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IL-10 IN HUMAN NEWBORNS

Ontogeny of IL-10 secretion and relation to the secretion of IFN-γ, immunoglobulin M, G, and A, and mononuclear cell composition in newborns

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Helsinki 2006

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By

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ACADEMIC DISSERTATION

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1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by Roman numerals:

I Kotiranta-Ainamo A, Rautonen J, Rautonen N. Interleukin-10 Production by Cord Blood Mononuclear Cells. Ped Res 41:110-113, 1997.

II Kotiranta-Ainamo A, Rautonen J, Rautonen N. Imbalanced Cytokine Secretion in Newborns. Biol Neonate 85:55-60, 2004.

III Kotiranta-Ainamo A. The Effect of IL-10 and Anti-IL-10 on Immunoglobulin Secretion in Newborns. Submitted to Scandinavian Journal of Immunology.

IV Kotiranta-Ainamo A, Apajasalo M, Pohjavuori M, Rautonen N, Rautonen J. Mononuclear Cell Subpopulations in Preterm and Full-term Neonates: Independent Effects of Gestational Age, Neonatal Infection, Maternal Pre-eclampsia, Maternal Betamethason Therapy, and Mode of Delivery. Clin Exp Immunol 115:309-314, 1999.

Some previously unpublished data are also presented.

2. ABBREVIATIONS

APC	antigen presenting cell
BCR	B cell receptor
CB	cord blood
CD	cluster of differentiation
ConA	concanavalin A
ELISA	enzyme-linked immunoassay
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GA	gestational age (weeks from the last menstrual period)
IFN-γ	interferon-gamma (or gamma-interferon)
Ig(s)	immunoglobulin(s)
IgM, IgG, IgA	immunoglobulin M, G, and A
IL	interleukin
LPS	lipopolysaccharide
MC	mononuclear cells
MHC I, MHC II	class I and II major histocompatibility complex
NK cell	natural killer cell
PB	peripheral blood
PC	plasma cell
PE	phycoerythrin
PWM	pokeweed mitogen
TCR	T cell receptor
Th0, Th1, Th2, Th3, Th-17	T helper-cell type 0, 1, 2, 3, and -17
Tregs	T regulatory cells

3. ABSTRACT

The purpose of this work was to elucidate the ontogeny of interleukin-10 (IL-10) secretion from newborn mononuclear cells (MCs), and to examine its relation to the secretion of interferon-γ (IFN-γ) and immunoglobulins (Igs). The initial hypothesis was that the decreased immunoglobulin (Ig) synthesis of newborn babies was the result of immature cytokine synthesis regulation, which would lead to excessive IL-10 production, leading in turn to suppressed IFN-γ secretion.

Altogether 57 full-term newborns and 34 adult volunteers were enrolled. Additionally, surface marker compositions of 29 premature babies were included. Enzyme-linked immunoassays were used to determine the amount of secreted IL-10, IFN-γ, and Igs, and the surface marker composition of MC were analyzed with a FACScan flow cytometer.

The three most important findings were:

1. Cord blood MC, including CD5+ B cells, are able to secrete IL-10. However, when compared with adults, the secretion of IL-10 was decreased. This indicates that reasons other than excessive IL-10 secretion are responsible of reduced IFN-γ secretion in newborns.

2. As illustrated by the IL-10 and IFN-γ secretion pattern, newborn cytokine profile was skewed towards the Th2 type. However, approximately 25% of newborns had an adult like cytokine profile with both good IL-10 and IFN-γ secretion, demonstrating that fullterm newborns are not an immunologically homogenous group at the time of birth.

3. There were significant differences in the surface marker composition of MCs between individual neonates. While gestational age correlated with the proportion of some MC types, it is evident that there are many other maternal and fetal factors that influence the maturity and nature of lymphocyte subpopulations in individual neonates.

In conclusion, the reduced ability of neonates to secrete Ig and IFN-γ is not a consequence of high IL-10 secretion. However, individual newborns differ significantly in their ability to secrete cytokines as well as Igs.

4. INTRODUCTION

Serious neonatal infections are an important factor causing morbidity and mortality in both full-term and preterm babies. The increased risk of infections in newborns may partly be due to their immaturity and decreased ability to produce immunoglobulins (Ig) and various cytokines, especially IFN-γ.

While the regulation of Ig production in mice and human adults is well characterized, very little is known about the regulation of Ig production in human newborns or during fetal life. One hypothesis explaining the defective Ig and cytokine production is the specific immaturity of neonatal T and B cells. Newborns have very few memory T cells (CD45RO+ T cells) and up to 99% of the B-cell population of human newborns consists of immature, CD5 positive cells (Antin et al 1986). In mice, similar B cells are important secretors of IL-10 (O'Garra (1) et al 1992), a known immunosupressor. Moreover, a large amount of spontaneously secreted IL-10 has been detected in cultured human cord blood (CB) mononuclear cell (MC) supernatants (Abrams et al 1992).

Our hypothesis was that the low capacity of neonates to produce Igs partly results from the relative excess of immature B cells (e.g. CD5+ B cells) and increased production of IL-10. That would in turn suppress the secretion of IFN-γ and increase general vulnerability to infections. The present study was undertaken to investigate the ontogeny of IL-10 production by cord blood mononuclear cells, and to assess the relationship between IL-10, IFN-γ and Ig secretion in human newborns.

5. REVIEW OF THE LITERATURE

5.1 GENERAL OVERVIEW OF THE HUMAN IMMUNE SYSTEM

5.1.1 Innate and adapted immunity

The immune system is crucial for humans, and animals, in protecting the host against invading pathogens. It can be divided into two general systems: the innate and the adapted immunity. Innate or ”natural” immunity is a rapidly activated host defense, which recognizes conserved microbial structures not expressed by the host, and mounts an unspecific immune response against these structures (often specific carbohydrates or lipoproteins). The activated effectors of innate immunity - phagocytic cells, natural killer cells (NK cells), and the complement system - are able to destroy the invader (Suffredini et al 1999, Borregaard et al 2000).

The adapted (often also called acquired or specific) immune system is a more specific and powerful tool against pathogens, but the primary response mounts slower than in innate immunity. Crucially, adapted immunity develops a memory, which enables a rapid and effective response in a re-infection. Adapted immunity recognizes antigenic structures (often peptides), not expressed on the host, as non-self. Antigens are presented to the effector cells of the adaptive immunity by antigen presenting cells (APC). The activation of these lymphocytes requires not only signaling through a specific antigen receptor but also through co-stimulatory molecules on APC. The APC can be a part of the innate (dendritic cells and macrophages) or the adapted immunity system (B cells). Lately a new model, ”the Danger model”, suggests, that discrimination between self and non-self is not the trigger of immunity, but that an additional “danger signal” from an infected or damaged tissue would be needed for the activation of immune defense (Anderson et al 2000).

Recent investigations have emphasized the role of dendritic cells (DC) as the most potent antigen presenting cell-type and the prime initiator of the immune response (Ridge et al 1998, Gallucci et al 1999). DC capture and process antigens in the periphery, actively migrate to lymphoid organs, and secrete cytokines to initiate immune responses. Dendritic cells have also a crucial role in maintaining self tolerance by presenting self antigens to T cells (Banchereau et al 1998). Bone-marrow derived DC are in fact a heterogeneous cell-population since at least four types have been defined: the

monocyte-derived DC, the dermal DC-interstitial DC, the Langerhans cell, and the plasmacytoid DC. (Reviewed in (Rossi et al 2005)). Although innate and adaptive immunity represent two separate arms of immunity, a close relationship exists between them. The initiation and direction (cellular or humoral) of adaptive immunity is influenced by innate immunity, which regulates its direction via cytokines, T and B cell co-stimulatory mechanisms, and most of all, antigen presentation (Palucka et al 1999).

Both innate and adaptive immunity are considered to be immature at the time of birth due to low complement levels, as well as impaired neutrofil (English et al 1992), and phagocytic cell functions (Weston et al 1977, Levy 2005). Moreover, the T (Hodge et al 2001) and B cell functions (Wilson et al 1990, Gathings et al 1981) are immature, and maturely functioning APC are scarce in human neonates (Trivedi et al 1997). Although mature functions of neonatal immune cells can be achieved under some circumstances, their responses are often non-protective or dampened (Adkins et al 2004)

5.2 LYMPHOCYTE DIFFERENTIATION

The adapted immunity can, analogously with the general immunity, be divided into two arms: the humoral and the cell-mediated immunity. The humoral immunity is created by B lymphocytes which secrete immunoglobulins, whereas cell-mediated immunity is mediated by direct cell-to-cell contacts and via soluble mediators. T-helper lymphocytes (Th cells) can be skewed toward either humoral or cell-mediated functions (Mosmann et al 1996).

5.2.1 Mononuclear cell CD markers

During lymphocyte development towards the destined phenotype, various surface markers are expressed on the surface of the cell. Some surface markers are solely expressed on one lymphocyte subtype, others are more ubiquitous. These “Cluster of Differentiation”, CD, markers can be identified and used in order to distinguish between different cell types (for example CD19 on B cells and CD3 on mainly T cells). Some CD markers are expressed during activation, like CD25 on T cells. CD45 is a leukocyte common antigen with two isoforms, e.g. CD45RA and CD45RO. Most B cells and naïve T cells are CD45RA positive, while during maturation through antigenic encounter, T cells switch to CD45RO expression. Macrophages/monocytes, B, T, and NK cells all originate from

a common CD34+ precursor stem cell (Srour et al 1991). The patterns, expression, and function of some of the most relevant CD markers are listed in Table I.

Table I. Commonly characterized CD markers, cells where generally expressed, and identified main function(s).

CD number	Expressed on	General function	Useful references
CD45	Leukocyte common antigen	Transmembrane protein tyrosine phosphatase	(Thomas 1989, Irie-Sasaki et al 2003)
Mainly T cell markers			
CD45RA	Naïve T cells, most B cells	Transmembrane protein tyrosine phosphatase	(Morimoto (2) et al 1985, Hermiston et al 2003)
CD45RO	Mature T cells	Transmembrane protein tyrosine phosphatase	(Morimoto (1) et al 1985, Hermiston et al 2003)
CD3	T lymphocytes, NK cell-subset	Part of the TCR complex, signal transduction	(Chetty et al 1994, Malissen et al 1999)
CD4	Helper lymphocytes	Part of the TCR complex, MHCII recognition	(Fabbri et al 2003)
CD8	Killer lymphocytes	Part of the TCR complex, MHCI recognition	(Fabbri et al 2003)
CD25	T lymphocytes	Alpha chain in the IL-2 receptor	(Nelson et al 1998, Zola 2000)
Mainly B cell markers			
CD19	B lymphocytes	Regulates B cell growth and responses	(Tedder, et al 1997, Otero et al 2003)
CD20	B lymphocytes	Regulates proliferation by transmembrane Ca^{++} movement	(Tedder, et al 1994)
CD21	B lymphocytes	Part of signal transduction complex, a receptor for C3	(Tedder, et al 1997)
CD23	B lymphocytes	Receptor for Fc-IgE	(Ikuta et al 1987)
CD5	T and B lymphocytes	Negative regulator of antigen mediated signals	(Tarakhovsky et al 1995, Brossard et al 2003)
CD11a	Mature leukocytes	Adhesion molecule, crucial in Ig secretion (B cells)	(Marlin et al 1987, Katada et al 1996)
CD40	B lymphocytes, APC	Important co-stimulatory molecule	(Lee et al 2003)
Additional markers			
CD16	NK cells, granulocytes, macrophages	Fc-IgG receptor	(Ritz et al 1988, Lanier (1) et al 1989)
CD56	NK cells, T lymphocytes	Adhesion molecule	(Lanier (2) et al 1989)
CD14	macrophages/monocytes	Differentiation antigen, a receptor for LPS	(Wright et al 1990)
HLA-DR	macrophages, lymphocytes	Isoform of MHC II, not a CD marker	(Amlot et al 1996, Andersson 1998)

5.2.2 B lymphocytes (B cells)

The immune defense of B lymphocytes constitutes mainly of Ig production. The first Ig class to be secreted is polyreactive, low affinity natural IgM, which functions as a part of innate immunity. The switch to the secretion of monoreactive and high affinity IgG, IgA, and IgE antibodies is a hallmark of adapted humoral immunity. Recently, B cells were suggested to possess regulatory functions in immune regulation as well (Milner et al 2005). It has been reported, that B cells can, under certain cytokine environment, differentiate into effector-like cells (Be1 and Be2). These effector B cells can, in turn, regulate the differentiation of naïve CD4+ T cells to Th1 and Th2 cells. Be1 cells are reported to secrete IFN-γ and IL-12, and Be2 cells IL-4 (Harris et al 2000, Harris et al 2005). Recently, another report suggested, that under inflammatory conditions, regulatory B cells ("Bregs") were specifically induced to suppress the exacerbation of inflammation and/or enhanced the recovery process (Mizoguchi et al 2006). Although these above mentioned results are from experiments done with murine B cells, some evidence of human regulatory B cells exists as well. If CD40 on B cells was stimulated without antigen/BCR engagement, adult B cells were reported to secrete IL-10, which suppressed the inflammatory response (Duddy et al 2004).

