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DIPLOMARBEIT

Titel der Diplomarbeit

EFFECTS OF ALLERGENS AND BACTERIAL STRAINS ON CORD BLOOD MONONUCLEAR CELLS

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DEFINING THE KINETICS WITH SPECIAL EMPHASIS
ON CYTOKINE PRODUCTION AND SOCS EXPRESSION

angestrebter akademischer Grad

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Abstract German

Hintergrund: Der schrittweise Übergang von der Th2-geprägten Schwangerschaft zum ausgewogenen Th1/Th2-Milieu, der in den ersten Lebensjahren stattfindet, kann durch Umweltfaktoren, wie die mikrobielle Exposition und die Besiedlung und Zusammenstellung der Darmflora, beeinflusst werden. Die zytokinvermittelte Zellaktivierung wird von negativen Regulatoren, wie den Suppressors Of Cytokine Signaling (SOCS), kontrolliert. Die Hoch- oder Herunterregulation der SOCS-Expression beeinflusst entscheidend das Ausmaß der Aktivierung als auch die Art der Zytokinantwort der Effektorzellen.

Ziel der Studie: In der vorliegenden Studie wurde der Einfluss von Nahrungsmittelallergenen sowie zweier probiotischer und eines Gram-negativen Bakterienstammes auf die Zytokin- und SOCS-Expression der mononukleären Zellen des Nabelschnurblutes untersucht.

Methoden: Die mononukleären Nabelschnurblutzellen von reifen, gesunden Neugeborenen wurden unter Zugabe von Nahrungsmittelallergenen ((Arachis hypogea (Ara h) 1 und 2, β-Laktoglobulin (BLG) und Ovalbumin (OVA)) sowie inaktivierten Bakterien (Lactococcus lactis (LL), Lactobacillus plantarum (LP) und Escherichia coli (EC)) kultiviert. Auswirkungen auf die Proliferation ([^{3}H]-Thymidineinbau), Zytokinproduktion (ELISA) und SOCS mRNA-Expression (RPA) wurden bestimmt.

Resultate: Die Nahrungsmittelallergene übten keinen Einfluss auf die SOCS mRNA-Expression von Nabelschnurblutzellen aus. In Gegenwart der Gram-positiven Bakterien LL und LP konnte allerdings ein dosisabhängiger, Th1-gerichteter Effekt beobachtet werden. LP erwies sich in dieser Hinsicht als das effektivere Bakterium. Obwohl E. coli ebenfalls einen klaren Th1-gerichteten Einfluss ausübte, war dieser schwächer als bei den Gram-positiven Bakterien. Die von EC induzierte IL-5- und IL-10-Produktion war, verglichen mit LL und LP, deutlich erhöht, während die IL-12- und IFNγ-Produktion erniedrigt war. Die Zytokinproduktion erreichte ihren Höchstwert nach 24 (IL-10, IL-12) und 72 Stunden (IL-5, IFNγ), wohingegen die SOCS mRNA-Expression bereits nach 1 (EC) beziehungsweise 2 Stunden (LP) nachgewiesen werden konnte. Darüber hinaus induzierten die unterschiedlichen Bakterienstämme spezifische SOCS mRNA-Expressionsmuster.

Schlussfolgerungen: Verschiedene Bakterienstämme führen zu differenzierter Zytokinexpression in Nabelschnurblutzellen. Dieser Stamm-spezifische Einfluss wird in Folge auch bei der SOCS-Expression deutlich. Doch bereits vor dem Ausschütten von Zytokinen kann eine durch Gram-positive und Gram-negative Bakterien induzierte SOCS-Expression der Nabelschnurblutzellen beobachtet werden, was auf eine direkte Aktivierung der SOCS-Gene mittels unspezifischer Immunantwort (Toll-ähnliche Rezeptoren) hinweist.

Abstract English

Background: Environmental factors, such as microbial exposure and the colonization of the gut in terms of composition, may influence the sequential switch from a Th2-skewed pregnancy into a balanced Th1/Th2 milieu within the first years of life. However, cytokine-mediated cell activation is controlled by negative regulators, like the suppressors of cytokine signaling (SOCS). Up- or down-regulation of SOCS expression is decisive in defining the extent of activation and also the type of cytokine response of an effector cell population.

Objective: The purpose of the study was to evaluate the effects of food allergens, two probiotic lactic acid bacteria and one gram-negative bacterial strain on the cytokine and SOCS expression of cord blood mononuclear cells (CBMCs).

Methods: CBMCs from randomly chosen full-term healthy neonates were co-cultured either with food allergens (Arachis hypogea (Ara h) 1 and 2, β-Lactoglobulin (BLG) and Ovalbumin (OVA)) or inactivated bacteria (Lactococcus lactis (LL), Lactobacillus plantarum (LP) and Escherichia coli (EC)). Proliferation ([^{3}H]-thymidine incorporation assay), cytokine production (ELISA) and SOCS mRNA expression (ribonuclease protection assay) were measured.

Results: Food allergens did not impact SOCS expression. The gram-positive bacterial strains LL and LP exerted a strong Th1-skewing effect that was dose-dependent, and more prominent in the presence of LP. Although E. coli clearly induced a Th1-type response, it was weaker than those induced by the gram-postive strains and characterized by higher IL-10 and IL-5 production, as well as lower INFγ and IL-12 production. While cytokine expression peaked at 24 hours (IL-10, IL-12) and 72 hours (IL-5, IFNγ), SOCS mRNA expression was induced at 1 and 2 hours by EC and LP, respectively. Furthermore, we observed strain-specifity in SOCS mRNA expression.

Conclusion: Different bacteria lead to differential cytokine expression in cord blood which is reflected in a distinct SOCS pattern in CBMCs. However, there is some evidence for a modulation of SOCS expression by gram-positive and gram-negative bacteria before the release of cytokines, pointing to direct activation of the SOCS gene via the Toll-like receptor.

Abbreviations

^{32}P	^{32}P-phosphate
^{3}H	tritium
all	allergen
APC	antigen presenting cell
Ara h1	Arachis hypogea (peanut) allergen 1
Ara h2	Arachis hypogea (peanut) allergen 2
BLG	β-lactoglobulin
C	control
CAM	cell adhesion molecule
CBMC	cord blood mononuclear cell
CD	cluster of differentiation
CDR	complementarity-determining region
CFU	colony forming unit
CIS	cytokine-inducible SH2 domain-containing protein
CpG	Cytosine plus Guanine dinucleotide motif
Cpm	counts per minute
Crude	crude peanut extract
DNA	Desoxyribonucleic acid
DNase	Desoxyribonuclease
EC	Escherichia coli
Foxp3	Forkhead Box P3
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GH	growth hormone
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IU	international unit
JAK	janus kinase
KIR	kinase inhibitory region
L32	ribosomal protein L32
LAB	lactic acid bacteria
LIF	leukocyte inhibitory factor

LL	Lactococcus lactis
LP	Lactobacillus plantarum
LPS	Lipopolysaccharide
MAP	mitogen-activated protein
MHC	major histocompatibility complex
mRNA	messenger RNA
NFκB	nuclear factor κB
NK cell	natural killer cell
OVA	Ovalbumin
PBMC	peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PHA	Phytohemagglutinin
PIAS	protein inhibitors of activated STAT
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
RTE	recent thymic emigrant
SEA	Staphylococcal enterotoxin A
SH2	Src-homology 2
SHP	SH2 domain-containing tyrosine phosphatase
SOCS	suppressor of cytokine signaling
Socs	SOCS gene
STAT	signal transducer and activator of transcription
Tc cell	cytotoxic T cell
TCR	T cell receptor
TGF	transforming growth factor
Th	helper T cell
TLR	Toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T cell
tRNA	transfer RNA
TYK	tyrosine kinase
UCC	Ultra Culture complete medium
γc cytokine	common γ cytokine
μCi	microcurie

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1 Literature review

1.1 Allergy

1.1.1 Definition of allergy

Allergic reactions occur when an individual who has produced immunoglobulin E (IgE) antibodies in response to an innocuous antigen, or allergen, subsequently encounters the same allergen. The allergen triggers the activation of IgE-binding mast cells in the exposed tissue, leading to a series of responses that are characteristic for allergy. (Janeway, 2005)

About 40% of people in Western populations are atopic, a state which is influenced by several gene loci. Atopic individuals have higher total levels of IgE and eosinophils in the circulation than non-atopic individuals.

Initial priming of the immune system against allergens possibly occurs as early as in the prenatal period, which has been documented by the detection of proliferative responses to allergens in cord blood (Kondo, Kobayashi et al. 1992).

1.1.2 T cells in allergy

Utilizing the CD cell surface markers, T cells can be divided into two different populations: $CD4^+$ T cells (Th cells), which facilitate the response of B- and T cells or induce the cytotoxic function in $CD8^+$-cells, and $CD8^+$-cells (Tc cells), which are able to lyse virus infected targets.

1.1.2.1 $CD4^+$ T cells

Based on the cytokines $CD4^+$ T cells produce there are several subgroups to be differentiated.

1.1.2.1.1 The Th1/Th2 paradigm

In 1986, Mosmann et al. reported that long term murine T cell clones could be segregated according to their patterns of cytokine production (Mosmann, Cherwinski et al. 1986). The following year Snapper and Paul (Snapper and Paul 1987) reported that IL-4 was a switch factor for the expression of the IgE heavy chain gene and that this process could be inhibited by INFγ. Meanwhile, subsets comparable to murine Th1 and Th2 cells have been reported in rat and human (Del Prete, De Carli et al. 1991).
The Th1-pattern is characterized by IL-2, IL-12, INFγ and TNFα production and the Th2-pattern is defined by IL-4, IL-5, IL-6, IL-9, and (later) IL-13 production (Maino and Picker 1998; Romagnani 2000). Expression of these cytokines is regulated by transcription factors, primarily signal transducer and activator of transcription 4 (STAT4) and T-bet for Th1 cells and their antagonists STAT6, GATA3 and c-maf, which control Th2 cells (Romagnani 2006).

The development of Th1 or Th2 cells from naive $CD4^+$ T cells is mainly influenced by the cytokine milieu created at the level of antigen presentation. Antigen presenting cells (APC) produce these "conditional" cytokines early in infection. Infection by intracellular pathogens and the subsequent expression of IL-12 by APC and IFNγ by NK cells drives Th1 differentiation (Wu, Demeure et al. 1993), whereas helminth infection and the expression of IL-4 induces Th2 differentiation (Romagnani 2000).

Th1 cells promote mainly cellular immunity, activating macrophages to a microbicidal state as well as supporting $CD8^+$ antiviral effector T cells. Th1 cells induce the release of IgG antibodies by B cells, which mediate opsonization and phagocytosis.

By contrast, Th2 cells promote mainly humoral immunity, stimulating the growth and differentiation of mast cells and eosinophils directly and indirectly via induction of the production of IgE by B cells, which can mediate the activation of these cells.
Additionally, Th2 cells play an important role in induction and maintenance of the allergic inflammatory cascade. Cytokines and chemokines produced by Th2 cells, as well as those produced by other cells in response to Th2 cytokines or as a reaction to Th2 related tissue damage, account for most pathophysiologic aspects of allergic disorders (IgE-production, IL-4 or IL-13), recruitment or activation of mast cells, basophils (through IL-4, IL-9 and IL-10) and eosinophils (IL-5), mucus hypersecretion, subepithelial fibrosis and tissue remodeling. Moreover, IL-4, IL-10 and IL-13 inhibit several macrophage functions and the development of Th1-cells (Romagnani 2000).

1.1.2.1.2 Other $CD4^+$ T cell subgroups

Aside from Th1 and Th2 cells, other $CD4^+$ subsets have been described.

Th0 cells are an intermediate between naïve $CD4^+$ T cells and differentiated Th1/Th2 cells. They exhibit an unrestricted cytokine profile (Firestein, Roeder et al. 1989) while having the potential of either becoming Th1 or Th2 cells.

Th3 cells secrete IL-10 and high levels of TGFβ, functioning as regulatory cells in the mucosal immune system. They have suppressive properties for both Th1 and Th2 cells (Weiner 2001).

The IL-17 producing *Th17 cells* have been recently described by Harrington et al. (Harrington, Hatton et al. 2005). IL-17 is also expressed by other $CD4^+$ T cells, as well as $CD8^+$ and γδ T cells. In addition to IL-17, Th17 cells produce IL-17F, IL-22, IL-26, TNFα and various chemokines, which mediate the pro-inflammatory effects of this population. It has been shown that TGFβ and IL-6 are required for naïve $CD4^+$ T cells to differentiate into Th17 cells (Bettelli, Carrier et al. 2006).
Th17 cells may have a specific role in combating certain bacterial gut infections, thus complementing the activities of Th1 and Th2 cells in their responses against

intracellular pathogens and helminths, respectively. On the other hand, Th17 cells have been shown to play an important role in the induction and propagation of autoimmunity in animal models. In addition, links to several human autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and psoriasis have been documented (Steinman 2007).

Regulatory T cells (Tregs) control self reactive T cells as well as excessive T cell response. They are characterized by the expression of the transcription factor FoxP3 (Forkhead box 3).
Tregs contribute to the regulation of Th1 and Th2 responses either via contact-dependent mechanisms or by secreting IL-10 and TGFβ.

1.1.2.2 $CD8^+$ T cells

Classical effector $CD8^+$ T cells have been defined by their ability to lyse virus infected targets and to produce high levels of INFγ. Functionally much less is known about the role of $CD8^+$ cells in allergic disease.

Comparable to $CD4^+$ cells, $CD8^+$ cells are also capable of producing different cytokine patterns, depending on the cytokines present during primary stimulation. These $CD8^+$ subpopulations can be compartmentalized along broadly similar lines to $CD4^+$ T cells with INFγ secreting $CD8^+$ cells (Tc1), IL-4 secreting $CD8^+$ cells (Tc2) (Carter and Dutton 1996) and $CD8^+$ cells with an unrestricted cytokine profile (Tc0). Tc1 and Tc2 memory cells are stable and retain their cytokine profile after restimulation (Cerwenka, Carter et al. 1998).
Although most of the experiments in this context were performed in a mouse model or in vitro, there were also studies performed which demonstrated that freshly isolated $CD8^+$ cells from human blood have the potential to produce IL-4 at similar (Ying, Humbert et al. 1997), or even higher (Stanciu, Shute et al. 1996) levels than those produced by comparable $CD4^+$ cells. In vitro models demonstrated an inhibitory effector function (Holmes, MacAry et al. 1997), as well as the potential to induce IgE switching (Punnonen, Yssel et al. 1997; Yanagihara, Kajiwara et al. 1999). These

studies also discuss the possibility that $CD8^+$ T cells could be the initial source of a Type 2 cytokine profile switch. There is strong evidence that Th2-like $CD8^+$ T cells are present at sites of allergic inflammation (Ying, Humbert et al. 1997) but it is not clear whether there is enough antigen to activate them or to which extent they contribute to allergic inflammation (Kemeny 1998).

