the FOXP3-CD4⁺ population, the FOXP3⁺CD4⁺ Treg population had a higher proportion of proliferation marker Ki67⁺ cells, with a significant increase following thymectomy (Figure 1E). Thus, these data suggest that there is a compensatory, selective expansion of Treg leading to an increased percentage of Foxp3⁺CD4⁺ T-cells.

Next we studied the dynamics of two distinct Treg subpopulations; gating of the FOXP3⁺ fractions is illustrated in Figures E2 and E3 (OR). The naive subpopulation CD45RO-FOXP3low (Fr. I) and the memory population with the highest FOXP3 expression CD45RO+FOXP3high (Fr. II) have been shown to be "true" Treg, whereas the memory population with low FOXP3 expression (Fr. III) are non-suppressive[4]. We observed that the percentage of FOXP3 cells expressing the naive CD45 isoform (Fr. I) remained stable, whereas both memory fractions increased significantly in thymectomizd patients (Figure 2A). The relative increase in the heterogeneous and non-suppressive CD45RO+FOXP3low population (Fr. III) may be a reflection of activation-induced transient upregulation of FOXP3, as has been shown *in vitro*.[7] Absolute numbers of both "true" Treg fractions decreased marginally, though not significantly

(Figure 2B). While cell numbers of CD45RO-FOXP3low Treg (Fr. I) were within the range of healthy controls, the majority of patients had low numbers (Figure 2B). Thus, neonatal thymectomy affects the composition of the Treg population.

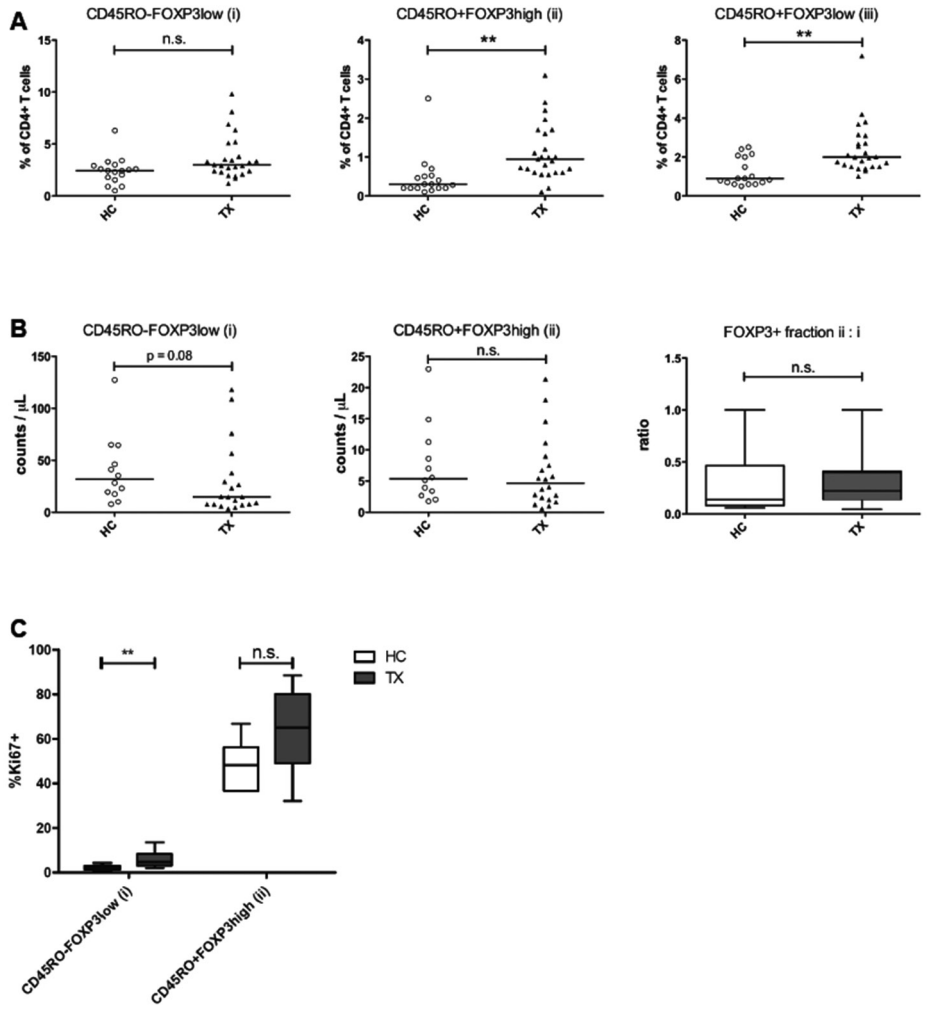

Figure 2. FOXP3$^+$ subpopulation dynamics after thymectomy. (**A**) Percentage of the three FOXP3$^+$ subpopulations within CD4 T cell population; (**B**) Cell count of the two FOXP3$^+$ Treg populations in healthy controls (HC) (○) and thymectomized patients (TX) (▲), and ratio of fraction ii : i; (**C**) Expression of Ki67 per FOXP3$^+$ Treg population. n.s. no significant difference, *P<0.05, **P<0.001.

When we examined the proliferation of the two "true" Treg subpopulations, a clear hierarchy was observed with a 10-fold higher percentage of Ki67 expressing cells in CD45RO+FOXP3high (Fr. II) compared to the naive CD45RO-FOXP3low Treg population in both thymectomized and healthy individuals. Following thymectomy, an increase in proliferation of both subpopulations was found compared to the healthy controls, reaching statistical significance in the naive Treg fraction (Fr. I) (Figure 2C). Together, these data suggest that peripheral proliferation of both naive and activated Treg compensated for the loss of thymic output, resulting in maintenance and increase of percentages of naive Treg and activated Treg populations respectively (Figure 2A). Although it is most likely that increased expansion of the activated Treg population is responsible for maintaining numbers of activated Treg in these thymectomized children (Figure 2B), we cannot exclude the possibility of additional increased conversion of naive Treg to activated CD45RO+FOXP3high Treg.[4] In a subgroup of patients naive CD45RO-FOXP3low Treg were low despite increased proliferation, which appeared most prominent in the children above 6 months of age (Figure E4). A shift in the balance between naive and memory Treg has been associated with several pathological conditions. Reduced naive Treg with a compensatory increase in memory Treg has been associated with multiple sclerosis[8] and sarcoidosis,[4] while an increase in naive Treg, albeit with impaired suppressive function, has been observed in active systemic lupus erythematosus.[4;9] Thus, removal of the thymus in the first month of life may affect immune regulation later in life. Overall, it is prudent to spare thymic tissue in patients requiring congenital heart surgery when technically possible.

This study demonstrates the specific homeostatic control of two distinct $FOXP3^+$ Treg populations. Peripheral proliferation of Treg cells counteracted the effect of loss of thymopoiesis, which illustrates the relative plasticity of the human immune system. However, changes in composition of the Treg population do warrant further investigation in the long-term functional effects of neonatal thymectomy following cardiac surgery.

Methods

Study population and blood specimens

Twenty-six patients were included in this study that all had undergone complete thymectomy at neonatal age during surgical correction for a congenital heart defect at the children's heart center, University Medical Center Utrecht, The Netherlands. The thymus is routinely removed during surgery involving the major vessels, such as transposition of the great arteries, hypoplastic heart syndrome, and hypoplastic aortic arch due to its anatomical obstruction in relation to the heart. A healthy, age-matched, control group was included from both patients admitted for correction of a heart defect which did not necessitate removal of the thymus such as ventricular septum defects (n=9) and healthy children who visited the University Medical Center Utrecht to undergo elective urologic or plastic surgery (n=8). All included patients were considered immunologically healthy because they did not have a recent history of infectious disease or a

hematologic or immunologic disorder. Patients with a known syndrome or genetic disorder were excluded (e.g. 22q11 deletion, trisomy 21). Characteristics of the 26 included patients and healthy controls are depicted in Table 1. As cell counts were not available for all samples, absolute numbers of cell populations are not shown for all study subjects.

Cell preparation and flow cytometry

Peripheral blood mononuclear cells were isolated from heparinized blood samples using the Ficoll Isopaque density gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden), and viably frozen and stored in liquid nitrogen until further processing. Characterization of the T-cell compartment was performed on thawed cryopreserved peripheral blood mononuclear cells that were washed in FACS buffer (PBS containing 2% FCS and 0.1% sodium azide) and blocked with normal mouse and rat serum. The cells were incubated in 50 microliter FACS buffer containing the appropriately diluted antibodies against human CD3, CD4, CD45RO and CD31. For intracellular staining of Ki-67 and FOXP3, the cells were first surface stained, followed by fixation and permeabilization according to the manufacturer's protocol. Antibodies against CD4 (clone SK3), CD31 (WM59) were obtained from BD Bioscience, CD45RO (UCHL1) from Caltag, Ki67 (MIB-1) from Immunotech, FOXP3 (PCH101) from eBioscience. Finally, stained mononuclear cells were washed twice in FACS buffer and run on a LSRII and analyzed by FACSDiva software (BD Biosciences). The gates for the different populations were kept identical for each experiment containing both thymectomized patients and healthy controls.

Statistics

To analyze the quantitative differences between thymectomized patients and healthy age-matched controls only data from after thymectomy was included. Statistical significance between the two groups was assessed using the Mann-Whitney U test for unpaired data, and chi-squared test for dichotomous data. Statistical difference is indicated with * for $P<0.05$ and ** for $P<0.001$.

Acknowledgements

We thank Sigrid Otto for assistance in performing flow cytometry assays.

References

1. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell 2008;133:775-787.
2. Long SA, Rieck M, Tatum M et al. Low-dose antigen promotes induction of FOXP3 in human $CD4^+$ T cells. J.Immunol. 2011;187:3511-3520.
3. Tulic MK, Andrews D, Crook ML et al. Changes in thymic regulatory T-cell maturation from birth to puberty: differences in atopic children. J.Allergy Clin.Immunol. 2012;129:199-206.
4. Miyara M, Yoshioka Y, Kitoh A et al. Functional delineation and differentiation dynamics of human $CD4^+$ T cells expressing the FoxP3 transcription factor. Immunity. 2009;30:899-911.
5. Gent van R, Schadenberg AW, Otto SA et al. Long-term restoration of the human T-cell compartment after thymectomy during infancy: a role for thymic regeneration? Blood 2011;118:627-634.
6. Braber den I, Mugwagwa T, Vrisekoop N et al. Maintenance of peripheral naive T cells is sustained by thymus output in mice but not humans. Immunity. 2012;36:288-297.
7. Wang J, Ioan-Facsinay A, van der Voort E, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory $CD4^+$ T cells. Eur.J.Immunol. 2007;37:129-138.
8. Haas J, Fritzsching B, Trubswetter P et al. Prevalence of newly generated naive in regulatory T cells (Treg) is critical for Treg suppressive function and determines Treg dysfunction multiple sclerosis. J.Immunol. 2007;179:1322-1330.
9. Pan X, Yuan X, Zheng Y et al. Increased CD45RA+ FoxP3(low) regulatory T cells with impaired suppressive function in patients with systemic lupus erythematosus. PLoS.One. 2012;7:e34662.

Supporting information (Online respository)

Table E1. Study population characteristics.

	Thymectomy	Control	P value
Number of subjects	26	17	
Age (months)	11.4 ± 10.9	10.8 ± 12.6	0.62
Age at TX (days)	10.0 ± 9.0	-	
Female : Male	9 : 17	6 : 11	0.96

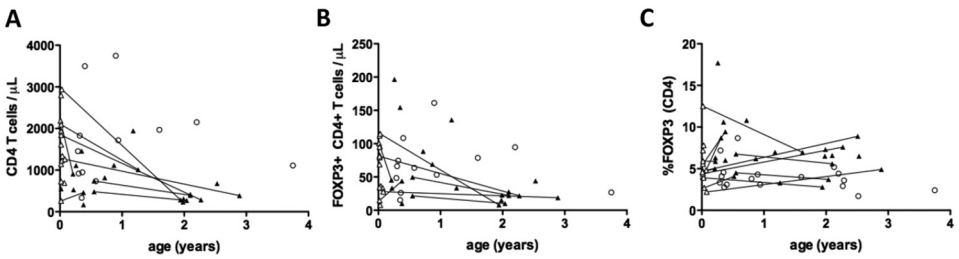

Figure E1. FOXP3 percentages and counts in the first years after neonatal thymectomy. (A) Absolute $CD4^+$ T cell counts per microliter of blood; (B); $FOXP3^+CD4^+$ Treg cell counts per microliter of blood (C) Percentage $FOXP3^+$ cells in $CD4^+$ T cell populations. ▲ represents values after thymectomy; △ samples taken just prior to thymectomy and ○ healthy controls. Lines connect longitudinal samples.

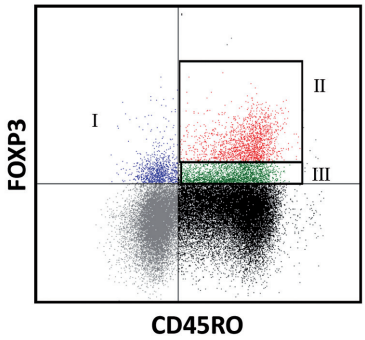

Figure E2. Gating strategy of subpopulations $FOXP3^+$ T cells. $FOXP3^+$ subpopulations after gating for $CD4^+$ lymphocytes. Fraction I; CD45RO-$FOXP^{3low}$, fraction II; CD45RO+$FOXP3^{high}$, fraction III; CD45RO+$FOXP3^{low}$.

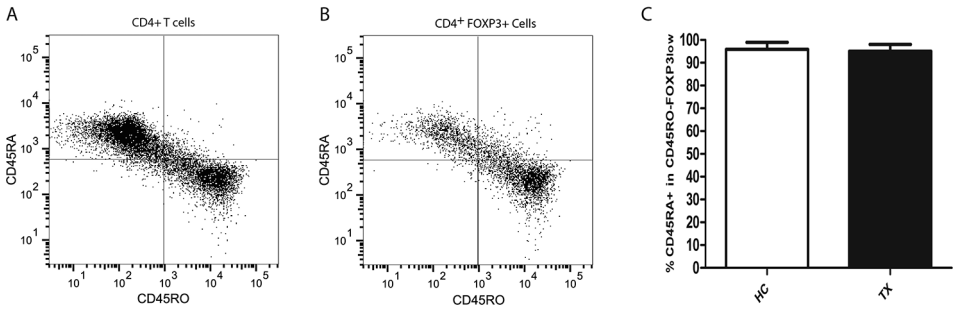

Figure E3. CD45RO-Foxp3$^+$ T cells represent CD45RA$^+$Foxp3$^+$ T cells. (**A**) Dot plot of CD45RO (memory) and CD45RA (naive) expression on CD3$^+$CD4$^+$ T cells. (**B**) Dot plot of CD45RO and CD45RA expression on CD3$^+$CD4$^+$Foxp3 (Treg) cells. (**C**) Expression of CD45RA$^+$ in CD45RO-FOXP3$^+$ subpopulation. Healthy control group (HC, n=7) thymectomized patients (TX, n=8). Data represented as mean percentage ± SD.

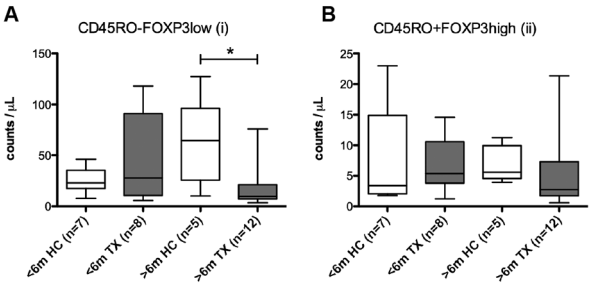

Figure E4. Subgroup analysis of naive and memory Treg populations in 0 – 6 months and >6 months old subjects. (**A**) CD45RO-FOXP3low naive Treg and (**B**) CD45RO+FOXP3high memory Treg cell numbers in the subgroups <6 months and >6 months of age in healthy controls (HC) and thymectomized patients (TX). Data represented as median, 25% and 75% percentile boxes and range. *P<0.05

Chapter

6

LONG-TERM RESTORATION OF THE HUMAN T-CELL COMPARTMENT AFTER THYMECTOMY DURING INFANCY: A ROLE FOR THYMIC REGENERATION?

R van Gent*[1], AWL Schadenberg*[2,3], SA Otto[1], RAJ Nievelstein[4], GT Sieswerda[5], F Haas[6], F Miedema[1], K Tesselaar[1], NJG Jansen[2], JAM Borghans[1]

[1] Dep. of Immunology,
[2] Dep. of Pediatric Intensive Care
[3] Dep. of Pediatric Immunology
[4] Dep. of Pediatric Radiology
[5] Grown-up Congenital Heart Center,
[6] Dep. of Pediatric Cardiothoracic Surgery
University Medical Center Utrecht, The Netherlands

* These authors contributed equally to this paper

Blood 2011. 118: 627-634

Abstract

Thymectomy during early childhood is generally thought to have serious consequences for the establishment of the T-cell compartment. In the present study, we investigated the composition of the T-cell pool in the first 3 decades after thymectomy during infancy due to cardiac surgery. In the first 5 years after thymectomy, naive and total $CD4^+$ and $CD8^+$ T-cell numbers in the blood and T-cell receptor excision circle (TREC) levels in $CD4^+$ T cells were significantly lower than in healthy age-matched controls. In the first years after thymectomy, plasma IL-7 levels were significantly elevated and peripheral T-cell proliferation levels were increased ~ 2-fold. From 5 years after thymectomy onward, naive $CD4^+$ and $CD8^+$ T-cell counts and TRECs were within the normal range. Because TREC levels are expected to decline continuously in the absence of thymic output, we investigated whether normalization of the naive T-cell pool could be due to regeneration of thymic tissue. In the majority of individuals who had been thymectomized during infancy, thymic tissue could indeed be identified on magnetic resonance imaging scans. Whereas thymectomy has severe effects on the establishment of the naive T-cell compartment during early childhood, our data suggest that functional regrowth of thymic tissue can limit its effects in subsequent years.

Introduction

The thymus is essential for the establishment of the peripheral T-cell population during childhood. Although the perivascular space in the thymus is progressively replaced by fat and thymic naive T-cell production declines significantly with age, even in adulthood the thymus has been shown to be able to produce new naive T cells.[1-8] There is a lot of controversy, however, as to what extent thymic output contributes to naive T-cell maintenance during adulthood. Based on T-cell receptor excision circle (TREC), the by-products of V(D)J recombination in the thymus, data from healthy adults, we have recently estimated that in young adults, approximately 10% of the naive T-cell pool was originally formed by the thymus, while the remaining 90% was produced through peripheral T-cell proliferation (Den Braber et al. submitted). Also in childhood, peripheral T-cell proliferation plays an important role in the establishment of the naive T-cell compartment.[9-11] Nevertheless, the thymus is thought to be crucial in T-cell generation, especially at younger ages, since it is the only source of T-cell diversity.