Immunoglobulin, the antibody molecule that is produced and secreted only by B cells, is the most important tool for the humoral arm of the adapted immunity. One B cell produces only one kind of antibody of given specificity. An immunoglobulin molecule is composed of four polypeptide chains: two identical heavy chains with four or five domains and two identical light chains with two domains (Nisonoff et al 1964). Both the heavy and the light chains include constant (Fc) and variable (Fv) domains. The variable domain is able to recognize a specific antigenic peptide and the constant domain is responsible for the biological functions of the antibody. When an antigen is bound to the Fv domain, a signal can be delivered through the Fc domain through its interaction with specific receptor, called Fc receptor (FcR). All Ig classes have FcRs and the biological diversity lie, partly, in their ability to bind to different FcR in various cells and systems (e.g. B cells, macrophages/monocytes, granulocytes, basophils, eosinophils, and/or the complement system) (Fridman 1991). The different Ig classes are listed in Table II.

Table II. Five Ig classes, IgM, IgG, IgA, IgE, and IgD, have been recognized in humans according to structural differences in the constant region of the heavy polypeptide chains (Spiegelberg 1974, Spiegelberg 1989).

Immunoglobulin	Functions	Useful Reviews
IgM	Antigen receptor for B cells	(Davis et al 1989)
	Activates the complement cascade	(Boes 2000)
	Is secreted during primary immune responses	
IgG	Targets antigens for phagocytosis	(Papadea et al 1989)
	Activates complement cascade	(Brekke et al 1995)
	Binds to Fc receptors in neutrophils	(Herrod 1993)
	Four subclasses	
IgA	Covers mucosal surfaces	(Lamm 1988)
	Inhibits antigen uptake from mucosal surfaces	(Fagarasan et al 2003)
	Is secreted to body fluids	
	Binds to Fc receptors in neutrophils	
	Two subclasses	
IgE	Ag receptor for basophils and eosinophils	(Spiegelberg 1989)
	Vital in parasite clearance	(Negrao-Correa 2001)
	Mediates anaphylaxis if triggered inappropriately	(Gould et al 2003)
IgD	Antigen receptor for B cells	(Preud'homme et al 2000)
	Marker for mature B cells	(Kim et al 1995)

Each naïve B cell expresses hundreds of B cell receptors (BCR). The BCR consists of a membrane bound Ig associated with assisting signaling molecules. B cells are effectively stimulated by DCs but they can also directly recognize native antigens through their BCR (Banchereau 1998). Activation through BCR leads to further effector activities.

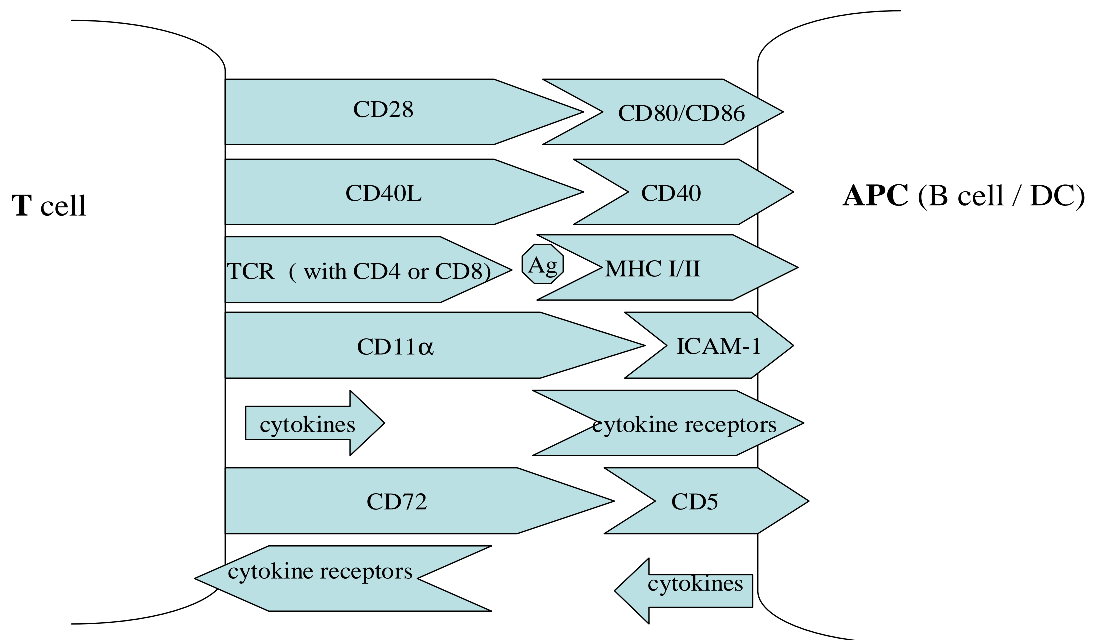
5.2.2.1 Independent B cells activation

B cell activation can be either T cell dependent or T cell independent. In T cell independent activation, either an intrinsically mitogenic antigenic peptide or a multivalent antigen, such as a pneumococcal polysaccharide, activates the B cell. The Ig classes secreted after this relatively simple form of B cell activation are IgM, IgG2, and IgG3.

5.2.2.2 T cell dependent B cells activation

The T cell dependent activation is more complex, since before T cells can provide help for antigen activated B cells, they have to be activated to express the CD40-ligand which then interacts with an important B cell activating molecule CD40 (van Essen et al 1995). Additional crucial ligand interactions include CD28 on T cell, which acts as a counter receptor for CD80/CD89 on APCs (Linsley et al 1993) and CD5-CD72 (T cell-B cell) ligand pair (Luo et al 1992, Van de Velde et al 1991). These ligand-pair interactions form a circuit which, when properly connected, directs B and T cells to full immune defense functions. A simplified schematic representation of APC-T-cell interactions is presented in Figure 1.

Figure 1. A schematic representation of the many ligand pair-interactions between T cell-APC interaction. The primary signal is delivered through TCR and major histocompatibility complex (MHC) ligation where the APC presents the processed antigenic peptide, bound to MHC complex, to the T cell. This interaction up-regulates additional accessory molecules on T cells, e.g. CD40-ligand (CD40L) which ligates with CD40 on APC, CD28 which interacts with CD80/CD86 (on APC), adhesion molecule CD11alpha (formerly LFA-1) which ligates with ICAM-1 (Grewal 1996). CD4 or CD8, as part of the TCR interact with the MHC. The CD5 is expressed on B cells but not on DC.



Additionally, various cytokines, (especially IL-4, IL-5, (Splawski et al 1989, Clark et al 1994) and IL-10 (Rousset et al 1992)), assist in different stages of B cell development. The germinal centers in the spleen and lymph nodes are the sites where an antigen can be presented for a prolonged time on follicular DC. Only a few oligoclonal B cells colonize each follicle, but they are expanded rapidly to form the germinal center. These B cell blasts are able to activate a hypermutation mechanism that acts on their immunoglobulin-variable (Ig-v)-region genes. During this process, Ig-v regions undergo

somatic mutations in a high rate to create high affinity antibodies. From these B cell clones are both Ig secreting terminal plasma cells, and long living memory B cells, generated (MacLennan 1994).

5.2.3 T lymphocytes (T cells)

T lymphocytes express on their surface a T cell antigen receptor (TCR), which has specificity for a single antigenic peptide. A specific antigen is thus able to activate only a small number of T cells, which are then clonally expanded upon an antigenic encounter. However, additional signals are a prerequisite for the activation and expansion of T cells (Figure 1.). The primary signal is delivered through TCR and major histocompatibility complex (MHC) ligation where an APC, typically a DC, presents to the T cell the processed antigenic peptide bound to MHC molecule. These interaction up-regulates additional accessory molecules on T cells (Grewal et al 1996). The CD40L-CD40 interaction seems compulsory for antigen specific T cell responses of CD4+ T cells (Grewal et al 1995) but is not as important for primary CD8+ T cells (Whitmire et al 1999).

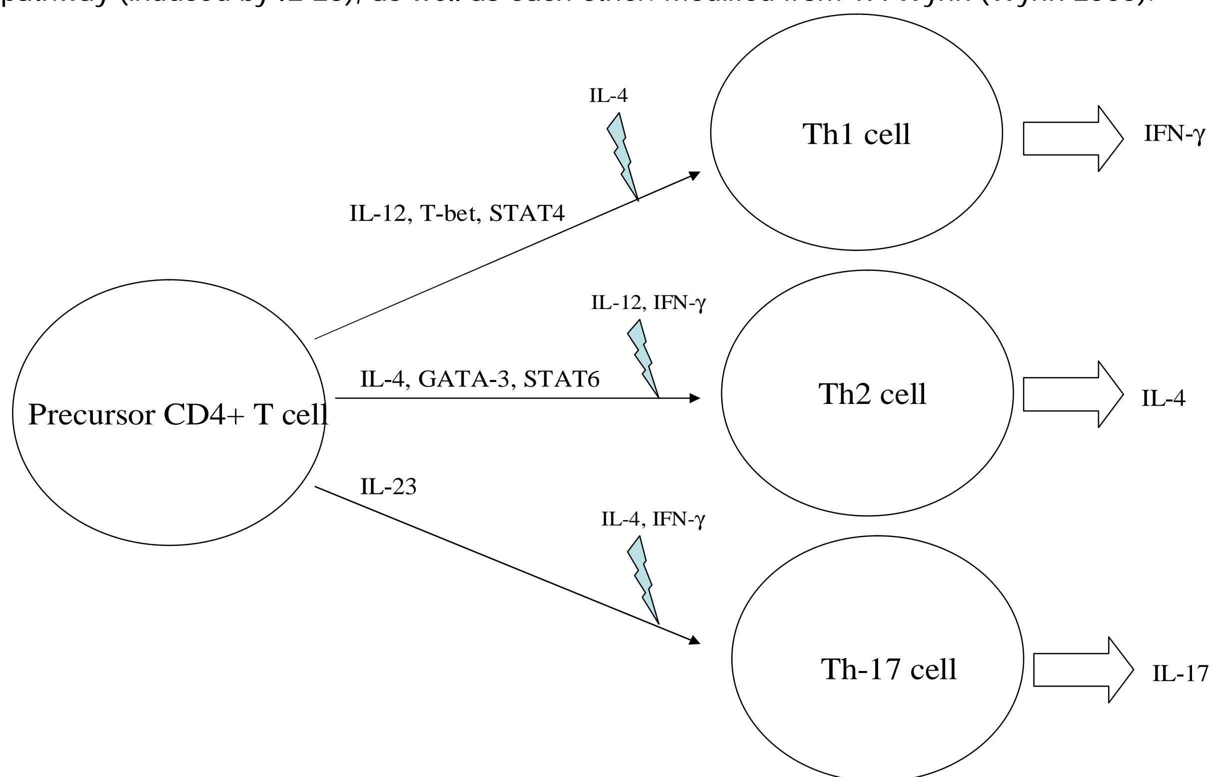
The differentiation towards helper T cell (CD4+) or killer T cell (CD8+) begins already in the bone marrow. As indicated by their name, T helper cells help to mediate humoral and cellular immune responses while T killer cells are cytotoxic and mediate target lysis. After the activation of a naïve T cell by cell-cell mediated contacts, the concomitant up regulation of surface cytokine receptors and cytokine secretion by adjacent cells direct the T cell towards full effector function.

5.2.3.1 Th-subtypes

In 1986, Mosmann et al reported that murine Th-cells could be divided into two types, named Th1 and Th2 cells (Mosmann et al 1986). The Th1 clones secreted mainly IL-2 and IFN-γ but no IL-4 or IL-5, whereas the Th2 clones secreted IL-4, IL-5 and a cytokine initially called cytokine synthesis inhibitory factor (CSIF), because it inhibited cytokine secretion of the Th1 clones (Fiorentino (2) et al 1989). CSIF was later re-named IL-10. Soon afterwards it was reported that human T helper cells had the same properties (Wierenga et al 1991) although the division was not strictly dichotomous (Th1 and Th2), but more complex, including a Th0 cell-type (Mosmann et al 1989, Abbas et al 1996). This Th0 cell clone secreted some amounts of both Th1 and Th2 type cytokines. A fourth T helper subset has been proposed, a regulatory Th3 cell type, which mainly secretes TGF-β which inhibits both Th1 (Powrie et al 1996, Strober et al 1997) and Th2 (Pakala et al 1997) type immune

responses (Bridoux et al 1997). Recently more types of naturally arising T cells have been identified. One is CD25+CD4+ regulatory T cell contributing to the maintenance of immunologic self-tolerance and negative control of various immune responses (Sakaguchi 2004), and one associated with IL-17 secretion and linked with autoimmune diseases (Park et al 2005, Harrington et al 2005), broadening the diversity of T cells even more. A schematic representation, modified from TA Wynn (Wynn 2005) of the signals driving the development of the well-characterized Th1, Th2, and the latest Th-17 cells, is presented in figure 2.