In summary $CD8^+$ cells may play a major role in determining whether IgE response will occur or not, but their role remains to be specified.

1.1.2.3 Th1/Th2 polarization in the perinatal period and the development of allergy

The development of IgE-mediated allergy is determined by both genetic and environmental factors. Atopy is influenced by several gene loci, and the immunological events that lead to the development of allergen-related Th memory may be initiated already in utero.

1.1.2.3.1 The prenatal period

The maternal adaptive and innate immune system is radically altered during pregnancy. Its appropriate regulation is essential for the fetal survival. From the critical time of implantation onwards, a constitutive polarization of the feto-maternal interface towards a more Th2-type profile takes place (Wegmann, Lin et al. 1993; Raghupathy 1997).
Although studies reject the Th2 paradigm as the major mechanism providing fetal allograft survival (Erlebacher 2001), there is no doubt about the abortificant role of typical Th1-cytokines (Raghupathy 1997). Elevated TNFα, INFγ and IL-2 levels are associated with recurrent spontaneous abortion (Raghupathy, Makhseed et al. 2000), while IL-4, IL-10, and IL-13 counterbalance their effects (Piccinni, Beloni et al. 1998). These Th1-damping effects may be amplified by the placenta through the synthesis of high levels of prostaglandin E_2 (Linnemeyer and Pollack 1993), which selectively inhibits INFγ production and local progesterone production, stimulating IL-4 production (Piccinni, Giudizi et al. 1995).

1.1.2.3.2 The postnatal period

During the first few years of life a universal skewing of the above mentioned intrauterine Th2 cytokine profile towards a Th1 cytokine profile, as present in adult non-atopic individuals, takes place. In people developing an allergy phenotype, this crucial time period differs substantially, leading to a Th2 dominated cytokine profile (Prescott, Macaubas et al. 1998).

At birth T lymphocytes exhibit a relative Th2-profile, characterized by a limited ability to produce Th1 as well as Th2-type cytokines (Prescott, Macaubas et al. 1999). Throughout the first months after birth these Th-2 skewed responses are modified into low-level immunity predominantly expressing Th1-cytokines and IgG-antibodies, particularly of the IgG1 subclass. This very time seems to be essential for further development of allergic disease (Bjorksten 1999).

Children with a family history of atopic disease seem to have a generally decreased capacity to produce Th1 and Th2 cytokines as well as allergen-specific Th1/Th2 cytokines (Cairo, Suen et al. 1992). Referring to Prescott et al., the defect is most apparent with regard to INFγ production (Prescott, Macaubas et al. 1999). Neonates at high risk to develop allergy showed a reduced capacity to produce IFNγ in cord blood mononuclear cells (CBMCs) stimulated with phytohemagglutinin (PHA) (Tang, Kemp et al. 1994) and allergens (Kondo, Kobayashi et al. 1998). Additionally, Schaub et al. demonstrated that IL-10 expression of CBMCs exposed to microbial stimuli via the toll-like receptor (TLR) was significantly lower in high risk neonates (Schaub, Campo et al. 2006). Furthermore, elevated levels of Th2 cytokines in CBMCs of term babies have been correlated to atopy in several studies. Elevated IL-13 levels (Spinozzi, Agea et al. 2001; Ohshima, Yasutomi et al. 2002; Lange, Ngoumou et al. 2003) and IL-4 levels, an increased IL-4/ IFNγ ratio in response to PHA at a protein level, as well as lower numbers of IL-12-producing cells after allergen stimulation could be observed in CBMCs of neonates at risk of atopy (Gabrielsson, Soderlund et al. 2001).
In contrast, Prescott et al. (Prescott, Macaubas et al. 1998) demonstrated reduced allergen-specific IL-13 levels in high-risk children at a protein level. In a later study,

these findings were expanded by the additional Th2 cytokines IL-6 and IL-10 at protein level as well as IL-4 at RNA level, showing markedly reduced responses to allergens, compared to a non-atopic group, as well. While Th2 responses where rapidly suppressed in non-atopic children during their first year of life, IFNγ production which was low in both atopic and non-atopic children at birth was raised with age in the non-atopic group only (Prescott, Macaubas et al. 1999).

In summary, the development from the Th2 skewed pregnancy into a balanced Th1/Th2 milieu in the first years of life seems to be detrimental for the development of allergic phenotypes later on. However, skewing factors and the underlying regulatory mechanisms remain to be evaluated.

1.1.2.4 The hygiene hypothesis and lactic acid bacteria

The development of type I allergy, characterized by a dominance of Th2 cytokines (IL-4, IL-5, IL-13), has been linked to several factors, such as genetic predisposition and the nature of the allergenic protein. These factors, however, cannot solely explain the increased prevalence of atopic diseases. Evidence is accumulating that "western life style", which is associated with reduced microbial exposure due to high hygienic standards, significantly contributes to the constant increase of allergies (Strachan 2000).

In this context, it has been demonstrated that early childhood exposure to livestock on a farm and ingestion of unpasteurized milk inversely correlate with the incidence of atopic diseases (Braun-Fahrlander, Riedler et al. 2002). Furthermore, children living on a farm lose their allergic sensitization more frequently than other children (Radon, Windstetter et al. 2004).

Other evidence for the relevance of the hygiene hypothesis is coming from studies focusing on the significance of gut microbiology and the strain specific impact on the atopic phenotype. Based on epidemiologic data, a relationship between the composition of the intestinal flora and the prevalence of allergic diseases has been documented. Infants from countries with a high prevalence of allergy, such as Sweden, significantly differ with regard to intestinal colonization with certain lactic acid bacteria (LAB)

strains from countries where allergic diseases are less prevalent, such as Estonia. Additionally, it was shown that 2-year-old allergic children were less often colonized with lactobacilli, compared to nonallergic children, but nonallergic children had higher counts of coliforms and *Staphylococcus aureus* (Bjorksten, Naaber et al. 1999).

Specific strains of LAB have been reported to exert health-beneficial or probiotic effects. In clinical trials, oral administration of a particular LAB strain (*Lactobacillus rhamnosus GG*) led to reduced atopic dermatitis in children with a positive family history of type I allergy (Kalliomaki, Salminen et al. 2001; Kalliomaki, Salminen et al. 2003). Consequently, an anti-allergic effect was considered. In this context, it has been demonstrated in a mouse model of type I allergy, that selected LAB strains (Lactococcus lactis and Lactobacillus plantarum) have Th1-promoting capacities in vitro and in vivo (Repa, Grangette et al. 2003). Other studies have shown that bacteria are important in down-regulating inflammation associated with hypersensitivity reactions in patients with atopic eczema and food allergy (Isolauri 2004). Interestingly, clinical improvement of atopic dermatitis patients could not be linked to immunological changes (Kalliomaki, Salminen et al. 2001; Kalliomaki, Salminen et al. 2003).
Although beneficial effects of LAB on atopic diseases have been suggested, evidence for clinical use strongly depends on the strains used and relates to studies with a relatively small sample size (Isolauri, Arvola et al. 2000; Kalliomaki, Salminen et al. 2003; Rosenfeldt, Benfeldt et al. 2003). Recently published trails raised concerns about early probiotic supplementation reducing the risk to develop atopic dermatitis in high-risk infants (Brouwer, Wolt-Plompen et al. 2006; Taylor, Dunstan et al. 2007). Consequently, LAB have not reached an evidence level for regular therapeutic application.

1.1.3 Regulatory mechanisms of Th1/Th2 cells

To assure an adequate Th1- or Th2-dominated response, cytokines produced by these subsets, predominantly IFNγ and IL-4, play an important role. However, the whole repertoire of immune cells is able to interfere via the secretion of Th1/Th2 favoring cytokines or regulatory cytokines. Moreover, both responses can be suppressed by Tregs and their associated cytokines. On a lower level, the JAK-STAT pathway regulates cellular responses to cytokines and growth factors, playing a central role in regulating the processes of proliferation, differentiation and apoptosis of Th1 and Th2 cells.

1.1.3.1 Cytokines involved in the regulation of Th1/Th2 cells

Interferons (IFNs) are produced by cells which have been infected with a virus (IFNα and IFNβ) or certain antigen-activated T cells (IFNγ). IFNs are among the first defense mechanisms activated after a virus infection.
Generally, IFNs create a higher resistance to viruses in the host cells, but IFNγ has numerous additional properties as well, one of them being the inhibition of proliferation of Th2 cells.

Interleukins (ILs) are mainly produced by T cells, but also by B cells, macrophages and other cells of the immune system. By the expression of certain ILs Th and Tc cells are classified into Th1/Tc1 and Th2/Tc2, IL-2 and IL-12 being Th1/Tc1 cytokines, IL-4, IL-5, IL-6, IL-9 and IL-13 representing the Th2/Tc2 profile (Romagnani 2000).
The various ILs control immune cells by influencing their proliferation, differentiation and activation. Their target cells include Th cells, B cells, NK cells, macrophages and many others.

The tumor necrosis factors (TNFα and TNFβ) mediate inflammatory and cytotoxic responses, TNFα being a Th1 cytokine.

Most cytokines use the JAK-STAT pathway to transmit signals, but IL-1 uses a much more complex pathway.

See *Table 1-1* for selected cytokines and their activities.

1.1.3.2 The JAK-STAT pathway

The JAK-STAT pathway regulates cellular responses to cytokines and growth factors that play a central role in the regulation of cell proliferation, differentiation and apoptosis. The Janus Kinases (JAKs) and Signal Transducers and Activators of Transcription (STATs) transduce signals from the cell surface receptor to the nucleus, where transcription of their target genes is activated.
JAKs, having tyrosine kinase activity, bind to the cytoplasmatic domains of the cell surface receptor. This event triggers their activation. The so activated JAKs phosphorylate tyrosine residues on the receptor, creating interaction sites for proteins that contain phosphotyrosine-binding SH2 domains. STATs, possessing SH2 domains, are attracted to the receptor and are themselves tyrosine-phosphorylated by JAKs. This triggers STAT dimerization via their SH2 domains and the STAT dimers move into the nucleus, acting as activator of transcription of their target genes (see *Table 1-2* for STATs and their functions).

Negative regulation of the pathway occurs at multiple levels, including removal of phosphates from receptors and activated STATs by protein tyrosine phosphatases and direct inhibition of transcriptional activation in the nucleus by Protein Inhibitors of Activated STATs (PIAS)).
More recently identified Suppressors of Cytokine Signaling (SOCS) interact with and inhibit JAKs or compete with STATs for binding sites on cell surface receptors (Chen and Khurana Hershey 2007).

1.1.3.3 Tregs

Regulatory T cells (Tregs) are defined as a T cell population that can influence other cell types with suppression of the immune response, controlling unwanted immune responses in vivo. They are characterized by expression of the transcription factor Foxp3 (Forkhead box P3).
Tregs help to maintain homeostasis in the lymphoid organs by suppressing autoreactive T cells. After the onset of an immune response, Tregs migrate to inflamed tissues to limit tissue damage by inflammation. In the intestine, dendritic cells induce naïve T cells to differentiate into Foxp3-expressing Tregs. Induction of Tregs in the intestine influences, amongst others, Th1/Th2 responses and relates closely to the hygiene hypothesis mentioned above.

Tregs utilize several mechanisms of suppression. Locally, effector T cells are shut down by cytokine deprivation and secretion of IL-10. Through direct interaction with APCs, Tregs are down-regulating the antigen-presenting activity of these cells and/or promoting the secretion of suppressive factors. Additionally, through expression of TGFβ, new Tregs are produced which spread out and promote a regulatory environment beyond local infection (Tang and Bluestone 2008).

1.2 Cord blood mononuclear cells (CBMCs)

1.2.1 CBMCs and allergy

Allergic sensitization is initiated by the development of Th2 cells reactive to allergens. Development of effector T cells, including Th2 cells, in the neonate is initiated by activation through antigen-presenting dendritic cells. The mechanisms leading to Th2-mediated allergic responses are assumed to be promoted by low activity of regulatory T cells, which are responsible for tolerogenic responses during the perinatal phase in healthy children, or poor Th1-type favouring actions. Cord blood mononuclear cells include premature naïve T cells and antigen-presenting cells as well as Tregs, thus serving as a useful model for the status of the immune system at birth (Allam, Zivanovic et al. 2005).

1.2.2 Special features of CBMCs

Cord blood T cells can be considered to represent a transitional population between thymocytes and adult T cells. Their immaturity becomes apparent in their proliferative activity to allergens, which is conducted mostly without the aid of memory cells, and the alleviation of cytokine production. Thus it remains unclear whether allergen-specific proliferation of CBMCs relates to intra-uterine priming or rather resembles an unspecific response of recent thymic emigrants (Thornton, Upham et al. 2004). Interestingly, a recent study provided evidence for antigen specific priming in utero following vaccination against influenza. Antigen specific T cells were detectable in cord blood (Rastogi, Wang et al. 2007).

The cytokine profile of cord blood T cells is skewed towards a Th2 phenotype, exhibiting higher levels of IL-4, IL-5 and IL-13, and diminished levels of IFNγ, compared to adult T cells. A better understanding of decisive events in the development of pathognomonic T cell populations from “naïve” cord blood T cells may help to invent preventive strategies (Cohen, Perez-Cruz et al. 1999).

However, T cell based differences are more or less apparent depending on the stimulation system. Evidence is increasing that the aberrant cytokine responses of CBMCs rather relate to different regulatory properties and immaturity of the APC (Allam, Zivanovic et al. 2005).
There are also pronounced differences between $CD4^+$ $CD25^+$ regulatory T cells from cord and adult blood, the former being a naïve ($CD45RO^-$ $CD45RA^+$) population, contrasting with the memory ($CD45RO^+$ $CD45RA^-$) population found in adults (Wing, Ekmark et al. 2002).