Thymectomy is an accepted treatment for patients with myasthenia gravis (MG). It was previously shown that thymectomy of adult MG patients did not affect the absolute number of T cells in the peripheral blood, whereas it could lead to reduced numbers of TREC-containing T cells.[12;13] These data suggested that maintenance of the naive T-cell compartment during adulthood does not heavily rely on thymic output. It is important to realize, however, that insights obtained from thymectomy in MG patients may be confounded by the autoimmune features of the disease and/or by the immunosuppressive treatment that patients receive.

Because of the crucial role of the thymus in the establishment of the peripheral T-cell compartment in early life, several studies have investigated the effect of thymectomy at an early age on the developing immune system.[14-18] In pediatric cardiac surgery, thymectomy is performed to gain an unrestricted view of the operation site. Especially in neonates, in whom the thymus is relatively large, surgical procedures involving the large vessels necessitate complete removal of the thymus. Thymectomy at an early age has been shown to result in reduced $CD4^+$ and $CD8^+$ T-cell numbers, largely due to reduced naive T-cell counts, in the first years after thymectomy. These changes in the T-cell pool at an early age occur without obvious clinical consequences,[14;19;20] although diminished responses to tick-borne encephalitis have been reported.[18] The long-term effects of thymectomy during early childhood on the composition of the T-cell compartment are less unequivocal. Whereas some thymectomized individuals showed reduced total and naive $CD4^+$ and $CD8^+$ T-cell counts[15;21;22] and clear signs of premature immunosenescence[22] in the second and third decades of life, many other thymectomized individuals have peripheral T-cell pools comparable to those of age-matched healthy controls.[15;22] It remains to be elucidated how the T-cell pool can be maintained after removal of the thymus during early childhood. Increased understanding of the mechanisms involved in T-cell maintenance could help us understand why disparities in the T-cell compartment after thymectomy during early childhood persist in some patients but not in others.

In the present study, we investigated both the short-term and long-term effects of thymectomy during infancy on the establishment and maintenance of the naive T-cell compartment in 39 patients who were thymectomized between 2 months to 31 years previously. By measuring T-cell subsets, Ki67 expression levels, IL-7 levels in plasma, and TRECs, we investigated the mechanisms by which the naive T-cell pool is maintained after removal of the thymus during infancy.

Materials and methods

Study population and blood specimens

Thirty-nine patients, who had undergone complete thymectomy during infancy because of surgery to treat a congenital heart defect at the Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands, were included in this study. Surgery involving the major vessels, such as transposition of the great arteries (TGA), hypoplastic left heart syndrome (HLHS), and hypoplastic aortic arch with or without coarctation of the aorta routinely necessitates thymectomy. The age at which these patients were thymectomized ranged from 0.0 to 1.5 years (median age: 0.03 years). Blood samples were taken prior to thymectomy if feasible, and during clinical follow-up, ranging from 2 months to 31 years after thymectomy. Exclusion criteria were clinical signs of infection at time of blood draw and the presence of a syndrome or genetic disorder (e.g. 22q11 deletion). Characteristics of the 39 thymectomized participants are shown in Table 1.

In addition to the above study group, we retrospectively evaluated magnetic resonance imaging (MRI) scans from another 24 patients who underwent complete thymectomy during infancy because of an arterial switch operation for a TGA. The age at which these patients were thymectomized ranged from 0.0 to 1.5 years (median age: 0.03 years).

A healthy control group consisted of 102 age-matched healthy children, aged 0.1 – 18.0 years, who visited the University Medical Center Utrecht to undergo elective urologic or plastic surgery. The children were considered immunologically healthy because they did not have a history of infectious diseases or a hematologic or immunologic disorder. Adult blood samples were collected from 52 healthy volunteers, aged 21.0 – 39.7 years.

The study was approved by the medical ethical committee of the University Medical Center Utrecht and written informed consent was obtained from all study participants or their legal guardians in agreement with the Helsinki Declaration of 1975, revised in 1983.

Visualization of thymic tissue after thymectomy

To determine whether the thymus remained absent after thymectomy, the presence of thymic tissue in patients was evaluated during follow-up. Fourteen of the 39 patients underwent a secondary operation as part of a multistage procedure during which the surgical team determined the macroscopic presence or absence of thymic tissue. For 8 patients who did not

require additional surgery, presence of the thymus after thymectomy was evaluated by MRI; in total, 14 MRI scans were performed in this group (Table 1). In addition, we retrospectively evaluated 34 MRI scans from another 24 patients who all underwent thymectomy during infancy for an arterial switch operation for a TGA, for the presence of thymic tissue. The presence or absence of thymic tissue on MRI scans was evaluated by an experienced pediatric radiologist. If present, the size of the thymic mass was quantified as either normal or smaller than expected for the age of the patient.[23;24]

Cell preparation and flow cytometry

PBMCs were obtained by Ficoll-Paque density gradient centrifugation, and viably frozen and stored in liquid nitrogen until further processing. Characterization of the T-cell compartment was performed on thawed cryopreserved PBMCs, which were incubated with mAb to CD4-Pacific Blue, CD8-AmCyan, CD8-PerCP-Cy5.5, CD27-APC, CD45RO-PE (Becton Dickinson), CD3-Pacific Blue, CD4-APC-AF750, CD8-APC-AF750, CD45RO-PE-Cy7 (eBioscience). Within the $CD4^+$ and $CD8^+$ T-cell compartment, naive ($CD27^+CD45RO^-$) and memory ($CD45RO^+$) subsets were identified.[25] To determine T-cell proliferation levels, thawed PBMCs were stained intracellularly with Ki67-FITC (Dako) after fixation and permeabilization with Cytofix/Cytoperm™ and Perm/Wash™ according to manufacturer's instructions (Becton Dickinson). After washing with PBS, cells were analyzed on an LSRII and analyzed by FACSDiva version 6.1.3 software (Becton Dickinson).

Absolute lymphocyte numbers were determined with a Cell-Dyn Sapphire™ Hematology Analyzer (Abbott Diagnostics, Hoofddorp, The Netherlands) and were used to calculate absolute numbers of signal joint T-cell receptor excision circles (TRECs), total T-cell counts and cell numbers within the different T-cell subsets.

MACS cell separation

To measure the total number of TRECs and TREC content of $CD4^+$ T cells, these subsets were purified from thawed PBMCs by magnetic-bead separation using the MiniMACS multisort kit according to manufacturer's instructions (Miltenyi Biotec Inc). Purity of MACS-sorted $CD4^+$ T cells was >90%.

TREC analysis

DNA was isolated using the NucleoSpin Blood QuickPure kit according to manufacturer's instructions (Macherey-Nachel). TREC numbers were quantified by real-time PCR, as described previously.[26;27] The TREC content per T cell was calculated by dividing the TREC content by 150.000 (assuming that 1µg DNA corresponds to 150.000 T cells).

Table1. Patient and sample characteristics

Patient	Sex	Heart defect	Age at TX, y	Age at blood draw, y			Age at MRI, y (results)‡			
T01*	m	TGA	0.0	0.2						
T02	m	HLHS	0.0	0.3†						
T03*	m	HRHS	0.3	0.5†	3.4	4.5†				
T04	f	HLHS	0.0	0.6	2.0†					
T05	m	PA	0.0	0.7†						
T06	m	CoA	0.3	0.9						
T07	m	TGA	0.1	1.0						
T08*	f	PA	0.0	1.3						
T09	m	TGA	0.0	1.9†						
T10*	m	TGA	0.0	1.9						
T11	m	TGA	0.1	2.0						
T12	f	HLHS	0.0	2.0†						
T13	m	TGA	0.0	2.1†						
T14	m	HLHS	0.0	2.1†						
T15*	m	TGA	0.0	2.3						
T16	f	HLHS	0.0	2.4†						
T17	m	CoA	0.1	2.5†						
T18*	m	TGA	0.1	2.9						
T19	f	AoH	0.0	3.6†						
T20	f	TvA	0.6	6.7†						
T21	m	TGA	0.0	7.6						
T22	f	TGA	0.0	8.0						
T23	m	TvA	0.1	8.6†						
T24	m	TGA	0.0	10.4						
T25	f	TGA	0.0	10.7			10.6(–)			
T26	m	TGA	0.1	12.4†						
T27	m	TGA	0.0	12.8						
T28	m	TGA	0.0	15.6						
T29	m	TGA	0.0	18.2			19.3(+)			
T30	m	TGA	0.0	18.6			3.9(s)	7.7(s)	16.5(+)	18.2(+)
T31	m	TGA	0.2	20.7						
T32	m	TGA	0.0	21.8						
T33	m	TGA	0.0	23.1			18.9(+)	21.6(+)	22.9(+)	
T34	m	TGA	0.0	23.9			19.7(–)	22.3(–)		
T35	m	TGA	0.0	24.1						
T36	f	TGA	0.6	24.7			21.2(+)			
T37	m	TGA	0.6	25.1			22.3(+)			
T38	f	TGA	1.1	31.5			28.8(+)			
T39	f	TGA	1.5	32.9						

HLHS indicates hypoplastic left heart syndrome; HRHS: hypoplastic right heart syndrome; PA: pulmonary atresia; CoA: aortic coarctation; AoH: aortic hypoplasia; TvA: tricuspid valve atresia; and TX: thymectomy. *Patients for whom a sample was collected prior to thymectomy. †Samples collected just prior to a secondary operation. During none of these operations could thymic tissue be observed macroscopically. ‡For MRI results, (−) indicates no thymic tissue on MRI; (s), thymus visible on MRI, but small for the age of the individual; and (+), thymus visible on MRI and normal for the age of the individual.

Plasma IL-7 levels

IL-7 in heparinized plasma from patients after thymectomy and age-matched healthy controls was determined by multiplex immunoassay as described previously.[28]

Statistics

To assess quantitative differences between thymectomized individuals and healthy controls while taking into account age-related changes in various immunologic parameters, the study group and the control group were separated into 2 age-matched groups. The first group contained individuals younger than 5 years of age, and consisted of 19 individuals (mean age, 1.9 ± 1.1 years; range, 0.2 – 4.5 years) who had been thymectomized at an age between 0.0 and 0.3 years, and 48 healthy controls (mean age, 1.8 ± 1.3 years; range, 0.1 – 4.6 years). The second group contained individuals older than 5 years of age, and consisted of 17 individuals (mean age, 16.6 ± 7.6 years; range, 6.7-31.5 years) who had been thymectomized at an age between 0.0 and 1.5 years, and 50 healthy controls (mean age, 14.3 ± 6.4 years; range, 5.1 – 35.0 years). Differences in T-cell (subset) counts and percentages, IL-7 levels in plasma, average TREC contents, TREC numbers per microliter of blood, and Ki67 expression levels in naive $CD4^+$ and $CD8^+$ T cells were assessed using the Mann-Whitney *U* test for unpaired data. Correlation between IL-7 levels in plasma and the percentage of $Ki67^+$ cells in the naive $CD4^+$ T-cell compartment was determined by the non-parametric Spearman's rank correlation coefficient (as denoted by r_s). To avoid any biases from dependent data in our analyses, of patients for whom longitudinal data were available, only the last data point was included in the statistical analyses, unless indicated otherwise. Differences were considered to be statistically significant when $P<0.05$.

Results

Impact of thymectomy during infancy on the $CD4^+$ and $CD8^+$ T-cell compartments

We studied the composition of the $CD4^+$ and $CD8^+$ T-cell compartments of 39 individuals who were thymectomized between 2 months and 31 years previously (Table 1). All participants underwent a complete thymectomy for surgery to treat a congenital heart defect at 0.0 – 1.5 years of age (median age, 0.03 years); 30 of the 39 individuals where younger than 1 month of age when the thymus was removed. None of the thymectomized individuals had any history of symptomatic infections nor did any develop opportunistic infections during follow-up.

First we determined the early impact of thymectomy on the constitution of the lymphocyte population. Blood samples before thymectomy (median age: 0.03 years) were available from 6 individuals, and showed that total, naive and memory $CD4^+$ and $CD8^+$ T cell counts per microliter of blood were similar to those of age-matched controls (Figure 1). Cross-sectional data showed that in the first 5 years after thymectomy, $CD4^+$ and $CD8^+$ T-cell counts had significantly declined to levels below those of age-matched controls ($P<0.001$ and $P<0.001$ respectively, Figure 1A-B), which was mainly due to a rapid decline of naive $CD4^+$ ($P<0.001$)

and CD8$^+$ (P<0.001) T-cell numbers (Figure 1A-B). In contrast, memory CD4$^+$ (P=0.12) and CD8$^+$ (P=0.06) T-cell counts per microliter of blood had increased to a similar extent as observed for healthy age-matched controls. As a result, the percentages of naive cells in the CD4$^+$ and CD8$^+$ T-cell pools of thymectomized individuals were significantly lower than healthy control values (P<0.001 and P=0.014, respectively, Figure 1C). Total CD4$^+$ and CD8$^+$ T-cell counts were similarly affected in the first 5 years after thymectomy because CD4:CD8 ratios in thymectomized individuals were comparable to those in healthy controls (P=0.23; data not shown). The effects on the constitution of the CD4$^+$ and CD8$^+$ T-cell compartments in the first 5 years after thymectomy were confirmed in the individuals for whom longitudinal data were available (Figure 1).

Despite the clear impact of thymectomy on naive and total CD4$^+$ and CD8$^+$ T-cell numbers during the first years of life, from 5 years after thymectomy onward, the majority of thymectomized individuals had normal total, naive and memory CD4$^+$ and CD8$^+$ T-cell numbers in the blood (P>0.15 Figure 1A-B). Even the percentages of naive cells in the CD4$^+$ and CD8$^+$ T-cell pools, which were so clearly affected in the first 5 years after thymectomy, normalized in the long term (P=0.46 and P=0.41, respectively; Figure 1C). In addition, CD4:CD8 ratios were comparable to healthy control values (P=0.19; data not shown).

Effect of thymectomy on naive T-cell proliferation levels

Since IL-7 is known to be essential for the survival and homeostatic proliferation of naive T cells, and because its availability has been shown to be inversely related to the size of the naive T-cell population,[29,30] we hypothesized that increased IL-7 levels in the first years after thymectomy might contribute to restoration of the naive T-cell compartment in the long term. In the present study, the level of IL-7 in plasma from thymectomized children in the first 2.5 years after thymectomy was indeed significantly higher (P=0.012) than in healthy age-matched controls (Figure 2A). In agreement with previous findings,[16,30] the IL-7 levels in thymectomized children were inversely correlated with naive CD4$^+$ T-cell numbers (r_s=- 0.73, P=0.01; Figure 2B).

To investigate whether elevated levels of IL-7 in thymectomized individuals are correlated with increased levels of peripheral T-cell proliferation, we measured the fraction of naive CD4$^+$ and CD8$^+$ T cells expressing the proliferation marker Ki67 in the first 5 years after thymectomy. Despite the clear depletion of the naive T-cell pool and increased plasma levels of IL-7 during the first years after thymectomy, median naive CD4$^+$ and CD8$^+$ T-cell proliferation levels were significantly (P=0.03 and P=0.01, respectively) but not drastically elevated compared with healthy controls (Figure 2C-D). There was no significant correlation between the percentage of Ki67$^+$ expressing naive CD4$^+$ T cells and plasma IL-7 levels (r_s=-0.03, P=0.94; data not shown).

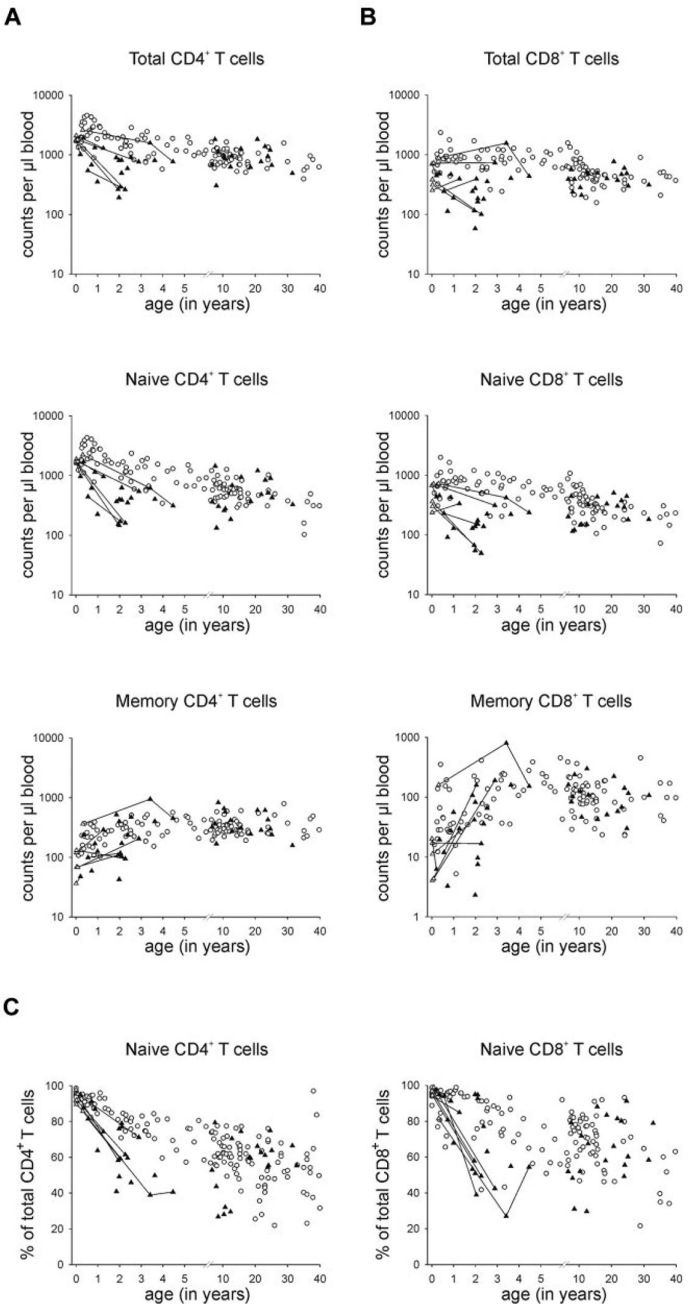

Figure 1. T-cell counts and percentages of naive T-cell subsets. (**A**) Total, naive and memory $CD4^+$ T cell numbers in counts per µl blood. (**B**) Total, naive and memory $CD8^+$ T cell numbers in counts per µl blood. (**C**) Percentage of naive $CD4^+$ and naive $CD8^+$ T cells. Filled triangles (▲) represent values after thymectomy, open triangles (△) represent samples taken just prior to thymectomy and open circles (○) represent healthy controls. Lines connect longitudinal samples (n=7).