Figure 2. The newest model of the development of T effector cells. The cytokines and signaling pathways of Th1 (induced by IL-12 which activates transcription factors T-bet and STAT4) and Th2 (induced by IL-4 which activates transcription factors GATA-3 and STAT6) antagonize the Th-17 pathway (induced by IL-23), as well as each other. Modified from TA Wynn (Wynn 2005).



The discovery of effector cells with distinct cytokine profiles quickly led to the recognition of their distinct, polarized functions. The maturation of Th1 cells predominantly directs the immune defense towards cellular immune responses, such as delayed-type hypersensitivity (Cher et al 1987) and macrophage activation (Stout et al 1989) whereas mature Th2 cells direct the immune defense towards a humoral response by providing B cell help in antibody production (Boom, W.H. et al 1988, Toellner et al 1998). Inappropriate skewing towards either Th1 or Th2 cytokine secretion is associated with many diseases, Th1 skewing with auto-immune diseases (Adorini et al 1996,

Heurtier et al 1997, Segal et al 1998, Segal et al 1998), and Th2 skewing with atopy and allergies (Wierenga et al 1990, Romagnani 1990, Koning et al 1996).

5.3 CYTOKINE INTERACTIONS DURING LYMPHOCYTE MATURATION

5.3.1 IL-10

Firstly described as a cytokine secretion inhibitory factor (CSIF), IL-10 was soon found to exhibit various immunomodulatory functions. It is mainly secreted by Th2 type T cells (Fiorentino et al 1989), but also macrophages/monocytes (de Waal Malefyt et al 1991), DCs (Yee et al 2005), B cells (Benjamin et al 1992), and even Th1 cells (Del Prete et al 1993), Th0 cells, lung mast cells (Ishizuka et al 1999), B cell derived tumor cell lines (Benjamin et al 1992), and keratinocytes (Rivas et al 1992) are also able to secrete IL-10.

IL-10 is an 18.5 kD protein of 160 amino acids. It exists in the form of a non-covalent homodimer (Vieira et al 1991). The immunomodulatory functions of IL-10 are various, ranging from supporting B cell differentiation and Ig secretion to inducing a strong anti-inflammatory response. A number of newly recognized cytokines, with diverse biological effects, have also been found to belong to the IL-10 related family of cytokines (reviewed in (Fickenscher et al 2002)).

5.3.1.1 Specific effects of IL-10 on B cell maturation

As a humoral (Th2 type) cytokine IL-10 supports a number of B cell responses and acts as a growth and differentiation factor for activated B cells (Rousset et al 1992). However, it also induces apoptosis of initially activated, purified adult B cells in the absence of other cytokines. The IL-10-induced apoptosis can be prevented by IL-2, leading to B cell differentiation into PC (Itoh et al 1995). IL-10 induces resting murine B cells to express class II MHC molecule (Go et al 1990). Furthermore, proliferation (Rousset et al 1992) and IgM, IgG1, IgG3, and IgA secretion of anti-CD40 antibody activated naïve human B cells is greatly accelerated by the presence of IL-10 (Defrance et al 1992, Briere et al 1994, Burdin et al 1995). IL-10 has also an important role in IgA secretion. Ig class switching to IgA is induced by both TGF-β (van Vlasselaer et al 1992) and IL-10 (Kitani et al 1994). Additionally, IL-10 is in charge of the expansion of the switched cells (Defrance et al 1992). Human IL-10 resembles closely murine IL-10, which acts as an autocrine growth factor

for murine B-1 cells, the homologue of human CD5+ B cells (O'Garra (1) et al 1992, O'Garra (2) et al 1992).

5.3.1.2 Specific effects of IL-10 on T cell maturation

The secretion of IL-10 from T cells and monocytes starts later than, and inhibits the secretion of, pro-inflammatory cytokines (de Waal Malefyt et al 1991), such as TNF-α (Sato et al 2003), IL-1α (Fiorentino (1) et al 1991), and IFN-γ (Fiorentino (2) et al 1991, Sher et al 1991, Hsu et al 1992, Yin et al 2003). Therefore, IL-10 can be considered as a natural suppressor of an inflammatory response (de Waal Malefyt et al 1992). The role of IL-10 as a suppressor of the Th1-type immune reaction is mainly mediated by inhibition of antigen-presenting capacity of APCs. IL-10 downregulates the expression of surface molecules on APCs, thus indirectly inhibiting T cell activation and Th1 type cytokine secretion. (Fiorentino (2) et al 1991) More specifically, this occurs by down-regulation of class I and II MHC molecules on the surface of APCs, thereby preventing the antigen specific T cell activation of both CD4+ and CD8+ T cells (de Waal Malefyt et al 1991, Bejarano et al 1992, Koppelman et al 1997, Groux et al 1998). Additionally, IL-10 downregulates other necessary molecules on APCs such as the adhesion molecule ICAM-1 (Willems et al 1994), CD86, and CD80 (Ding et al 1993, Willems et al 1994), the latter two being necessary ligands for T cells co-stimulatory molecule CD28 (Kubin et al 1994). IL-10 has also APC-independent inhibitory effects on T cells, e.g., reduction of IL-2 secretion from responding T cells (de Waal Malefyt et al 1993, Perrin et al 1999).

5.3.1.3 Specific effects of IL-10 on Monocyte/macrophage maturation

Monocytes/macrophages are able to secrete IL-10 among other, mainly pro-inflammatory, cytokines. Interestingly, the secretion of IL-10 seems to be self-limited, since while IL-10 inhibits the secretion of pro-inflammatory cytokines (IL-1, IL-6 and TNF-alpha) from activated macrophage cell-lines (Fiorentino (1) et al 1991) its own production is down-regulated as well (de Waal Malefyt et al 1991). However, the anti-inflammatory effect of IL-10 is not solely due to down-regulation of pro-inflammatory cytokines. IFN-γ activated monocytes/macrophages induce the production of nitric oxide by nitric oxide synthase (NOS). Pre-incubation of murine macrophages with IL-10 inhibits NOS induction and production of nitric oxide (Cunha et al 1992). This IL-10-mediated suppression

of macrophages activity inhibits parasite and fungi killing (Oswald et al 1992, Gazzinelli et al 1992, Cenci et al 1993).

Besides down-regulatory effects on monocyte/macrophages, IL-10 can influence human monocyte differentiation at the precursor level. When monocytes are incubated with granulocyte-macrophage colony-stimulating factor and IL-13 they differentiate into DC. IL-10 can direct this differentiation into macrophages (Allavena et al 1998).

5.3.1.4 Clinical aspects of IL-10 function

The immunosuppressive and anti-inflammatory properties of IL-10 that are crucial in preventing harmful inflammatory self-destruction have been well revealed in many experimental studies (Souza et al 2003). IL-10-deficient mice develop a chronic inflammatory bowel disease (Kuhn et al 1993), but on the other hand, when infected with *Pneumocystis carinii*, IL-10 knock-out mice can respond to the organism significantly more efficiently than the wild type mouse (Qureshi et al 2003). Thus in spite of the protective nature against an overwhelming inflammatory response by Th1 cytokines, increased IL-10 secretion is associated with disorders where an infection or malignancy is not eliminated because of depressed cell-mediated response (Zhu et al 2003). Examples of such disease states are lepromatous leprosy (Yamamura et al 1991), experimental listeriosis (Groux et al 1999), and basal cell carcinoma (Yamamura et al 1993). Interestingly, two human herpes viruses (Epstein-Barr virus (Moore et al 1990, Hsu et al 1990, Niiro et al 1992) and the human cytomegalovirus (Kotenko et al 2000)) have a homologous IL-10 gene in their genome. This viral IL-10 might induce local immunosuppression and thus provide additional advantage for viral spread during an infection (Fickenscher et al 2002).

5.3.2 IFN-γ

IFN-γ, a virus-inhibiting agent secreted by human leukocytes, was reported already in 1965 (Wheelock 1965). Human IFN-γ protein consists of 166 amino acids and the functional molecule exists in the form of a non-covalent homodimer (Rinderknecht et al 1984). IFN-γ is mainly produced by T cells, especially by Th1 type T cells (Kasahara et al 1983). In addition, IFN-γ is secreted by various other cells like NK cells (Young et al 1987), B cells (Pang et al 1992), DCs (Sun et al 2003), and macrophages (Fultz et al 1993). Mature T cells expressing the CD45RO marker are considered

to be the main source of secreted IFN-γ (Sanders et al 1988), although in newborns naïve T cells expressing CD45RA+ seem to be the major IFN-γ secreting cell population (Chalmers et al 1998).

The major stimulus for CD4+ T cells to secrete IFN-γ is the recognition of antigen, presented by the MHC II complex, and for CD8+ cells, antigen associated with MHC I complex (Young et al 1995). The production of IFN-γ is regulated by a network of other cytokines: IL-2, IL-12, IL-15, and IL-18 enhance (Kasahara et al 1983, Jung et al 1999, Barbulescu et al 1998, Lau et al 1996, Gosselin et al 1999), whereas IL-10 and TGF-β down-regulate the synthesis of IFN-γ (D'Andrea et al 1993, Fiorentino (2) et al 1989). The role of IL-4 is more complex as it can either suppress or, via IL-12 regulation, enhance IFN-γ production (D'Andrea et al 1995). The role of prostaglandin E2 (PGE_2), which inhibits IFN-γ secretion in adult PB MC (Snijdewint et al 1993), is less clear. Moreover, it seems that PGE_2 does not have a role in neonatal IFN-γ production (Jones et al 1999).

5.3.2.1 Specific effects of IFN-γ on B cell maturation

IFN-γ has multiple effects on B cells, depending on their maturity and on other cellular signals. Suppressive effects include the inhibition of LPS-induced B cell proliferation (Abed (1) et al 1994), downregulation of IL-4 induced MHC II expression (Mond et al 1986), decrease in IgG1 and IgE secretion (Abed (2) et al 1994), and inhibition of murine pre-B cells growth *in vitro* (Grawunder et al 1993). IFN-γ inhibits both T cell-dependent and T cell-independent B cell activation in both conventional (CD5- B cells) and CD5+ B cells (Abed (1) et al 1994). In contrast to these effects, IFN-γ protects mature B cells from apoptosis (Buschle et al 1993), and enhances IgG2a secretion by stimulating the precursor frequency of IgG2a-secreting cells (Snapper et al 1988, Bossie et al 1991).

5.3.2.2 Specific effects of IFN-γ on T cell maturation

IFN-γ has profound effects on the development of CD4+ T cells towards the Th1 direction. IFN-γ suppresses the growth of IL-4 secreting CD4+ cell population (Gajewski et al 1988) and inhibits IL-10 production by monocytes (Chomarat et al 1993). IFN-γ acts as an autocrine growth factor for CD4+ Th1 population synergistically with IL-2 and IL-12. The need of additional signals is essential for Th1 cell differentiation and is well underlined in studies demonstrating that if other accessory signals (like IL-2) are missing, IFN-γ causes apoptosis of antigen activated T cells (Liu et al 1990).

5.3.2.3 Specific effects of IFN-γ on Monocyte/macrophage maturation

IFN-γ is a major activating factor also for monocytes/macrophages (Schultz et al 1983) working in various ways and thus playing an important role in the first line immune defense. IFN-γ activates macrophages by enhancing their NO-dependent microbe killing (Liew et al 1991, Liew et al 1999) and tumor-cell cytotoxicity (Pace et al 1983. Pace et al 1985). IFN-γ increases the killing of intracellular pathogens (Torrico et al 1991), and, by up-regulating HLA-DR on monocytes, increases the presentation of antigens to lymphocytes (Basham et al 1983). Additionally, IFN-γ enhances production of pro-inflammatory cytokines (TNF-α, IL-1) from LPS activated macrophages/monocytes (Hart et al 1989), and stimulates macrophages/monocytes to secrete IL-12. The latter, in turn, is a powerful IFN-γ inducer and thus forms a positive feedback loop for the secretion of IFN-γ (Lee et al 1998). On the other hand, IL-10 production by macrophages/monocytes is inhibited by IFN-γ (Chomarat et al 1993).

5.4 CHARACTERISTICS OF THE NEONATAL IMMUNE PROFILE

As mentioned in section 4.1, both innate and adaptive immunity are considered to be immature at the time of birth. This is thought to increase the risk of serious infections during the neonatal period.

5.4.1 CD-marker expression

Term neonates tend to have proportionally less CD4+ cells and CD3+ cells (Maccario et al 1993, Milosevits et al 1995) than adults. The expression of CD25 and CD45 isoforms (i.e. CD45RA and CD45RO), differ also significantly between adults and newborns. Newborn lymphocytes are reported to express significantly less CD25, (which is up-regulated in activated T cells,) than adult T cells. The CD45RA, a marker of immaturity in T cells, is expressed significantly more, and CD45RO significantly less, than in adult T cells (Erkeller-Yuksel et al 1992, Aldhous et al 1994, Zola et al 1995, Amlot et al 1996). Up to 99% of the neonatal B cells can be CD5 positive, which are known to secrete mainly low affinity, autoreactive natural Ig (Casali et al 1989, Berland et al 2002).