1.2.3 Immune responses in cord blood

1.2.3.1 Allergen-induced specific response in cord blood

The response of CBMCs to antigens/allergens differs clearly from the adult T cell response. Firstly, more than 90% of $CD3^+$ cells in cord blood are naïve, $CD45RA^+$ cells, while in adults $CD45RO^+$ memory T cells become increasingly dominant with age (Cossarizza, Ortolani et al. 1996). Neonatal $CD45RA^+$ T cells also express CD38, which indicates a similarity to recent thymic emigrants (RTEs). Interestingly, it was shown that even upon removal of the low numbers of so-called memory cells from cord blood, a responsiveness to allergens was measurable in 50% of the donors (Devereux, Seaton et al. 2001). Therefore, the proliferative reactivity of CBMCs to allergen appears not to depend on conventional memory T cells. It has been proposed that these unprimed reactions to allergen in cord blood are part of a mechanism providing a "broad range" response to antigens during the early postnatal period, preceding the development of conventional T cell memory (Thornton, Upham et al. 2004). Functionally immature T cells of cord blood have structural differences, such as reduced CDR3 length (Schelonka, Raaphorst et al. 1998) and have been observed to show reactivity to multiple regions of an allergen, as opposed to an average of one site or less in PBMCs from 5 year olds (Yabuhara, Macaubas et al. 1997).

Moreover, it has been demonstrated that cytokine production is down-regulated in neonatal T cells. In comparison to adult T cells, TNFα production was reduced, and

expression of IL-3, IL-4, IL-5 and IFNγ was barely observed. These differences were explained through the immaturity of cord blood T cells (Allam, Zivanovic et al. 2005). However, it is not clear whether these down-regulations rather relate to immaturity of antigen presenting cells, since polyclonal responses to T cells (e.g., PHA induced proliferation) is not diminished in cord blood (Chipeta, Komada et al. 2000).

1.2.3.2 Bacteria-induced immune response in cord blood

1.2.3.2.1 Effects of probiotic on allergy

The hygiene hypothesis suggests that insufficient exposure to microbes is one of the reasons for elevated frequency of allergic diseases in Western societies. Initially, the direct immunomodulatory impact of a Th1-driven infection as well as the influence of the diversity of microbes during establishment of the intestinal flora was thought to direct development away from the allergic phenotype. At present, the beneficial effect of environmental factors is thought to rather relate to an induction of tolerance than to a Th2-opposing Th1-type response. The high degree of hygiene in industrial countries, and the resulting absence of environmental microbial exposure may pose a problem for immune maturation and tolerance development in infancy. One possible approach is the administration of probiotics which are thought to provide the necessary microbial stimulation of the child's developing immune system (Ouwehand 2007).

Various effects of probiotics on individuals have been hypothesized. Firstly, probiotics are able to modulate the intestinal microbial flora and improve the barrier function of the intestinal mucosa, leading to reduced antigen exposure (Malin, Verronen et al. 1997). Secondly, a direct modulation of the immune system through the induction of Th1 cytokines and inhibition of Th2 cytokines has been demonstrated in the mouse model (Pochard, Gosset et al. 2002; Niers, Timmerman et al. 2005). Moreover, an increased production of secretory IgA which can contribute to an exclusion of antigens from the intestinal mucosa has been described (Fukushima, Kawata et al. 1998).

1.2.3.2.2 Lactic acid bacteria

Dietary lactic acid bacteria (LAB) are non-invasive and non-pathogenic gram-positive bacteria with GRAS (generally regarded as safe) status that have been used for food processing and preservation for centuries. The major species of LAB are used in dairy manufacturing: *Lactobacillus* (milk, meat, vegetables, and cereal), *Lactococcus* (milk), *Streptococcus* (milk), and *Leuconostoc (*vegetables, milk). Other members of LAB, notably lactobacilli, occupy important niches in the gastrointestinal tracts of humans and animals and have been reported to exert health beneficial or probiotic effects. A possible role of LAB in the prevention of of type I allergy and atopic dermatitis has been suggested (Kalliomaki, Salminen et al. 2001; Kalliomaki, Salminen et al. 2003).

Escherichia coli are gram-negative anaerobe bacteria found in the human intestinal system where they constitute an important part of the essential intestinal flora. Although most strains of E. coli are not regarded as pathogens, they can cause infections in immunocompromised hosts. The pathogenic strains cause gastrointestinal illness when ingested.

Endotoxins are intrinsic components of microbial structure. The gram-positive cell wall consists of the endotoxins peptidoglycan and lipoteichoic acid, while the gram-negative cell wall contains the endotoxins lipopolysaccharide (LPS) and lipoproteins.

1.2.3.2.3 Lactic acid bacteria and the modulation of the innate immune response

The innate immune response directs T cell differentiation, and thereby probably influences the development of immunological tolerance to environmental antigens. In particular, postnatal colonization of the gut is crucial for tolerance development. As mentioned above, the composition of the gastrointestinal flora seems to play an important role.

Overview of the innate immune response

The innate or non-antigen-dependent immune response forms a front line protection against pathogens. Bacteria, viruses and parasites that overcome the epithelial surfaces are firstly recognized by tissue macrophages. In the gastrointestinal tract these macrophages are situated in the mucosal lining. They express various receptors for bacterial components, including CD14 and receptors of the Toll-like receptor (TLR) family. TLRs are able to recognize bacterial CpG-DNA, exotoxins and conserved microbial structures (endotoxins) such as peptidoglycan and lipopolysaccharide (LPS), the cell wall components of gram-positive and gram-negative bacteria. TLRs are expressed by monocytes, dendritic cells and various others including the intestinal epithelial cells. Binding of an antigen to this receptor triggers signaling pathways resulting in the production of inflammatory cytokines, which recruit neutrophils and macrophages to the site of infection. If the pathogen persists, the development of an adaptive immune response is initiated by transporting the antigen to the lymphoid organs via dendritic cells and macrophages (antigen-presenting cells), where they can be recognized by naïve B and T cells (Ouwehand 2007).

Toll like receptors and their "instructive" cytokines

Ten members of the human TLR-family have been described so far (Leulier and Lemaitre 2008). Among those that recognize Bacteria, TLR2, 4 and 9 are thought to be most important. TLR2 plays an essential role in the response to bacterial lipoprotein (gram-positive and gram-negative) and peptidoglycans (gram-positive), while TLR4 mediates activation through lipopolysaccharide (gram-negative) and TLR9 reacts to CpG-DNA of certain bacteria. Through the recognition of pathogens or their products, TLRs can induce the production of Th1 cytokines in APC. Specifically, release of IFNγ and IL-12 has been shown to be induced via TLR (Yang, Mark et al. 1998). These cytokines function as "instructive" cytokines and drive naïve T cells to differentiate into Th1 cells.

Additionally, counter-regulatory molecules like SOCS1 and SOCS3 were found to be induced by LPS or CpG–DNA stimulation in macrophages (Bode, Nimmesgern et al. 1999; Crespo, Filla et al. 2000).

The innate immune system and the hygiene hypothesis

Children who grow up on a farm, and are therefore exposed to higher levels of endotoxin than children of non-farming families, are at reduced risk of developing allergic diseases (Riedler, Braun-Fahrlander et al. 2001). It has been speculated that binding of microbial products to TLR activates antigen-presenting cells, inducing modifications of the adaptive immune response. These modifications are speculated to lead to a Th1 skewed environment. In this way, innate immunity is expected to play an important role in the sensitation of children to specific allergens. In another publication, Braun-Fahrlander et al demonstrated an association between a higher level of LPS exposure and a decreased risk for atopic diseases in schoolchildren (Braun-Fahrlander, Riedler et al. 2002).

After long-term exposure to heightened levels of endotoxins, the responsiveness of the innate immune system to LPS is reduced, a phenomenon known as lipopolysaccharide tolerance. This study suggests that schoolchildren with high levels of environmental exposure to endotoxins show a reduced capacity of cytokine production in response to activation via the innate immune system. Consequently this also leads to a down-regulation of the resulting inflammatory responses through adaptive immunity. This state of tolerance may prevent the development of atopic diseases in these children (Braun-Fahrlander, Riedler et al. 2002).
Furthermore, children with an increased environmental exposure to microbial compounds were shown to have an amplified gene expression of CD14 and TLR2 (Lauener, Birchler et al. 2002). Interestingly, TLR4 expression was not heightened in the farmers' children which parallels *in vitro* experiments investigating the effect of LPS on human blood cells (Flo, Halaas et al. 2001).

Regarding regulatory T cells, it has been demonstrated that Tregs selectively express TLRs, directly responding to LPS via TLR4 (Caramalho, Lopes-Carvalho et al. 2003).
Sutmuller et al. established a direct link between TLRs and regulatory T cells by demonstrating that TLR2 negatively controls the function of Tregs in vivo (Sutmuller, den Brok et al. 2006).

Additionally, a link between maternal atopy and Foxp3 (a gene specific for Tregs) induction was pointed out by Schaub et al. in 2006. The group demonstrated that IL-10 and Foxp3 expression induced via TLR2 stimulation was decreased in CBMCs of atopic mothers. These potentially less effective Tregs in atopic mothers were speculated to have a diminished capacity to respond to microbial stimuli. (Schaub, Campo et al. 2006).

Finally, it has to be noted that the hygiene hypothesis is regarded as a model to explain certain observations connecting the innate immune system with the development of atopic diseases. Neither the exact mechanisms nor the relevant endotoxins or environmental factors are to date known.

The innate immune system and lactic acid bacteria

In contrast to pathogens, commensals in the healthy gut have been shown to control inflammatory responses by turning the signaling cascades down. Recognition of these bacteria by TLRs ensures intestinal homeostasis, integrity of the individual and has been shown to prevent allergic inflammation (Bashir, Louie et al. 2004).

Karlsson, Hessle et al. demonstrated that LAB, being gram-positive commensals, induce high levels of IL-12, a key cytokine for the differentiation of naïve cells toward a Th1 cytokine pattern, and TNFα, a proinflammatory Th1 cytokine, in both cord blood mononuclear cells and adult peripheral mononuclear cells (PBMC). In contrast, gram-negative bacterial strains (e.g. E. coli) were shown to be poor inducers of IL-12 and TNFα. Interestingly, high levels of IL-10 and IL-6, both Th2 cytokines, were induced equally by gram-positive and gram-negative bacteria in cord blood.
However, CBMCs and PBMCs have to be regarded as model systems for the immune system which do not allow to draw conclusions about the intestinal immune response. The collected data therefore has to be treated as indirect evidence and most importantly highlights strain specific differences.

Concerning the differences between cord blood and adult mononuclear cells, it has been shown that neonatal cells produce higher levels of IL-6 than adult cells in response to most bacterial strains, including *L. plantarum* and *E.coli*. No statistically significant differences in the levels of IL-12, TNFα, and IL-10 were evaluated (Karlsson, Hessle et al. 2002).

Pochard et al. complemented these observations by using a Th2 cytokine-producing cellular model (i.e., PBMCs stimulated with the superantigen SEA) to assess the change in the cytokine profile of atopic and non-atopic patients exposed to different LAB (Lactobacillus plantarum, Lactococcus lactis, Lactobacillus casei, and Lactobacillus rhamnosus GG) and E.coli. It was shown that IL-4 and IL-5 were reduced in all LAB treated probes, while E.coli didn't have this effect. Interestingly, the PBMCs of atopic patients which were incubated with the related allergen reacted to LAB in the same way; IL-4, IL-5 and IL-13 levels were markedly reduced, even in patients allergic to aeroallergens. LAB were capable of inducing IL-12 secretion in PBMCs, as well as in monocytes via their TLRs. The large amounts of IL-12 produced by LAB-stimulated APCs lead to activation of STAT4, which is known to transactivate IFNγ directly (Pochard, Gosset et al. 2002). Recently, Niers et al showed that different LAB strains are able to decrease Th2 cytokines in PHA-stimulated PBMC cultures, mainly via expression of IL-10 by monocytes (Niers, Timmerman et al. 2005).

In short, antigen-presenting cells in newborns seem to have the ability to respond to probiotics efficiently. The cytokine pattern ultimately expressed by CBMCs suggests a role for lactic acid bacteria in the maturation of the immune system by inhibition of an overwhelming Th1- as well as Th2-type response.
On the other hand, as discussed above, an evidence level for regular therapeutic use of LAB in atopic and autoimmune diseases has not been reached to date.

1.3 Suppressors of Cytokine Signaling (SOCS)

1.3.1 Introduction

Regulation of the initiation, duration and magnitude of cytokine signaling occurs at multiple levels; one of the simplest means of attenuating a response is via a negative feedback loop. An important class of negative feedback inhibitors are the Suppressors of Cytokine Signaling (SOCS) proteins.
SOCS1, SOCS2, SOCS3 and CIS mRNA and proteins are generally present at low levels in unstimulated cells, and mRNA and protein levels are rapidly induced by a broad spectrum of cytokines, both in vitro and in vivo, with the STATs playing an important part in regulating SOCS gene transcription. Apart from cytokines, pathogens and their products such as LPS and CpG-DNA have been shown to induce SOCS expression as well. However, the underlying mechanisms of activation seem to differ between individual SOCS (Alexander and Hilton 2004).

1.3.2 The SOCS protein family

The SOCS protein family consists of eight members: cytokine-inducible SH2 domain-containing protein (CIS) and SOCS1-7. Common familial features include a central SH2 domain and a conserved C-terminal SOCS box. SOCS1 and SOCS3 have an additional kinase inhibitory region (KIR) adjacent to the SH2 domain. All SOCS proteins contain only few introns and can be characterized as immediate-early genes (Starr, Willson et al. 1997).

SOCS family members have been documented to act as negative feedback regulators that are induced by cytokine signaling itself and subsequently shut down the respective signaling cascade. Numerous studies show that inhibition of signal transduction occurs through interaction of the SH2 domains of SOCS proteins with key phosphotyrosine residues in activated signaling components. SOCS1 directly interacts with JAK1, JAK2, JAK3, and TYK2 and, in doing so, inhibits their kinase activity by interacting with a key regulatory tyrosine in the activation loop of JAKs with its SH2 domain.

For SOCS2, SOCS3 and CIS, the evidence points towards phosphotyrosines in the cytoplasmatic domains of cytokine receptors being the primary site of interaction. The KIR of SOCS1 and SOCS3, located next to the SH2 domain, is believed to inhibit JAK activity. Another way SOCS proteins might inhibit signaling is by competing with STATs for common phosphotyrosine binding sites within the cytoplasmic domains of cytokine receptors. Additionally, the SOCS box acts to couple the substrate-specific interactions of the SH2 domains to generic components of the ubiquitin ligation machinery. The SOCS box interacts with Elongin-C and -B to form an E3 ubiquitin ligase complex with further proteins. This complex is thought to attach ubiquitin to key signaling proteins as JAK2 which interact with the SH2 domain of SOCS proteins, leading to the degradation of these proteins in the proteasome, and the termination of signaling (Yasukawa, Sasaki et al. 2000).