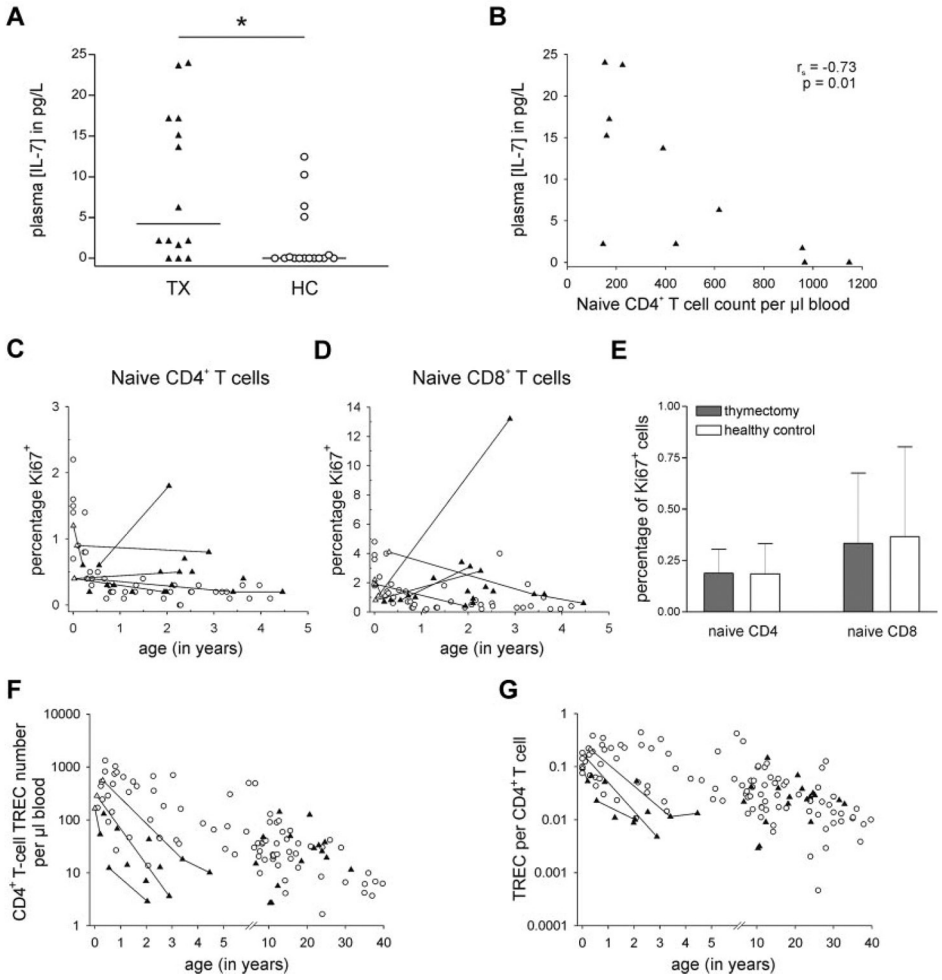

Figure 2. Plasma IL-7 levels, Ki67 expression and TRECs after thymectomy during infancy. (**A**) Plasma IL-7 levels in the first 2.5 years post-thymectomy (TX, n=14) compared to age-matched healthy controls (HC, n=16). Asterisk denotes statistical significance; horizontal line represents median value for each group. (**B**) Correlation between plasma IL-7 levels and the number of naive $CD4^+$ T cells per µl blood in thymectomized individuals during the first 2.5 years post-thymectomy (n=11). (**C**) The percentage of proliferating ($Ki67^+$) cells in the naive $CD4^+$ and (**D**) naive $CD8^+$ T-cell populations in the first 5 years following thymectomy compared to healthy age-matched control values. Lines connect longitudinal samples. (**E**) The percentage of proliferating ($Ki67^+$) cells (median value + standard deviation) in the naive $CD4^+$ and naive $CD8^+$ T-cell populations in thymectomized individuals from 5 years post-thymectomy onward (n=9) compared to age-matched healthy controls (n=47). (**F**) The average TREC content of $CD4^+$ T cells, and (**G**) the total number of $CD4^+$ T-cell TRECs per µl blood in thymectomized individuals and healthy controls as a function of age. Filled triangles (▲) represent values after thymectomy, open triangles (△) represent samples taken just prior to thymectomy and open circles (○) represent healthy controls. Lines connect longitudinal samples.

In the long term, when naive T-cell numbers had been restored to normal levels despite thymectomy during infancy, the percentages of proliferating naive $CD4^+$ and $CD8^+$ T cells in individuals who had been thymectomized were no longer elevated (P=0.60 and P=0.52, respectively; Figure 2E).

Changes in TRECs after thymectomy

To investigate whether the eventual restoration of the T-cell pool after thymectomy was (in part) due to *de novo* T-cell production, we measured TRECs, the by-products of V(D)J rearrangement that are uniquely formed during T-cell development, in $CD4^+$ T cells of thymectomized children and age-matched healthy controls. Changes in the total number of TRECs per microliter of blood reflect changes in *de novo* T-cell production or in T-cell death rates. Conversely, the average number of TRECs per T cell (the so-called TREC content) is also strongly affected by peripheral T-cell division.[27]

In thymectomized individuals, total $CD4^+$ T-cell TREC numbers per microliter of blood had declined more rapidly (P<0.001) during the first 5 years after thymectomy than in healthy age-matched controls (Figure 2F). Such an accelerated decline was to be expected, because in the absence of new cells from the thymus, total TREC numbers per microliter of blood decrease with every cell that dies.[31] Nevertheless, we observed that total $CD4^+$ T-cell TREC numbers per microliter of blood normalized in the long term (P=0.66), and showed no further significant decline from 5 years after thymectomy onward (P=0.72, Figure 2F). Similarly, the average TREC content of $CD4^+$ T cells in thymectomized individuals had declined faster in the first 5 years after thymectomy than in healthy age-matched controls (P<0.001; Figure 2G). This accelerated decline of TREC contents was to be expected, not only because T-cell proliferation levels in thymectomized individuals were somewhat higher than in healthy controls (Figure 2C-D), but also because in the absence of *de novo* T-cell production, TREC contents are diluted whenever a cell divides in the periphery.[31] Remarkably, from 5 years after thymectomy onward, we observed no further significant decrease in $CD4^+$ T-cell TREC contents in thymectomized individuals (P=0.72), such that $CD4^+$ T-cell TREC contents of thymectomized individuals became similar to age-matched healthy control values (P=0.80; Figure 2G). These data suggest that after an initial large impact of thymectomy during early childhood, the T-cell compartment restored through production of new TREC-containing naive T cells.

Recurrence of thymic tissue long term after thymectomy

We investigated whether regrowth of thymic tissue could have been responsible for the generation of new TREC-containing naive T cells by analyzing MRI scans of the chest (Figure 3). These scans had been performed for clinical reasons in 8 patients from our study group (median age, 19.5 years; range, 3.9 – 28.8 years). Thymic tissue could be identified on scans from 6 of the 8 patients (Table 1). To further substantiate this finding, MRI scans from an additional group of 24 patients thymectomized during an arterial switch operation (median age

at thymectomy, 0.03 years) were assessed to determine the presence of thymic tissue (median age, 9.6 years; range, 4.0 – 28.0 years). Combined with the MRI scans of the study group, a total of 48 scans from 32 patients were available. In 4 of the 32 patients, no thymic tissue could be observed. From one of these patients, a second scan was available, made 2.5 years later, which still showed no evidence of thymic tissue. In 7 individuals, thymic tissue was present but smaller than expected for the age of the individual. In one of these patients, the thymus size remained small on subsequent scans, whereas 5 of these patients eventually reached a normal thymus sizes on subsequent scans. In 26 individuals, thymic tissue eventually reached a size comparable to age-matched healthy controls (Figure 4). Whenever thymic tissue could be identified on an MRI scan, any follow-up scans from the same individual always reconfirmed the evidence for thymic tissue. Whenever the size of the thymic tissue had become normal for the age of the individual, the thymus size on follow-up scans always remained normal.

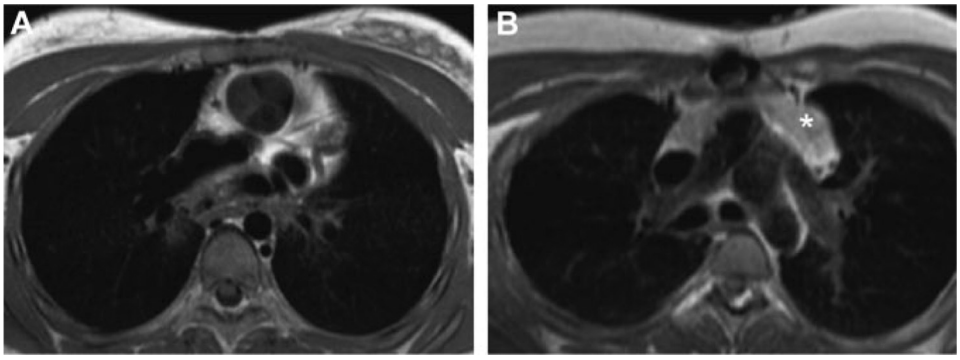

Figure 3. Visualisation of thymic tissue in thymectomized individuals. (**A**) MRI of a patient (T25) 10.6 years post-thymectomy with no evidence of thymic tissue. (**B**) MRI of a patient (T30) 16.5 years after neonatal thymectomy showing thymic tissue (*).

We also investigated whether thymic tissue could already be identified at younger ages during secondary surgical procedures. In none of the 14 individuals who underwent a secondary operation (at a median age of 2.1 years) could the surgical team observe thymic tissue at the site of operation (Table 1).

These data suggest that slow regrowth of thymic tissue was responsible for the eventual normalization of the initially strongly affected peripheral T-cell compartments of individuals thymectomized during infancy.

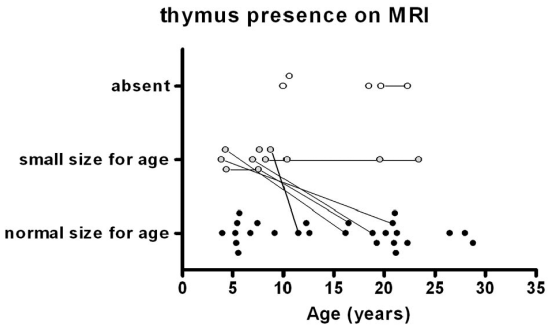

Figure 4. Presence of thymic tissue on MRI scans of patients thymectomized during infancy. Summary of MRI scans of 32 patients (study group: 8 patients, and additional group: 24 patients) after surgery for a CHD. Open circles represent MRI scans with no evidence of thymic tissue. Grey dots represent scans with evidence of thymic tissue, but small for the age of the individual. Filled circles represent MRI scans with evidence of thymic tissue of similar size as healthy age-matched controls. Lines connect consecutive scans of the same patient (n=7). Only 42 of the 48 MRI scans that were made are plotted in this figure; once an MRI scan of a patient showed thymic tissue of normal size for the age of the individual, any follow-up MRI scans (which consistently reconfirmed the normalization of thymic tissue) were not plotted in this figure.

Discussion

Thymectomy at an early age is frequently performed during surgical correction of congenital heart defects. Several studies have shown that in the first years after thymectomy at an early age, the composition of the T-cell compartment is dramatically affected.[14-18] The long-term effects of thymectomy at an early age are much less unequivocal, however, and aberrations in size and composition of the T-cell compartment have been reported in some patients but not in others.[15;21;22] In the present study, we investogated the mechanisms responsible for the long-term restoration of the T-cell compartment after thymectomy during infancy. In agreement with earlier reports, we found that in the first years after thymectomy, T-cell numbers were severely reduced compared to healthy age-matched controls, with naive T-cell counts affected most severely. From 5 years after thymectomy onward, however, the T-cell compartment in most individuals had a normal size and composition.

We investigated whether increased survival or proliferation of naive T cells contributed to the normalization of the T-cell compartment. It was previously shown that IL-7 positively affects naive T-cell survival and proliferation in mice.[32;33] Although a potential role for IL-7 in naive T-cell homeostasis has been observed,[30;34;35] its effect on naive T-cell survival and proliferation in humans remains unclear. The negative correlation that we found between naive T-cell numbers and IL-7 levels in plasma in the first 2.5 years after thymectomy is in agreement with previous

observations in lymphopenic settings,[16;30] suggesting reduced consumption of IL-7 when naive T-cell numbers are low. Naive T-cell proliferation levels (as measured by Ki67-expression) were approximately 2-fold increased in the first years after thymectomy, suggesting that increased T-cell proliferation may contribute to the maintenance of the T-cell compartment after thymectomy. Remarkably, the elevated IL-7 levels shortly after thymectomy were not correlated with naive T-cell proliferation levels. We cannot exclude the possibility that the increased IL-7 levels in the first years after thymectomy nevertheless contributed to the restoration of the $CD4^+$ T-cell pool by increasing the survival of naive T cells.

Our TREC data strongly suggested that the eventual restoration of the T-cell pool after thymectomy during infancy was to a large extent due to *de novo* T-cell generation. The average TREC content of $CD4^+$ T cells and total $CD4^+$ T-cell TREC numbers per microliter of blood, which were clearly affected in the first 5 years after thymectomy, were found to be normal at later ages. In the absence of *de novo* T-cell production, TREC contents and total TREC numbers per microliter of blood are expected to continuously decline because of T-cell proliferation and T-cell death, respectively. The observed lack of decline in total $CD4^+$ T-cell TREC numbers per microliter of blood could in principle be explained by an extremely low death rate of $CD4^+$ T cells in thymectomized individuals. The absence of further TREC content dilution from 5 years after thymectomy onward would imply, however, that $CD4^+$ T-cells should also have stopped proliferating. The most likely explanation for our TREC findings is therefore that newly generated TREC-containing cells had been produced. In agreement with this, the majority of MRI scans available from individuals thymectomized during infancy showed evidence for thymic tissue as early as 4 years after thymectomy. Thymic tissue could never be observed during secondary surgeries. Since 11 of the 14 children who underwent a secondary surgery were under 4 years of age, the regrowing thymus in these children may have been too small or not visually accessible to be identified. Although the presence of thymic tissue on MRI scans does not imply that the tissue is capable of thymopoiesis, in combination with our TREC data and the normalization of the T-cell compartment in the long term, the most likely explanation for our findings is that renewed thymopoiesis was responsible for the long-term recovery of the T-cell compartment after thymectomy during infancy.

Although some studies have suggested enlargement of thymic mass after cessation of chemotherapy or following stem-cell transplantation,[3;36] to the best of our knowledge, formation of thymic tissue at the anatomical location of the thymus after its complete removal has not been reported previously. Recent studies in mice, however, have shown the potential of postnatal epithelial progenitor cells to generate functional thymic lobules.[37] If sufficient numbers of such progenitor cells are left behind during thymectomy, then these cells might be responsible for the regrowth of functionally competent thymic tissue in subsequent years. A likely reason why other studies did not find evidence for the *de novo* formation of thymic tissue after thymectomy is that previous studies have mainly used radiography to identify thymic tissue.[21;22] It has recently been shown that whereas thymic tissue should be identifiable during the first 2 decades of life

on MRI or computed tomography imaging in healthy individuals, identification by thoracic radiography is unreliable after the age of 3 years.[24]

The eventual restoration of the peripheral T-cell compartment that we observed in almost all participants is in agreement with previous studies reporting normal size, composition and functionality of the T-cell compartment in the second and third decade after thymectomy during early childhood in the majority of individuals.[15;22] However, the latter studies also showed that in some thymectomized individuals normalization did not occur. Moreover, a recent study reported that thymectomy resulted in diminished naive T-cell counts and naive T-cell TREC contents well into the third decade of life.[21] We can only speculate on the cause of these differences. A clear difference between the latter and the current study is the age at which the children were thymectomized. Whereas almost all patients (87%) included in our study were thymectomized within the first 4 months of life, and all before the age of 1.5 years (mean age at thymectomy, 0.16 years), the patients included in the study of Prelog et al were thymectomized at an average age of 2.6 years. Similarly, the patients with no residual thymus and decreased TREC contents in the study by Halnon et al were either monitored in the first 5 years after thymectomy (similar to our study) or had been thymectomized beyond the age of 4 years. It is tempting to speculate that the regenerating capabilities of the thymus may be age dependent and that the long-term effects of removal of thymic tissue in the first months of life may (rather surprisingly) be less dramatic than the long-term effects of thymectomy during later childhood. However, further studies, including analyses of T-cell repertoire diversity, are needed to confirm this proposition.

In summary, we have shown that whereas thymectomy during early childhood clearly affected the T-cell compartment during the first 5 years after thymectomy, such deviations from age-matched controls were not observed during later life. Because the normalization of the T-cell pool coincided with *de novo* T-cell production, as suggested by TREC data and by recurrence of thymic tissue on MRI scans, the most likely explanation for our data is that thymic regeneration was responsible for the long-term restoration of the T-cell compartment. Evidence that thymectomy early in life can lead to exacerbations in the T-cell compartment in cytomegalovirus-seropositive individuals[22] suggests that, despite the apparent ability of the T-cell compartment to recover from removal of the thymus during infancy, it may nevertheless be desirable to spare as much thymic tissue during cardiac surgery as possible.

Acknowledgements

This work was financially supported by the Netherlands Organisation for Scientific Research (NWO, grants 917.96.350 and 836.07.002 to J.A.M.B.), and the Wilhelmina Children's Hospital Research Fund (grant OZF 2007 to A.W.L.S.). We thank S.O. Algra and M.A. Siemelink from the Dept. of Pediatric Intensive Care and F. de Roo from the Dept. of Pediatric Cardiothoracic Surgery for their assistance in patient sampling, and B.J. Prakken for his valuable input in the design of this study.