The way of delivery has been reported to influence the proportion of certain cell-types in CB. Vaginal delivery, when compared with caesarean section, increased the proportion of NK cells and decreased the proportion of CD4+ cells (Samelson et al 1992). Very little is known about the phenotypes of the immune cells in preterm newborns. Since at 18-22 weeks of gestational age (GA)

blood samples are sometimes drawn for perinatal diagnosis of severe inherited diseases, some data exist on lymphocyte subtypes at this GA (Lucivero et al 1991, Peakman et al 1992). The composition of the lymphocyte subtypes seems already at that time to be remarkably similar to that in full-term newborns (Baker et al 1987, Moretta et al 1991, Muller et al 1996, Motley et al 1996). However, proportions of lymphocyte subtypes are quite different from adults, and further maturation is thought to take place upon multiple antigen contacts after birth.

5.4.2 Immunoglobulin secretion

The fetal spleen is the primary origin for IgG and IgM synthesis as early as 10 weeks of GA (Gitlin et al 1969). Since Ig secreting B cells (PC) are scarce in the fetal blood or tissue until 15-16 weeks of GA, Ig secretion before the mid second trimester is marginal (Gathings et al 1981).

Pokeweed-mitogen (PWM), a T cell dependent B cell mitogen (Keightley et al 1976), induces predominantly secretion of IgM in cord blood (Tosato et al 1980, Miyagawa et al 1980). The number of Ig secreting cells in newborns at term after PWM stimulation is less than 5% of the amount recovered in adults. IgA and IgG secreting B cells are relatively scarce in neonates. By the age of three, the number of Ig producing cells has increased to about one half of the mean amount of adults, and at the age of five the amount of Ig producing cells reaches adult levels (Miyawaki et al 1981). In spite of the equal number of Ig secreting cells, it takes until adolescence for the levels of antibody production to reach adult levels (Stiehm et al 1966).

Neonatal B cells, besides being smaller in number, seem to be functionally and phenotypically different from adult B cells, although the precise nature of the difference in signaling pathways remains uncovered. Experiments with mature type B cells from a neonatal environment that fail to proliferate after activation via surface Ig molecules, has also raised a theory of extrinsic negative signals that affect B cell activation in the neonatal environment (Marshall-Clarke et al 2000). Recent experimental studies on mice indicate that B cell responses correlate with the development of neonatal follicular DC. These professional APC do not mature until the age of two months in humans (Pihlgren et al 2003).

The results of studies attempting to induce neonatal Ig secretion have been conflicting, presumably because the *in vitro* conditions have been variable. For example, cytokines combined with polyclonal

activators and/or B cell receptor antibodies have been used to activate B cells, and neonatal T cells have been replaced with adult T cells or with culture supernatant from adult MC cultures. The replacement of neonatal T cells with adult T cells has increased the population of Ig producing cells in newborn B cells (Hayward et al 1977, Miyagawa et al 1980). When neonatal B cells were activated with a polyclonal activator (e.g. ConA) and IL-2 and were helped by adult T cells, IgM secretion was enhanced, but only to approximately one eighth of the levels secreted by adult cells in similar conditions. IgM secretion could be further up-regulated adding various surface activation molecules and additional cytokines, although not above one seventh of the respective adult IgM levels (Howard et al 1997). Howard et al concluded that poor Ig secretion in a neonatal T cell dependent response is due to naïveté of both T and B cells.

It seems clear that signalling through CD40 is critically required for specific Ig secretion (Nonoyama et al 1993). In a murine model, neonatal B cell CD40-ligation prevented transplantation tolerance (Flamand et al 1998) and blocking the CD40 receptor resulted into complete (newborns) or nearly complete (adults) blocking of Ig secretion (Howard 1997). The CD40L expression on neonatal T cells has been intensely investigated but the results are controversial since some investigators have reported that the expression of CD40L is insufficiently induced (Brugnoni 1994, Durandy et al 1995), whereas others report that the upregulation of CD40L in neonatal T cells is sufficient (Splawski et al 1996, Matthews et al 2000). Exposure to IL-10 combined with CD40-ligand or with anti-CD40 antibody has been reported to induce IgM secretion from newborn MC (Splawski et al 1998, Durandy et al 1995). However the levels attained were still less than one tenth of the amount of IgM secreted by adult cells in similar culture conditions.

Induction of secretion of other Ig classes by neonatal B cells has been more challenging and results have been poor with very little or no increase in IgG, IgA, or IgE levels (Howard 1997, Splawski et al 1998). However, CD40-activated neonatal B cells were able to undergo isotype switching and secrete IgG when supernatant from activated adult T cells was added to the *in vitro* model (Servet-Delprat et al 1996). Additionally, clinical experience from congenitally infected newborns as well as some *in vitro* works have shown the fetal immune system to be capable of secreting IgG, IgA, and IgE (Punnonen et al 1992, Punnonen et al 1994, Splawski et al 1998, King et al 1998, Numazaki et al 1998). Taken together, still, these experiments have strengthened the hypothesis of general naïveté of the newborn immune system (Adkins et al 2004).

5.4.3 Th1/Th2 balance

It is not completely understood what events dictate the progression of naïve precursor Th cells towards either Th1 or Th2 direction (or towards immunity or tolerance). There are most likely multiple determining factors, such as the type of microbe/antigen invading the host, genetic background of the host, dose of the microbe/antigen, avidity of the antigen, and the route of the infection (Murray 1998, Jakobsen et al 2002, Aliberti et al 2003, Szabo et al 2003). For example, bacterial invasion predominantly activates macrophages and eventually NK cells which produce IL-12 (Hsieh et al 1993). IL-12 is a potent inducer of IFN-γ (Trinchieri 1993, Manetti et al 1994). This kind of cytokine environment drives the naïve TCR activated Th cell towards Th1 direction (O'Garra 1998). The presence of IL-4 in the milieu of TCR activated naïve Th cell is associated with skewing towards Th2 direction but the source of IL-4 and mechanisms leading to its secretion are not clearly understood (O'Garra 1998, Glimcher et al 2000). It has been suggested, that in the absence of Th1 initiation, eg IL-12, Th2 transcription factor GATA-3 autoactivates itself and induces the Th2 phenotype development (Glimcher et al 2000).

Th2 deviation has been suggested to be a phenomenon unique to the neonatal period (Adkins et al 2001, Zhang et al 2005), as suggested by allergy (Holt (1) 1996, Holt (2) 1996) and transplantation tolerance (Donckier et al 1998, Flamand et al 1998) studies. The underlying reasons of neonatal Th2 skewing are controversial, T cell immaturity being unable to explain this phenomenon alone. Neonatal T cells are unable to secrete IL-4 upon a primary stimulation and are thus not a good source of IL-4 (Demeure et al 1994, Servet-Delprat et al 1996). Based on murine studies, Th2-type cytokine secretion at the materno-fetal interface has been postulated to be essential for successful pregnancy (Wegmann et al 1993, Uthoff et al 2003). In humans the primary source of IL-4, possibly influencing the fetal immune system, has been suggested to be amnion epithelium (Jones et al 1995) and the placenta (de Moraes-Pinto et al 1997). On the other hand, also many cytokines other than IL-4 have been detected from the amniotic fluid. IL-10, IFN-γ, IL-1, IL-6, and TNF-α have all been detected from amniotic fluid collected at term (Opsjln et al 1993, Olah et al 1996, Jones et al 1997). Recently, the T cell inhibitory placental protein 14, occurring in high concentrations in amniotic fluid, has been suggested to preferentially inhibit Th1 responses and thus be able to skew Th polarization (Mishan-Eisenberg et al 2004).

As mentioned above, there is growing evidence that, in mice, B cells can differentiate into effector cells capable of regulating the differentiation of naïve CD4+ cells and control DC cytokine secretion (Harris et al 2000, Sun et al 2005). Sun and colleagues reported, that during a harmful systemic inflammation, the toll-like receptor on neonatal murine CD5+ B cells was triggered, leading into excess production of IL-10. IL-10 seemed to prevent the secretion of IL-12 from neonatal DC, thus skewing the immune reaction towards Th2 direction in spite of the systemic inflammation (Sun et al 2005).

5.4.4 Cytokine secretion

Studies on human newborn cytokine production indicate that most cytokines are secreted or expressed considerably less by CB than by adult PB lymphocytes (Cohen et al 1999).

The secretion of IL-2 in neonates, however, is fairly well established in mixed MC cultures, and both naïve and mature T cells are able to produce IL-2 in neonates (Hauser et al 1985, Andersson et al 1990).

In 1992, Abrams et al reported that a number of cord blood samples produced large amounts (over 3000 pg/mL) of IL-10 spontaneously to the culture medium (Abrams et al 1992), but the cellular origin of this production remained unsolved. Enriched T cell cultures of neonatal origin stimulated with PMA or immobilized anti-CD3 failed to secrete large amounts of IL-10 and in mixed cultures the amount of secreted IL-10 after LPS stimulation was only 20% of adult equivalent (Chheda et al 1996, Splawski et al 1998).

Neonatal IFN-γ production is reduced at the secreted protein level when compared with adults (Wilson et al 1986, Frenkel et al 1987). Further experiments revealed that also IFN-γ specific intracellular mRNA levels were reduced in newborns (Lewis et al 1986). Later, studies have strengthened these observations by showing that the amount of intracellular IFN-γ protein is reduced in neonates also at a single cell level (Chalmers et al 1998). In particular, those neonates with a familial history of atopy show reduced mitogen induced IFN-γ secretion (Tang et al 1994, Macaubas et al 2003). Postulated reasons for defective IFN-γ secretion have been numerous: naïveté of crucial cell-types (Sanders et al 1988, Takahashi et al 1995), an intrinsic T cell deficiency (Wilson 1986), lack of cytokines (IL-12) that induce IFN-γ secretion (Lee et al 1996, Barbulescu et al 1998)

(although a conflicting report on IL-12 secretion exists (Scott et al 1997)), and immaturity of APCs (Trivedi et al 1997). The decreased ability to secrete IFN-γ, during the neonatal period, is suggested to be a main contributor to the increased vulnerability to infections.

The balance between the Th1 dominated and Th2 dominated immune reactions is important. Overall, the proper functioning of the both entities is crucial in protective immunity and an inappropriate skewing towards either Th1 or Th2 direction can lead to harmful diseases. IL-10 and IFN-γ are both important cytokines dictating the “opposite” direction of the immune reactions. The well-documented decreased ability to secrete IFN-γ and the reported excess of IL-10, if universal to all newborns, could explain in part the vulnerability of newborns to infections.

6. AIMS OF THE STUDY

This study was undertaken to determine whether regulation of cytokine synthesis in newborns leads into excessive production of IL-10 and whether this has an effect on Ig and IFN-γ secretion. The specific aims were to:

1. **Analyze the amount of secreted IL-10** from unstimulated and stimulated mononuclear cell cultures and study the role of CD19+CD5+ B cells in the secretion of IL-10 in newborns and adult controls.

2. **Evaluate the effect of IL-10 on IFN-γ secretion** by analyzing the amount of secreted IFN-γ from unstimulated and stimulated mononuclear cell cultures.

3. **Determine the effect of endogenous and exogenous IL-10 on Ig secretion** by measuring the amount of secreted Ig from unstimulated and stimulated mononuclear cell-cultures.

4. **Explore the relationship between the maturation stage of the MC and secretion of IL-10, IFN-γ and Ig** by analyzing the relative abundance of various CD markers on neonatal MC and correlating those findings with the measured secretion patterns.

7. MATERIAL AND METHODS

The ethical committee of the Children's Hospital, and of the Department of Obstetrics and Gynecology, University of Helsinki, approved the study protocol.

7.1 CLINICAL MATERIAL

Cord blood from altogether 57 newborns and peripheral blood from 35 adult volunteers, were obtained during the course of this study, to determine cytokine secretion, immunoglobulin secretion and MC composition. In addition, arterial blood (1.2mL) from 29 preterm babies was collected to study MC composition in relation to pre- and perinatal factors.

The full term newborns (born at gestational weeks 37-43) were all healthy. Twenty of them were delivered vaginally and 37 by caesarean section. Over 90% of the full term newborns were of Caucasian origin.

The diagnoses for the preterm babies were determined by a senior neonatologist after evaluating maternal case history (symptoms such as high blood pressure, proteinuria, oedema, and fever, and laboratory findings), clinical status of the baby (respiratory and circulatory symptoms and irritability), and laboratory findings (leukocyte, thrombocyte, CRP values, aerobic and anaerobic blood culture, and arterial blood gas analysis).

Blood samples (1.2mL) from 29 preterm babies were obtained from intra-arterial lines established for routine clinical tests. The average age of the newborns at the time of sample collection was 16.3 hours (range 3-39h). Ten of the babies were born at 24-26, eleven at 27-29, and eight at 30-32 weeks of gestation; twenty (20/29) newborns were delivered by caesarean section. All but four (25/29 or 86%) of the mothers of the preterm newborns had received betamethason (12mg, once or twice at twelve hour interval) treatment prior to the delivery. Nine (9/29) of the preterm babies were born to mothers with severe pre-eclampsia, and ten (10/29) had a neonatal systemic infection. The diagnoses of the other ten preterm babies included nonidentical twins (2 pairs + 1 = 5), premature birth due to cervix insufficiency (2), placental ablation (1), and prematurity of unknown reason (2).