The first member of the SOCS family, CIS, was identified as a negative feedback regulator of the STAT5 pathway in response to erythropoietin, IL-2 and IL-3.
The second member, SOCS1, binds to all Jak family tyrosine kinases, thereby acting as a negative regulator of a wide range of cytokine signaling pathways which utilize STAT1, most importantly IFNγ.
SOCS3 expression is induced by a wide variety of inflammatory and anti-inflammatory cytokines, including IFNγ, IL-3, IL-6 and IL-10 and works via the inhibition of STAT3.
However, it has to be kept in mind that since intracellular JAK/STAT pathways are shared between different cytokine signaling cascades, induction of individual SOCS will also inhibit cytokine pathways different from the inducing signal cascade (cross-talk inhibition) (Heeg and Dalpke 2003).

1.3.3 SOCS functions

The in vivo actions of SOCS proteins have been investigated by production of transgenic mice expressing various SOCS proteins. Additionally, studies of mice genetically engineered to lack functional *Socs* genes have been undertaken, revealing SOCS proteins to be important biological regulators in the adaptive and innate immune response. Results from these studies have clearly defined key physiological roles of

individual SOCS proteins, such as the essential role for SOCS1 in regulating IFNγ signaling and T cell homeostasis. In other areas, it is clear that no consensus has yet become apparent.

See *Table 1-2* and *Table 1-3* for an overview of STATs and SOCS and their functions.

1.3.3.1 SOCS1

SOCS1 appears to be the most potent inhibitor of cytokine signaling and *Socs1*$^{-/-}$ mice show the most severe phenotype. The pathology of sick *Socs1*$^{-/-}$ mice shows similarities to wild-type mice administered with IFNγ, leading to the hypothesis that the disease of *Socs1*$^{-/-}$ mice might be due to excessive responses to IFNγ (Alexander, Starr et al. 1999). However, long-term studies have revealed that combined S*ocs1*$^{-/-}$*Ifn*γ$^{-/-}$ mice eventually succumb to a range of inflammatory diseases, which are only detectable in the absence of IFNγ (Metcalf, Mifsud et al. 2002). These symptoms might be due to abnormal signaling of other inflammatory cytokines, including IL-2, -4, -6, -12, -15, -23 and TNFα, associating SOCS1 with a wide range of acute and chronic inflammatory disorders.

1.3.3.1.1 Regulation of IFNγ signaling

Mice lacking the *Socs1*$^{-/-}$ gene die within the first 3 weeks of life due to an uncontrolled fatal inflammatory disease. They display low body weight and complex pathology. The most striking defects in these mice are found in the acquired and innate immune system (Starr, Metcalf et al. 1998). *Socs1*$^{-/-}$ mice display evidence of an ongoing response to IFNγ including constitutive activation of STAT1 in the liver, and markedly elevated expression of IFNγ-inducible genes in several SOCS1-deficient tissues (Alexander, Starr et al. 1999).

The reasons for the symptoms of *Socs1*$^{-/-}$ mice are rather related to the increased sensitivity of SOCS1-deficient tissue to IFNγ than to elevated circulating concentrations of IFNγ. This hypersensitivity leads to an immune response induced by much lower doses of IFNγ than required by wild-type cells (Alexander, Starr et al. 1999).

Together, these observations suggest that the actions of SOCS1 are necessary to attenuate the duration of IFNγ signaling in cells, preventing negative effects of an uncontrolled immune response while allowing IFNγ to exert its beneficial effects.

1.3.3.1.2 Regulation of IL-4 signaling

Dickensheets et al revealed that SOCS1 overexpression can block activation of STAT6, which is involved in IL-4 and IL-13 signaling, in macrophages (Dickensheets, Venkataraman et al. 1999). In a later study by the same group it was shown that IL-4 and the related cytokine IL-13 directly induce SOCS1 gene expression in monocytes and macrophages in a STAT6 dependent process. In addition, forced expression of SOCS1 inhibits IL-4 signaling (Dickensheets, Vazquez et al. 2007).

1.3.3.1.3 Regulation of IL-10 signaling

IL-10, a key cytokine in regulating inflammatory responses, induced SOCS1 up-regulation in various human and mouse cell lines (Ding, Chen et al. 2003). A partial inhibition of IL-10 signaling through STAT3 inhibition has been proposed, while neither SOCS1 nor SOCS3 have been shown to directly interact with the IL-10 receptor.

1.3.3.1.4 Regulation of T cell homeostasis via the γc-dependent family of cytokines

SOCS1 also has an important IFNγ-independent role in T lymphoid development and function. The common gamma (γc) cytokines, namely IL-2, IL-4, IL-7 and IL-15, potently induce SOCS1 via the γc receptor. T cells lacking SOCS1 showed hypersensitivity to signals from cytokines that act through γc, and activation of STAT5 was evident following stimulation with significantly lower concentrations of cytokines than in wild type cells (Cornish, Davey et al. 2003).

While the *Socs1* gene appears to be transcribed at all major stages of T cell development in the thymus, SOCS1 expression is down-regulated during immature T cell development and becomes particularly high in double positive $CD4^+$ $CD8^+$ thymocytes.

At various stages of T cell homeostasis, up-regulation of SOCS1 expression has been hypothesized to keep T cells in a cytokine unreceptive state until they receive the appropriate developmental triggers to proliferate and differentiate. During positive selection, double positive cells cannot survive in the absence of pro-survival cytokines, such as IL-7. It has been speculated that SOCS1 maintains a fail-safe mechanism to prevent inadvertent signaling by pro-survival cytokines in double positive cells (Alexander and Hilton 2004).

1.3.3.1.5 Regulation of T helper cell polarization

SOCS1 expression has been reported to be fivefold higher in Th1 cells than in Th2 cells (Egwuagu, Yu et al. 2002). Nevertheless, it has been hypothesized that SOCS1 acts as a mutual suppressor for Th1 and Th2 cells in Th cell differentiation, depending on the cytokine milieu present. Fujimoto et al. showed that naïve *Socs1*$^{+/-}$ CD4^{+} cells underwent enhanced differentiation in vitro under either Th1- or Th2-polarizing conditions. These enhanced Th cell functions were attributed to enhanced effects of IL-12 and IL-4 on the cells due to lack of suppression by SOCS1 (Fujimoto, Tsutsui et al. 2002). Specifically, in case of high IL-12 (or IFNγ) levels, SOCS1 acts as a suppressor of STAT6, thus blocking IL-4 signaling, whereas in case of high IL-4 levels STAT1 and subsequent IFNγ signaling is suppressed. Additionally, IL-6-induced SOCS1 has been shown to prevent Th1 cell differentiation via blockade of IFNγ signaling (Diehl, Anguita et al. 2000), possibly via regulatory Th17 cells (Yoshimura, Naka et al. 2007).
Taken together, these findings suggest that SOCS1 is playing a part in the resistance of mature Th cells to antagonistic cytokines.

In 2002, Federici et al. demonstrated that biopsies from patients with psoriasis or allergic contact dermatitis showed high levels of SOCS1, SOCS2, and SOCS3 (Federici, Giustizieri et al. 2002). Recently, a significant connection between SOCS1 and allergic diseases has been found in asthmatic patients, who shared a polymorphism in the SOCS1 promoter which enhances the transcription of SOCS1. The resulting higher levels of SOCS1 in the T cells of these patients might have led to suppression of

Th1 cells and promotion of Th2 cells by alleviating the levels of IL-12 and IFNγ (Harada, Nakashima et al. 2007). However, the underlying mechanisms in the connection between SOCS1 and asthma are probably more complicated, because, as already mentioned above, SOCS1 suppresses signaling by Th2 cytokines as well.

Although evidence is suggesting that SOCS1 may be involved in with the differentiation of naïve $CD4^+$ T cells into Th1 cells (Cornish, Davey et al. 2003), a consistent model has not yet been found.

1.3.3.2 SOCS3

The deletion of SOCS3 resulted in embryonic lethality at midgestation, because of defects in the structure of the placenta possibly caused by aberrant leukocyte inhibitory factor (LIF) signaling (Takahashi, Carpino et al. 2003). Croker et al. revealed the key role for SOCS3 in the regulation of IL-6 signaling through analysis of *Socs3*$^{-/-}$ macrophages. Stimulation of macrophages with IL-6 lead to prolonged activation of both STAT3 and STAT1 as well as SH2 domain-containing tyrosine phosphatase 2 (SHP2), relative to that observed in wild type cells (Croker, Krebs et al. 2003). Additionally, SOCS3 has been shown to inhibit STAT4 activation by binding to the STAT4 docking area on the IL-12 receptor (Yamamoto, Yamaguchi et al. 2003). Finally, Seki et al. observed that overexpression of SOCS3 in T cells leads to strong Th2 polarization (Seki, Inoue et al. 2003).

1.3.3.2.1 Regulation of IL-6 and IFNγ signaling

SOCS3 expression is induced by IFNγ and IL-6, comparable to SOCS1. The difference lies in the function of SOCS1 and SOCS3: while *Socs1*$^{-/-}$ cells show prolonged responses to IFNγ, but not IL-6, responses of *Socs3*$^{-/-}$ cells are reciprocal: although enforced expression of SOCS3 can inhibit responses to IFNγ, the regulation of IFNγ was shown to be unperturbed in livers lacking SOCS3 and IFNγ-induced STAT1 activation was indistinguishable from wild-type livers (Croker, Krebs et al. 2003). There seems to exist a significant overlap between the signaling pathways triggered by

IL-6 and IFNγ via the restriction of STAT1 activation. Analysis of cytokine-responsive genes in IL-6 stimulated *Socs3*$^{-/-}$ cells revealed that a large group of these genes are usually attributed to IFNγ stimulation which are induced by prolonged activation of STAT1 (Croker, Krebs et al. 2003). A possible key role for SOCS3 is to sculpt the specific response observed in cells exposed to IL-6, perhaps particularly by restricting activation of STAT1.

Apart from STAT1, SOCS3 interacts with STAT3 to suppress the IL-6 signaling cascade induced by TLR signaling (Bode, Nimmesgern et al. 1999). SOCS3 is induced by both IL-6 and IL-10, but inhibits selectively IL-6 because SOCS3 is not able to bind the IL-10 receptor (Yasukawa, Ohishi et al. 2003). Nevertheless, it has been shown that forced constitutive expression of SOCS3 inhibits IL-10 signaling via STAT3 activation (Berlato, Cassatella et al. 2002).

Furthermore, SOCS3 suppresses inflammatory cytokine production by TLR ligands trough a yet unknown protein, which declares SOCS3 as a negative regulator of TLR signaling, and a negative regulator of the pro-inflammatory cytokine cascade (Yoshimura, Naka et al. 2007).

1.3.3.2.2 Regulation of T helper cell polarization

Like SOCS1, SOCS3 is expressed in naïve T cells. However, while SOCS1 is supposed to play an important role in differentiation to Th1 cells, SOCS3 protein is expressed in Th2 cells in 23-fold higher quantity than in Th1 cells (Egwuagu, Yu et al. 2002). This observation has led to an examination of the role of SOCS3 in allergies such as atopic asthma, which is characterized by extensive infiltration of the airways by T cells expressing Th2 cytokines. A positive correlation was evident between SOCS3 expression and asthma pathology as well as serum IgE levels in patients with allergy (Seki, Inoue et al. 2003).

Socs3$^{+/-}$ mice or transgenic mice expressing a dominant-negative form of SOCS3 exhibited decreased Th2 development. Conversely, transgenic mice constitutively

expressing the wild-type SOCS3 protein in splenic cells showed increased Th2 responses, and in a mouse airway hypersensitivity model of asthma exhibited enhanced pathological features (Seki, Inoue et al. 2003). These observations support a role for SOCS3 as a positive regulator of Th2 development and suggest that modulation of SOCS3 may represent a worthwhile therapeutic strategy in immunological diseases characterized by a Th1/Th2 imbalance.

Together, this data suggests a model in which SOCS3 contributes to maintaining quiescence in T lymphocytes and that antigen-stimulated down-regulation of SOCS3 expression allows T helper cell activation. SOCS3 appears to have an important supplementary role in controlling CD28-mediated responses in differentiated Th2 cells.

1.3.3.3 CIS

CIS (cytokine-inducible SH2 domain-containing protein) is induced by cytokines that activate STAT5, such as erythropoietin, growth hormone, prolactin, IL-2 and IL-3. Widespread expression of CIS in transgenic mice resulted in symptoms which closely resembled abnormalities evident in mice lacking STAT5a and/or STAT5b, like failure to activate STAT5 in response to IL-2 and resulting attenuation of proliferation (Alexander and Hilton 2004). Li et al. demonstrated that the expression of CIS is selectively induced in T cells after TCR stimulation. In transgenic mice, with selective expression of CIS in $CD4^+$ T cells, elevated CIS has been shown to strongly promote TCR-mediated proliferation and cytokine production in vitro, and superantigen-induced T cell activation in vivo (Li, Chen et al. 2000). Additionally, T cells over-expressing CIS exhibited a tendency for Th2-polarized differentiation in vitro (Matsumoto, Seki et al. 1999).

It has been suggested that CIS uses the proteasome to negatively regulate growth hormone (GH) signaling, likely by targeting the GH receptor/JAK2/CIS complex for degradation (Krebs and Hilton 2001). Nevertheless, T cell development appeared to occur normally in these transgenic mice and so the biological consequences on immune cells remain unclear.

1.3.3.4 SOCS and Innate Immunity

The phagocytes of the innate immune system are controlled by several cytokines that are controlled by SOCS proteins, including IL-12 and the IFNs. Therefore, SOCS proteins contribute substantially to the indirect regulation of the innate immune response.

The potent response of SOCS1 and SOCS3 to TLR ligands such as LPS and CpG-DNA has been extensively documented (Dalpke, Opper et al. 2001; Naka, Fujimoto et al. 2005). SOCS3 has been shown to be one of the most abundantly induced proteins in macrophages following stimulation with LPS. In addition to indirectly regulating TLR signaling via STAT3 activation (and being a key regulator for the divergent activity of IL-6 and IL-10 following TLR stimulation), SOCS3 might have some direct effects on TLR signaling as well.