References

1. Bertho JM, Demarquay C, Moulian N, Van Der Meeren A, Berrih-Aknin S, Gourmelon P. Phenotypic and immunohistological analyses of the human adult thymus: evidence for an active thymus during adult life. Cell Immunol. 1997;179:30-40.
2. Douek DC, McFarland RD, Keiser PH, et al. Changes in thymic function with age and during the treatment of HIV infection. Nature. 1998;396:690-695.
3. Hakim FT, Memon SA, Cepeda R, et al. Age-dependent incidence, time course, and consequences of thymic renewal in adults. J Clin Invest. 2005;115:930-939.
4. Jamieson BD, Douek DC, Killian S, et al. Generation of functional thymocytes in the human adult. Immunity. 1999;10:569-575.
5. Muraro PA, Douek DC, Packer A, et al. Thymic output generates a new and diverse TCR repertoire after autologous stem cell transplantation in multiple sclerosis patients. J Exp Med. 2005;201:805-816.
6. Poulin JF, Viswanathan MN, Harris JM, et al. Direct evidence for thymic function in adult humans. J Exp Med. 1999;190:479-486.
7. Steinmann GG. Changes in the human thymus during aging. Curr Top Pathol. 1986;75:43-88.
8. Vrisekoop N, den Braber I, de Boer AB, et al. Sparse production but preferential incorporation of recently produced naive T cells in the human peripheral pool. Proc Natl Acad Sci U S A. 2008;105:6115-6120.
9. Hazenberg MD, Otto SA, van Rossum AM, et al. Establishment of the $CD4^+$ T-cell pool in healthy children and untreated children infected with HIV-1. Blood. 2004;104:3513-3519.
10. van Gent R, van Tilburg CM, Nibbelke EE, et al. Refined characterization and reference values of the pediatric T- and B-cell compartments. Clin Immunol. 2009;133:95-107.
11. Bains I, Antia R, Callard R, Yates AJ. Quantifying the development of the peripheral naive $CD4^+$ T-cell pool in humans. Blood. 2009;113:5480-5487.
12. Sempowski G, Thomasch J, Gooding M, et al. Effect of thymectomy on human peripheral blood T cell pools in myasthenia gravis. J Immunol. 2001;166:2808-2817.
13. Storek J, Douek DC, Keesey JC, Boehmer L, Storer B, Maloney DG. Low T cell receptor excision circle levels in patients thymectomized 25-54 years ago. Immunol Lett. 2003;89:91-92.
14. Brearley S, Gentle TA, Baynham MI, Roberts KD, Abrams LD, Thompson RA. Immunodeficiency following neonatal thymectomy in man. Clin Exp Immunol. 1987;70:322-327.
15. Halnon NJ, Jamieson B, Plunkett M, Kitchen CM, Pham T, Krogstad P. Thymic function and impaired maintenance of peripheral T cell populations in children with congenital heart disease and surgical thymectomy. Pediatr Res. 2005;57:42-48.
16. Mancebo E, Clemente J, Sanchez J, et al. Longitudinal analysis of immune function in the first 3 years of life in thymectomized neonates during cardiac surgery. Clin Exp Immunol. 2008;154:375-383.
17. Wells WJ, Parkman R, Smogorzewska E, Barr M. Neonatal thymectomy: does it affect immune function? J Thorac Cardiovasc Surg. 1998;115:1041-1046.
18. Prelog M, Wilk C, Keller M, et al. Diminished response to tick-borne encephalitis vaccination in thymectomized children. Vaccine. 2008;26:595-600.
19. Eysteinsdottir JH, Freysdottir J, Haraldsson A, et al. The influence of partial or total thymectomy during open heart surgery in infants on the immune function later in life. Clin Exp Immunol. 2004;136:349-355.
20. Torfadottir H, Freysdottir J, Skaftadottir I, Haraldsson A, Sigfusson G, Ogmundsdottir HM. Evidence for extrathymic T cell maturation after thymectomy in infancy. Clin Exp Immunol. 2006;145:407-412.
21. Prelog M, Keller M, Geiger R, et al. Thymectomy in early childhood: significant alterations of the CD4(+) CD45RA(+)CD62L(+) T cell compartment in later life. Clin Immunol. 2009;130:123-132.
22. Sauce D, Larsen M, Fastenackels S, et al. Evidence of premature immune aging in patients thymectomized during early childhood. J Clin Invest. 2009;119:3070-3078.
23. Adam EJ, Ignotus PI. Sonography of the thymus in healthy children: frequency of visualization, size, and appearance. AJR Am J Roentgenol. 1993;161:153-155.

24. Nasseri F, Eftekhari F. Clinical and radiologic review of the normal and abnormal thymus: pearls and pitfalls. Radiographics;30:413-428.
25. Baars PA, Maurice MM, Rep M, Hooibrink B, van Lier RA. Heterogeneity of the circulating human $CD4^+$ T cell population. Further evidence that the $CD4^+CD45RA\text{-}CD27^-$ T cell subset contains specialized primed T cells. J Immunol. 1995;154:17-25.
26. Pongers-Willemse MJ, Verhagen OJ, Tibbe GJ, et al. Real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia using junctional region specific TaqMan probes. Leukemia. 1998;12:2006-2014.
27. Hazenberg MD, Otto SA, Cohen Stuart JW, et al. Increased cell division but not thymic dysfunction rapidly affects the T-cell receptor excision circle content of the naive T cell population in HIV-1 infection. Nat Med. 2000;6:1036-1042.
28. de Jager W, Prakken BJ, Bijlsma JW, Kuis W, Rijkers GT. Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies. J Immunol Methods. 2005;300:124-135.
29. Surh CD, Sprent J. Homeostasis of naive and memory T cells. Immunity. 2008;29:848-862.
30. Fry TJ, Connick E, Falloon J, et al. A potential role for interleukin-7 in T-cell homeostasis. Blood. 2001;97:2983-2990.
31. Ribeiro RM, Perelson AS. Determining thymic output quantitatively: using models to interpret experimental T-cell receptor excision circle (TREC) data. Immunol Rev. 2007;216:21-34.
32. Schluns KS, Kieper WC, Jameson SC, Lefrancois L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells *in vivo*. Nat Immunol. 2000;1:426-432.
33. Seddon B, Zamoyska R. TCR and IL-7 receptor signals can operate independently or synergize to promote lymphopenia-induced expansion of naive T cells. J Immunol. 2002;169:3752-3759.
34. Rosenberg SA, Sportes C, Ahmadzadeh M, et al. IL-7 administration to humans leads to expansion of $CD8^+$ and $CD4^+$ cells but a relative decrease of $CD4^+$ T-regulatory cells. J Immunother. 2006;29:313-319.
35. Sportes C, Hakim FT, Memon SA, et al. Administration of rhIL-7 in humans increases *in vivo* TCR repertoire diversity by preferential expansion of naive T cell subsets. J Exp Med. 2008;205:1701-1714.
36. Mackall CL, Fleisher TA, Brown MR, et al. Age, thymopoiesis, and $CD4^+$ T-lymphocyte regeneration after intensive chemotherapy. N Engl J Med. 1995;332:143-149.
37. Bleul CC, Corbeaux T, Reuter A, Fisch P, Monting JS, Boehm T. Formation of a functional thymus initiated by a postnatal epithelial progenitor cell. Nature. 2006;441:992-996.

Chapter 7

SUMMARY AND DISCUSSION

SUMMARY

The immune system is a dynamic system that is designed to respond rapidly to potential harmful stimuli. Following activation tight control mechanisms are in place to avoid collateral damage. Cardiac surgery is well known to induce an acute systemic inflammatory response and therefore, elective cardiac surgery creates an opportunity to study the inflammatory response in human in detail, from its initiation through the peak of inflammation up to recovery. So to restore the dynamic immunological equilibrium; 'What goes up must come down'.

In this thesis we illustrate how the immune system of children responds to two different aspects of pediatric cardiac surgery, both known to disturb the immunological equilibrium. In the first part of this thesis the immediate inflammatory response due to surgery is investigated with an emphasis on endogenous feedback mechanisms. In the second part of this thesis we investigate how the developing immune system copes with total thymectomy due to neonatal cardiac surgery.

Pediatric cardiac surgery induces an inflammatory response which contributes to outcome
Cardiac surgery is a major iatrogenic inducer of the immune system. **Chapter 2** gives an overview of the main activators of the inflammatory response following pediatric cardiac surgery. In the past decades surgical techniques and peri-operative management have improved substantially, which reduces immune activation. However, pre-operative clinical condition, the period in theatre that involves surgical tissue damage and cardiopulmonary bypass (CPB) amongst others, and the recovery period on pediatric intensive care unit (PICU), all continue to activate the immune system. Immune activation can potentially lead to morbidity due to increased vascular permeability and ultimately organ damage and to temporary immune deficiency. Finding the appropriate balance between these two, without compromising wound healing is the great challenge in the peri-operative care of these children. Pharmacological efforts to control the inflammatory response have overall been disappointing. Priority in enabling to manipulate this response is a thorough understanding of the post-operative inflammatory response. Herewith one needs to take into account that children may respond differently compared to adults.

Unraveling the mechanism behind monocyte unresponsiveness following cardiac surgery
A well-known phenomenon during systemic inflammation is a diminished responsiveness of monocytes to toll like receptor (TLR) stimulation. In **Chapter 3** we studied monocyte unresponsiveness following pediatric cardiac surgery, in particular whether this was due to tolerance to TLR stimulation, or due to active inhibition. We demonstrate that pediatric cardiac surgery with the use of CPB initiates a controlled systemic inflammatory response characterized by a cytokine storm, monocytosis and transient monocyte activation. The responsiveness of monocytes to TLR-mediated activation is diminished in the postoperative course. We demonstrate an important role for STAT3 in the plasma-mediated inhibition of LPS-induced

TNF-α production by monocytes. Plasma samples obtained 4 hours after surgery potently inhibit LPS-induced intracellular monocyte TNF-α synthesis, as compared to healthy control plasma and autologous plasma samples obtained 24 hours post surgery. This suppressed TNF-α production is not caused by attenuation of signaling pathways downstream of TLR. Rather, active inhibition through IL-10 / STAT3 pathway appears to be essential in the reduced monocyte responsiveness. Accordingly, pre-treatment of patient monocytes with a cell-permeable STAT3 inhibitor peptide largely restores LPS-induced TNF-α production in the presence of suppressive plasma. Altogether, our findings suggest that STAT3 plays a crucial role in the regulation of TNF-α synthesis by human monocytes in the course of systemic inflammation *in vivo*.

Functional ability of FOXP3$^+$ Treg during transient systemic inflammation

FOXP3$^+$ Treg are important regulators of inflammation, with effects on both adaptive and innate immune components. Their best-described function is maintaining peripheral tolerance. In **Chapter 4** we studied the Treg population during the transient inflammatory response following pediatric cardiac surgery. Twenty-four hours after surgery the proportion of FOXP3$^+$ T cells increase compared to the overall T cell population. Also, FOXP3$^+$ T cells contain the highest percentage of proliferating cells. This population has phenotypic characteristics of true Treg with high expression of CD25, CTLA-4 and GITR and remain anergic to *in vitro* T cell receptor (TCR) stimulation. However, their capacity to suppress effector T cells is markedly reduced. Again, plasma factors appear to play a significant role, as post-operative plasma clearly inhibits the suppressive capacity of 'healthy' Treg. Therefore, FOXP3 appears to be rapidly induced during systemic inflammation and may be important in regulating the systemic inflammatory response following cardiac surgery.

Neonatal thymectomy results in altered composition of Treg population despite compensatory peripheral proliferation

In neonatal cardiac surgery total thymectomy is commonly required to gain unrestrictive access of the operating site. However, the thymus is essential for the establishment and maintenance of T cells. Treg, a subpopulation of T cells essential for immune homeostasis, can originate from two possible sources and are hence divided into; thymus derived natural Treg and peripherally induced Treg. Peripherally circulating Treg can be functionally divided into different subsets based on expression of FOXP3 and CD45 isoforms. In **Chapter 5** we explored the impact of neonatal thymectomy on these selected Treg subpopulations in the first three years of life. Following thymectomy total FOXP3$^+$ Treg are reduced with a significantly lower number recently emigrated from the thymus (CD31$^+$FOXP3$^+$ T cells). Total numbers of memory Treg (CD45RO+FOXP3^{high}) remain stable, whereas naive Treg numbers decrease in patients older than 6 months of age. Reduced circulating numbers of naive Treg are compensated for by increased peripheral proliferation of Treg. While this illustrates the relative plasticity of the human immune system, one wonders whether a different composition of the Treg population

could have functional effects for immune tolerance later in life. Overall, peripheral proliferation of circulating Treg counteracts the effect of loss of thymopoiesis. The resulting altered composition of the Treg population needs further investigation.

Naive T cell compartment is affected in the first years following neonatal thymectomy, but reestablishes later in life alongside thymus regeneration

Several studies have previously shown that a total thymectomy in neonatal cardiac surgery affects the T cell compartment at an early age, without obvious clinical consequences. In **Chapter 6** we investigated the impact of neonatal thymectomy on the naive T cell compartment in the first 3 decades of life. Similar to previous reports we show that in the first 5 years following thymectomy T cell numbers are decreased, with naive T cells being most severely affected. T cell receptor excision circles (TREC), a direct measurement of thymic output) content of $CD4^+$ T cells was found to be significantly lower in the first years of life. In the first years after thymectomy, when the T cell numbers is reduced, compensatory measures are recognizable. Plasma IL-7 concentration is increased, which may contribute to the restoration of the $CD4^+$ T cell population. However, proliferation of naive T cells is only marginally increased. After approximately 10 years following thymectomy the T cell compartment resembles healthy, not thymectomized controls. T cell numbers return to normal levels and we found evidence of renewed thymopoiesis (normalization of TREC content of $CD4^+$ T cells). The *de novo* T cell production coincides with recurrence of thymic tissue on MRI, suggesting that normalization of the T cell compartment could be due to regeneration of functional thymus. Thus, the T cell compartment is able to recover from neonatal thymectomy through thymic regeneration. However, there is insufficient evidence to state that thymectomy at an early age does not have functional long term effects on the immune system which warrants further investigation.

GENERAL DISCUSSION

Pediatric cardiac surgery

Congenital heart disease affects a significant proportion of newborns, with a prevalence of 6 – 13 per 1000 live births.[1] This approximates 1250 new cases per year in the Netherlands. One quarter of these cases will require surgical or cardiac catheter intervention in the first year of life.[2] Recovery following pediatric cardiac surgery requires intensive treatment on a pediatric intensive care. Due to improved antenatal screening and treatment options many patients follow a relatively uncomplicated recovery and can be discharged home within days of the surgical procedure. However, all patients remain at risk of serious morbidity and mortality. While innovative treatment options have improved outcome to many congenital heart defects in the last decades, it has also resulted to surgery being performed on more complex defects in very young patients. These advances in surgical options enhance the challenges in post-operative care. Hence, increased surgical options, parallel an ethical responsibility in offering intense curative or palliative treatment to the appropriate patients.[3;4] Advanced treatment options with high peri-operative risk of severe morbidity or mortality necessitate very careful patient selection. While pre-operative risk factors can be taken into account when identifying which patient is at risk of prolonged recovery time and risk of morbidity, unexpected events occurring intra-operatively and post-operatively also contribute to subsequent risk.[5]

Defining the inflammatory response following pediatric cardiac surgery

Regardless of the type of surgical procedure, the age of the patient, or length of CPB, every cardiac surgical procedure will initiate an inflammatory response. As discussed in chapter 2, immune activation results from a peri-operative multi hit model of triggers including anesthesia, surgical tissue injury, ischemia-reperfusion, CPB and mechanical ventilation. The accumulation of these insults results in a systemic inflammatory response. Severe systemic inflammation or systemic inflammatory response syndrome (SIRS) is associated with hemodynamic instability and organ failure and occurs in up to 10% of adult patients following cardiac surgery.[6] Numerous studies illustrate a systemic inflammatory response following pediatric cardiac surgery.[7-14] This systemic inflammatory response can result in hemodynamic instability, impaired function of multiple organs and early morbidity.[15] Hemodynamic instability may cause variable systemic blood pressures, dysrhythmias, edema, poor urine output, and decreased perfusion. If persistent, such instability can lead to a low cardiac output syndrome (LCOS) state with poor oxygen delivery, as manifested by low mixed venous oxygen saturation, elevated serum lactate, and metabolic acidosis. LCOS is associated with early postoperative morbidity and mortality.[16;17]

While initially most emphasis of the inflammatory response was on the pro-inflammatory mediators and the possible damaging effects, the counter anti-inflammatory component of this response is nowadays also recognized for contributing to post-operative morbidity. This has lead to the concept of a dual response, with initially a hyper-inflammatory phase followed by a hypo-

inflammatory phase, or immune paralysis.[18;19] This bimodal response is further recognized in clinical translation of these events. Typically, clinical phenomena resulting from an unbalanced inflammatory response in the direction of hyper-inflammation (increased vascular permeability with hypotension and organ failure) occur early on in the post-operative period. On the other hand, effects of immune deactivation (secondary infections) become clinically apparent after several days post-operatively.

While categorizing the inflammatory response into a pro- and anti-inflammatory phase has guided research into looking at both aspects of the response,[20] there remain inconsistencies with this concept. Looking at release of various types of cytokines, it is apparent that there is no clear-cut chronological division in release of pro- followed by anti-inflammatory mediators. Based on our and other's work, IL-10 (both mRNA and protein) for instance appears very rapidly following the surgical insult alongside the release of typical pro-inflammatory cytokines such as TNFa and IL-6.[8;10;21-24]

In addition, laboratory signs of immune dysfunction appear already early on following surgery. For example, decreased monocyte responsiveness to TLR stimulation occurs as early as 4 hours after surgery.[22] One might speculate that the typical infectious complications seen following cardiac surgery need time to manifest and that the anti-inflammatory sequelae leading to increased infectious risk occur already quite soon after surgery. In terms of the inflammatory balance, numerous immediate negative feedback mechanisms are available to keep the inflammatory response in control. Activation of intracellular inflammatory signaling cascades results in translation of pro-inflammatory proteins, and simultaneous release of negative feedback mediators to shut this cascade down (such as STAT3 and SOCS3). Therefore, the bimodal clinical response may not be as distinct on a cellular level. Both the pro- and anti-inflammatory sides of a response act promptly and continue throughout the inflammatory response.

Inflammation is primarily triggered by tissue damage, while beyond the site of cellular stress activated cells should be inhibited (Figure 1). The concept of tissues guiding the inflammatory response, rather than immune cells themselves goes back to the danger theory by Matzinger.[25] This model of how the immune system is triggered, suggests that the immune system responds to danger signals from tissues, rather than recognition of 'non-self' pathogens. The problem in systemic inflammation is that multiple tissues beyond the surgical site can send and maintain ongoing danger signals. Differential exposure of danger signals by tissues may explain why some tissues are more susceptible to inflammatory damage than others. This is further substantiated by the knowledge that compartmentalized expression of pro-inflammatory gene programs determines different local effects during systemic inflammation.[26] During SIRS specific pro-inflammatory genes are switched on in the lungs while these same genes are actively silenced in systemically circulating leukocytes.[27;28] Thus, organ damage can occur due to exaggerated inflammation, while simultaneously, systemically circulating leukocytes may be hypo-responsive to subsequent invading pathogens.

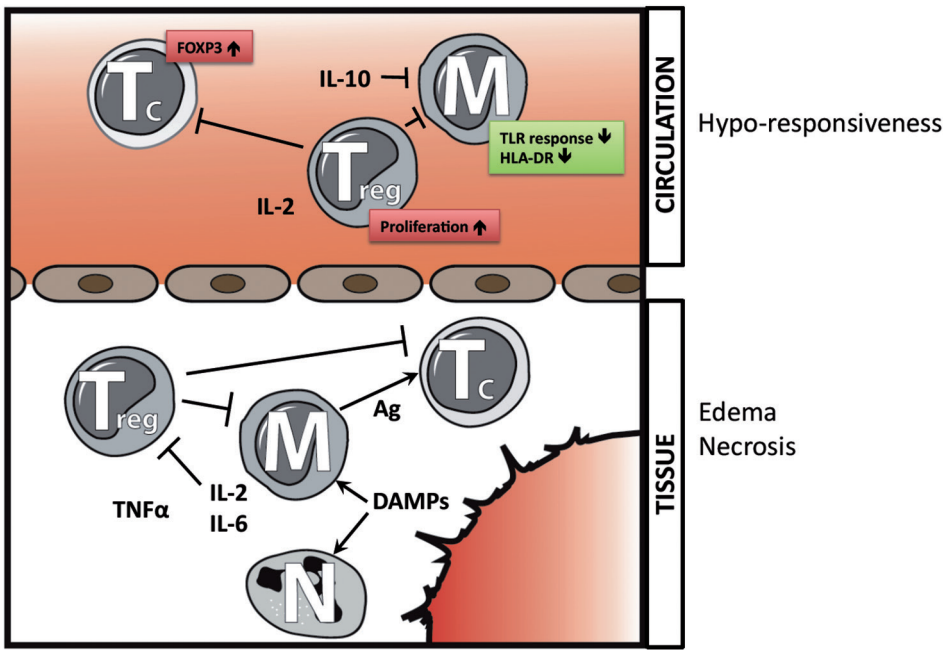

Figure 1. Differential local and systemic inflammation. Inflammation is triggered by local tissue damage through release of damage-associated molecular patterns (DAMPs). Processed antigen (Ag) can be presented to T cells to further enhance the inflammatory response. Local release of pro-inflammatory cytokines can inhibit Treg cells to maintain the inflammatory response. Ideally, beyond the site of tissue damage, inflammatory cells need to be suppressed to prevent systemic inflammation with potential collateral damage. Possible means of immune regulation include 1) transient up-regulation of FOXP3 in conventional T cells to inhibit proliferation; 2) proliferation of functional Treg to actively suppress unwanted inflammation; 3) inhibition of monocytes to respond to Toll like receptor (TLR) stimulation with HLA-DR down-regulation. The down-side of this immune regulation however is systemic hypo-responsiveness to pathogens.