7.2 CELL ISOLATION AND CULTURES

Cord blood samples were obtained from umbilical cord vessels within minutes after a normal delivery or an elective caesarean section. The clamped cord was punctured with a sterile needle attached to a syringe without any suction (Venoject; Terumo Europe N.V., Leuven, Belgium). When the needle was in the umbilical vessel, a tube with negative pressure was attached to the syringe. This technique assured that CB was not contaminated with maternal blood. Mononuclear cells were isolated from peripheral (healthy adult volunteers) or cord blood by a Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient. The cells ($1x10^6$/mL) were cultured at +37°C in RPMI 1640 (Gibco, Paisley, Scotland) + 10% FCS + 1% glutamine + 100U/mL penicillin G and 100µg/mL streptomycin sulfate (all from Gibco), for 72h (cytokines) or 240h (=10 days) (Igs). The cells were stimulated at 0 hour with lipopolysaccharide (LPS) (final concentration 5 µg/mL) (from E. coli 0127:B8, Sigma Chemical Co., St Louis, MO), concanavalinA (ConA) (5µg/mL) (Sigma), anti-IL-10-antibody (1:250 v:v) (PharMingen, San Diego, CA), polyclonal anti-IgM antibodies (10 µg/mL) (Protos ImmunoResearch, San Francisco, CA), PWM (1µg/mL) (Sigma Chemicals Co., St. Louis, MO), IL-10 (100 ng/mL) (PharMingen, San Diego, CA), or with IL-10+PWM.

CD5+ B cell isolation (three neonates and two adults, Study I) was performed in two steps by first incubating mononuclear cells with magnetizable polystyrene beads coated with anti-CD19 Mab (Dynabeads® M-450 PanB (CD19), Dynal, Oslo, Norway) for 20 min at +4°C. Cells attached to the magnetic beads were captured with a magnetic particle concentrator MPC©-6 (Dynal). Magnetic beads were removed by applying polyclonal antibody specially made for detachment of these beads (DETACHaBEAD®, Dynal). The proportion of CD19+ cells of all recovered cells, as analyzed by flow cytometry, was 85-98%. For further isolation of CD5+ B cells, magnetic beads coated with rat anti-mouse IgG1 were first incubated with mouse anti-human CD5 antibodies (DAKO, Glostrup, Denmark) and then with CD19+ cells as advised by the manufacturer (Direct technique, Dynal). Cells attached to the beads were captured with MPC-6.

7.3 CYTOKINE DETECTION

Concentration of IL-10 protein in the culture supernatants was determined by an enzyme-linked immunoassay (ELISA). Microtiter plates (Nunc-Immuno Plate, Roskilde, Denmark) were coated

overnight at +4°C with rat anti-human IL-10 (10µg/mL) (Pharmingen, San Diego, CA). Samples (1/1 and 1/5) and standards (Pharmingen) were diluted in RPMI 1640 + 5% FCS. Biotinylated rat anti-human IL-10 (2µg/mL) (Pharmingen) and streptavidin-HRP (Zymed, South San Francisco, CA) were used for detection of IL-10. The detection limit was 200-350 pg/mL.

Concentration of IFN-γ protein in the culture supernatants was determined by a commercially available ELISA kit according to the manufacturer's instructions (Human IFN-γ kit, Endogen, Cambridge, MA). All standards and samples were done in duplicates. The detection limit ranged from 7 to 12 pg/mL.

7.4 IMMUNOGLOBULIN DETECTION

The concentration of total IgG, IgM, and IgA in the supernatants was determined by an ELISA where two affinity purified anti-Ig-antibodies (all from Protos ImmunoResearch, San Francisco, CA) were connected with a HRP-dependent enzyme-reaction. Microtiter plates were coated overnight at +4°C with goat anti-human Ig (5 mg/µL). Samples and standards (polyclonal human Ig, Protos ImmunoResearch) were diluted in RPMI 1640 + 2% FCS. HRP-conjugated goat anti-human- Ig antibody (Protos ImmunoResearch) was used for detection of Igs. The detection limit ranged, depending on the resemblance of the duplicate dilutions measurements on the standard curve, from 0.5-25 ng/mL. All ELISA measurements were performed with a multiscan counter (Orion Diagnostica, Espoo, Finland).

7.5 FLOW CYTOMETRY

Immediately after sample collection, 50 µL of whole blood was incubated with 10 µL of fluorescein isothiocyanate- (FITC-) and/or phycoerythrin- (PE-) conjugated mouse anti-human monoclonal antibodies (MAbs) for 30 minutes in room temperature (in the dark). The MAbs were specific for CD4, CD8, CD3, CD21, CD25, CD45RA, CD45RO, CD11α, CD16+CD56, CD19, CD5, CD14, HLA-DR, IgG1/IgG2 (Immunotech, Marseille, France), and CD20, CD23 and CD40 (Serotec, Oxford, England). Red cells were lysed with FACS lysing solution (Becton Dickinson, San Jose, CA). The cells were washed twice in a phosphate buffer (Orion Diagnostica), fixed with 0.5%

paraformaldehyde, and stored at 4°C until analyzed. Lymphocytes were gated from an unstained sample and the accuracy of the gate was monitored by a pan-leukocyte marker CD45 (FITC) combined with a myeloid marker CD14 (PE). For each sample 10 000 gated events were analyzed with a FACScan flow cytometer (Becton Dickinson) using LYSYS II® or CellQuest® softwear.

7.6 STATISTICS

The association between cytokine secretion (IL-10 and IFN-γ), Ig secretion (IgM, IgG, and IgA), and the proportion of T, B, NK cells, and macrophages, was assessed by linear correlation analysis. To compare the results between adults and newborns, t test (a logarithmic transformation of IL-10 and IFN-γ concentration was used to normalize the distribution) or Mann Whitney U test (when comparing the proportions of different MC sub-types) was used. A paired t test was used to analyze the effect of different stimulants on Ig secretion.

The associations between the proportions of different cell types and gestational age, neonatal infection, maternal pre-eclampsia, maternal betamethason therapy, and mode of delivery were assessed with a forward stepping multiple regression analysis. Gestational age was treated as continuous variable, whereas the other factors were coded as dichotomous (yes/no) variables.

A result was considered statistically significant if the value of $P < 0.05$.

8. RESULTS

The results are based on four studies, which included 57 full-term newborns and 35 adult volunteers. The number of newborns and controls in individual experiments may vary due to availability of sample material or other technical issues. Additionally, in study IV, the results of surface marker composition of 29 premature babies are included.

8.1 IL-10 SECRETION (I, II)

The amount of IL-10 in unstimulated MC samples (newborns 600 pg/mL vs. adults 1200 pg/mL, p=0.003) as well as in the LPS-stimulated MC samples (1300 pg/mL vs. 2700 pg/mL, p=0.004) was significantly lower in newborns than in adults (see Table III, that shows IL-10, IFN-γ, IgM, IgG, and IgA concentrations). However, there was no significant difference in the relative magnitude of the response (2.1 vs. 2.0, p=0.902) (article II, Table 1, page 57). When cord blood cells were stimulated with ConA, neither the magnitude of the response (2.3 vs. 1.7, p=0.180) nor the actual level of IL-10 secretion (1400 pg/mL vs. 2200 pg/mL, p=0.061) were statistically significantly different from those of the adults.

To examine the role of CD5+ B cells in IL-10 production, we separated CD19+CD5+ cells from two adult and three CB samples. This separation process itself resulted in an activation of the baseline IL-10 production in four out of the five individual experiments: the unstimulated adult CD5+ B cells produced 53% and 210% more IL-10 than unstimulated unfractioned mononuclear cells, whereas the cord blood CD5+ B cells produced 110%, 380%, and 515% as much IL-10 as unfractionated cells. No further activation in response to either LPS or anti-IgM antibodies occurred in any of the five experiments. Nevertheless, our results verified that both newborn and adult CD5+ B cells can produce IL-10. On the other hand, the magnitude of this production was only an average of 16% (in adults, 11% and 12%, and in newborns, 4%, 20%, and 33%) of the total stimulated IL-10 production by unfractionated MCs, indicating that the CD5+ B cells were not the primary source of IL-10 in this culture system.

8.1.1 Correlation to MC sub-populations

In newborns, the proportions of macrophages and B cells did not correlate with the LPS induced IL-10 production, but when analyzed in more detail, the proportion of CD5- B cells had the positive correlation with IL-10 production and the proportion of CD5+ B cells actually had a negative correlation with both LPS stimulated IL-10 secretion and response to LPS (Table IV). Further, the proportion of CD45RA+ cells was inversely correlated with IL-10 response in newborns but not in adults. In adult controls, the proportions of both macrophages and B cells were correlated with the LPS induced IL-10 production, indicating that these cells are a significant source of IL-10 in adults. Again, when analyzed in more detail, only the proportion of CD5- B cells, but not the proportion of CD5+ B cells, was correlated with the IL-10 response. No significant correlations between surface marker distribution and IL-10 production in response to ConA were observed in either newborns or in adults. Correlation coefficients and *P* values for both newborns and adults are listed in Table IV.

8.2 IFN-γ SECRETION (II)

The baseline levels of IFN-γ secretion in newborns was similar to adults (68 pg/mL vs. 63 pg/mL, p=0.833, Table III). In contrast, significant differences in stimulated IFN-γ levels were readily apparent. LPS stimulated IFN-γ secretion was higher in newborns than in adults both in absolute terms (400 pg/mL vs. 140 pg/mL, p=0.016) and relative to the baseline (5.8 vs. 2.1, p=0.009), whereas ConA stimulated IFN-γ secretion was lower in newborns than in adults both in absolute (1200 pg/mL vs. 6300 pg/mL, p<0.001) and relative terms (19 vs. 100, p<0.001) (article II, page 57, Table 1.). Anti-IL-10 treatment increased IFN-γ secretion over 20-fold in both groups (26-fold in adults and 28-fold in newborns, p=0.886), but no differences between newborns and adults could be demonstrated (1200 pg/mL vs. 1900 pg/mL p=0.418).

8.2.1 Correlation to MC sub-populations

In newborns the proportion of T cells (both CD4+ and CD3+) and the proportion of cells harbouring CD11α+, had a significant positive correlation with both baseline IFN-γ secretion as well as ConA stimulated IFN-γ secretion (Table IV). Interestingly, the proportion of CD45RA+ cells had a significant positive correlation with baseline and ConA stimulated IFN-γ secretion whereas the

proportion of CD45RO+ cells had a significant negative correlation with IFN-γ secretion after ConA stimulation. In adults, the proportion of CD4+ cells had a negative correlation whereas CD8+ cells had a positive correlation with ConA stimulated IFN-γ secretion.

Table III. IL-10, IFN-γ, IgM, IgG, and IgA concentrations at baseline (Bl) and after stimulation in neonatal and adult cell cultures.

	Newborns Geometric mean	Min/max	**Adults** Geometric mean	Min/max	***p***
IL-10 Bl pg/ml	600	200/6900	1200	200/14000	0.003
LPS	1300	400/13000	2700	400/21000	0.004
ConA	1400	200/10000	2200	350/16000	0.061
IFN-γ Bl pg/ml	68	7/4100	63	7/3600	0.833
LPS	400	12/8800	140	7/62000	0.016
ConA	1200	51/18000	6300	910/29000	<0.001
IgM Bl ng/ml	17	2/545	66	4/4419	0.002
PWM	66	1/2249	501	5/8441	<0.001
IL-10	35	4/220	14	4/250	0.106
IL-10 + PWM	224	5/7190	537	93/4634	0.264
Anti-IL-10	7	1/79	23	2/753	0.007
IgG Bl ng/ml	49	1/746	372	80/2570	<0.001
PWM	46	1/1063	1259	69/15000	<0.001
IL-10	54	23/210	372	93/1750	<0.001
IL-10 + PWM	87	27/285	1097	174/5052	<0.001
Anti-IL-10	89	15/1057	309	15/1008	<0.001
IgA Bl ng/ml	3	1/140	282	47/1953	<0.001
PWM	3	1/140	309	10/2113	<0.001
IL-10	3	1/145	224	2/1745	<0.001
IL-10 + PWM	5	2/185	363	80/2800	<0.001
Anti-IL-10	2	1/10	71	35/1467	<0.001

Table IV. The correlation coefficients and corresponding p values between cytokine secretion and mononuclear cell subtypes (of all lymphocytes unless otherwise stated).