The possibility that SOCS proteins are directly induced by microbial infection has been raised in various studies. LPS has been proposed to directly induce SOCS3 expression in macrophages (Stoiber, Kovarik et al. 1999). Expression of SOCS1 and SOCS3 induced by bacterial CpG-DNA in macrophages and dendritic cells does not require protein synthesis and is observed independent of JAK-STAT signaling (Dalpke, Opper et al. 2001). Similarly, exposure to *Leishmania donovani*, a parasite, appears to directly induce expression of SOCS3 in human macrophages (Bertholet, Dickensheets et al. 2003). Induction of SOCS1 and particularly SOCS3 by bacterial lipoproteins (e.g., peptidoglycan) via TLR2 has been demonstrated in mouse macrophages (Dennis, Jefferson et al. 2006).

Some components of this activity may be indirect and due to autocrine factors induced by microbial infection, e.g., IFNs or IL-10. Nevertheless, it has been demonstrated that *Socs1*$^{+/-}$ and *Socs1*$^{-/-}$ mice show dramatically increased sensitivity to the lethal effects of LPS (Nakagawa, Naka et al. 2002). Further investigations have revealed that phosphorylation of NFκB and MAP kinase is increased in LPS-stimulated *Socs1*$^{-/-}$ macrophages compared to wild-type cells, and enforced SOCS1 expression blocks LPS-mediated activation of NFκB. These observations were confirmed by Mansell et al., who demonstrated that SOCS1 regulates the phosphorylation of NFκB directly

(Mansell, Smith et al. 2006). This indicates that SOCS1 is induced by LPS and acts as a feedback mechanism to inhibit signals from TLR4 via a direct inhibitory loop.

SOCS3 protein levels are upregulated by IL-10, a potent anti-inflammatory cytokine. It has been speculated that SOCS3 might have some direct effects on TLR signaling (Stoiber, Kovarik et al. 1999). Qasimi et al. observed suppression of LPS-induced TNFα and CD40 expression by SOCS3 in macrophages at a physiological level (Qasimi, Ming-Lum et al. 2006). Additionally, a recent report indicates that SOCS3 might be involved in the signal cascade controlling TLR- and IL-1-induced responses (Frobose, Ronn et al. 2006).

Thus, in addition to the well established role of SOCS proteins as classic negative feedback inhibitors of signaling from the hematopoetin class cytokine receptors, SOCS are also a part of feedback regulation of distinct receptor classes including the TLR.

Cytokine	Producing Cell	Target Cell	Function
IL-1a IL-1b	monocytes macrophages B cells dendritic cells	Th cells	co-stimulation
		B cells	maturation and proliferation
		NK cells	activation
		various	inflammation, acute phase response, fever
IL-2	Th1 cells	activated T and B cells, NK cells	growth, proliferation, activation
IL-3	Th cells NK cells	stem cells	growth and differentiation
		mast cells	growth and histamine release
IL-4	Th2 cells	activated B cells	proliferation and differentiation IgG1 and IgE synthesis
		macrophages	MHC Class II
		T cells	proliferation
IL-5	Th2 cells	activated B cells	proliferation and differentiation IgA synthesis
IL-6	Th2 cells monocytes macrophages	activated B cells	differentiation into plasma cells
		plasma cells	antibody secretion
		stem cells	differentiation
		various	acute phase response, inflammation
IL-7	marrow stroma thymus stroma	stem cells	differentiation into progenitor B and T cells
IL-8	macrophages endothelial cells	neutrophils	chemotaxis
IL-9	Th2 cells	activated T cells	proliferation of T cells
IL-10	Th2 cells Tregs	macrophages	inhibition of cytokine production (anti-inflammatory properties)
		B cells	activation
		T cells	feedback inhibition
IL-12	macrophages B cells dendritic cells	Naïve T cells	Differentiation into Th1 cells
		activated Tc cells	differentiation into Tc cells (with IL-2)
		T cells	activation of IFNγ production
		NK cells	activation
IFN-α	leukocytes	various	Inhibition of viral replication, MHC I expression
IFN-β	fibroblasts	various	Inhibition of viral replication, MHC I expression
IFNγ	Th1 cells, Tc cells, NK cells	various	Inhibition of viral replication
		macrophages	MHC expression
		activated B cells	Ig class switch to IgG2a
		Th2 cells	Inhibition of proliferation
		macrophages	pathogen elimination
TNFα	macrophages, mast cells, NK cells	macrophages	CAM (cell adhesion molecule) and cytokine expression
		tumor cells	cell death
TNFβ	Th1 and Tc cells	phagocytes	phagocytosis, nitride oxide production
		tumor cells	cell death

Table 1-1: **Selected immune cytokines and their activities.**

Gene	Involved cytokines	Phenotype of knockout mice
STAT1	IFNα, -β, -γ	No response to IFNs
STAT2	INFα, -β	Defective immunity to viruses
STAT3	IL-6, IL-10	Embryonic lethal
STAT4	IL-12	Defect in Th1-development
STAT5a	IL-2, IL-7, IL-9, prolactin	Impaired prolactin signaling, defective T cell proliferation in response to IL-2
STAT5b	IL-2, IL-7, IL-9, growth hormone	Impaired GH signaling, defective T cell proliferation in response to IL-2
STAT6	IL-4, IL-13	Defect in Th2-development, dysregulation in Asthma and Atopic rhinitis

Table 1-2: **Signal Transducers and Activators of Transcription (STATs).**

	High transcription rate induced by:	Inhibits signaling by:	Consequences:
CIS	Cytokines that activate STAT5: erythropoietin, growth hormone, prolactin, **IL-2** and **IL-3**	IL-2 via STAT5	High CIS levels lead to attenuation of proliferation
	Microbial components like LPS, peptidoglycan and CpG-DNA		
	T cells over-expressing CIS exhibit a tendency for Th2 polarized differentiation in vitro.		
+ SOCS1	T cell cytokines: **IFNγ**, **IL-6, IL-10.** *Socs1*$^{-/-}$ cells show prolonged responses to IFNγ, but not IL-6.	IFNγ via STAT1 Inflammatory cytokines like IL-2, -4, (-6), -12, -15, -23 (similar to IL-12) and TNFα	Induction of high SOCS1 levels by IL-6 leads to prevention of Th1 differentiation via blockade of IFNγ signal transduction *Socs1*$^{-/-}$ mice show severe diseases resulting from IFNγ hypersensitivity *Socs1*$^{-/-}$*Ifnγ*$^{-/-}$ mice display a wide range of acute and chronic inflammatory disorders.
	During the T cell development in the thymus: via the common gamma (γc) receptor and its associated cytokines, namely **IL-2, IL-4, IL-7** and **IL-15**	During the T cell development in the thymus: γc cytokines IL-2, IL-4, IL-7 and IL-15.	SOCS1 keeps the cells in a cytokine-unreceptive state at various stages of T cell homeostasis until they receive the triggers to proliferate/differentiate.
	SOCS1 expression is fivefold higher in **Th1 cells** than in Th2 cells.	IL-4 via STAT6	Restriction of IL-4 signaling in a Th1 environment
		IFNγ via STAT1	Restriction of IFNγ signaling in a Th2 environment
	LPS binds the Toll receptor TLR4 and directly induces expression of SOCS1, additionally to indirect activation via LPS induced autocrine factors such as IFNs.	Signals from TLR4 in a direct inhibitory loop	SOCS1 expression negatively regulates LPS signaling.
	Peptidoglycan binds to TLR2 and directly induces SOCS1 independent of JAK-STAT signaling.	Cytokine and TLR signaling in macrophages	
	CpG-DNA binds to TLR9 and directly induces SOCS 1 independent of protein synthesis.	Regulation of JAK/STAT signaling after triggering of Toll-like receptor signal pathways.	
	High SOCS1 levels correspond with a.) differentiation of naïve CD4+ T cells into Th1 cells (some studies deny this), b.) prevention of Th1 differentiation via blockade of IFNγ signal transduction, induced by IL-6 and c.) restriction of IL-4 signaling in Th1 cells.		

	High transcription rate induced by:	Inhibits signaling by:	Consequences:
SOCS3	T cell cytokines: **IFNγ, IL-6, IL-10** Reciprocal to SOCS1, IFNγ signaling is (almost) unperturbed by over-/under-expression of SOCS3, whereas IL-6 signaling is inhibited.	IL-6 via STAT1 and STAT3.	High SOCS3 expression corresponds with inhibition of IL-6 signaling.
	Th2 cells contain 23-fold higher levels of SOCS3 protein than Th1 cells.	IL-12 via STAT4	Through blocking of STAT4, high levels of SOCS3 impair Th1 development in Th2 cells.
	LPS binds the Toll receptor TLR4 and directly induces expression of SOCS3, additionally to indirect activation via LPS induced autocrine factors such as IFNs.	IL-6 via STAT1 and STAT3 in macrophages	Mice in which the *Socs3* gene has been deleted in macrophages show resistance to challenge with LPS because of unchecked IL-6 levels which enhance inhibition of macrophage activation.
	Peptidoglycan binds to TLR2 and directly induces SOCS3 independent of JAK-STAT signaling.	Cytokine and TLR signaling in macrophages	
	CpG-DNA binds to TLR9 and directly induces SOCS 3 independent of protein synthesis.	Regulation of JAK/STAT signaling after triggering of Toll-like receptor signal pathways.	
		Signaling cascade of proliferative response to T cell mitogens CD28-mediated cytokine production, especially production of IL-2, and NFκB activation.	SOCS3 specifically binds to the phosphorylated form of CD28 and blocks signal transduction.
	SOCS3 is almost selectively expressed in Th2 cells and appears to be a positive regulator of Th2 development.		

Table 1-3: **Selected SOCS and their functions.**

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2 Aims of the thesis

The aim of the thesis is to determine the response of the immune system to food allergens and probiotic as well as gram-negative bacterial strains at birth and to delineate differences with regard to cytokines patterns and key regulators of cytokine signaling (suppressors of cytokine signaling (SOCS)) in vitro.

To achieve this, the following approaches will be undertaken:

- Proliferation of cord blood mononuclear cells (CBMCs) will be assayed in the presence of a panel of food allergens by thymidine incorporation assay.

- Cytokine mRNA and SOCS mRNA will be detected and semi-quantified utilizing the ribonuclease protection assay (RPA) after stimulation with the same panel of food allergens and a polyclonal T cell activator (phytohemagglutinin (PHA)).

- Cytokine production in response to 2 strains of probiotic gram-positive bacteria (Lactobacillus plantarum, Lactococcus lactis), a gram-negative strain (Escherichia coli BL 21(DE3)) and a superantigen (staphylococcal enterotoxin A (SEA)) will be assayed, and strain specific patterns at 3 different time points will be measured utilizing enzyme-linked immunosorbent assay (ELISA).

- SOCS mRNA expression will be detected and semi-quantified utilizing RPA after stimulation with the above mentioned bacterial strains, and strain specific patterns at 4 different time points will be analyzed.

3 Materials and methods

3.1 Materials

3.1.1 Plastic Ware and Filters

Product	Company
Cellstar® Test tubes, sterile, 50mL	Greiner Austria
Cryovial® sterile, 2mL	Bibby Sterilin Staffordshire, UK
Safe-lock micro test tubes, PCR clean, 1,5mL	Eppendorf, Hamburg, Germany
96-well round plates	Iwaki, Tokyo, Japan
48-well flat bottom plates	BD Falcon, San José, CA
Bottle top filter, 50mm filter unit	Nalge Nunc International, Rochester, NY
Cellulose acetate syringe filter, sterile, 0,20µm	Iwaki, Tokyo, Japan
Wallac filter mat	Perkin Elmer, Weiterstadt, Germany
Wallac sample bag	Perkin Elmer, Weiterstadt, Germany
Chromatography paper	Whatman International, Maidstone, England

3.1.2 Buffers and Cell culture media

Experiments were performed using sterile buffers and media listed in the next paragraph. Sterile filtration was carried out using bottle top filters for PBS, UCC and ammonium chloride buffer, and syringe filters for freezing medium.

Buffer/Medium	Abbreviation	Ingredients
Phosphate-buffered saline	PBS	8g NaCl 0,2g KCl 1,8g Na_2HPO_4 $2H_2O$ 0,24g KH_2PO_4 A.d. ad 1000mL pH=7,2-7,4
Serum-free Ultra Culture complete medium	UCC	UC-medium 2mM L-glutamine 170 mg/L gentamycinsulphate
Isotonic ammonium chloride buffer		155mM NH_4Cl 10mM $KHCO_3$ 0,1mM EDTA
ELISA Wash buffer		0.05% Tween 20 PBS
ELISA Saturation buffer		5% BSA PBS
ELISA Biotinylated Diluent buffer		1% BSA PBS
ELISA Antibody Diluent buffer		1% BSA PBS
ELISA HRP-Streptavidin Diluent buffer		1% BSA 0,1% Tween 20 PBS
10x TrisBase-EDTA buffer	10x TBE	53,9g TrisBase 3,72g EDTA (Titriplex III) Boric acid until pH=8,3 A.d. ad 500mL
Acrylamide gel stock solution		111,780mL Rotiphorese® Gel 40 50mL 10xTBE 240,405 g Urea A.d. ad 500mL

3.1.3 Chemicals and Kits

Product	Company
Ethanol abs.	Carl Roth, Karlsruhe, Germany
EDTA (Ethylenediamine tetraacetic acid)	Sigma Sciences, St. Louis, MO
CBMC isolation and cell culture	
Ficoll-Paque™ PLUS	GE Healthcare, Munich, Germany
NaCl	Fluka Chemica, Buchs, Switzerland