Prompt activation of feedback mechanisms not only occurs in the innate immune system. We show in this thesis that T cells also respond rapidly to cardiac surgery. An increased percentage of T cells expressed FOXP3 24 hours after surgery, and subsequently return to pre-operative levels. The remaining question to be answered is whether the observed FOXP3$^+$ T cells are true Treg, with reduced suppressive action due to inflammatory milieu,[29] or that the population of FOXP3$^+$ T cells consisted of a mixture of true Treg and conventional T cells which transiently expressed FOXP3, without acquiring suppressive capacity (Figure 1).

If indeed there is an increase in Treg following cardiac surgery, one can speculate that during inflammation, Treg act to limit collateral damage. It has been shown that Treg are actually the first T cells (within hours of priming) to respond to IL-2.[30] By being first-responders, Treg can

potentially prevent excessive activation of other effector immune cells. In a recent mouse study by Jia et al, Treg are protective in a mouse model of induced systemic inflammation.[31] They report that depletion of Treg prior to an induced SIRS profoundly increases lung tissue injury and mortality. However, the exact role of Treg in infectious SIRS remains controversial. While other studies also report improved survival in septic mice following adoptive transfer of Treg,[32] other studies show the opposite. Two mouse studies of induced sepsis through cecal ligation show an association between increased Treg numbers and immune deficiency during SIRS. Furthermore, depletion of Treg restores bacterial clearance and improves survival.[33;34]

Alternatively, the increased FOXP3 T cells following cardiac surgery may not be an increase in true Treg, but a transient expression of FOXP3 in conventional T cells. The population with Treg phenotype is less suppressive, suggesting that the population may be enhanced with non-Treg cells. These cells are however anergic, like true Treg and this may illustrate an intrinsic regulation mechanism for conventional T cells. While transient expression of FOXP3 (without acquiring suppressive function) in conventional T cells has been illustrated before, its function remains unclear.[35;36] Recently McMurchy, et al illustrated that FOXP3 expression acts as an intrinsic regulator for conventional T cells and may prevent unwanted proliferation and cytokine production.[37] As such, FOXP3 expression during the inflammatory response following surgery may act as a regulator to minimize activation of potentially harmful conventional T cells. Subsequently, this may also contribute to immune dysfunction in these children post-operatively.

Overall, the immune system responds to inflammatory stress with activation of both pro- and anti-inflammatory pathways simultaneously. Resultant clinical outcome probably does not rely on simply the ratio between the two, but more likely can both be hampered by excessive pro-inflammation with subsequent edema, hypotension and organ failure, and immune deficiency resulting in infection (or both simultaneously). Thus, treatment options solely intended to non-specifically inhibit either side of the inflammatory response could have dangerous side effects.

Lessons to be learnt for systemic inflammation?

In the past decade much insight has been gained in understanding the inflammatory response following pediatric cardiac surgery. Due to technical advances, tissue damage and immune activation has been reduced, resulting in improved outcome. Nevertheless, there are still patients whose post-operative recovery is hampered by excessive inflammation.

The single most relied on pharmacological entity to inhibit the inflammatory response following cardiac surgery is corticosteroid. Corticosteroids have been shown to effectively reduce the pro-inflammatory response.[38-41] While hypothetically a useful agent to limit the potential damaging effects of the inflammatory response, accumulating evidence suggests that for most pediatric patients peri-operative corticosteroid exposure has limited added benefit and is independently associated with poor outcome including post-operative infections.[42;43] In

adults also no immediate benefit could be proven on outcome, but interestingly was associated with reduced post-operative infection rate and length of stay.[44;45]

Lessons learnt from research on controlling inflammation following cardiac surgery may well initiate new insight on excessive inflammation due to major trauma, surgery, infections or burns. In the past numerous attempts have been made to try to control inflammation during sepsis. Upon recognition of the correlation between levels of certain cytokines and outcome, anti-inflammatory agents were developed, inhibiting specific pro-inflammatory cytokines (e.g. TNFa, IL-1 antagonists[46-48]). Similarly, attempts have been undertaken to block TLR activation by endotoxin with antibodies against endotoxin[49] and more recently the TLR4 antagonist eritoran.[50] The most recent, strongly debated, anti-inflammatory agent to treat sepsis is recombinant human activated protein C (rhAPC). Developed from in-vitro studies, through promising animal studies, large human trials however remained controversial for a decade.[51;52] Finally the drug drotrecogin was withdrawn from the market in 2011 following reports it showed insufficient efficacy in both adult or pediatric sepsis.[53;54] The overall conclusion of numerous large trials on inhibiting specific pro-inflammatory cytokines turns out to be disappointing. No trial showed any improvement in outcome and some even resulted in increased mortality.

The anti-inflammatory component of the immune response has also been recognized to correlate to clinical outcome. High levels of IL-10 are found in both septic and post-operative patients with typically a reduced monocyte response to endotoxin.[22;55;56] Circulating monocytes in systemic inflammation show reduced expression of HLA-DR, which is also correlated to subsequent morbidity.[57-59] Furthermore, increased numbers of potent anti-inflammatory T cells ($FOXP3^+$ Treg) are found during systemic inflammation.[60] Further evidence that not only the pro-inflammatory response may be detrimental in systemic inflammation was shown in post-mortem studies of deceased septic patients. Patients, both adult and pediatric, who succumbed to sepsis show loss of both innate and adaptive immune cells.[61-63] Taken together, effectively using immunomodulatory therapy for systemic inflammation depends on which side of the immune response is detrimental and thus needs to be tackled. This is probably highly patient specific and time dependent. Acknowledging the diverse nature of an ensuing inflammatory response has resulted in the first promising animal studies on blocking the anti-inflammatory side of the response. Specific antibodies against negative co-stimulatory molecules (Programmed cell Death protein-1 (PD-1) and Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4)) improve survival when administered at the appropriate time in the sequence of inflammation.[64;65] Moreover, new studies are investigating to restore the loss of immune effector cells during sepsis. In a murine sepsis model rhIL-7 restores lymphocyte effector function and improves survival.[66]

Currently, limited evidence based treatment options are available to manipulate the human inflammatory response successfully during sepsis. The prevailing negative outcome of human trials following promising experimental models of an agent has raised concerns over the applicability of animal studies for human use. Indeed, a recent transcriptome analysis of mouse models versus human sepsis indicates very poor correlation between the pathophysiology of

septic shock in mice and humans.[67] This could explain why nearly 150 drugs tested in human sepsis failed to replicate the encouraging results from mouse studies. Moreover, the study by Seok, et al.[67] also reveals that human acute inflammatory responses from different etiologies result in very similar genomic responses. Thus, although the triggers resulting to infectious and non-infectious inflammation e.g. surgery and sepsis are very different, the resulting response is remarkably similar. This would implicate that the way forward to new treatment strategies for sepsis should more strongly rely on translation of human studies from other fields of inflammation, rather than animal studies.

Studying sepsis is complicated by the fact that the initial trigger has already occurred, and at the time of presentation an overwhelming inflammatory response is ongoing. In the case of cardiac surgery, the trigger is exactly timed (initiation of surgery) and patients are under close monitoring during the whole successive recovery period. So, although research of the inflammatory response following pediatric cardiac surgery can probably benefit a select group of cardiac patients, this knowledge can also be translated to the benefit of other patients suffering from severe inflammation, including sepsis.

Neonatal thymectomy, so what?

Removing the thymus, the primary gland for development of new T cells, occurs routinely at a young age during cardiac surgery to gain access to the large vessels. Although this has caught the attention of several immunology research groups, it remains controversial whether neonatal thymectomy causes any immune disorders later in life. From a surgical point of view thymectomy in these procedures is a necessity, enabling life-saving surgical correction of congenital heart diseases. Furthermore thymectomy in these patients has been performed for decades with no obvious clinical concerns.

Numerous studies demonstrate reduced T cell numbers, most notably naive T cells, in the first years following neonatal thymectomy.[68-72] Despite these changes, *in vitro* immune responses to exposed antigen remain intact.[69;73] The study by Prelog et al was the first to indicate that loss of thymopoiesis at a young age can result in decreased immunity.[74] They report diminished IgG responses to tick-borne encephalitis vaccination, most notably in patients thymectomized at a young age.

Although the human adaptive immune system is remarkably competent at birth,[75] it still requires further maturation and is unable to mount significant and lasting T cell dependent antibody responses to vaccines.[76] The presence of large numbers of recent thymic emigrants (RTE) in neonates has been implicated in their vulnerability to infections due to the impaired effector function of RTE.[77] Since neonatal thymectomy results in a relative sparing of memory T cells compared to RTE, this could (counter-intuitively) limit the risk of infections during lymphopenia following thymectomy.

Also beyond the first decade after neonatal thymectomy, alterations in T cell numbers have been described. While a subgroup of patients continue to show low T cell numbers, clearly not all patients older than ten years have low T cell numbers after thymectomy. Our study

on the long term effects of neonatal thymectomy would concur with previous studies and suggest that in the majority of patients, T cell numbers return to normal levels and the thymus regenerates.[78;79] Age of thymectomy is possibly important in determining whether thymus regeneration occurs. In the study of Prelog, et al.,[80] showing long term reduced T cell numbers and reduced T cell receptor excision circles (TREC), thymectomy occurred significantly later (mean 2.6 years) than in our study. One can speculate that the capacity of thymus to regenerate is age-dependent and (unexpectedly) limited to the first months after birth. Alternatively, regrowth of thymus may simply depend on how much thymus remains following thymectomy, i.e. surgical thoroughness. In any case, also in our study we found two 10-year-old individuals, with low $CD4^+$ numbers in combination with decreased levels of thymic output, suggesting that not in all cases the thymus is able to regenerate adequately following neonatal thymectomy.

Patients in which no or insufficient return of thymopoiesis occurs would need close follow-up to investigate the clinical impact. In healthy aging adults, maintenance of naive T cells appears to rely predominantly on increased peripheral proliferation and prolonged survival.[81-83] However, in conditions of T cell depletion such as following bone marrow transplantation or HIV infection, the thymus remains important for reconstituting the T cell compartment in both children and adults.[84-86] Following thymectomy, T cell numbers are decreased, so one can expect that without regeneration through thymopoiesis, T cell immunity will be affected. If the circulating T cell population is not replenished through thymopoiesis, the T cell repertoire is at risk of oligoclonality (Figure 2B). This occurs in the elderly, which has been associated with decreased immunity.[87;88] It has been speculated that reduced TCR diversity can be compensated for by increased cross-reactivity, possibly leading to increased auto-immunity.[89;90]

While T cell numbers generally return to normal levels after the first decade following neonatal thymectomy, a subgroup of young patients (median age 22 years) in the study by Sauce et al exhibit altered T cell profiles usually seen in the elderly.[79] They reveal that a multiple hit on the naive T cell repertoire through Cytomegalovirus infection plus loss of thymopoiesis results in oligoclonality, together with increased inflammatory markers. These immunological changes, seen 20 years after thymectomy, resemble deficiencies seen in the elderly. This raises concerns about whether neonatal thymectomy can indeed be compensated for with peripheral proliferation and thymic regeneration in all patients. Thus after neonatal thymectomy some patients show signs of immunosenescence early in life, which can result in increased risk of infections as seen in the elderly.

Treg subpopulations depend on thymopoiesis for a balanced maintenance

In chapter 5 we studied the effect of neonatal thymectomy on the maintenance of the Treg population. The general consensus is that two sets of Treg are required to achieve a healthy immunological homeostasis; *de novo* derived Treg through thymopoiesis (nTreg) and peripherally induced Treg (iTreg). nTreg are thought to maintain peripheral self-tolerance, while iTreg are induced in response to foreign antigens to regulate non-pathogenic tolerance.[91;92] However, the mainstay of evidence for peripherally induced Treg comes from experimental

settings. *In vitro* experiments show that under specific circumstances FOXP3$^+$ can be induced in human naive FOXP3- T cells and render them suppressive.[93;94] In mice, naive T cells can be converted *in vivo* to functional Treg through presentation of antigen in subimmunogenic conditions[95] or upon adoptive transfer into lymphopenic hosts[96]. Besides the possibility of peripheral induction of FOXP$^+$ Treg from FOXP3- naive T cells, thymic derived Treg can also convert to an effector (non-Treg) phenotype following transfer into lymphopenic recipients and during ex vivo stimulation with inflammatory cytokines.[97;98] Apart from FOXP3 expression, epigenetic changes appear to be critical for the stability of Treg cell lineage. In particular hypo-methylation of specific DNA sites determines the fate of FOXP3$^+$ T cells.[99] Thus, FOXP3 is indispensible but not sufficient for conferring and maintaining Treg function and phenotype.

While FOXP3$^+$ Treg undoubtedly play an essential role in maintaining immune homeostasis in humans, distinguishing nTreg from iTreg *in vivo*, based on phenotypic markers remains a challenge. So, the overall contribution of iTreg to the total pool of peripheral Treg in human under basal and inflammatory conditions remains under debate (Figure 2). Human FOXP3$^+$ T cells can be phenotypically divided into different functional subsets as was elegantly described by Miyara et al.[100] They report two functionally suppressive Treg populations; resting Treg (FOXP3^{low}CD45RA$^+$RO-) and activated Treg (FOXP3^{high}CD45RA-RO+) with distinct properties (Figure 2). In aged donors the resting Treg decrease, whereas activated Treg increase, which corresponds to our data following thymectomy where resting Treg numbers decrease significantly 6 months after thymectomy. Interestingly, their results suggest that in adults the majority of resting Treg originate from the thymus. In addition, tracing clonotypes of each Treg cell fraction show that activated Treg predominantly derive from resting Treg and that conversion of FOXP3-CD4$^+$ T cells to FOXP3$^+$ Treg can only contribute for a minor part, if any, to the total Treg population. The maintenance of the pool of activated Treg depends on a constant development of proliferating cells from the resting Treg pool, balanced by death of activated Treg following suppression. These observations have important implications for thymectomized individuals. This would suggest that the Treg pool following thymectomy depends on the thymic derived Treg pool at the time of thymectomy, which from a resting state continuously replenishes the activated Treg pool (Figure 2). Interestingly, up to 90% of naive T cells in adults have been estimated to rely on peripheral proliferation rather than thymic output.[81] This would imply that the Treg population depends more on thymus output than naive T cells.

Taken together, neonatal thymectomy reduces naive T cell numbers in the first years of life. Although this has not been associated with increased risk of infections, antibody responses are reduced to some vaccinations. Limited data is available on the longer term effects of neonatal thymectomy and needs further follow-up. In the majority of patients the thymus probably regenerates and continues to replenish the T cell pool. However, in a small subgroup of patients this does not occur. Therefore, sparing thymic tissue during pediatric cardiac surgery should be advised.

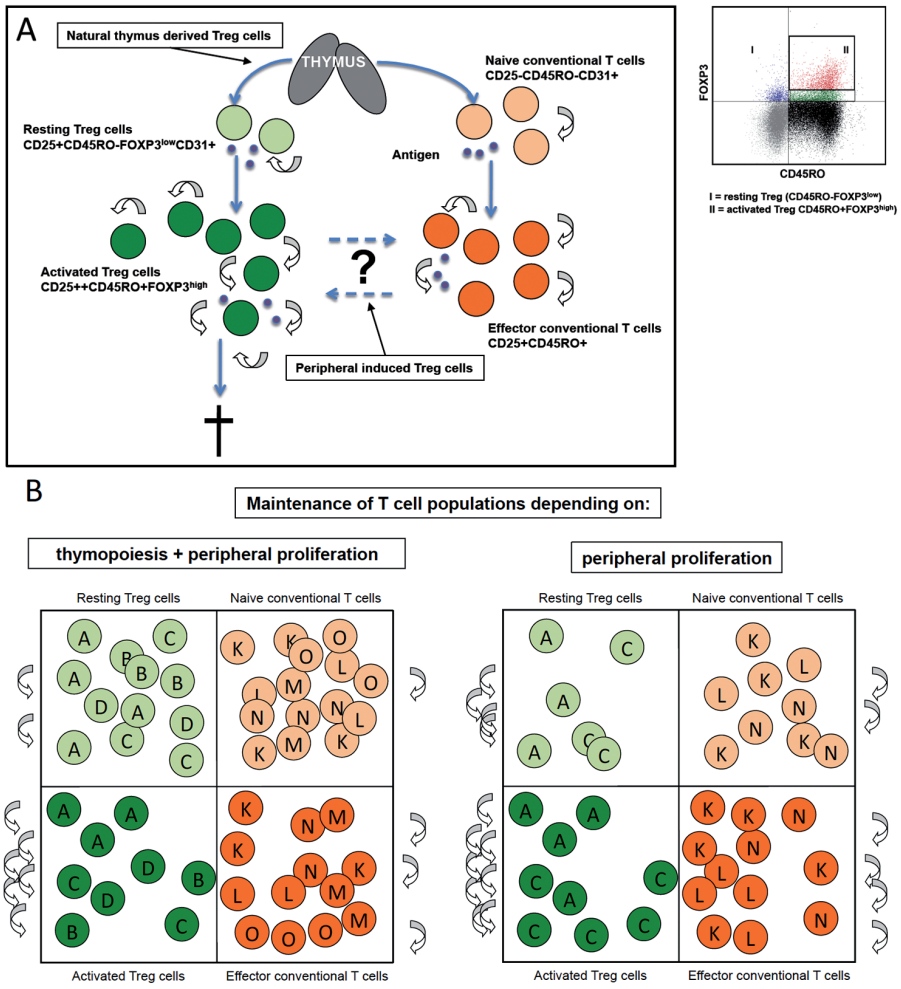

Figure 2. Differentiation and maintenance of human Treg and conventional T cells. (**A**) FOXP3$^+$ Treg cells depart from the thymus as resting Treg (rTreg) cells expressing CD31$^+$, CD45RO-, CD25$^+$, and low levels of FOXP3. Upon peripheral Ag encounter, rTreg cells proliferate, upregulate CD45RO, CD25 and FOXP3 and become activated Treg (aTreg) cells. aTreg cells are short lived and rapidly die after activation and suppression. Naive conventional T cells depart from the thymus expressing CD31$^+$, CD45RO-, CD25- and FOXP3-. After Ag stimulation naive T cells upregulate CD45RO$^+$ and CD25$^+$, but remain FOXP3- to become effector memory conventional T cells. It remains uncertain whether human Treg cells are induced from conventional T cells *in vivo*, and if so, to what degree these induced Treg cells contribute to the maintenance of the total Treg population. Dotplot illustrates rTreg and aTreg expression of FOXP3 and CD45RO. (**B**) The maintenance of both Treg and conventional T cells depends on thymopoiesis for a polyclonal output of naive cells and peripheral proliferation to achieve adequate clonal size. With loss of thymopoiesis (after thymectomy) proliferation of all T cells is increased to maintain cell numbers. However, the naive compartment of Treg and conventional T cells can not be maintained and ultimately TCR diversity is affected

Future directions

In this thesis we show that studying the immune system of children following cardiac surgery can aid our understanding of inflammation. In addition, follow-up of patients after thymectomy can improve our knowledge of how the immune system maintains homeostasis throughout life. Though answering some questions, this research also raises new questions that require further investigation.