Newborns

	IL-10						**IFN-γ**					
	Baseline		**LPS**		**Resp# LPS**		**Baseline**		**ConA**		**Resp# ConA**	
	r	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
CD45RO+	-0.45	**0.003**	-0.16	0.336	0.18	0.287	-0.21	0.162	-0.39	**0.017**	-0.13	0.438
CD45RO+/CD4*	-0.30	0.054	-0.09	0.591	0.11	0.508	-0.19	0.206	-0.34	0.036	-0.07	0.697
CD45RO+/CD8**	-0.35	0.050	-0.12	0.528	0.08	0.685	-0.06	0.716	-0.29	0.138	-0.31	0.108
CD45RA+	0.06	0.699	-0.33	**0.038**	-0.39	**0.013**	0.48	**0.001**	0.39	**0.015**	-0.22	0.185
CD45RA+/CD4*	0.00	0.997	-0.24	0.134	-0.18	0.267	0.16	0.283	0.11	0.498	-0.11	0.506
CD45RA+/CD8**	-0.10	0.580	-0.41	**0.027**	-0.23	0.233	0.33	0.050	0.19	0.338	-0.19	0.335
CD4+	0.35	**0.024**	-0.32	0.045	-0.49	**0.002**	0.37	**0.012**	0.40	**0.014**	-0.34	**0.042**
CD8+	0.25	0.108	-0.11	0.521	-0.24	0.147	0.29	0.054	0.29	0.079	-0.07	0.690
CD3+	0.37	**0.037**	-0.28	0.132	-0.52	**0.003**	0.42	**0.010**	0.38	**0.045**	-0.33	0.090
CD11α+	0.23	0.325	-0.49	**0.033**	-0.75	**0.000**	0.54	**0.011**	0.67	**0.002**	-0.10	0.698
CD11α+CD20-	0.19	0.401	-0.47	**0.044**	-0.75	**0.000**	0.63	**0.002**	0.62	**0.006**	-0.39	0.108
CD11α+CD20+	-0.17	0.462	0.04	0.871	0.20	0.396	0.03	0.892	-0.06	0.819	-0.07	0.792
CD19+	-0.05	0.768	-0.04	0.797	-0.10	0.553	0.03	0.835	0.10	0.560	0.20	0.221
CD19+CD5-	0.02	0.913	0.39	**0.016**	0.34	0.035	-0.18	0.248	-0.03	0.857	0.37	**0.026**
CD19+5+/19+§	-0.04	0.824	-0.37	**0.020**	-0.34	0.031	0.24	0.115	0.02	0.928	-0.31	0.057
CD21+CD23+	-0.65	**0.001**	-0.22	0.370	0.38	0.112	-0.15	0.462	-0.53	0.028	0.03	0.903
CD3+16+56+	-0.35	0.027	-0.03	0.838	0.24	0.148	-0.16	0.300	-0.37	0.025	-0.16	0.338
CD14+§§	-0.18	0.262	0.29	0.070	0.42	**0.007**	-0.30	0.044	-0.25	0.144	0.19	0.270

#Resp = relative magnitude of the response calculated as % of baseline, *of all CD4+ cells, **of all CD8+ cells, §of all CD19+ cells, §§of all cells

Table IV continued.

Adults

	IL-10						**IFN-γ**					
	Baseline		**LPS**		**Resp# LPS**		**Baseline**		**ConA**		**Resp# ConA**	
	r	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**	**r**	**p**
CD45RO+	-0.35	0.054	-0.15	0.442	0.13	0.498	-0.16	0.396	0.14	0.525	0.21	0.324
CD45RO+/CD4*	-0.29	0.121	-0.10	0.608	0.10	0.622	-0.00	0.992	0.18	0.412	0.09	0.683
CD45RO+/CD8**	-0.61	**0.003**	0.14	0.551	0.67	**0.001**	-0.31	0.202	0.20	0.505	0.44	0.130
CD45RA+	0.11	0.552	0.03	0.863	0.13	0.487	0.04	0.855	-0.33	0.117	-0.14	0.527
CD45RA+/CD4*	0.06	0.735	0.00	0.999	0.06	0.774	-0.11	0.572	-0.37	0.079	0.00	0.994
CD45RA+/CD8**	0.37	0.103	-0.00	0.990	-0.21	0.378	0.10	0.700	-0.31	0.297	-0.20	0.505
CD4+	0.40	**0.025**	0.31	0.100	-0.03	0.891	-0.25	0.188	-0.47	**0.021**	0.07	0.731
CD8+	-0.10	0.587	-0.44	**0.016**	-0.41	**0.026**	0.22	0.252	0.43	**0.043**	-0.06	0.778
CD3+	0.31	0.167	0.02	0.931	-0.24	0.302	-0.12	0.620	0.11	0.704	0.20	0.467
CD11α+	-0.10	0.716	-0.11	0.704	-0.16	0.582	0.36	0.208	0.47	0.166	-0.09	0.815
CD11α+CD20-	0.19	0.476	0.01	0.960	-0.23	0.411	0.24	0.383	0.35	0.328	-0.07	0.855
CD11α+CD20+	0.05	0.874	0.29	0.320	0.15	0.603	0.10	0.725	0.44	0.200	-0.08	0.829
CD19+	-0.01	0.966	0.56	**0.001**	0.66	**0.000**	-0.09	0.644	-0.03	0.904	0.09	0.677
CD19+CD5-	-0.03	0.893	0.57	**0.001**	0.64	**0.000**	-0.09	0.651	-0.07	0.754	0.09	0.700
CD19+CD5+/19+§	-0.02	0.899	-0.10	0.623	-0.02	0.902	-0.01	0.951	-0.19	0.386	-0.12	0.581
CD21+CD23+	-0.37	0.131	0.06	0.829	0.43	0.082	-0.32	0.235	-0.31	0.385	-0.26	0.467
CD3+16+56+	-0.26	0.167	-0.27	0.166	0.15	0.434	-0.35	0.072	-0.12	0.587	0.21	0.341
CD14+§§	0.01	0.941	0.42	**0.020**	0.41	**0.023**	0.11	0.563	0.16	0.448	-0.17	0.429

#Resp = relative magnitude of the response calculated as % of baseline, *of all CD4+ cells, **of all CD8+ cells, §of all CD19+ cells, §§of all cells

8.3 IFN-γ/IL-10 BALANCE IN NEWBORNS

To compare the individual orientation of responses by PBMC between newborns and adults, we calculated the mean ratios of IFN-γ/IL-10 secretion for baseline, LPS, and ConA stimulated values for each newborn and adult. The baseline ratios did not differ between adults and newborns, mean ratios being 1/10 (i.e., IL-10 levels being 10-fold the IFN-γ levels) in both groups (article II, page 57, Table 2). The significantly higher mean IFN-γ/IL-10 ratio in LPS stimulated secretion levels ($p=0.004$) in newborns indicated a more marked shift towards Th1 direction after LPS stimulation than in adults. Nevertheless, mean IL-10 levels were still three times higher than mean IFN-γ levels.

In adults, a clear shift towards Th1 direction was observed after ConA stimulation, IFN-γ secretion exceeding IL-10 secretion approximately 2.8-fold. Among the newborns, a shift towards Th1 direction was detected as well, but the mean ratio was only 0.9 (i.e. IL-10 levels still being a little higher than IFN-γ levels). The difference between adults and newborns was significant ($p=0.007$), indicating greater overall impairment in IFN-γ secretion than in IL-10 secretion in neonates when compared with adults.

8.4 TH1/TH2 ORIENTATION

To further classify neonates and adults on the basis of their IFN-γ and IL-10 secretion, we drew a quadrant plot, using 1000 pg/mL as the threshold level for IL-10 secretion after LPS stimulation, and IFN-γ secretion after ConA stimulation. Four different groups were formed; high IL-10/low IFN-γ quadrant being the "IL-10-oriented" and low IL-10/high IFN-γ being the ” IFN-γ-oriented”. High IL-10/high IFN-γ was labeled ”balanced good secretors” and low IL-10/low IFN-γ as the ”nonresponders” (Table V). This plot revealed a strikingly different pattern in newborns than in adults (article II, figure 1., page58). All but one of the adults with high levels of LPS stimulated IL-10 secretion were also good ConA stimulated secretors of IFN-γ, whereas many newborns with comparable IL-10 levels were poor secretors of IFN-γ. The majority of adults, 77%, belonged to balanced good secretors quadrant, and only 1 adult (5%) belonged to the IL-10-oriented quadrant. In contrast, only 25% of the newborns were in the adult like balanced good secretors quadrant and 31% in the IFN-γ oriented quadrant, and more than one third (36%) remained in the IL-10 oriented

quadrant. We did not find significant differences in ConA stimulated IL-10 secretion between IL-10 oriented and IFN-γ oriented individuals but interestingly 10 out of the 11 of the IL-10 oriented newborns increased their IFN-γ secretion to levels comparable to those in the other quadrants after anti-IL-10 supplementation (article II, figure 2., Page 58). Additionally, the only adult belonging to the IL-10 oriented group increased the IFN-γ secretion by 3.8 fold (from 910 to 3530 pg/ml) in response to anti-IL-10.

Table V. Cytokine secretion patterns in adults and neonates. The classification is based on ConA stimulated IFN-γ levels and LPS stimulated IL-10 levels.

	IFN-γ Low (<1000pg/ml)	**IFN-γ** High (>1000pg/ml)
IL-10 High (>1000pg/ml)	IL-10 oriented Newborns 36% Adults 5%	Balanced good secretors Newborns 25% Adults 77%
IL-10 Low (<1000pg/ml)	Balanced low secretors Newborns 8% Adults 0%	IFN-γ oriented Newborns 31% Adults 18%

Regarding both IFN-γ and IL-10, neither the way of delivery (vaginal delivery or caesarean section), nor the gender of the neonate influenced the results.

8.5 Ig SECRETION IN NEONATES (III)

As expected, we detected significant differences between newborns and adults in all baseline Ig classes (Table III). The mean baseline IgM secretion in neonates and adults was 17 vs. 66 ng/mL, respectively ($p = 0.002$), IgG secretion 49 vs. 372 ng/mL ($p<0.001$), and IgA secretion 3 vs. 282 ng/mL ($p<0.001$).

To study the effect of IL-10 on Ig secretion we added IL-10, anti-IL-10 and IL-10 + PWM to cell cultures. In newborns, IL-10 supplementation increased (p=0.024), and anti-IL-10 decreased (p=0.003) IgM secretion (article III, figure 1., page 12). A reverse pattern was observed in IgG secretion: IL-10 stimulation resulted in a significant decrease in IgG secretion (p=0.026). Anti-IL-10 increased IgG secretion on an average by 58%, although this increase did not reach statistical significance (p=0.11) (III, figure 1., page 12). In adults, neither IL-10 nor anti-IL-10 had any effect on IgG or IgM secretion (III, figure 2., page 13). IgA secretion in neonates was too often below the assay detection limit to allow for assessment of any stimulation effects. In adults, however, anti-IL-10 decreased IgA secretion by an average of 78% (from 324 ng/mL to 71 ng/mL, p=0.004).

8.5.1 Correlation between Ig and cytokine secretion

In newborns, IFN-γ secretion was associated with the ability to secrete IgM: PWM stimulated IgM levels correlated with both baseline (r=0.56, p=0.001) and ConA stimulated IFN-γ production (r=0.46, p=0.012; III, page 14, fig.3). Further, baseline IL-10 secretion had an inverse correlation with baseline IgM secretion (r=-0.44, p=0.014). No other associations were observed in the neonate group. Two significant correlations were noted between cytokine secretion and Ig secretion in adults. The baseline IgG secretion correlated inversely with ConA stimulated IFN-γ secretion (r=-0.43, p=0.042) and the baseline IgM secretion correlated inversely with the LPS stimulated IL-10 secretion (r=-0.51, p=0.009).

The "balanced good secretors", with adult-resembling cytokine production capabilities, had 260% higher baseline (p=0.030) and 269% higher PWM stimulated IgG secretion (p=0.050) than the other neonates. The ten neonates with more immature cytokine pattern (adult-like IFN-γ, but low IL-10 secretion) tended to have an IgM-emphasized response to all stimulants: at baseline, the IgM/IgG ratio was 1.5 vs. 1.4 for these 10 neonates and for the others, respectively (p=0.96), whereas after PWM stimulation the ratios were 8.4 vs. 2.8 (p=0.011), after IL-10 stimulation 4.5 vs. 1.0 (p=0.007), and after PWM+IL-10 stimulation 19.6 vs. 4.1 (p=0.039), respectively. Corresponding results were not found in adults.

8.5.2 Correlation to MC sub-populations

Various correlations between distribution of lymphocyte subtypes and Ig secretion were detected in newborns and in adults (Table VI). In newborns, the proportion of RO+ cells (of all lymphocytes) was inversely associated with both PWM-stimulated IgM (r=-0.46, p=0.011) and IgG (r=-0.43, p=0.015) secretion, whereas the proportion of RA+ cells had a positive association with IL-10 stimulated IgG secretion (r=0.522, p=0.018). There were also correlations between the baseline IgM secretion and the proportion of macrophages (CD14+ cells of all cells, r=0.43, p=0.015) and CD11α+ B-cells (r=0.59, p=0.027). In adults, but not in the neonates, the proportion of immature CD5+ B cells had a positive correlation with baseline IgM secretion (r=0.57, p=0.002), and the proportion of mature CD11α+ B cells with PWM-stimulated IgG secretion (r=0.75, p=0.002). Further, in adults the proportion of CD4+ cells was associated with baseline (r=0.37, p=0.046) PWM stimulated (r=0.44, p=0.017), IL-10 stimulated (r=0.60, p=0.031), and anti-IL-10 stimulated (r=0.71, p=0.001) IgG production.