KCl	Merck, Darmstadt, Germany
Na_2HPO_4 $2H_2O$	Merck, Darmstadt, Germany
KH_2PO_4	Merck, Darmstadt, Germany
Ultra culture-medium	Bio Whittaker, Walkersville, MD
L-glutamine	Sigma Sciences, St. Louis, MO
Gentamycine sulphate	Sigma Sciences, St. Louis, MO
NH_4Cl	Merck, Darmstadt, Germany
$KHCO_3$	Merck, Darmstadt, Germany
RPMI 1640 medium	Sigma Sciences, St. Louis, MO
DMSO (Dimethylsulfoxide)	Merck, Darmstadt, Germany
FCS (Fetal calf serum)	Gibco, Life Technologies Inc., Rockville, MD
Tuerk solution	Fluka Chemica, Buchs, Switzerland
Trypane blue solution 0,4%	Sigma Sciences, St. Louis, MO
GenElute™ Mammalian Total RNA Miniprep Kit	Sigma Sciences, St. Louis, MO
RNaseZap® RNase Decontamination Solution	Ambion Inc., Austin, TX
Proliferation assay	
Amersham [5'-^{3}H] Thymidine, 12,8 Ci/mmol	GE Healthcare, Munich, Germany
Scintillation solution	
ELISA	
HybriDomus Human IL-4 ELISA Pair	HybriDomus, Eubio, Vienna, Austria
Human IL-5 ELISA	Bender Medsystems, Vienna, Austria
HybriDomus Human IL-10 ELISA Pair	HybriDomus, Eubio, Vienna, Austria
HybriDomus Human IL-12 ELISA Pair	HybriDomus, Eubio, Vienna, Austria
OptEIA™ Human IFNγ ELISA Set	BD Biosciences, San Diego, CA, USA
Tween 20	
BSA (Bovine serum albumin)	Sigma Sciences, St. Louis, MO
Ribonuclease protection assay	
α-^{32}P-UTP, 250µCi, 25µl	New England Nuclear (PerkinElmer Life), USA
RiboQuant™ RPA Starter Package	BD Biosciences, San José, CA, USA
hCK1 Multi-Probe Template Set	BD Biosciences, San José, CA, USA
hSOCS Multi-Probe Template Set	BD Biosciences, San José, CA, USA
Tris-saturated phenol	Carl Roth, Karlsruhe, Germany
Chloroform	Merck, Darmstadt, Germany
Isoamylalcohol	Carl Roth, Karlsruhe, Germany
Mineral oil	Sigma Sciences, St. Louis, MO
TrisBase (Trishydroxy-	Merck, Darmstadt, Germany

methylaminomethane)	
Boric acid	Sigma Sciences, St. Louis, MO
Rotiphorese® Gel 40 (19:1) 40 % acrylamide/bisacrylamide stock solution at a ratio of 19:1	Carl Roth, Karlsruhe, Germany
Urea	Sigma Sciences, St. Louis, MO
TEMED	Sigma Sciences, St. Louis, MO
Ammonium persulfate	Sigma Sciences, St. Louis, MO
PlusOne Repel-Silane	Amersham Biosciences, NJ, USA
Chromatography paper	Whatman International, Maidstone, England

3.1.4 Allergens

All allergens are endotoxin free.

Allergens		Abbreviation	Source/Company	Preparation
Peanut	Arachis hypogea allergen 1	Ara h1	Clare Mills (IFR Norwich)	Allergen obtained from whole Virginia red variety peanuts as described by Eiwegger et al. (Eiwegger, Rigby et al. 2006).
Peanut	Arachis hypogea allergen 2	Ara h2	Jean-Michelle Wal (INRA, Laboratoire d'Immuno-Allergie Alimentaire, Paris, France)	Allergen obtained from ground, roasted whole peanuts as described by Adel-Patient et al. (Adel-Patient, Bernard et al. 2005).
Milk	β-Lacto-globulin	BLG	Susanne Brix (BioCentrum-DTU, Technical University of Denmark, Lyngby, Denmark)	Protein obtained from raw milk as described by Brix et al (Brix, Bovetto et al. 2003).
Egg	Ovalbumin	OVA	Sigma Sciences, St. Louis, MO	Product No. A5503

3.1.5 Bacteria

Bacteria were inactivated with 1% phosphate-buffered formalin for 3 hours at room temperature, washed twice with sterile phosphate-buffered saline, and stored at -20°C. Successful inactivation was confirmed by the absence of bacterial growth after plating on selective agar.

Bacteria	Specification	Abbreviation	Source	Preparation
Lactobacillus plantarum	NCIMB8826	LP	Ursula Wiedermann (Department of Pathophysiology, Medical School, Vienna, Austria)	Originally isolated from human saliva.
Lactococcus lactis	MG1363	LL	Ursula Wiedermann (see above)	As described by Gasson et al. (Gasson 1983)
Escherichia coli	BL 21(DE3)	EC	Ursula Wiedermann (see above)	As described by Repa et al. (Repa, Grangette et al. 2003)

3.1.6 Other stimulants

Antigen	Specification	Abbreviation	Company	Product No.
Phytohemagglutinin	M Form	PHA	Gibco Invitrogen Corporation, NJ, USA	10576-015
Human Interleukin 2		IL-2	Roche Applied Science, IN, USA	1 147 528
Staphylococcal Enterotoxin A	Staphylococcus aureus	SEA	Sigma	S9399
Lipopolysaccharide	Serotype 055:B50	LPS	Sigma	L2880

3.1.7 Cord blood samples

Human umbilical cord blood from randomly chosen full-term healthy infants (> 37 weeks of gestation) was analyzed. Cord blood was obtained by venopuncture of the umbilical vein immediately after delivery and placed in sterile sodium heparin tubes. The protocol was approved by the local ethical committee of the University of Vienna.

3.2 Methods

3.2.1 Cord blood mononuclear cell preparation

At a maximum delay of five hours after birth the heparinized cord blood was diluted 1:1 with phosphate-buffered saline (PBS) and drawn over Ficoll-Paque (Pharmacia, Uppsala, Sweden). Cord blood mononuclear cells (CBMCs) were isolated by density-gradient centrifugation (30 min, 1400 rpm, no brake and room temperature, Beckman centrifuge). Afterwards the monocyte band was recovered and the cells were washed three times with PBS through centrifugation (15 min, 1400 rpm, with brake and room temperature, Beckman centrifuge). Isotonic ammonium chloride buffer was added to eliminate remaining erythrocytes.
The CBMCs were stained with Tuerk solution and quantified using a Buerker-Tuerk counting chamber. Total cell count of the sample was determined using the following equation:

500.000 x *counted cells* x *total mL of sample* x *2 (if stained)* = *total cell count*

3.2.2 Proliferation assay

CBMCs ($5x10^4$ /200µl) were cultured in Ultra Culture complete medium (UCC) in 96-well plates. In the presence of different allergen concentrations - Ara h1 (0,5µg/mL; 5µg/mL; 50µg/mL), Ara h2 (0,5µg/mL; 5µg/mL; 50µ/mL), BLG (1µg/mL; 10µg/mL,

100µg/mL), OVA (1µg/mL; 10µg/mL, 100µg/mL) - cells were incubated in triplicates at 37°C in a humidified atmosphere with 5% CO_2 for 7 days.
IL-2 (25 IU/mL) was used as positive control and medium alone as negative control. After 6 days of culture, tritiated thymidine (Amersham; 0,5µCi per well) was added, and after 16 hours of incubation incorporated radioactivity (counts per minute (cpm)) was measured by liquid scintillation.
Proliferation is expressed as stimulation index (SI) of the geometric mean of the triplicates. The SI is determined by using the following equation:

SI = cpm in stimulated cultures / cpm in unstimulated cultures

According to the literature, an SI>2 was considered positive (Szepfalusi, Nentwich et al. 1998).

3.2.3 Cytokine protein measurement

3.2.3.1 Cell culture conditions

$2x10^6$ CBMCs per mL UCC were cultured in 48-well plates, the total volume per well being 0,5mL. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

CBMCs were cultured with 10^5, 10^6 and 10^7 CFU (colony forming units) of formalin inactivated L. lactis, L. plantarum, or E. coli as well as SEA (2µg/mL) as positive control and medium alone as negative control. Cell free supernatants were obtained after 24, 48 and 72 hours and stored at -70°C until analysis.

3.2.3.2 Enzyme-linked immunosorbent assay (ELISA)

Quantitative detection of total IL-4, IL-5, IL-10, IL-12 and IFNγ in the cell free supernatant was performed using ELISA (Bender Medsystems, Vienna, Austria; HybriDomus, Eubio, Vienna, Austria; BD Biosciences – Pharmingen, San Diego, CA, USA) according to the manufacturers' protocols.

The limits of detection were as follows:

IL-4: 1,1pg/mL (HybriDomus)

IL-5: 1,45pg/mL (Bender Medsystems)

IL-10: 12,5pg/mL (HybriDomus)

IL-12: 6,25pg/mL (HybriDomus)

IFNγ: 12,5pg/mL (BD)

3.2.4 Cytokine and SOCS mRNA measurement

3.2.4.1 Cell culture conditions

10^6 CBMCs per mL UCC were cultured in 48-well plates, the total volume per well being 2mL. Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2.

Allergens

CBMCs were cultured in the presence of different allergen concentrations - Ara h1 (0,5µg/mL; 5µg/mL; 50µg/mL), Ara h2 (0,5µg/mL; 5µg/mL; 50µ/mL), BLG (1µg/mL; 10µg/mL, 100µg/mL), OVA (1µg/mL; 10µg/mL, 100µg/mL) - as well as 1% PHA as positive control and medium alone as negative control.

Total RNA was extracted after 1, 2, 4, 16 and 24 hours, according to the manufacturer's instructions (Sigma), and stored at -70°C until analysis.

Bacteria

CBMCs were cultured with 10^4 CFU (colony forming units) of formalin inactivated L. lactis, L. plantarum, or E. coli as well as SEA (0,5µg/mL) as positive control and medium alone as negative control.

Total RNA was extracted after 10, 30, 60, 90 minutes and 2, 4, 16 and 24 hours, according to the manufacturer's instructions (Sigma), and stored at -70°C until analysis.

3.2.4.2 Total RNA extraction and quantification by UV-Spectroscopy

Total cellular RNA was isolated from stimulated CBMCs and the negative control using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. All materials used for handling RNA were DNase and RNase free, and the workplace was treated with RNaseZap®.
Total RNA concentration was determined via UV spectroscopy (wavelengths 260nm and 280nm).
RNA concentration was determined by using the following equation:

$$40\mu g/mL \times ABS_{260nm} \times dilution\ factor = RNA\ concentration\ (\mu g/mL)$$

RNA concentrations were standardized to 2µg/probe and dried in a SpeedVac® concentrator.

3.2.4.3 Ribonuclease protection assay (RPA)

For detection and quantification of cytokine RNA and SOCS (suppressors of cytokine signaling) RNA the RiboQuant MultiProbe RPA System (BD Biosciences - Pharmingen, San Diego, CA, USA) was used.

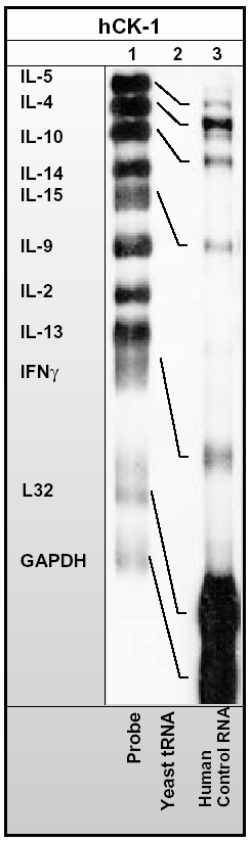
A [^{32}P]-labeled anti-sense RNA probe was transcribed *in vitro* from a DNA template using T7 RNA polymerase. Two different templates were used in the course of this study, hCK-1 (Human Cytokine multiprobe template set) and hSOCS (Human Suppressor of Cytokine Signaling multiprobe template set) (*Fig. 3-1*).
The hCK-1 multiprobe template set was used to synthesize RNA probes for the human cytokine genes IL-2, IL-4, IL-5, IL-9, IL-10, IL-13, IL-14, IL-15 and IFNγ as well as the housekeeping genes L32 (ribosomal protein L32) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase).
The hSOCS multiprobe set included templates for the signal transduction regulation genes SOCS1, SOCS2, SOCS3, SOCS5, SOCS6, SOCS7 and CIS as well as the housekeeping genes L32 and GAPDH.

Human Control RNA and yeast tRNA (2 µg) were included as positive and negative controls, respectively. [^{32}P]-labeled probe, diluted to 1000 - 2000 cpm, was included as a marker.

3×10^5 cpm of labeled probe was hybridized with 2,5µg of total RNA for 16 h at 56°C. After hybridization, free riboprobes and single-stranded RNA were digested with an RNase A plus T1 mix at 30°C for 45 min. Proteinase K treatment was used to inactivate the ribonucleases. The probe (protected fragment) and target RNA were resolved by denaturing polyacrylamide gel electrophoresis (8m urea, 4.75% acrylamide (19:1 acrylamide/bisacrylamide)), including positive and negative controls, as well as [^{32}P]-labeled probe, diluted to 1000 – 2000 cpm (unprotected probe).

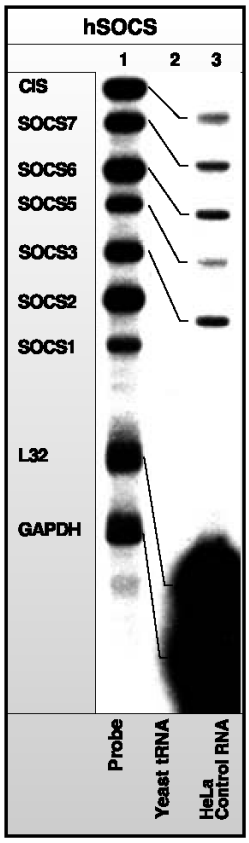
The gel was absorbed to filter paper, dried, and exposed to a Molecular Dynamics detection screen overnight (Molecular Dynamics, Sunnyvale, CA, USA).