The present research regarding regulation of the inflammatory response after pediatric cardiac surgery was performed in children with a favorable recovery course. To truly understand how this knowledge can benefit patients, the next step will be to study the inflammatory response of patients with an excessive response. Clinical parameters may then be associated with functional immunological tests to guide which part of the inflammatory response may benefit from intervention. Subsequently, it will be worthwhile to compare these results with other clinical manifestations of systemic inflammation, such as sepsis and burns. Of importance for future translation to potential pharmacological interventions will be to understand what occurs in different compartments over time. Local sites of inflammation (such as the myocardium and pulmonary tissue) will likely illustrate a different response compared to what can be measured systemically. Unraveling these different responses sequentially after cardiac surgery can help our understanding why children are affected with organ failure while simultaneously show systemic signs of immune unresponsiveness. In addition this can guide research in how to target these different responses without exaggerating the opposing inflammatory response. Though studying the inflammatory response in different compartments will be challenging in patients, it may prove crucial to successfully manipulate the inflammatory response without causing additional harm. Existing research that looks into local and systemic inflammation in a PICU population includes studies on inflammatory response due to mechanical ventilation. Here local alveolar inflammation can be compared to systemically circulating inflammation by sampling blood and alveolar lavage simultaneously. Overall, immunological research in different patient groups in an intensive care setting can work synergistically to an understanding of harmful inflammation and how to guide potential treatment options successfully.

Previous work by others and our studies presented in this thesis underline the need to continue research on the impact of neonatal thymectomy in human. Although, limited data is available on clinical abnormalities, there is sufficient evidence that thymectomy affects the immune system in all children in the first years of life and some patients into adulthood. All patients need to be followed up clinically with an emphasis on possible immune senescence. New research should be aimed in particular at those patients showing no recovery of thymic output. These patients should be particularly scrutinized for immunological changes later in life connected with oligoclonality and reduced naive Treg. With new techniques a wide range of autoimmune dysregulation can be studied including TCR repertoires and (auto)antibodies. As

long as it remains unclear what risks total neonatal thymectomy inflict on patients, preservation of thymus, where technical possible, must be advocated.

This thesis illustrates the plasticity of the immune system and its robustness in regaining a balance despite the impact of pediatric cardiac surgery. Hence, Newton's law: "*What goes up must come down*" also applies to the immune system.

References

1. van der Linde D, Konings EE, Slager MA et al. Birth prevalence of congenital heart disease worldwide: a systematic review and meta-analysis. J.Am.Coll.Cardiol. 2011;58:2241-2247.
2. Botto LD, Correa A, Erickson JD. Racial and temporal variations in the prevalence of heart defects. Pediatrics 2001;107:E32.
3. Mavroudis C, Mavroudis CD, Farrell RM et al. Informed consent, bioethical equipoise, and hypoplastic left heart syndrome. Cardiol.Young. 2011;21 Suppl 2:133-140.
4. McHaffie HE, Laing IA, Parker M, McMillan J. Deciding for imperilled newborns: medical authority or parental autonomy? J.Med.Ethics 2001;27:104-109.
5. Brown KL, Ridout DA, Goldman AP, Hoskote A, Penny DJ. Risk factors for long intensive care unit stay after cardiopulmonary bypass in children. Crit Care Med. 2003;31:28-33.
6. Cremer J, Martin M, Redl H et al. Systemic inflammatory response syndrome after cardiac operations. Ann.Thorac.Surg. 1996;61:1714-1720.
7. Ashraf SS, Tian Y, Zacharrias S et al. Effects of cardiopulmonary bypass on neonatal and paediatric inflammatory profiles. Eur.J.Cardiothorac.Surg. 1997;12:862-868.
8. Hovels-Gurich HH, Schumacher K, Vazquez-Jimenez JF et al. Cytokine balance in infants undergoing cardiac operation. Ann.Thorac.Surg. 2002;73:601-608.
9. Liu KD, Altmann C, Smits G et al. Serum interleukin-6 and interleukin-8 are early biomarkers of acute kidney injury and predict prolonged mechanical ventilation in children undergoing cardiac surgery: a case-control study. Crit Care 2009;13:R104.
10. Madhok AB, Ojamaa K, Haridas V et al. Cytokine response in children undergoing surgery for congenital heart disease. Pediatr.Cardiol. 2006;27:408-413.
11. Pathan N, Burmester M, Adamovic T et al. Intestinal injury and endotoxemia in children undergoing surgery for congenital heart disease. Am.J.Respir.Crit Care Med. 2011;184:1261-1269.
12. Stiller B, Sonntag J, Dahnert I et al. Capillary leak syndrome in children who undergo cardiopulmonary bypass: clinical outcome in comparison with complement activation and C1 inhibitor. Intensive Care Med. 2001;27:193-200.
13. Tarnok A, Hambsch J, Emmrich F et al. Complement activation, cytokines, and adhesion molecules in children undergoing cardiac surgery with or without cardiopulmonary bypass. Pediatr.Cardiol. 1999;20:113-125.
14. Zupancich E, Paparella D, Turani F et al. Mechanical ventilation affects inflammatory mediators in patients undergoing cardiopulmonary bypass for cardiac surgery: a randomized clinical trial. J.Thorac.Cardiovasc. Surg. 2005;130:378-383.
15. Roth SJ, Adatia I, Pearson GD. Summary proceedings from the cardiology group on postoperative cardiac dysfunction. Pediatrics 2006;117:S40-S46.
16. Neuhof C, Walter O, Dapper F et al. Bradykinin and histamine generation with generalized enhancement of microvascular permeability in neonates, infants, and children undergoing cardiopulmonary bypass surgery. Pediatr.Crit Care Med. 2003;4:299-304.
17. Parr GV, Blackstone EH, Kirklin JW. Cardiac performance and mortality early after intracardiac surgery in infants and young children. Circulation 1975;51:867-874.
18. Bone RC. Sir Isaac Newton, sepsis, SIRS, and CARS. Crit Care Med. 1996;24:1125-1128.
19. Mannick JA, Rodrick ML, Lederer JA. The immunologic response to injury. J.Am.Coll.Surg. 2001;193:237-244.
20. Allen ML, Peters MJ, Goldman A et al. Early postoperative monocyte deactivation predicts systemic inflammation and prolonged stay in pediatric cardiac intensive care. Crit Care Med 2002;30:1140-1145.
21. Allen ML, Hoschtitzky JA, Peters MJ et al. Interleukin-10 and its role in clinical immunoparalysis following pediatric cardiac surgery. Crit Care Med. 2006;34:2658-2665.

22. de Jong PR, Schadenberg AW, van den Broek T et al. STAT3 regulates monocyte TNF-alpha production in systemic inflammation caused by cardiac surgery with cardiopulmonary bypass. PLoS.One. 2012;7:e35070.
23. Duggan E, Caraher E, Gately K et al. Tumor necrosis factor-alpha and interleukin-10 gene expression in peripheral blood mononuclear cells after cardiac surgery. Crit Care Med. 2006;34:2134-2139.
24. Schadenberg AW, Vastert SJ, Evens FC et al. FOXP3$^+$ CD4$^+$ Tregs lose suppressive potential but remain anergic during transient inflammation in human. Eur.J.Immunol. 2011;41:1132-1142.
25. Matzinger P. Tolerance, danger, and the extended family. Annu.Rev.Immunol. 1994;12:991-1045.
26. McCall CE, Yoza BK. Gene silencing in severe systemic inflammation. Am.J.Respir.Crit Care Med. 2007;175:763-767.
27. Coldren CD, Nick JA, Poch KR et al. Functional and genomic changes induced by alveolar transmigration in human neutrophils. Am.J.Physiol Lung Cell Mol.Physiol 2006;291:L1267-L1276.
28. Guo RF, Riedemann NC, Sun L et al. Divergent signaling pathways in phagocytic cells during sepsis. J.Immunol. 2006;177:1306-1313.
29. Goodman WA, Levine AD, Massari JV et al. IL-6 signaling in psoriasis prevents immune suppression by regulatory T cells. J.Immunol. 2009;183:3170-3176.
30. O'Gorman WE, Dooms H, Thorne SH et al. The initial phase of an immune response functions to activate regulatory T cells. J.Immunol. 2009;183:332-339.
31. Jia W, Cao L, Yang S et al. Regulatory T cells are protective in systemic inflammation response syndrome induced by zymosan in mice. PLoS.One. 2013;8:e64397.
32. Heuer JG, Zhang T, Zhao J et al. Adoptive transfer of *in vitro*-stimulated CD4$^+$CD25$^+$ regulatory T cells increases bacterial clearance and improves survival in polymicrobial sepsis. J.Immunol. 2005;174:7141-7146.
33. Nascimento DC, Alves-Filho JC, Sonego F et al. Role of regulatory T cells in long-term immune dysfunction associated with severe sepsis. Crit Care Med. 2010;38:1718-1725.
34. Venet F, Chung CS, Kherouf H et al. Increased circulating regulatory T cells (CD4(+)CD25 (+)CD127 (-)) contribute to lymphocyte anergy in septic shock patients. Intensive Care Med. 2009;35:678-686.
35. Tran DQ, Ramsey H, Shevach EM. Induction of FOXP3 expression in naive human CD4$^+$FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. Blood 2007;110:2983-2990.
36. Wang J, Ioan-Facsinay A, van der Voort E, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4$^+$ T cells. Eur.J.Immunol. 2007;37:129-138.
37. McMurchy AN, Gillies J, Gizzi MC et al. A novel function for FOXP3 in humans: intrinsic regulation of conventional T cells. Blood 2013;121:1265-1275.
38. El Azab SR, Rosseel PM, de Lange JJ et al. Dexamethasone decreases the pro- to anti-inflammatory cytokine ratio during cardiac surgery. Br.J.Anaesth. 2002;88:496-501.
39. Heying R, Wehage E, Schumacher K et al. Dexamethasone pretreatment provides antiinflammatory and myocardial protection in neonatal arterial switch operation. Ann.Thorac.Surg. 2012;93:869-876.
40. Jansen NJ, van Oeveren W, van den Broek L et al. Inhibition by dexamethasone of the reperfusion phenomena in cardiopulmonary bypass. J.Thorac.Cardiovasc.Surg. 1991;102:515-525.
41. Kawamura T, Inada K, Nara N, Wakusawa R, Endo S. Influence of methylprednisolone on cytokine balance during cardiac surgery. Crit Care Med. 1999;27:545-548.
42. Pasquali SK, Hall M, Li JS et al. Corticosteroids and outcome in children undergoing congenital heart surgery: analysis of the Pediatric Health Information Systems database. Circulation 2010;122:2123-2130.
43. Mastropietro CW, Barrett R, Davalos MC et al. Cumulative corticosteroid exposure and infection risk after complex pediatric cardiac surgery. Ann.Thorac.Surg. 2013;95:2133-2139.
44. Dieleman JM, van Paassen J, van Dijk D et al. Prophylactic corticosteroids for cardiopulmonary bypass in adults. Cochrane.Database.Syst.Rev. 2011CD005566.
45. Dieleman JM, Nierich AP, Rosseel PM et al. Intraoperative high-dose dexamethasone for cardiac surgery: a randomized controlled trial. JAMA 2012;308:1761-1767.

46. Abraham E, Wunderink R, Silverman H et al. Efficacy and safety of monoclonal antibody to human tumor necrosis factor alpha in patients with sepsis syndrome. A randomized, controlled, double-blind, multicenter clinical trial. TNF-alpha MAb Sepsis Study Group. JAMA 1995;273:934-941.
47. Fisher CJ, Jr., Dhainaut JF, Opal SM et al. Recombinant human interleukin 1 receptor antagonist in the treatment of patients with sepsis syndrome. Results from a randomized, double-blind, placebo-controlled trial. Phase III rhIL-1ra Sepsis Syndrome Study Group. JAMA 1994;271:1836-1843.
48. Fisher CJ, Jr., Agosti JM, Opal SM et al. Treatment of septic shock with the tumor necrosis factor receptor:Fc fusion protein. The Soluble TNF Receptor Sepsis Study Group. N.Engl.J.Med. 1996;334:1697-1702.
49. Ziegler EJ, Fisher CJ, Jr., Sprung CL et al. Treatment of gram-negative bacteremia and septic shock with HA-1A human monoclonal antibody against endotoxin. A randomized, double-blind, placebo-controlled trial. The HA-1A Sepsis Study Group. N.Engl.J.Med. 1991;324:429-436.
50. Tidswell M, Tillis W, Larosa SP et al. Phase 2 trial of eritoran tetrasodium (E5564), a toll-like receptor 4 antagonist, in patients with severe sepsis. Crit Care Med. 2010;38:72-83.
51. Marti-Carvajal AJ, Sola I, Gluud C, Lathyris D, Cardona AF. Human recombinant protein C for severe sepsis and septic shock in adult and paediatric patients. Cochrane.Database.Syst.Rev. 2012;12:CD004388.
52. Bernard GR, Ely EW, Wright TJ et al. Safety and dose relationship of recombinant human activated protein C for coagulopathy in severe sepsis. Crit Care Med. 2001;29:2051-2059.
53. Annane D, Timsit JF, Megarbane B et al. Recombinant human activated protein C for adults with septic shock: a randomized controlled trial. Am.J.Respir.Crit Care Med. 2013;187:1091-1097.
54. Nadel S, Goldstein B, Williams MD et al. Drotrecogin alfa (activated) in children with severe sepsis: a multicentre phase III randomised controlled trial. Lancet 2007;369:836-843.
55. Munoz C, Carlet J, Fitting C et al. Dysregulation of *in vitro* cytokine production by monocytes during sepsis. J.Clin.Invest 1991;88:1747-1754.
56. Weighardt H, Heidecke CD, Emmanuilidis K et al. Sepsis after major visceral surgery is associated with sustained and interferon-gamma-resistant defects of monocyte cytokine production. Surgery 2000;127:309-315.
57. Docke WD, Randow F, Syrbe U et al. Monocyte deactivation in septic patients: restoration by IFN-gamma treatment. Nat.Med. 1997;3:678-681.
58. Oczenski W, Krenn H, Jilch R et al. HLA-DR as a marker for increased risk for systemic inflammation and septic complications after cardiac surgery. Intensive Care Med. 2003;29:1253-1257.
59. Wolk K, Docke WD, von Baehr V, Volk HD, Sabat R. Impaired antigen presentation by human monocytes during endotoxin tolerance. Blood 2000;96:218-223.
60. Venet F, Chung CS, Monneret G et al. Regulatory T cell populations in sepsis and trauma. J.Leukoc.Biol. 2008;83:523-535.
61. Bommhardt U, Chang KC, Swanson PE et al. Akt decreases lymphocyte apoptosis and improves survival in sepsis. J.Immunol. 2004;172:7583-7591.
62. Felmet KA, Hall MW, Clark RS, Jaffe R, Carcillo JA. Prolonged lymphopenia, lymphoid depletion, and hypoprolactinemia in children with nosocomial sepsis and multiple organ failure. J Immunol. 2005;174:3765-3772.
63. Hotchkiss RS, Tinsley KW, Swanson PE et al. Depletion of dendritic cells, but not macrophages, in patients with sepsis. J.Immunol. 2002;168:2493-2500.
64. Brahmamdam P, Inoue S, Unsinger J et al. Delayed administration of anti-PD-1 antibody reverses immune dysfunction and improves survival during sepsis. J.Leukoc.Biol. 2010;88:233-240.
65. Inoue S, Bo L, Bian J et al. Dose-dependent effect of anti-CTLA-4 on survival in sepsis. Shock 2011;36:38-44.
66. Unsinger J, McGlynn M, Kasten KR et al. IL-7 promotes T cell viability, trafficking, and functionality and improves survival in sepsis. J.Immunol. 2010;184:3768-3779.
67. Seok J, Warren HS, Cuenca AG et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. Proc.Natl.Acad.Sci.U.S.A 2013;110:3507-3512.

68. Brearley S, Gentle TA, Baynham MI et al. Immunodeficiency following neonatal thymectomy in man. Clin. Exp.Immunol. 1987;70:322-327.
69. Eysteinsdottir JH, Freysdottir J, Haraldsson A et al. The influence of partial or total thymectomy during open heart surgery in infants on the immune function later in life. Clin.Exp.Immunol. 2004;136:349-355.
70. Madhok AB, Chandrasekran A, Parnell V et al. Levels of recent thymic emigrant cells decrease in children undergoing partial thymectomy during cardiac surgery. Clin Diagn.Lab Immunol. 2005;12:563-565.
71. Mancebo E, Clemente J, Sanchez J et al. Longitudinal analysis of immune function in the first 3 years of life in thymectomized neonates during cardiac surgery. Clin.Exp.Immunol. 2008;154:375-383.
72. Wells WJ, Parkman R, Smogorzewska E, Barr M. Neonatal thymectomy: does it affect immune function? J.Thorac.Cardiovasc.Surg. 1998;115:1041-1046.
73. Torfadottir H, Freysdottir J, Skaftadottir I et al. Evidence for extrathymic T cell maturation after thymectomy in infancy. Clin.Exp.Immunol. 2006;145:407-412.
74. Prelog M, Wilk C, Keller M et al. Diminished response to tick-borne encephalitis vaccination in thymectomized children. Vaccine 2008;26:595-600.
75. Hermann E, Truyens C, onso-Vega C et al. Human fetuses are able to mount an adultlike CD8 T-cell response. Blood 2002;100:2153-2158.
76. PrabhuDas M, Adkins B, Gans H et al. Challenges in infant immunity: implications for responses to infection and vaccines. Nat.Immunol. 2011;12:189-194.
77. Haines CJ, Giffon TD, Lu LS et al. Human CD4$^+$ T cell recent thymic emigrants are identified by protein tyrosine kinase 7 and have reduced immune function. J.Exp.Med. 2009;206:275-285.
78. Halnon NJ, Jamieson B, Plunkett M et al. Thymic Function and Impaired Maintenance of Peripheral T Cell Populations in Children with Congenital Heart Disease and Surgical Thymectomy. Pediatr.Res. 2004;.:
79. Sauce D, Larsen M, Fastenackels S et al. Evidence of premature immune aging in patients thymectomized during early childhood. J.Clin.Invest 2009;119:3070-3078.
80. Prelog M, Keller M, Geiger R et al. Thymectomy in early childhood: significant alterations of the CD4(+) CD45RA(+)CD62L(+) T cell compartment in later life. Clin.Immunol. 2009;130:123-132.
81. den Braber I, Mugwagwa T, Vrisekoop N et al. Maintenance of peripheral naive T cells is sustained by thymus output in mice but not humans. Immunity. 2012;36:288-297.
82. Freitas AA, Rocha B. Population biology of lymphocytes: the flight for survival. Annu.Rev.Immunol. 2000;18:83-111.
83. Naylor K, Li G, Vallejo AN et al. The influence of age on T cell generation and TCR diversity. J Immunol. 2005;174:7446-7452.
84. Hakim FT, Memon SA, Cepeda R et al. Age-dependent incidence, time course, and consequences of thymic renewal in adults. J Clin Invest 2005;115:930-939.
85. Muraro PA, Douek DC, Packer A et al. Thymic output generates a new and diverse TCR repertoire after autologous stem cell transplantation in multiple sclerosis patients. J Exp.Med. 2005;201:805-816.
86. Vrisekoop N, van Gent R, de Boer AB et al. Restoration of the CD4 T cell compartment after long-term highly active antiretroviral therapy without phenotypical signs of accelerated immunological aging. J.Immunol. 2008;181:1573-1581.
87. Blackman MA, Woodland DL. The narrowing of the CD8 T cell repertoire in old age. Curr.Opin.Immunol. 2011;23:537-542.
88. Yager EJ, Ahmed M, Lanzer K et al. Age-associated decline in T cell repertoire diversity leads to holes in the repertoire and impaired immunity to influenza virus. J.Exp.Med. 2008;205:711-723.
89. Nikolich-Zugich J, Slifka MK, Messaoudi I. The many important facets of T-cell repertoire diversity. Nat. Rev.Immunol. 2004;4:123-132.
90. King C, Ilic A, Koelsch K, Sarvetnick N. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. Cell 2004;117:265-277.
91. Long SA, Rieck M, Tatum M et al. Low-dose antigen promotes induction of FOXP3 in human CD4$^+$ T cells. J.Immunol. 2011;187:3511-3520.