Regarding the Igs studied, neither the way of delivery (vaginal delivery or caesarean section), nor the gender of the neonate, influenced the results.

Table VI. The correlation coefficients (r) between IG secretion and mononuclear cell subtypes (of all lymphocytes unless otherwise stated), and corresponding p values when p< 0.1 (r/p).

Newborns			**IgM**		
Surfacemarkers	**Baseline**	**PWM**	**antiIL10**	**IL-10**	**PWM+IL10**
CD45RO+	.325	-.459/**.011**	.365/**.073**	.122	-.122
CD45ro+/CD4+[†]	.224	-.307	.366/**.072**	-.063	-.184
CD45ro+/CD8+[‡]	.124	-.293	.370	.252	-.564
CD45RA+	-.021	.308	.141	-.108	-.113
CD45ra+/CD4+[†]	.134	-.022	.215	-.385/**.094**	-.399
CD45ra+/CD8+[‡]	.099	-.182	.418	-.202	NA
CD4+	-.025	.161	-.213	-.130	-.256
CD8+	-.145	-.062	-.201	-.201	-.307
CD3+	-.045	.293	.041	-.583/**.077**	-.564
CD11a+	-.030	.268	-.234	.126	NA
CD11a+20+/CD20+[°]	.589/**.027**	.175	.488	.805/**.016**	NA
CD11a+20-	-.121	.156	-.108	-.360	NA
CD19+	-.021	.066	.190	.351	.440/**.101**
CD19+5-	.269	.195	.131	.072	.121
CD19+5+/CD19+[§]	-.212	-.091	-.014	.063	.068
CD23+/CD21+[¶]	.280	-.051	.704/**.077**	NA	NA
CD16+56+	.066	-.162	.291	.024	.202
CD3+16+56+	.532/**.002**	-.354/**.055**	.433/**.031**	.134	.081
CD14+**	.427/**.015**	-.158	.226	-.178	-.026

[†]Of all CD4+ cells, [‡]of all CD8+ , [§]of all CD19+ cells, [°]of all CD20+ cells, [¶]of all CD21+ cells , **of all cells, NA = not available.

Table VI continued.

Newborns			**IgG**		
Surfacemarkers	**Baseline**	**PWM**	**antiIL10**	**IL-10**	**PWM+IL10**
CD45RO+	-.066	-0.433/**.015**	-.125	.365	-.275
CD45ro+/CD4+[†]	.128	-.270	-.096	.383/**.096**	-.102
CD45ro+/CD8+[‡]	.451/**.027**	-.158	.018	.576/**.082**	-.600
CD45RA+	-.033	.126	.310	.522/**.018**	.118
CD45ra+/CD4+[†]	.268	.025	.168	.475/**.034**	.029
CD45ra+/CD8+[‡]	.345	.040	.214	.539/**.108**	-.200
CD4+	-.189	-.138	-.149	-.111	-.218
CD8+	.004	.003	.137	-.083	-.359
CD3+	-.218	.018	NA	.115	NA
CD11a+	-.552/**.033**	.048	.071	.143	NA
CD11a+20+/CD20+[°]	-.044	-.127	.071	.500	.500
CD11a+20-	-.334	.154	.286	.179	NA
CD19+	-.186	.063	.011	.131	-0.539/**.038**
CD19+5-	.046	.342	.143	.018	.020
CD19+5+/CD19+[§]	-.077	-.319/**.075**	-.134	.104	.275
CD23+/CD21+[¶]	-.180	-.298	-.214	.321	NA
CD16+56+	-.014	.107	.140	.313	.132
CD3+16+56+	-.190	-0.475/**.007**	-.368	.164	.170
CD14+**	-.004	-.053	-.429/**.032**	-.053	-.038

[†]Of all CD4+ cells, [‡]of all CD8+ , [§]of all CD19+ cells, [°]of all CD20+ cells, [¶]of all CD21+ cells , **of all cells, NA = not available.

Table VI continued.

Adults			**IgM**		
Surfacemarkers	**Baseline**	**PWM**	**antiIL10**	**IL-10**	**PWM+IL10**
CD45RO+	.067	.006	-.064	-.148	.242
CD45ro+/CD4+†	-.058	-.385	-.408	-.449	-.022
CD45ro+/CD8+‡	-.341	-.171	-.700	-.800	NA
CD45RA+	.272	.096	.095	.171	-.082
CD45ra+/CD4+†	.163	.227	.308	.239	.258
CD45ra+/CD8+‡	.556/**.025**	-.009	NA	.200	NA
CD4+	.076	.103	.036	.119	.396
CD8+	.015	.184	-.022	-.091	.066
CD3+	.181	.437	.536	.400	NA
CD11a+	.204	.493	NA	NA	NA
CD11a+20+/CD20+°	.441	.301	.500	NA	NA
CD11a+20-	.093	.615/**.025**	NA	NA	NA
CD19+	-.066	-.296	-.023	.158	-.301
CD19+5+/CD19+§	.574/**.002**	.217	.326	.172	.203
CD19+5-	-.312	-.334	-.235	.100	-.308
CD23+/21+¶	.196	-.213	NA	NA	NA
CD16+56+	.276	.065	-.055	-.256	-.209
CD3+16+56+	-.042	-.089	-.133	.240	-.126
CD14+**	.037	.005	.188	.028	-.176

†Of all CD4+ cells, ‡of all CD8+ , §of all CD19+ cells, °of all CD20+ cells, ¶of all CD21+ cells , **of all cells, NA = not available.

Table VI continued.

Adults			**IgG**		
Surfacemarkers	**Baseline**	**PWM**	**antiIL10**	**IL-10**	**PWM+IL10**
CD45RO+	-.201	.285	-.130	-.159	.374
CD45ro+/CD4+[†]	-.281	-.144	-.358	-.291	.088
CD45ro+/CD8+[‡]	-.400	.017	-.314	.600	NA
CD45RA+	.223	.178	.387	.330	.242
CD45ra+/CD4+[†]	.205	.350/**.063**	.547/**.023**	.516/**.071**	.242
CD45ra+/CD8+[‡]	.344	.075	NA	NA	.200
CD4+	.373/**.046**	**.441/.017**	.713/**.001**	.599/**.031**	.291
CD8+	-.066	-.149	-.265	-.198	-.192
CD3+	.126	.568/**.009**	.119	NA	-.400
CD11a+	-.113	.417	-.800	NA	NA
CD11a+20+/CD20+[°]	-.090	.754/**.002**	-.200	NA	NA
CD11a+20-	-.050	.314	NA	NA	NA
CD19+	.127	.036	-.071	-.252	.028
CD19+5+/CD19+[§]	.323/**.093**	.352/ **.066**	.282	.280	.056
CD19+5-	.115	-.103	-.179	-.357	.070
CD23+/21+[¶]	.221	.347	.200	NA	NA
CD16+56+	-.139	-.219	-.331	-.286	-.313
CD3+16+56+	.038	.132	.118	.086	.448
CD14+**	.079	.083	-.137	.082	.286

[†]Of all CD4+ cells, [‡]of all CD8+ , [§]of all CD19+ cells, [°]of all CD20+ cells, [¶]of all CD21+ cells , **of all cells, NA = not available.

8.6 MC SUB-POPULATIONS (I-IV)

8.6.1 MC sub-populations in full term neonates (I-IV)

The proportions of different cell types in full term newborns and adults are summarized in Table VII. As expected, the expression of most surface markers varied significantly between newborns and adults.

Table VII. Proportion of different cell types (of all lymphocytes, unless otherwise indicated) in term newborns (cord blood) and adults.

	Newborns		**Adults**		*p*
Cells	mean(%)	range(%)	mean(%)	range(%)	
CD3+	68	36-85	76	60-89	0.003
CD25+/CD3+*	8	5-13	11	2-27	0.020
CD4+	49	25-74	45	27-65	0.113
CD45RA+/CD4+[†]	75	2-96	44	5-88	<0.001
CD45RO+/CD4+[†]	2	0-7	40	9-81	<0.001
CD8+	17	8-24	25	13-48	<0.001
CD45RA+/CD8+[‡]	90	42-98	60	25-96	<0.001
CD45RO+/CD8+[‡]	1	0-6	27	9-56	<0.001
CD4+CD8+	4	1-15	1	0-3	<0.001
CD19+	14	3-29	11	2-33	0.020
CD5+/CD19+[§]	81	44-100	35	1-68	<0.001
CD19+CD5-	3	0-9	7	1-18	<0.001
CD11α+/CD20+[°]	75	46-95	76	32-99	0.904
CD23+/CD21+[¶]	3	0-12	1	0-7	0.072
NK (CD16+CD56+CD3-)	13	1-40	12	2-33	0.400
CD16+CD56+CD3+	1	0-11	5	1-17	<0.001
CD14+**	5	0-20	5	2-10	0.201
CD45RA+	84	47-99	61	31-90	<0.001
CD45RO+	1	0-8	27	7-62	<0.001
CD11α+	91	68-100	97	89-100	0.017

*Of CD3+ cells, [†]Of CD4+ cells, [‡]Of CD8+ cells, [§]Of CD19+ cells, [°]Of CD20+ cells, [¶]Of CD21+ cells , **Of all cells

8.6.2 Effect of GA on MC sub-populations (IV)

Table 1 in article IV, page 309, summarizes the effect of GA and other factors on the proportion of surface markers. The proportion of CD3+ cells, CD8+ cells, CD11α+ and CD45RA positive cells increased with gestational age while the proportion of CD45RO+ cells was not associated with any of the variables studied. A significant proportion of the CD14+ cells of the newborns were HLA-DR

negative (article IV, figure 3., page 312) and there was an inverse correlation between gestational age and the proportion of HLA-DR negative CD14+ cells.

8.6.3 Effects of neonatal infection, maternal pre-eclampsia, and betamethason treatment

The proportion of CD3+ cells, CD45RA+ cells, and CD11α+ cells was correlated with the presence of neonatal infection. Maternal betamethason therapy was associated with higher CD3+ cell proportion (IV, Table 1, page 311). Interestingly, the proportion of those CD3+ cells that also expressed CD25 was significantly higher in newborns with than in those without infections (IV, Figure 1., page 312).

Maternal pre-eclampsia had a significant effect on T cell distribution: neonates born to mothers with pre-eclampsia had significantly less CD4+ cells and CD4+CD8+ double positive cells than other neonates (IV, figure 2., page 312). Furthermore, the CD4/CD8 ratio was decreased in these babies. Quite oppositely, the CD4+/CD8+ ratio was increased in neonates with infection.

In all newborns, more than 50% - up to 99% - of CD19+ B cells were also CD5 positive. Maternal pre-eclampsia decreased the proportion of CD5+CD19+ cells by approximately 15 percentage points. A substantial proportion of all B cells were CD11α negative: the lowest levels of CD11α positive B cells were observed in those prematures who had an infection (IV, figure 4., page 312). Another interesting phenomenon was the occurrence of CD19-CD40+ cells: in three infected neonates, all very immature born at 25 weeks of gestational age, 12-14% of all lymphocytes were CD40+ despite being CD19 negative. A significant proportion of the CD14+ cells of the newborns were HLA-DR negative (IV, Figure 3., page 312). The highest proportions of these cells were observed in those premature infants who also had a neonatal infection. Maternal pre-eclampsia and treatment with betamethason had opposing effects on the proportion of HLA-DR positive CD14+, as well as on the proportion of all CD14+ cells, pre-eclampsia diminishing and betamethason increasing these proportions.

9. GENERAL DISCUSSION

9.1 CYTOKINE SECRETION

Our first study of IL-10 production from CB cells demonstrated that IL-10 secretion, on an average, was not as well established by neonatal MC than by adult MC. Although CD5+ B cells were able to secrete IL-10, they were not the cells responsible in either adults or newborns for most of the IL-10 secretion. This finding was further supported by the inverse correlation of the proportion of newborn CD5+ B cells and LPS stimulated IL-10 secretion. Subsequent studies have confirmed that human CD5+ B cells are capable of IL-10 production (Villasenor-Bustamante et al 1999) and another report suggests, that murine CD5+ and CD5- (conventional) B cells secrete IL-10 equally well (Gieni et al 1997).

The stimulated secretion of IFN-γ was on an average significantly lower among newborns than in adults. The previously suggested Th2-orientation (Adkins et al 2001) could be detected as well: Our results indicated that the regulation of IFN-γ secretion was more impaired than that of IL-10 in newborns when compared with adults. The mean ratios of secreted IFN-γ/IL-10 were all under 1, even after ConA stimulation. This is in accordance with other reports proposing that after a mitogenic stimulation the Th2-type cytokine secretion (IL-4, IL-5, and IL-10) is more easily established than the Th1-type cytokine secretion in newborns (Trivedi et al 1997), contributing to Th2 deviation.