Laser densitometry was performed using a Storm 840 PhosphorImager (Molecular Dynamics).



hCK-1
Cat. No. 556151

Template	Probe	Protected
IL-5	388	360
IL-4	351	325
IL-10	316	287
IL-14	283	256
IL-15	256	227
IL-9	231	203
IL-2	218	191
IL-13	190	161
IFN-γ	173	145
L32	141	113
GAPDH	124	96



hSOCS
Cat. No. 559927

Template	Probe	Protected
CIS	315	286
SOCS7	282	253
SOCS6	252	226
SOCS5	231	202
SOCS3	210	181
SOCS2	191	164
SOCS1	174	145
L32	141	112
GAPDH	124	96

Fig. 3-1: **Sample data for Human Cytokine and Human Cell Signaling Multi-Probe Template Sets.**

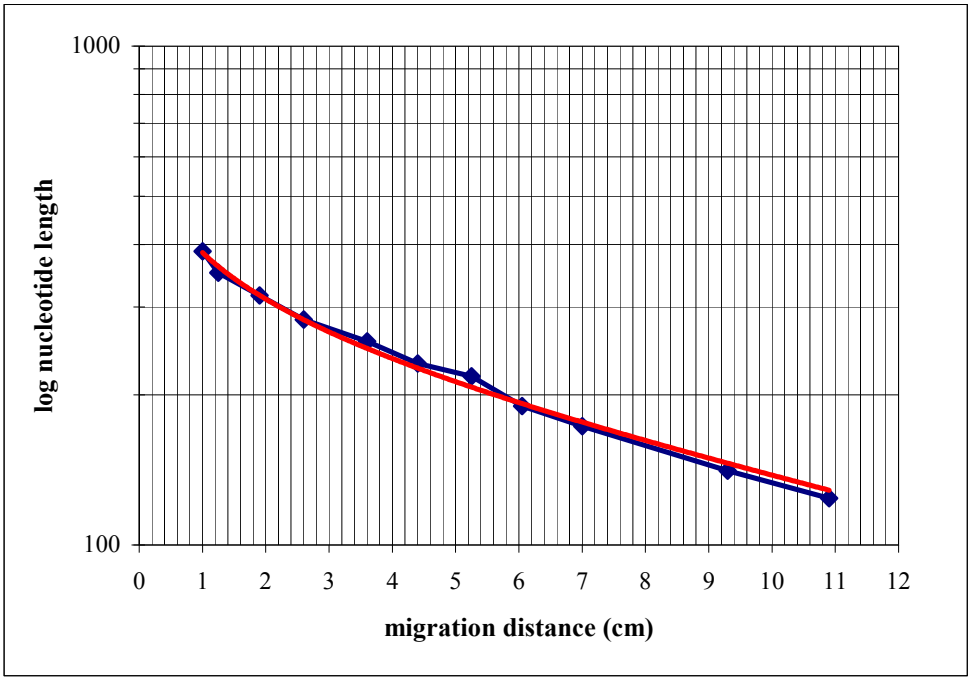


Fig. 3-2: **Standard curve for hCK1.**

To establish the identity of each protected fragment, the known sizes and migration distance of the unprotected probe was used to prepare a standard curve. On semi-log paper, nucleotide length was plotted against migration distance. The point where the migration of the protected fragments intersects the curve was determined to extrapolate the corresponding nucleotide length (*Fig. 3-1* and *Fig. 3-2*).

The software ImageQuant (Molecular Dynamics) was used for semi-quantitative analysis of mRNA expression. Values were normalized for loading differences as a percentage of internal housekeeping gene (L32) expression.

3.2.5 Statistical analysis

Wilcoxon signed-rank test was used for the evaluation of significant changes between repeated measurements on the same sample. Correlations were tested utilizing Pearson's correlation coefficient.

3.3 References

Adel-Patient, K., H. Bernard, et al. (2005). "Peanut- and cow's milk-specific IgE, Th2 cells and local anaphylactic reaction are induced in Balb/c mice orally sensitized with cholera toxin." Allergy **60**(5): 658-64.

Brix, S., L. Bovetto, et al. (2003). "Immunostimulatory potential of beta-lactoglobulin preparations: effects caused by endotoxin contamination." J Allergy Clin Immunol **112**(6): 1216-22.

Eiwegger, T., N. Rigby, et al. (2006). "Gastro-duodenal digestion products of the major peanut allergen Ara h 1 retain an allergenic potential." Clin Exp Allergy **36**(10): 1281-8.

Gasson, M. J. (1983). "Genetic transfer systems in lactic acid bacteria." Antonie Van Leeuwenhoek **49**(3): 275-82.

Szepfalusi, Z., I. Nentwich, et al. (1998). "Cord blood mononuclear cells and milk-specific T cell clones are tools to evaluate the residual immunogenicity of hydrolyzed milk formulas." J Allergy Clin Immunol **101**(4 Pt 1): 514-20.

4 Results

4.1 Allergen-induced immune response in cord blood

4.1.1 Proliferation assay

Allergen specific proliferation was assessed by ^{3}H thymidine incorporation assay. Cord blood mononuclear cells (CBMCs) were co-cultured for 6 days with the major peanut allergens Ara h1 and Ara h2, the major cows' milk allergen β-lactoglobulin (BLG) and hens' egg allergen ovalbumin (OVA). Dose dependency for each allergen was assessed by using 3 concentrations of allergen, ranging from 0,5µg to 50µg/mL (Ara h1 and Ara h2) and 1µg to 100µg/mL (BLG and OVA). According to the literature, a stimulation index (SI) >2 was regarded positive (Szepfalusi, Pichler et al. 2000).

CBMCs from 7 out of 19 individuals showed positive proliferative response to at least one allergen (36,8%). Ara h1, BLG and OVA induced similar percentages of positive proliferation (31,6%-36,8%), while Ara h2 elicited a lower proliferation frequency of 13,3% (*Table 4-1*).
Individual CBMCs with an SI>2 to one food allergen were observed to show positive proliferation to other food allergens as well. In 5 out of these 7 individuals the response was also positive to the other food allergens tested.
Initial stimulations with crude peanut extract (showing a proliferation frequency of 80% (n=5)) were withdrawn due to the impossibility of endotoxin removal to a level that does not affect proliferation itself (Eiwegger, Mayer et al. in press) (*Table 4-1*).

No clear dose dependency with higher allergen concentrations inducing increased proliferation was observed for any of the allergens tested (*Fig. 4-1*). Interestingly, some CBMCs showed positive proliferation to one or two concentrations of a certain allergen only, some to the lowest or middle concentration as well. Consequently, the mean stimulation index did not correlate with the dose of allergen used. Based on the applied

amounts of allergen in the literature we decided to use the highest allergen concentration for further RNAse protection assays on cytokine and SOCS expression.

n	Ara h1	Ara h2	Crude	BLG	OVA
1	+	+		+	+
2	+	+		+	+
3	+		+	+	+
4	+		+	+	+
5	+		+	+	+
6	+		+	+	-
7	-	-		+	+
8	-	-		-	-
9	-	-		-	-
10	-	-		-	-
11	-	-		-	-
12	-	-		-	-
13	-	-		-	-
14	-	-		-	-
15	-	-		-	-
16	-	-		-	-
17	-	-		-	-
18	-	-		-	-
19	-		-	-	-
	31,6 %	**13,3 %**	**80 %**	**36,8 %**	**31,6 %**

Table 4-1: **Proliferation assays of CBMCs of 19 individuals co-cultured with 3 concentrations of food allergens.**
CBMCs were cultured in the presence of 3 different concentrations of 4 different allergens (Ara h1, Ara h2, BLG and OVA) for 6 days. Medium alone served as negative control, IL-2 [100 IU/mL] as positive control. Allergen specific response was assessed via ^{3}H proliferation assay. Proliferation to an allergen is shown as positive (+) if at least one concentration of the allergen induced an SI>2. An SI<2 for all concentrations of an allergen was considered negative (-).

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; Ara h1: Arachis hypogea (peanut) allergen 1; Ara h2: Arachis hypogea (peanut) allergen 2; BLG: β-lactoglobulin; OVA: Ovalbumin; IL-2: Interleukin 2; SI: stimulation index

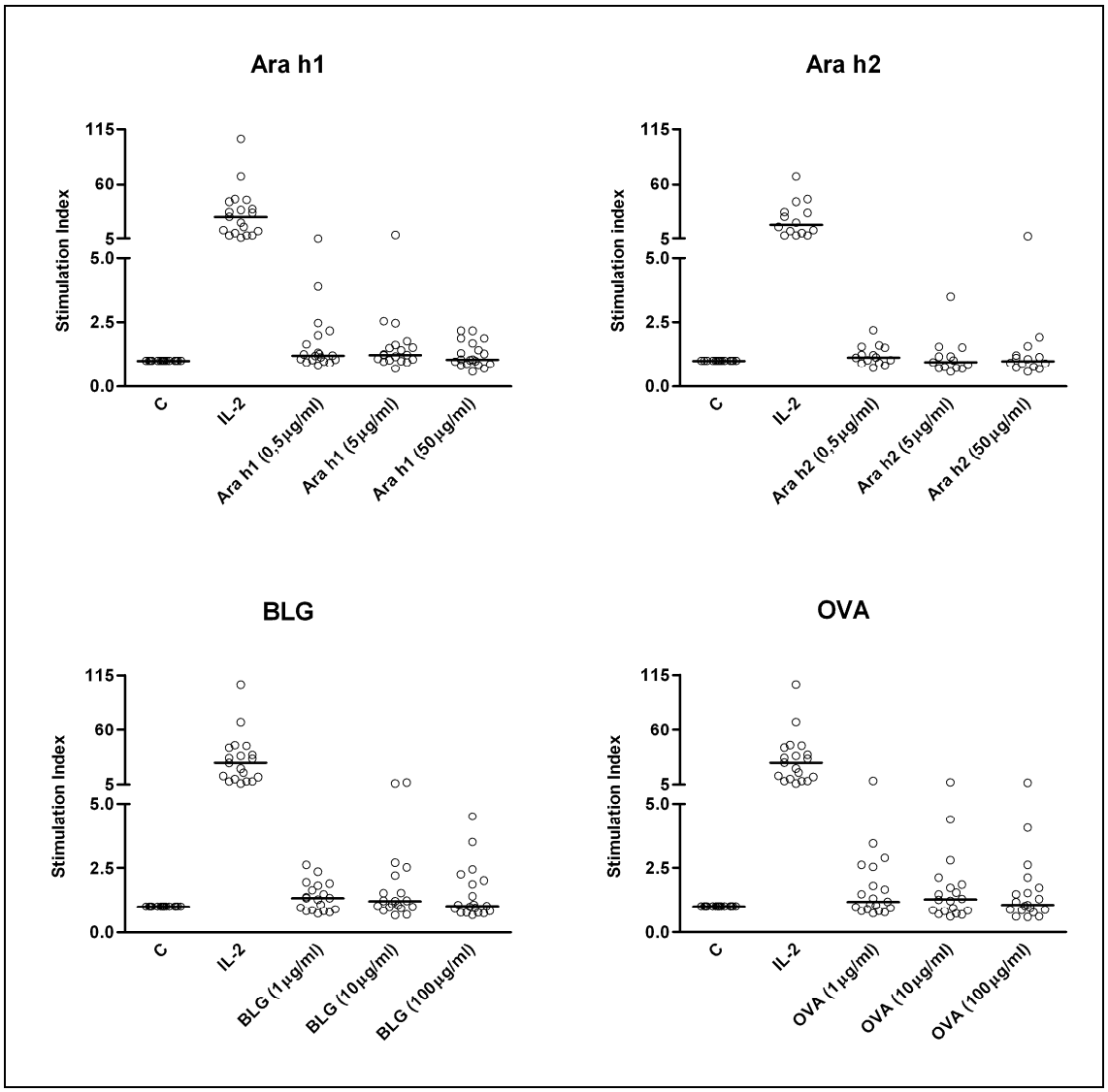


Fig. 4-1: **Dose dependency of allergen specific proliferative responses of cord blood.**
CBMCs were cultured in the presence of 3 different concentrations of 4 different allergens (Ara h1, Ara h2, BLG and OVA) for 6 days. Medium alone served as negative control, IL-2 [100 IU/mL] as positive control. Allergen specific response was assessed via ^{3}H proliferation assay. A SI of >2 was considered positive.

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; Ara h1: Arachis hypogea (peanut) allergen 1; Ara h2: Arachis hypogea (peanut) allergen 2; BLG: β-lactoglobulin; OVA: Ovalbumin; IL-2: Interleukin 2; SI: stimulation index

4.1.2 Allergen-induced Cytokine mRNA expression

To analyze Th1-type, Th2-type and regulatory cytokine expression, total RNA of CBMCs was prepared after stimulation with the highest concentration of the different allergens and the mitogen PHA for 24 hours. Cytokine mRNA expression was semi-quantified relative to the housekeeping gene L32, utilizing the ribonuclease protection assay (RPA) for the cytokines IL-2, IL-4, IL-5, IL-9, IL-10, IL-13, IL-14, IL-15 and IFNγ.

First, evaluability of cytokine expression via RPA using different quantities of RNA (1; 2,5; 5 and 10µg) was investigated. Cytokine production of CBMCs and PBMCs (peripheral blood mononuclear cells) was induced by LPS (*Fig 4-2*). Cytokine as well as housekeeping gene (L32) expression was found to be evaluable in PBMCs at lower quantities of RNA, compared to CBMCs. While cytokine expression of CBMCs was analyzable with 2,5µg of RNA used, 10µg of RNA yielded the best results (*Fig. 4-2*). Nevertheless, with a limited amount of CBMCs and therefore RNA available, we standardized the quantity of RNA analyzed via RPA to 2µg per probe.
Analyzing CBMCs co-cultured with the food allergens Ara h1, Ara h2, BLG and OVA, cytokine mRNA levels were found to be under the detection limit (*Fig. 4-3*). Likewise, the polyclonal T cell activator PHA did not elicit evaluable levels of cytokine mRNA, excepting IL-13 (*Fig. 4-3*).

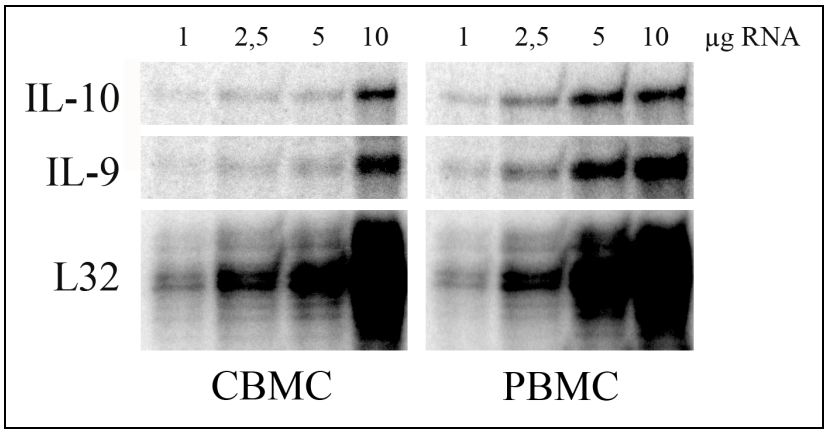


Fig. 4-2: **RNase protection assay of IL-9 and IL-10 expression in CBMCs and PBMCs.**
CBMCs and PBMCs were co-cultured with LPS [5µg/mL] for 6 hours. Total RNA of the CBMCs and PBMCs was prepared according to the protocol and quantified using UV spectroscopy. IL-10 and IL-9 gene expression was measured by ribonuclease protection assay (RPA) utilizing 4 different quantities of RNA (1; 2,5; 5 and 10µg). Cytokine expressions are shown above expression of the housekeeping gene L32.