92. Sun CM, Hall JA, Blank RB et al. Small intestine lamina propria dendritic cells promote *de novo* generation of Foxp3 T reg cells via retinoic acid. J.Exp.Med. 2007;204:1775-1785.

93. Allan SE, Song-Zhao GX, Abraham T, McMurchy AN, Levings MK. Inducible reprogramming of human T cells into Treg cells by a conditionally active form of FOXP3. Eur.J.Immunol. 2008;38:3282-3289.

94. Walker MR, Kasprowicz DJ, Gersuk VH et al. Induction of FoxP3 and acquisition of T regulatory activity by stimulated human $CD4^+CD25^-$ T cells. J.Clin.Invest 2003;112:1437-1443.

95. Kretschmer K, Apostolou I, Hawiger D et al. Inducing and expanding regulatory T cell populations by foreign antigen. Nat.Immunol. 2005;6:1219-1227.

96. Carpentier M, Chappert P, Kuhn C et al. Extrathymic induction of Foxp3 regulatory T cells declines with age in a T-cell intrinsic manner. Eur.J.Immunol. 2013

97. Komatsu N, Mariotti-Ferrandiz ME, Wang Y et al. Heterogeneity of natural $Foxp3^+$ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. Proc.Natl.Acad. Sci.U.S.A 2009;106:1903-1908.

98. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. $FOXP3^+$ regulatory T cells in the human immune system. Nat.Rev.Immunol. 2010;10:490-500.

99. Ohkura N, Hamaguchi M, Morikawa H et al. T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. Immunity. 2012;37:785-799.

100. Miyara M, Yoshioka Y, Kitoh A et al. Functional delineation and differentiation dynamics of human $CD4^+$ T cells expressing the FoxP3 transcription factor. Immunity. 2009;30:899-911.

Addenda

NEDERLANDSE SAMENVATTING

Regulatie van het afweersysteem na kinderhartchirurgie

De taak van het afweersysteem is om snel te reageren op potentieel schadelijke stimuli. Na activering van het afweersysteem wordt een afweerreactie nauwkeurig gecontroleerd om schadelijke bijwerkingen te voorkomen. Hartchirurgie is een sterke stimulans voor het optreden van een acute systemische afweerreactie en is daardoor uitermate geschikt om deze afweerreactie in mensen in detail te bestuderen. Na activering van het afweersysteem door de ingreep, kan de afweerreactie bestudeerd worden van de eerste verschijnselen van ontsteking totdat deze weer is uitgedoofd. Bij een gezonde afweer zal het afweersysteem na verloop van tijd weer in een natuurlijke balans terugkeren.

Dit proefschrift bestudeert hoe het afweersysteem van kinderen reageert op een tweetal aspecten van kinderhartchirurgie. Het eerste deel van dit proefschrift beschrijft de acute afweerreactie welke optreed na hartchirurgie waarbij er gericht wordt gekeken naar een aantal intrinsieke regelmechanismen die van belang zijn dat deze afweerreactie niet uit de hand loopt. In het tweede deel van dit proefschrift wordt het effect bestudeerd van het verwijderen van de zwezerik, of thymus (thymectomie), ten tijde van de hartoperatie op het zich ontwikkelende afweersysteem.

De afweerreactie ten gevolge van hartchirurgie bij kinderen beïnvloedt het post-operatieve herstel

Zoals eerder beschreven, veroorzaakt een hartoperatie een afweerreactie. **Hoofdstuk 2** geeft een overzicht van de belangrijkste prikkels van de afweerreactie na kinderhartchirurgie. In de afgelopen decennia zijn chirurgische technieken en peri-operatieve behandelmogelijkheden sterk verbeterd, met als gevolg minder activatie van het afweersysteem. Desondanks treedt er een onvermijdelijke postoperatieve afweerreactie op tijdens de operatie en gedurende de postoperatieve herstel periode op de kinderintensive care. Dit komt door een combinatie van de pre-operatieve klinische conditie van een patiënt en intra-operatieve stimulansen zoals chirurgische weefselschade en het gebruik van de hartlongmachine. Deze postoperatieve afweerreactie kan leiden tot complicaties als gevolg van een te sterke, ongecontroleerde afweerreactie. Een ongecontroleerde ontstekingsreactie kan bij een te sterk geactiveerd afweersysteem resulteren in toegenomen vaatpermeabiliteit en orgaanfalen, of bij het tekort schieten van de afweer een risico op infecties. De uitdaging in de postoperatieve behandeling is vervolgens het vinden van de juiste balans in het beïnvloeden van het afweersysteem, zonder het herstel nadelig te beïnvloeden. Tot op heden zijn er weinig medicamenteuze behandelingen succesvol gebleken om de uitersten van de postoperatieve afweerreactie gunstig te beïnvloeden. Een goed inzicht in de postoperatieve afweerreactie is cruciaal voor het succesvol ontwikkelen van nieuwe behandelmogelijkheden. Hierbij is het van belang te onderkennen dat het afweersysteem

bij kinderen waarschijnlijk niet identiek is als bij volwassenen. Hier dient dan ook specifiek onderzoek naar te gebeuren.

Verminderde reactiviteit van monocyten na hartchirurgie

Een bekend fenomeen tijdens een systemische afweerreactie is een verminderde reactiviteit van monocyten op mogelijke schadelijke prikkels. Deze schadelijke prikkels worden herkent door specifieke receptoren op de celwand, toll like receptoren (TLR). In **hoofdstuk 3** bestuderen we de verminderde reactiviteit van monocyten na kinderhartchirurgie. We waren met name geïnteresseerd of dit als gevolg van tolerantie voor TLR stimulatie was of door actieve remming. In dit hoofdstuk tonen wij aan dat kinderhartchirurgie, waarbij de hartlongmachine gebruikt wordt, een gecontroleerde systemische afweerreactie veroorzaakt. Deze afweerreactie wordt gekarakteriseerd door het vrijkomen van cytokinen en een toename in het aantal circulerende geactiveerde monocyten. Deze monocyten reageren gedurende enkele uren na de operatie minder op stimulatie via TLR. Wij lieten zien dat dit niet het gevolg van tolerantie is, maar door actieve remming, waarbij de transcriptiefactor STAT3 een cruciale rol speelt. Plasma van patiënten vier uur na de operatie remde krachtig het vermogen van monocyten om TNF-α (een belangrijk cytokine om een afweerreactie te initiëren) te produceren na stimulatie met lipopolysacchariden (LPS, een onderdeel van de celwand van bacteriën). Deze verminderde TNF-α productie is niet het gevolg van een verminderde TLR activatie aangezien de signaleringscascade direct na de TLR nog functioneerd. Actieve remming door IL-10 / STAT3 blijkt fundamenteel te zijn voor de verminderde monocyten reactie. Dit wordt bevestigd doordat toevoeging van STAT3 remmers aan het plasma, de reactiviteit van monocyten kan herstellen. Samengevat laten onze bevindingen zien dat STAT3 een belangrijke rol speelt in de regulatie van menselijke monocyten tijdens een systemische afweerreactie in vivo en mogelijkheden biedt om medicamenteus in te grijpen in een ongecontroleerde afweerreactie.

Functionaliteit van FOXP3$^+$ Treg tijdens systemische inflammatie

FOXP3$^+$ regulatoire T cellen (Treg) zijn belangrijke regelaars van inflammatie. Zij hebben een sterk regulerende rol in zowel het aangeboren (aspecifieke) afweersysteem als het verworven (specifieke) afweersysteem. In de afgelopen decennia hebben Treg met name faam verworven door hun functie in perifere tolerantie. In **hoofdstuk 4** wordt hun rol gedurende de afweerreactie na kinderhartchirurgie beschreven. De proportie FOXP3$^+$ T cellen stijgt vierentwintig uur na de operatie ten opzichte van de algemene T cel populatie. Daarnaast heeft de FOXP3$^+$ T cel populatie het hoogste percentage delende cellen. De beschreven populatie beschikt over alle kenmerken van echte Treg met hoge expressie van specifieke receptoren (CD25, CTLA-4 en GITR) en zijn niet in staat te delen na in vitro T cel receptor (TCR) stimulatie. Ondanks deze klassieke kenmerken van echte Treg is deze populatie FOXP3+ T cellen na hartchirurgie minder in staat om hun fundamentele taak uit te oefenen, namelijk het remmen van delende effector T cellen. Plasma factoren blijken ook hierin een belangrijke rol te spelen, aangezien plasma

afgenomen van patiënten 4 uur na de operatie, gezonde Treg remt in hun functie. Concluderend wordt FOXP3 snel geïnduceerd tijdens een systemische afweerreactie en is hierdoor van belang voor de endogene regulatie van de afweerreactie na kinderhartchirurgie.

De samenstelling van de Treg populatie verandert na thymectomie op neonatale leeftijd ondanks tekenen van compensatoire perifere celdeling

Thymectomie op jonge leeftijd is soms vereist tijdens neonatale hartchirurgie om toegang te verschaffen tot het operatiegebied. De thymus is echter cruciaal voor de ontwikkeling van het immuunsysteem op jonge leeftijd, maar ook voor het onderhoud van een gezond immuunsysteem later. Treg zijn een subpopulatie T cellen van belang voor homeostase van het afweersysteem. Deze populatie T cellen kunnen geproduceerd worden door twee verschillende mechanismen. Treg kunnen geproduceerd worden in de thymus of perifeer door inductie uit andere T cellen. Perifeer circulerende Treg kunnen onderverdeeld worden in functioneel verschillende subpopulaties op basis van expressie van de eiwitten FOXP3 en CD45. In **hoofdstuk 5** wordt het effect bestudeerd van neonatale thymectomie op deze verschillende Treg subpopulaties. Na thymectomie is het aantal $FOXP3^+$ Treg cellen in de circulatie verminderd met een significant lager aantal Treg die kortgeleden uit de thymus gemigreerd zijn ($CD31^+FOXP3^+$ T cellen). Het totale aantal memory Treg ($CD45RO^+FOXP3^{high}$) blijft stabiel, terwijl de naïeve Treg populatie ($CD45RO^-FOXP3^{low}$) in patiënten ouder dan 6 maanden significant verlaagd is ten opzichte van leeftijdsgenoten. Dit laat zien dat het afweersysteem in staat is om het verlies van de thymus tot op zekere hoogte te compenseren, maar dat de samenstelling van de Treg populatie uiteindelijk verandert. Het is belangrijk om het effect hiervan op de functionaliteit van het afweersysteem te bestuderen, in het bijzonder voor tolerantie later in het leven. Concluderend laat deze studie zien dat perifere proliferatie van Treg cellen voor een deel het verlies van functionele thymus opvangt. Wat het effect is van een veranderde samenstelling van deze populatie (relatief minder naïeve Treg ten opzichte van memory Treg), voor de functionaliteit later in het leven verdient verder onderzoek.

De naïeve T cel populatie is in de eerste jaren na neonatale thymectomie aangedaan, maar herstelt later in het leven met tekenen van thymus regeneratie

Verschillende studies laten zien dat thymectomie op jonge leeftijd ten tijde van een hartoperatie significante effecten heeft voor de zich ontwikkelende T cel populatie, zonder evidente klinische verschijnselen. In **hoofdstuk 6** wordt het effect van neonatale thymectomie bestudeerd op de naïeve T cel populatie in de eerste 30 jaar van het leven. Overeenkomstig met eerdere studies laten we zien dat in de eerste vijf jaar na neonatale thymectomie het aantal circulerende T cellen sterk verminderd is, met het grootste effect op de naïeve T cel populatie. T cell receptor excision circles (TRECs), een maat voor T cel productie door de thymus, is sterk verlaagd in $CD4^+$ T cellen in de eerste jaren na thymectomie. Naast een verminderde productie van T cellen door de thymus zijn er echter ook compensatie mechanismen meetbaar. De plasma concentratie

van IL-7, een cytokine belangrijk voor T cel homeostase, is verhoogd, wat mogelijk bijdraagt aan het herstel van de $CD4^+$ T cel populatie. Desondanks is het aantal perifeer delende T cellen marginaal verhoogd en onvoldoende om het verlies aan thymus geproduceerde T cellen te compenseren. Ongeveer tien jaar na neonatale thymectomie zijn de aantallen T cellen weer vergelijkbaar met die van gezonde personen die geen thymectomie hebben ondergaan. Tevens zijn er tekenen van regeneratie van thymus weefsel, met normalisering van de aantallen $TREC^+$ T cellen. Gelijktijdig met normalisatie van het aantal naïeve T cellen en $TREC^+$ T cellen wordt op MRI beelden teruggroei van thymus gezien. Dit suggereert dat de teruggroei van de thymus fundamenteel is voor de normalisatie van de T cel populatie vanaf 10 jaar na thymectomie.

Samengevat betekent dit dat de T cel populatie in staat is te herstellen door regeneratie van thymus weefsel. Van belang is nu om te onderzoeken of de periode direct na thymectomie (wanneer er onvoldoende thymus gegenereerde T cellen zijn), een effect heeft op de functionaliteit van het afweersysteem later in het leven. Voordat dit goed onderzocht is lijkt het verstandig om, indien mogelijk, deze patiënten groep te opereren met een thymus sparende techniek.

ABBREVIATIONS

ACC	aortic crossclamping
ADA	adenosine deaminase
AKI	acute kidney injury
AoH	aortic hypoplasia
ASD	atrial septum defect
ATP	adenosine triphosphate
aTreg	activated Treg
AVSD	atrioventricular septum defect
CABG	coronary artery bypass graft
C1inh	complement factor 1 esterase inhibitor
CD	cluster of differentiation
CHD	congenital heart disease
CNS2	conserved noncoding sequence 2
CoA	coarctation aorta
CPB	cardiopulmonary bypass
CRP	C-reactive protein
CTLA-4	cytotoxic T lymphocyte antigen-4
DAMPs	danger-associated molecular pattern
DNA	deoxyribonucleic acid
FACS	fluorescence-activated cell sorting
FOXP3	forkhead box P3
GITR	glucocorticoid-induced tumor necrosis factor receptor-related protein
HIF	hypoxia-inducible transcription factor
HIV	human immunodeficiency virus
HLA-DR	human leukocyte antigen DR
HLHS	hypoplastic left heart syndrome
HRHS	hypoplastic right heart syndrome
HSP	heat shock protein
ICAM-1	intercellular adhesion molecule-1
IKK	IkB kinase
IL	interleukin
IL1ra	interleukin-1 receptor antagonist
IFNγ	interferon γ
IRAK	interleukin-1 receptor-associated kinase
iTreg	peripherally induced Treg
JAK	jak family tyrosine kinase
LCOS	low cardiac output syndrome
LOS	length of stay
LPS	lipopolysaccharide = endotoxin

MAPK	mitogen-activated protein kinase
MCP	monocyte chemotactic protein
MG	myasthenia gravis
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MRI	magnetic resonance imaging
MS	multiple sclerosis
MyD88	myeloid differentiation factor 88
NFkB	nuclear factor kappa-B
NO	nitric oxide
nTreg	natural thymic derived Treg
PA	pulmonary atresia
PAMPs	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PD-1	programmed cell death receptor-1
PECAM-1	platelet endothelial cell adhesion molecule = CD31
PICU	pediatric intensive care unit
PRR	pattern recognition receptor
RANTES	regulated on activation, normal T cell expressed and secreted
RCT	randomized controlled trial
RNA	ribonucleic acid
ROS	reactive oxygen species
rTreg	resting Treg
SHP-1	Src homology region 2 domain-containing phosphatase-1
SIRS	systemic inflammatory response syndrome
SOCS	suppressor of cytokine signalling
STAT	signal transducer and activator of transcription
TCR	t cell receptor
Teff	effector T cell
TGA	transposition of the great arteries
TGFβ	transforming growth factor β
TIRAP	toll-interleukin 1 receptor domain-containing adaptor protein
TLR	toll like receptor
TNFα	tumor necrosis factor α
TREC	T cell receptor excision circle
Treg	regulatory T cell
TVA	tricuspid valve atresia
TX	thymectomy
TYK2	tyrosine kinase 2
VEGF	vascular endothelial growth factor
VSD	ventricular septum defect

DANKWOORD

Dit proefschrift is het resultaat van bijna 10 jaar succesvolle samenwerking met een groot aantal mensen en verschillende afdelingen. Het initiële onderzoeksidee, de patiënten inclusie en sample afnames in het kinderhartcentrum, de uitvoering van de experimenten in verschillende laboratoria maar voornamelijk het 'Prakkenlab', analyse van data en de uiteindelijke totstandkoming van dit manuscript, waren alleen mogelijk dankzij onuitputtelijke inzet van velen. Ik wil iedereen voor hun inzet hartelijk bedanken. In de volgende paragraaf wil ik een aantal personen in het bijzonder bedanken.

Dit onderzoek was niet mogelijk geweest zonder de medewerking van ruim 150 **patiënten en hun ouders**. Onze informed consent gesprekken moesten vaak op een zeer ongelukkig moment gebeuren, bijvoorbeeld wanneer ouders zich aan het voorbereiden waren op de hartoperatie van hun kind de volgende dag. Veel dank aan alle patiënten en ouders die het vertrouwen in ons als onderzoeksteam hadden om dit onderzoek mogelijk te maken.