The underlying reasons to the Th2 bias are controversial, universal T cell immaturity being unable to explain this phenomenon alone. Successful pregnancy is reported to require a more Th2 biased immune response (Wegmann et al 1993), but does this affect the immune system of the fetus remains unclear, since the amniotic fluid contains equal amounts of IL-4, IFN-γ, and IL-10 in samples collected before labor onset or during labor (Jones et al 1997). Purified naïve CD4+ T cells from adults and neonates have the same ability to secrete Th1 cytokines as well as switch towards Th2 effectors after repetitious stimulation (Delespesse et al 1998). One speculated reason is the small number of professional antigen presenting cells (e.g. DC) in the newborn system, that may favor Th2 orientation (Ridge et al 1996). Further, the neonatal DC have been suggested to be intrinsically

biased against Th1 orientation (Langrish et al 2002). Other immature or dysregulated cells have been proposed to play an important role as well (Marshall-Clarke et al 2000).

We found out that IL-10 oriented cytokine secretion was not a universal characteristic in all neonates. Our results are in concordance with those of Hassan and Reen (Hassan et al 2000) who stated that the reported Th2 orientation of neonatal murine lymphocytes couldn't always be generalized to neonatal human lymphocytes. The human T cell system seems to be more mature than the murine counterpart at birth (Adkins 1999). While the largest proportion (36%) of the newborns exhibited an IL-10 oriented cytokine secretion, 31% were IFN-γ oriented, and 25% "balanced good secretors" of both cytokines. This distribution differed markedly in adults, of whom 77% were "balanced good secretors" of both cytokines, and only one had an IL-10-oriented pattern. The significant increase of the IFN-γ secretion in response to anti-IL-10, in neonates with the IL-10-oriented cytokine pattern, indicates that the endogenous IL-10 production might be partly responsible for the impaired IFN-γ secretion, and that IL-10 oriented neonates were not intrinsically defective in IFN-γ secretion. An alternative explanation to the Th2 orientation could well be the immaturity and/or scarcity of other cells, especially DC.

Our quite large material and concomitant IL-10, IFN-γ and MC surface marker phenotype analysis enabled us to address correlation between mature and naïve cell populations and cytokine secretion, and thus indirectly identify cells contributing to IFN-γ/IL-10 oriented cytokine secretion. Overall, in newborns IFN-γ or IL-10 oriented cytokine secretion had a number of correlations with MC sub-populations, whereas in adults only a few correlations could be detected. The proportion of CD45RA+ cells increases with age (Muller et al 1996), which is detectable already during gestation (as reported in publication IV). The role of newborn CD45RA+ cells, exhibiting a positive correlation with IFN-γ secretion and a negative one with IL-10 secretion, was interesting, as usually CD45RA+ cells (especially T cells) are considered to be naïve and not be major contributors in IFN-γ secretion. In concordance with our finding is a study, although limited to analysis of three CB samples, suggesting that CD45RA cells produced more IFN-γ than CD45RO+ cells (Chalmers et al 1998). IL-10 secretion was not analyzed.

The positive correlation of newborn CD4+ cells with both baseline IL-10 and baseline and ConA stimulated IFN-γ secretion (Table IV) is clear as CD4+ cells can secrete both cytokines, depending

on the Th orientation (Mosmann et al 1986, Mosmann et al 1989). Previous studies have shown that Th1 CD4+ cells secrete IFN-γ and Th2 CD4+ cells secrete IL-10 in response to antigens (Mosmann et al 1989). Studies to find surface molecules able to discriminate these two cell types have been undertaken (Spinozzi et al 1997). A marker for murine Th2-type cells has been identified (Xu et al 1998, Savignac et al 2004), but direct identification of human Th1, or Th2 cells, by CD marker analysis, is not yet possible.

It was somewhat surprising that the proportion of CD45RO+ cells had a negative association with both cytokines, e.g. ConA stimulated IFN-γ secretion and baseline IL-10 secretion as well as Ig secretion. Although it has been reported that adult CD45RO+ cells are capable of secreting IFN-γ and IL-10 (Sanders et al 1988, Yssel et al 1992) our finding supports the notion that CB CD45RO+ cells are in an immature state and are not capable of similar cytokine secretion as adult CD45RO+ cells in an identical experimental setting (Takahashi et al 1995). It might be, that the CD45RO+ cells detected in small proportions in cord blood were, instead of true memory cells, very immature T cells that had leaked from the thymus (Fijii et al 1992). CD4+CD45RA+, instead of CD45RO+ cells, appear as the main produces of IFN-γ in neonates.

9.2 Ig SECRETION

The baseline secretion of all Ig classes was reduced in newborns when compared with adults, a finding confirmed by previous studies as well. The number of Ig secreting cells in the human newborn is smaller than in adults (Tosato et al 1980, Miyawaki et al 1981), which, together with relative cytokine deficiencies and naïve cell subpopulations offers one explanation to reduced Ig baseline secretion.

Supplementary IL-10 and anti-IL-10 had opposite effects on IgM and IgG secretion in newborns, but had no effect in adults, indicating differences in regulation of Ig synthesis between neonates and adults. Both baseline and ConA stimulated IFN-γ secretion correlated positively with baseline IgM secretion. Baseline IL-10 secretion had a negative correlation to baseline IgM and the IL-10 oriented newborns did not secrete any of the Ig better than the other newborns

Secretion of IgM is better established than that of other Ig classes in newborns (Splawski et al 1991, Watson et al 1991, Howard 1997), presumably because IgM secretion can proceed without mature helper activity (van Essen et al 1995). Previous experiments have used various IL-10 concentrations and different culture environments combined with anti-B cell ligand antibodies with super-antigens or additional cytokines. The results have been controversial, probably due to great variation in culture conditions. At most, IgM secretion has reached one seventh or one eighth of the corresponding adult amount of secreted IgM in these experiments.

None of the previous reports have studied the effect of anti-IL-10 on Ig secretion. The importance of IL-10 is underlined by the present results displaying that anti-IL-10 diminished significantly IgM secretion from the baseline in newborns. The decrease of IgG secretion after IL-10 supplementation might be the result of a risk of newborn B cells (switched to IgG) to undergo apoptosis after primary stimulation (e.g. IL-10 stimulation, (Itoh 1995)) in the absence of co-stimulatory signals. These signals are reported to be scarce in the neonatal environment (Brugnoni et al 1994, Adkins 1999). Anti-IL-10 can theoretically act in different ways: It can hinder the effect of endogenous IL-10 by neutralizing it or by blocking IL-10 receptors. By neutralizing the effects of endogenous IL-10, anti-IL-10 can induce IFN-γ secretion and thus shift the Th1/Th2 balance towards Th1 direction. Our results suggested that, in newborns, anti-IL-10 is a potent inducer of IFN-γ secretion, (even in newborns with low secretion of IFN-γ after ConA stimulation) resulting in 28-fold increase in IFN-γ secretion over the baseline (II). We speculated that the demonstrated increase of IgG secretion in newborns could be attributed to increased IFN-γ secretion after anti-IL-10 supplementation, since IFN-γ has previously been shown to increase IgG_2 secretion especially from CB MC, but not from adults. This IgG_2 represented the total increase from the baseline amount of secreted IgG in newborns (Kawano et al 1996).

Although IL-10 is reported to act as a growth factor for IgA secreting B cells (Defrance et al 1992), the secretion of one isotype of IgA, IgA_2, is reported to be strictly dependent on a direct interaction between the B cell and the DC (Fayette et al 1997). Since the maturation of neonatal DCs is on going up to the age of two months in humans (Pihlgren et al 2003), it may partly explain why we could induce IgA secretion above the baseline from only a few CB MC samples. However, the effect of anti-IL-10 in hampering adult IgA secretion clearly underlines the importance of IL-10 in IgA secretion. All in all, attempts to restore IgG and IgA secretion from CB B cells have not been very

successful, and our experiments suggest that supplementation with IL-10 does not overcome the diminished secretion. Probably the number of switched B cells is very low in newborns and only antigenic encounter will increase the amount of IgA and also IgG secreting cells. The scarcity of DC might play a very important role as well.

Our results suggest that neonates with ”balanced good secretion” of both IFN-γ and IL-10 were the best IgG producers. They exhibited 260% higher baseline IgG secretion levels than the rest of the neonates. Secondly, neonates with IFN-γ oriented response benefited most from the IL-10 supplementation. These results are not easily interpreted but it is tempting to speculate, that the ability to secrete IFN-γ appears to be a prerequisite for good IgM responses. Thus the route to improved responsiveness goes via IFN-γ secretion, associated with IgM responses, towards IFN-γ /IL-10 balanced secretion and IgG responses. Since baseline IL-10 had an inverse correlation with baseline IgM, it may be that neonates responding in an IL-10-biased manner, are as much at risk for lower Ig secretion levels, as are “the non-responders” (neonates with both low IL-10 and IFN-γ secretion patterns).

Interestingly the proportion of CD45RO+ cells did not have a positive correlation with any of the Ig classes studied, but was inversely associated with PWM stimulated IgG and IgM production. This might reflect the suggested immaturity of CB CD45RO+ cells already mentioned previously. The positive correlation between CD11α+ B cells and Ig secretion in both newborns and adults indicates logically that the proportion of mature B cells influences Ig secretion especially since CD11α is needed in B cell development into an Ig secreting cell (Katada et al 1996, Tohma et al 1992).

9.3 MC COMPOSITION IN THE NEWBORNS

Our results concerning the proportions of different mononuclear cell subtypes of full term babies were comparable with previous reports (Maccario et al 1993, Osugi et al 1995, Motley et al 1996). Although we did not have information on the Ig secreting or cytokine secreting abilities of the preterm infants in this study, the precence of CD11α- B cells in the very premature newborns with an infection raised a question whether these newborns contracted the infection partly because of the immaturity of their B cells? The effect of increasing GA was clear in the increasing proportions of CD11α+ B cells and activated HLA-DR+ macrophages. Further, the proportion of CD4+ cells and

CD8+ cells, cornerstones of both humoral and cell mediated immunity, increased with GA, which could contribute to the immunologic immaturity and subsequent vulnerability to infections of the very premature neonates.

The effect of maternal pre-eclampsia on the proportion of CD4+ cells and the resulting decreased CD4/CD8 ratio has been demonstrated before (Baker et al 1987). Baker et al suggested that the decreases in the proportion of these cell types and NK cells were merely reflecting intrauterine malnutrition, a factor delaying or inhibiting maturation. However, our finding that the proportions of two "hallmarks" of immature cell types (CD5+ B cells, and CD4+CD8+ double positive T cells) decrease with maternal pre-eclampsia does not support this notion; rather, it is indicative of a more rapidly advancing maturation. Since even the proportions of CD14+HLA-DR+ and CD11α+ (*in vitro* secretion of IFN-γ has been associated with the proportion of CD18/CD11α+ cells, (Muller et al 1996)) were decreased in these babies, it seems that maternal pre-eclampsia had an inhibitory effect on the cell-mediated immunity. Treatment of the mothers with a cortisone derivative, betamethason, prior to the delivery could theoretically cause such a suppression of the baby's immune system. This treatment, that is given in order to accelerate maturation of the lungs of the fetus, however, did not explain our findings, since betamethason had an opposite effect on the proportion of CD14+HLA-DR+ and NK cells, and a clear increasing effect on the proportion of T (CD3+) cells (article IV, Table 1, page 311).

Based on previous reports, vaginal delivery, when compared with caesarean section, increases the proportion of NK cells and decreases the proportion of CD4+ cells (Samelson et al 1992). We could not confirm these results. The only significant difference was the proportion of CD14+ cells being smaller in babies born by section. Vaginal delivery is considered to be beneficial for the maturation of the fetal lung, but in the light of our results, vaginal delivery vs. caesarean section does not critically influence the proportions of MC subtypes. The difference between previous reports and this study might have resulted from the differences in statistical analysis of the results. The use of multiple regression analysis, which we used, takes into account the numerous correlations between different factors and is more reliable and less prone to produce false positive results than univariate methods. After taking other factors into account, betamethason therapy did not have an independent effect on the MC composition of the newborn. Betamethason therapy of mothers should not be deferred in fear of immunosuppressive effects in the neonates.

In conclusion, the reduced ability of neonates to secrete Ig and IFN-γ is not a consequence of high IL-10 secretion. However, individual newborns differ significantly in their ability to secrete cytokines as well as Igs. It would be of interest to study whether babies with different cytokine secretion patterns (IL-10-oriented, IFN-γ-oriented, balanced-good-secretors, and nonresponders) differ in their susceptibility to infections or have different risk of developing allergic/atopic symptoms. This could be possible by longitudinal follow-up studies. Assessing the ability of premature babies to secret cytokines and Igs would be of great interest, since such clear differences were detected in the proportions of cell sub-types between, e.g., pre-eclamptic vs. infected premature neonates. In the future, improved understanding of the heterogeneity of the newborn immune system will hopefully help us identify babies more susceptible to environmental threats like infections or allergies.

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