Beste **Berent en Koos**, jullie waren vanaf het begin verantwoordelijk om mij op te leiden als onderzoeker en dit proefschrift tot een goed einde te brengen. *The best journey answers questions you didn't know exist.* **Berent**, jij bent degene die me heeft aangezet om me te blijven verwonderen over het immuunsysteem. Hoewel bij jou het credo 'publish or perish' nooit vooropstaat, moest ik uiteindelijk toch gaan focussen op die publicaties! Al veel promovendi zijn mij voorgegaan en nog velen zullen volgen. Jij maakt een promotieonderzoek voor een ieder een zeer persoonlijke periode, waarbij je het mogelijk maakt om iedereen de ruimte te geven om op zijn eigen mannier een promotietraject tot een goed einde te brengen. In zekere zin berust de samenwerking in jouw lab zich op de 'Gestalt filosofie', waarbij het geheel meer is dan de som van de losse onderdelen. Hoewel het lab over de tijd flink gegroeid is, heb je dit toch weten te behouden. Ik ben er trots op dat ik bij jou mag promoveren. **Koos**, ik kan me nog goed herinneren dat wij onze eerste gesprekken voerden welke uiteindelijk leidden tot dit promotie onderzoek. Ik heb grote waardering voor hoe jij je steeds weer voor dit onderzoek hebt ingezet en over de jaren hebt uitgebreid met onderzoekers en onderzoekslijnen. Ik was volgens mij jouw eerste promovendus, en heb het denk ik ook het langste weten te rekken. Tijdens deze periode heb ik je leren kennen als een zeer toegewijde onderzoeksleider. Naast je voortdurende motivatie in het onderzoek ben je ook altijd op de hoogte van het persoonlijke leven van iedereen met wie je samenwerkt. Nu we ook klinische collega's worden zie ik genoeg mogelijkheden voor de toekomst om deze samenwerking te continueren. Spoedig een lamsbout op Britse bodem proberen?

All men dream, but not equally. Those who dream by night, in the dusty recesses of their minds, awake in the day to find that it was vanity. But the dreamers of the day are dangerous men, for they may act their dreams with open eyes to make it reality (T.E. Lawrence).

Mijn dank gaat uit naar de **beoordelingscommissie; Prof. van Dijk, Prof. Hack, Dr. van Rossum, Prof. van Vught, Prof. Wulffraat**. Ik kijk uit naar onze gedachtenwisseling op 11 december.

Over de jaren hebben een aantal personen belangeloos zeer waardevolle kennis gedeeld. **Prof. Jan Kimpen, Prof. Wietse Kuis, Prof. Hans van Vught en Marianne Boes** wil ik hier in het bijzonder noemen voor die momenten dat ik even vanuit een totaal andere invalshoek naar mijn onderzoek kon kijken of voor persoonlijk advies kon aankloppen.

Dan veel dank aan mijn directe onderzoeksmaatjes. Lieve **Selma**, wat bijzonder dat we deze promotie periode samen kunnen afsluiten! Je hebt me gelukkig net voor laten gaan, aangezien ik ook enkele jaren eerder begon. Na eerst twee en een half jaar alleen het onderzoek vertegenwoordigd te hebben op OK, de PICU en het lab, kwam jij als zeer welkome onderzoekspartner. De sterkte van ons team zat misschien wel met name in hoe we elkaar kunnen aanvullen. Ons team beperkte zich echter niet alleen tot de kamer naast Koos, maar gelukkig stond jij ook altijd open voor een dieper gesprek in Kafé België, met of zonder een schaakbord als afleiding. Met je kritische blik en analytisch vermogen mag iedere radiologie vakgroep zich gelukkig prijzen.

Beste **Theo**, ik ben blij dat jij naast me wilt staan tijdens mijn verdediging. Onze samenwerking begon toen jij student was en er een klik bestond in het filosoferen over hoe het immuunsysteem werkt. Na een periode in San Diego kwam je terug om je eigen onderzoeksproject te organiseren en konden we nog altijd veel samenwerken met verschillende projecten. Ik ben blij dat je dat mooie pandje aan de Oudegracht hebt kunnen overnemen om een mooie traditie voort te zetten. Hopelijk wordt er nog altijd tot laat aan de keukentafel gefilosofeerd over immunologie en andere minder essentiële zaken van het leven. Ik kijk uit naar de afronding van jouw proefschrift.

Lieve **Femke**, in de tijd dat Berent steeds meer promovendi ging begeleiden kwam jij als welkome aanvulling in de groep. Sinds onze eerste ontmoeting op een congres, heb ik altijd makkelijk ideeën met je kunnen delen en van gedachten kunnen wisselen. Jij weet op een altijd charmante wijze precies de zere punten van een stuk te verwoorden, zonder schroom me op de juiste weg te helpen. Hopelijk kan ik blijven tappen uit je kennis.

Beste **Ruud**, na je eerste schreden in translationeel onderzoek vanuit de PICU naar het lab, liet je zien waar je kracht zit. Als student hield je mijn onderzoek draaiende toen ik in Eindhoven kindergeneeskunde leerde. Zonder veel ondersteuning had je in no time de nodige fijne lab technieken weten te beheersen en ons stuk een flinke boost weten te geven. Je gaat het vast nog ver schoppen in de wetenschap, ik kijk uit naar het vervolg na je tijd in de VS.

Het **Prakken lab** is een ruim begrip, dat niet zomaar in enkele woorden te vangen is. Waar te beginnen met dankbetuigingen? In de jaren dat ik voor korte of langere tijd in dit instituut me begaf heb ik veel bijzondere personen mogen ontmoeten. Mijn 'Prakken-periode' begon met **Wilco, Mark, Ismé en Bas**. **Wilco,** jij hielp me met het aanleren en vervolgens begeleiden van experimenten als een verstrooide professor, maar altijd op het cruciale moment heb jij de wijsheid in pacht waarom iets niet werkt. **Mark**, gedurende de 10 jaar dat ik in en uit het lab kwam was jij mijn rots in de branding. De groep veranderde steeds en soms kwam ik in het lab zonder ook maar iemand te kennen, waar jij dan altijd in de buurt was om even te checken hoe dingen ook al weer geregeld worden. Daarnaast heb ik mogen genieten van je indrukwekkende kookkunsten, waar ik jaloers op ben. **Ismé**, ongetwijfeld ben jij mijn eerste leermeester geweest in het lab-werk. Uren heb ik naast je gezeten en samen gepipetteerd, kweken ingezet, en gefacsd. Misschien op dat moment nog niet zo onderkend, werd dat de basis voor dit proefschrift. **Bas**, het eerste artikel samen geschreven met prachtig resultaat… na ontelbare keren opnieuw submitten. Jouw energie en optimisme in het werk is een voorbeeld voor velen. In de vervolg periode in het '**Prakken-lab'** verdienen een aantal personen nog extra aandacht. **Joost**, in het onderzoek was jij vaak mijn soul-mate. Dingen waar we samen van konden genieten, met name de meer filosofische kant van immunologie, nu nog de 'zakelijke' afronding! Met jouw gevoel voor humor kom je nog ver. **Sytze**, hoewel we in het lab weinig immunologische overeenkomsten hadden, heb ik altijd je kennis bewonderd. Als buitenmens staan we dichter bij elkaar. Blijf je verwonderen over de wereld. **Berber,** in het lab hebben we elkaar vaak net misgelopen. Nu we PIC collega's zijn wordt het wellicht tijd om ook met onderzoek meer overeenkomsten te vinden en uit te werken. Benieuwd welke PICU jij komt versterken, mazzelaars!

Vervolgens heb ik het voorrecht gehad om met een aantal zeer gedreven studenten te mogen werken. In order of appearance: **Judith, Eva**, **Marten, Theo, Ruud** en **Mieke**, allen op hun eigen mannier van onschatbare waarde belangrijk voor dit onderzoek en fijne gesprekpartners om over het immuunsysteem te verwonderen. En dan zijn er nog veel onderzoekers uit het **Prakken-lab** waar ik meer of minder mee gewerkt heb, maar een ieder hartelijk wil bedanken voor de altijd goede sfeer: **Annick, Annemarie, Arash, Ellen, Evelien, Eveline, Eva, Gijs, Henk, Huib, Jeffrey, Jenny, Jorg, Lianne, Lieke, Lise, Maja, Mariska, Marloes, Pleun, Sanne, Sarah, Sylvia**, **Yvonne.** Over de jaren heb ik veel bijzondere personen mogen ontmoeten, en is de kans aanwezig dat ik iemand ten onrechte vergeet. Last but not least in dit rijtje; **Erica en**

Angela, bewakers van Berent's agenda en alwetend hoe dingen geregeld moeten worden. Veel dank dat jullie mijn vele vragen steeds weer wilden beantwoorden.

Behalve de hulp die ik heb mogen krijgen uit mijn eigen lab, heb ik kunnen putten uit kennis van een heel aantal onderzoekers uit andere groepen. In het bijzonder van belang voor de totstandkoming van dit proefschrift wil ik noemen: **Kiki, José en Rogier** samen een prachtig hoofdstuk mogen schrijven dank zij de input van jullie invalshoek in de immunologie. Veel dank voor de praktische hulp uit diverse labs: **Ger, Gerrit, Koos, Pirko, Sigrid.**

Beste **Joke**, veel dank om in deze gehele periode altijd beschikbaar te zijn om een aantal onmogelijke agenda's te coördineren voor een bespreking. Jij was voor mij, waar ik je dan ook vandaan mailde het aanspreekpunt om dingen voor elkaar te krijgen, van onschatbare waarde!

Pelikaan, wat een bijzondere afdeling zijn jullie. Ik ben er ingelijfd als student, vervolgens onderzoeker en uiteindelijk als arts-assistent. Voor mijn onderzoek zijn jullie cruciaal geweest. Soms kritisch ten opzichte van bloedafnames, meestal geïnteresseerd in wat er vervolgens met het bloed gebeurt. Jullie zijn een groep verpleegkundigen met veel kennis en primair verantwoordelijk voor de zorg van de kinderen die op de intensive care liggen. Veel dank voor jullie medewerking en ik hoop op termijn dat dit soort onderzoek ingebed wordt in de patiëntenzorg op jullie afdeling.

OK team. Mijn dank gaat uit naar het chirurgisch team, **Prof. Hitchcock, Jola Evens** en later **Prof. Haas en Prof. Schoof**. Dat wij deze patiënten hebben kunnen includeren is grotendeels door het groot vertrouwen dat de ouders in jullie hebben. Tijdens de ingreep konden de vele bloedafnames alleen gebeuren door de medewerking van anesthesisten (**Ton, Nigel, Bart, Erik, Alex**) en **perfusionisten**. En dan in het bijzonder wil ik **Frank** hier noemen voor zijn nooit aflatende behulpzaamheid.

Dank ook voor de klinische ondersteuning op de poli's en afdeling **Leeuw**. Mijn dank gaat uit naar de gehele afdeling **kindercardiologie** waarbij vooral **Gertjan Sieswerda** vanaf de 'overkant' de follow-up TGA patiënten in de gaten hield en **Rutger-Jan Nievelstein** van essentiële waarde was voor de interpretatie van vele MRI scans.

PICU Bristol; the final stretch of finishing my PhD thesis coincided with my start as a PIC fellow in Bristol. I look forward to come back after my anaesthetic detour in Gloucester. In particular i want to thank **Pat Weir** for helping me get started in Bristol PICU life. Then, **Prof. Andy Wolf**, a great pleasure to be able to finish this scientific moment with you as an opponent! I look forward to discuss matters from this thesis with your sharp mind! *When I see an adult on a bicyle I do not despair of the human race (H.G. Wells).*

Deze gehele promotie periode was voor mij alleen mogelijk dankzij de nodige afleiding en continue ondersteuning van familie en vrienden. Vrienden, om over andere zaken dan onderzoek te praten en van avonturen te dromen; **Bertho, Herman, Joost, Peter, Timme, Tjerk en Wouter,** met jullie is het leuker. *The mountain is high, the ocean wide, and that which does not kill us makes us stronger (F. Nietzsche).*

What would I do without my **family**? Thanks to **Rowan, Bryony, Toby, James and Rosemary** for being my siblings and being considerate over the years. While moving across the channel has not improved the possibility of catching up regularly, I'm glad to know this will not change our feeling for each other, being family. I'm proud of every single one of you, following your own destination! *Family, that slippery word, a star to every wandering bark, and everyone sailing under a different sky (Mark Haddon).*
Extended family, **Marijke, Jos, Nienke, Mirjam** and **Niels**, and of course all the kids making the family gatherings even more fun and bustling; **Emily Rose, Eleonora, Heather, Mathew Owen, Louise Emma** and recently **Oliver**, thanks for all being there.

Dank ook aan **Theo, Annie, Noud** en **Robert**. Het boekje is af, fijn om samen te kunnen delen en op naar meer mooie gelegenheden om samen te vieren!

A special thanks to **Dad and Mum** for always supporting me, regardless of where this has brought me and too often further afield. Therefore, this thesis is dedicated to you. Although I keep telling myself that after this I will have more time to spend with family and friends, you probably know too well that this is life. You have equipped me very well to keep searching the horizon for new adventures and challenges. Alas, this does not always bring me closer to you, I love you very much. *I don't know where I'm going but I'm on my way (C. Sandburg).*

Ellen, lieverd, jij hebt als geen ander mijn hoogte en dieptepunten als onderzoeker moeten doorstaan. Ik ben heel trots op hoe jij steeds je eigen carrière pad weet te bepalen en flexibel genoeg bent om samen, waar dan ook op deze wereld, een mooi leven op te bouwen. Benieuwd wat volgend jaar in petto heeft! Met al dat prachtige landschap in onze directe omgeving kijk ik er naar uit om onze vrije momenten in plaats van achter een computer te spenderen, op ontdekking te gaan, op avontuur. Never a dull moment.
The longing for Africa, once contracted, is an incurable condition which like malaria recurs again and again (Peter Matthiessen).

I keep six honest serving-men
(They taught me all I knew);
Their names are What and Why and When
And How and Where and Who,
I send them over land and sea,
I send them east and west;
But after they have worked for me,
I give them all a rest.

I let them rest from nine till five,
For I am busy then,
As well as breakfast, lunch and tea,
For they are hungry men:
But different folk have different views;
I know a person small-
She keeps ten million serving-men,
Who get no rest at all!
She sends 'em abroad on her own affairs,
From the second she opens her eyes-
One million Hows, two million Wheres,
And seven million Whys!

The Elephant's Child, Rudyard Kipling

CURRICULUM VITAE

Alvin Wietse Leslie Schadenberg was born in the Noordoostpolder on January the 14th in 1977. He graduated from the Ichthus College in Veenendaal in 1995 after which he moved to Utrecht to study Medicine at the University of Utrecht. During his medical study he travelled to Ghana to gain clinical experience. This trip led to the Logba Tota Health Project, which he founded together with a friend to materialize a health centre. He interrupted his study to spend one year in the Volta Region of Ghana to organise the construction and implementation of this clinic. In the final stages of medical study he took an interest in research of inflammation in critical care. This led to a PhD project combing the clinical setting of paediatric intensive care (supervised by Koos Jansen), with fundamental research in the Center for Molecular and Cellular Intervention (led by prof. Berent Prakken) in Utrecht. During his PhD research he entered the training programme for paediatrics at the Wilhelmina Children's Hospital, in Utrecht (supervised by prof. Jan Kimpen and later on by dr. Joost Frenkel). The first steps in paediatric training were taken at the Catharina Ziekenhuis Eindhoven, supervised by dr. Hein Brackel. His paediatric training included a six-month rotation at the paediatric department of Tygerberg Hospital, Stellenbosch University in Cape Town, South Africa (supervised by prof. Mariana Kruger). In 2012 he completed his residency in paediatrics. Currently, Alvin is a Paediatric Intensive Care fellow at the Bristol Royal Hospital for Children, United Kingdom (supervised by dr. Patricia Weir). Alvin lives in Bristol with his fiancée Ellen Nelissen.

De wereld is mijn vaderland, de wetenschap mijn religie

(C. Huygens)

LIST OF PUBLICATIONS

This thesis

Schadenberg AWL, Algra SA, Prakken BJ, Jansen NJG. Inflammatory response to pediatric heart surgery.
Submitted

Schadenberg AWL, van den Broek T, Siemelink MA, Algra SA, de Jong PR, Jansen NJG, Prakken BJ*, van Wijk F*. Differential homeostatic dynamics of human Treg cell subsets following neonatal thymectomy.
Accepted JACI July 2013

de Jong PR*, **Schadenberg AWL***, van den Broek T, Beekman JM, Van Wijk F, Coffer PJ, Prakken BJ*, Jansen NJG*. STAT3 regulates monocyte TNF-alpha production in systemic inflammation caused by cardiac surgery with cardiopulmonary bypass.
PLoS One. 2012;7(4):e35070

van Gent R*, **Schadenberg AWL***, Otto SA, Nievelstein RA, Sieswerda GT, Haas F, Miedema F, Tesselaar K, Jansen NJG, Borghans JA. Long-term restoration of the human T-cell compartment after thymectomy during infancy: a role for thymic regeneration?
Blood. 2011 Jul 21;118(3):627-34.

Schadenberg AWL, Vastert SJ, Evens FC, Kuis W, van Vught AJ, Jansen NJG, Prakken BJ. FOXP3+ CD4+ Tregs lose suppressive potential but remain anergic during transient inflammation in human.
Eur J Immunol. 2011 Apr; 41(4):1132-42

* These authors contributed equally

Other publications

Algra SO, Groeneveld KM, **Schadenberg AWL**, Haas F, Evens FCM, Meerding J, Koenderman L, Jansen NJG and Prakken BJ. Cerebral ischemia initiates an immediate innate immune response in neonates during cardiac surgery.
J Neuroinflammation. 2013 Feb 7;10(1):24

Algra SO, Driessen MM, **Schadenberg AW**, Schouten AN, Haas F, Bollen CW, Houben ML, Jansen NJ. Bedside prediction rule for infections after pediatric cardiac surgery. *Intensive Care Med. 2012 Mar;38(3):474-81*

E.J. Anten-Kools, et al. Een professionele kijk op borstvoeding: Hoofdstuk 1.4 Moedermelk en immunologie, **Schadenberg AWL** *[ISBN 9789023246435] 2011*

de Jong PR, **Schadenberg AWL**, Jansen NJG, Prakken BJ. Hsp70 and cardiac surgery: molecular chaperone and inflammatory regulator with compartmentalized effects. *Cell Stress Chaperones. 2009 Mar;14(2):117-31*

Schadenberg AWL, Vastert SJ, Jansen NJ, Prakken BJ. The right circumscript populations. *Crit Care Med. 2005 Jun;33(6):1468*

Schadenberg AWL, Plötz FB, Fleer A, Van Vught HJ, Kimpen JLL, Wolfs TFW. Macroscopic aspect of tracheobronchial secretions from intubated children has no clinical value. *Clinical Intensive Care 2002, 13(4): 161-165*