Abbreviations: CBMC: cord blood mononuclear cell; PBMC: peripheral blood mononuclear cell; LPS: Lipopolysaccharide; IL-9: Interleukin 9; IL-10: Interleukin 10; L32: ribosomal protein L32

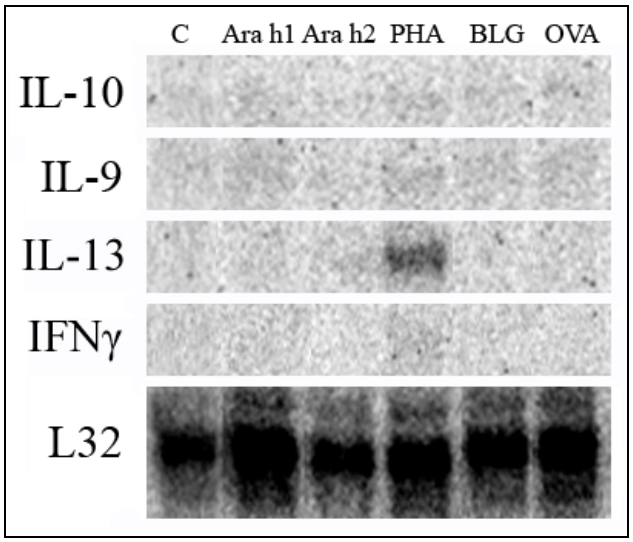


Fig. 4-3: **RNase protection assay of IL-9 and IL-10 expression in CBMCs co-cultured with food allergens.**
CBMCs were co-cultured with Ara h1, Ara h2 [50µg/mL], BLG and OVA [100µg/mL] and the mitogen PHA (1%). Total RNA of the CBMCs (2 µg/probe) was prepared after 24 hours according to the protocol and quantified using UV spectroscopy. IL-10 and IL-9 gene expression was measured by ribonuclease protection assay (RPA). Cytokine expressions are shown above expression of the housekeeping gene L32.

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; Ara h1: Arachis hypogea (peanut) allergen 1; Ara h2: Arachis hypogea (peanut) allergen 2; BLG: β-lactoglobulin; OVA: Ovalbumin; PHA: Phytohemagglutinin; IL-9: Interleukin 9; IL-10: Interleukin 10; L32: ribosomal protein L32

4.1.3 Allergen-induced SOCS mRNA expression

SOCS expression patterns have been linked to cytokine production and Th1/Th2-type response. To investigate the direct impact of allergen exposure on SOCS expression in a naïve in vitro system, expression of suppressors of cytokine signaling (SOCS) mRNA was analyzed. Total RNA of CBMCs after stimulation with the 4 allergens was prepared after 24 hours, and expression of CIS, SOCS1 and SOCS3 mRNA was semi-quantified relative to the housekeeping gene L32 utilizing RPA.
In order to highlight differences between the 4 allergens, significances were calculated comparing the SOCS expression of Ara h1, Ara h2, BLG, OVA as well as the polyclonal T cell activator PHA.

Mean SOCS mRNA expression was not significantly increased by any allergen applied, while PHA induced elevated CIS and SOCS1 as well as reduced SOCS3 expression, compared to the negative control (*data not shown*).
Allergen-specific differences were visualized via relative SOCS expression (ratios to the negative control; *Fig. 4-4*). Ara h1 elicited significantly higher levels of CIS mRNA compared to Ara h2, BLG and OVA (*Fig. 4-4a*). Expression of SOCS1 mRNA was significantly increased in CBMCs co-cultured with OVA, compared to BLG (*Fig. 4-4b*). No differences in SOCS3 expression were observed between the 4 allergens (*Fig. 4-4c*). All allergens tested induced a significantly lower CIS and SOCS1 expression than PHA (*Fig. 4-4a and b*). In contrast, PHA led to a significantly lower SOCS3 expression than Ara h1, Ara h2, BLG or OVA (*Fig. 4-4c*).

According to the literature that linked high CIS/SOCS1 expression to Th1-type and high SOCS 3 expression to Th2-type responses, ratios of SOCS1,CIS and SOCS3 expression were calculated. We utilized these ratios to highlight potential differences between the investigated food allergens not reflected by absolute SOCS expression defined by RPA. Data are presented in the log10 scale.

The influence of PHA on CIS expression was highlighted by the significantly decreased SOCS1/CIS ratio (*Fig. 4-5a*), compared to Ara h2, BLG, OVA and the negative control.

Additionally, the SOCS1/SOCS3 ratio compared to all allergens tested (*Fig. 4-5b*) mirrored the impact of PHA on SOCS3 expression. Ara h1 induced a significantly lower SOCS1/CIS ratio than all allergens and the negative control (*Fig. 4-5a*), while no difference was observed concerning the SOCS1/SOCS3 ratio (*Fig. 4-5b*).

Consistent with the Th1/Th2-type association of SOCS expression, the expression of SOCS1 and SOCS3 have been observed to be negatively correlated. On the other hand, SOCS1 and SOCS3 are induced by common cytokines, most importantly IL-6 and IFNγ. To assess if the expression of SOCS1 and SOCS3 is cytokine driven in our setup, SOCS1 mRNA and SOCS3 mRNA expression for each allergen and each cord blood was correlated using Pearson's correlation coefficient.

We found a positive correlation (Pearson r=0,4683; p=0,0018) between SOCS1 and SOCS3 expression (*Fig. 4-6*). No correlation between SOCS3 and CIS expression was observed, while a weak negative correlation between SOCS1 and CIS expression was found (Pearson r=-0,3212; p=0,0295; *data not shown*). Generally, a Th1/Th2-type influence was not apparent in relative SOCS expression or in the SOCS ratios.

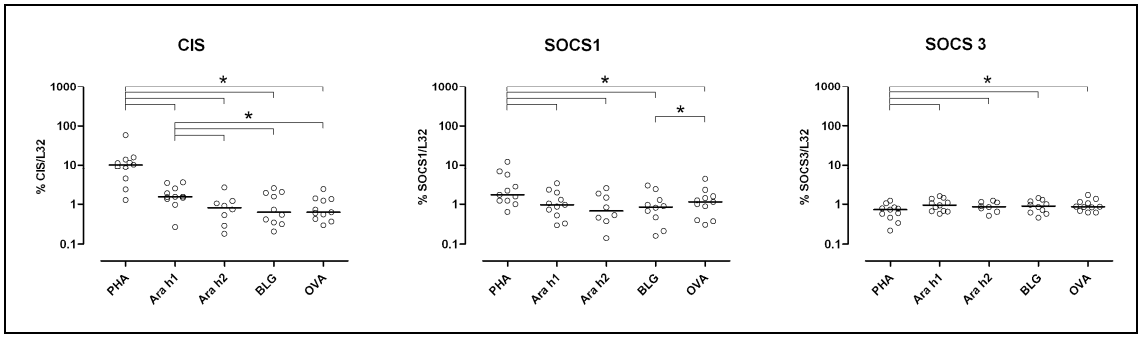


Fig. 4-4: **Allergen specific differences in the SOCS expression of CBMCs, diagrammed as ratios to the negative control.**
CBMCs were co-cultured with Ara h1, Ara h2 [50µg/mL], BLG and OVA [100µg/mL] and the mitogen PHA (1%). Medium alone served as negative control. Total RNA of the CBMCs was prepared after 24 hours of incubation according to the protocol. The CIS (*a*), SOCS1 (*b*) and SOCS3 (*c*) gene expression was measured by ribonuclease protection assay (RPA). Data are presented relative to the housekeeping gene L32 and as ratios to the negative control as the median of the separate patients in the log 10 scale.Significance was determined by Wilcoxon signed-rank test (*p<0.05).

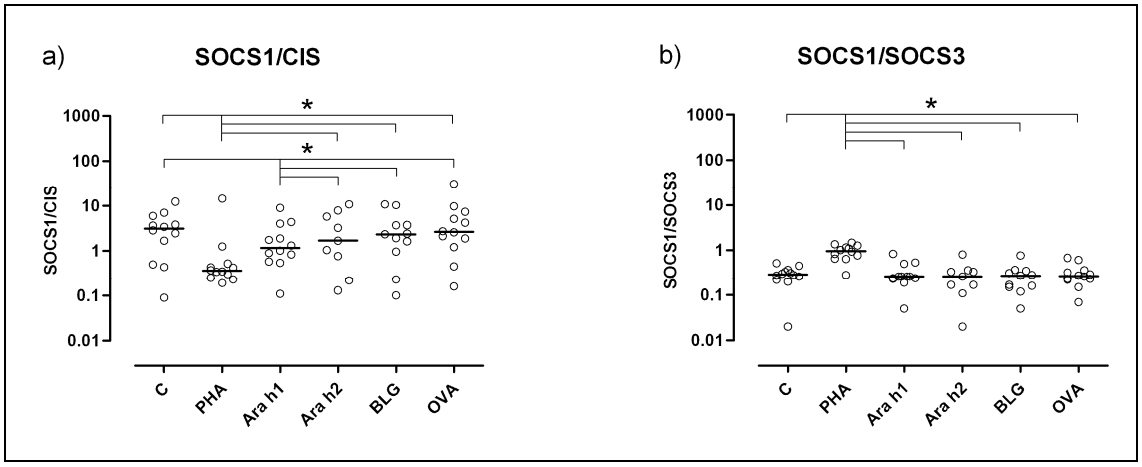


Fig. 4-5: **Ratio of SOCS1/CIS and SOCS1/SOCS3 expression of CBMCs induced by food allergens.**
CBMCs were co-cultured with Ara h1, Ara h2 [50µg/mL], BLG and OVA [100µg/mL] and the mitogen PHA (1%). Medium alone served as negative control. Total RNA of the CBMCs was prepared after 24 hours of incubation according to the protocol. The CIS, SOCS1 and SOCS3 gene expression was measured by ribonuclease protection assay (RPA) and presented relative to the housekeeping gene L32. Data are displayed as the SOCS1/CIS (*a*) and the SOCS1/SOCS3 (*b*) ratio as the median of the separate patients in the log 10 scale. Significance was determined by Wilcoxon signed-rank test (*p<0.05).

Abbreviations (*Fig. 4-4 and 4-5*): CBMC: cord blood mononuclear cell; C: control with medium alone; Ara h1: Arachis hypogea (peanut) allergen 1; Ara h2: Arachis hypogea (peanut) allergen 2; BLG: β-lactoglobulin; OVA: Ovalbumin; PHA: Phytohemagglutinin; L32: ribosomal protein L32; CIS: cytokine-inducible SH2 domain-containing protein; SOCS1 and 3: suppressors of cytokine signaling 1 and 3

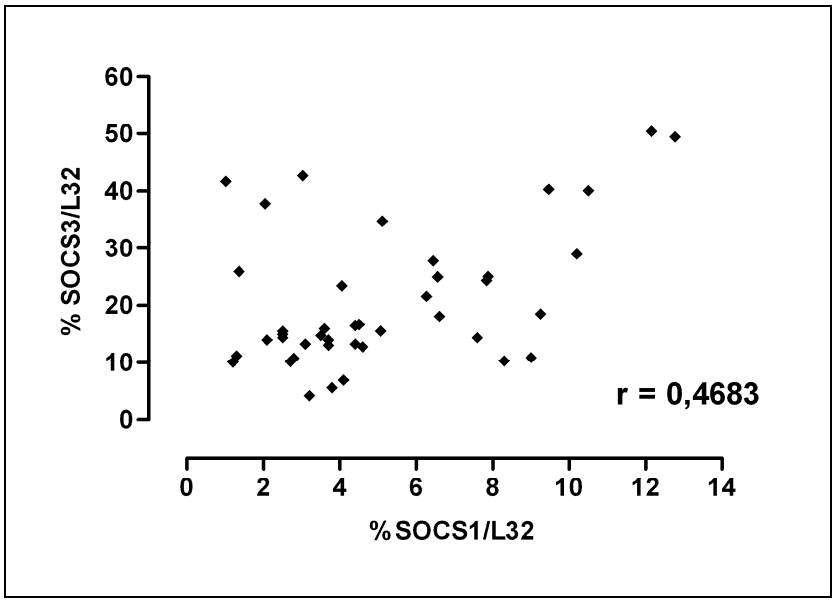


Fig. 4-6: **Correlation of SOCS1 and SOCS3 expression in CBMCs co-cultured with food allergens.**
CBMCs were co-cultured with Ara h1, Ara h2 [50µg/mL], BLG and OVA [100µg/mL]. Total RNA of the CBMCs was prepared after 24 hours of incubation according to the protocol. The SOCS1 and SOCS3 gene expression was measured by ribonuclease protection assay (RPA) and presented relative to the housekeeping gene L32. Data are presented as a correlation scatter plot. Significance was determined by Pearson's correlation coefficient ($p<0,01$).

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; Ara h1: Arachis hypogea (peanut) allergen 1; Ara h2: Arachis hypogea (peanut) allergen 2; BLG: β-lactoglobulin; OVA: Ovalbumin; PHA: Phytohemagglutinin; L32: ribosomal protein L32; SOCS1 and 3: suppressors of cytokine signaling 1 and 3

4.1.4 Relation between proliferation and SOCS mRNA expression

The relation between allergen specific proliferative responses and SOCS expression of CBMCs was evaluated. We compared intra-individual differences in individuals with positive proliferation to at least one allergen (allergen that induced positive proliferation (SI>2) compared to allergen that induced no positive proliferation (SI<2)) and inter-individual differences (individuals showing proliferation to at least one allergen compared to individuals with a negative response to any of the allergens).

Allergen-induced SOCS3 expression was higher than allergen-induced expression of the other SOCS in all individuals (*Fig. 4-7*). Regarding CIS and SOCS1 expression, we observed inter-individual differences independent from the proliferative status of the individual CBMCs (e.g., *Fig. 4-7f and Fig. 4-7g*).
CBMCs stimulated with PHA showed a clearly aberrant SOCS profile from CBMCs co-cultured with allergens and the negative control. With the exception of one individual (*Fig. 4-7g*), CIS expression was distinctly higher than SOCS1 and SOCS3 expression (*Fig. 4-7a-f and h*).

Concerning intra-individual differences, we found no distinction between the influence of proliferation positive and negative allergens on CBMCs (*Fig. 4-7*).

In summary, inter-individual differences in SOCS expression regardless of proliferative status were found to be much more distinct than any relation of proliferation to SOCS expression.

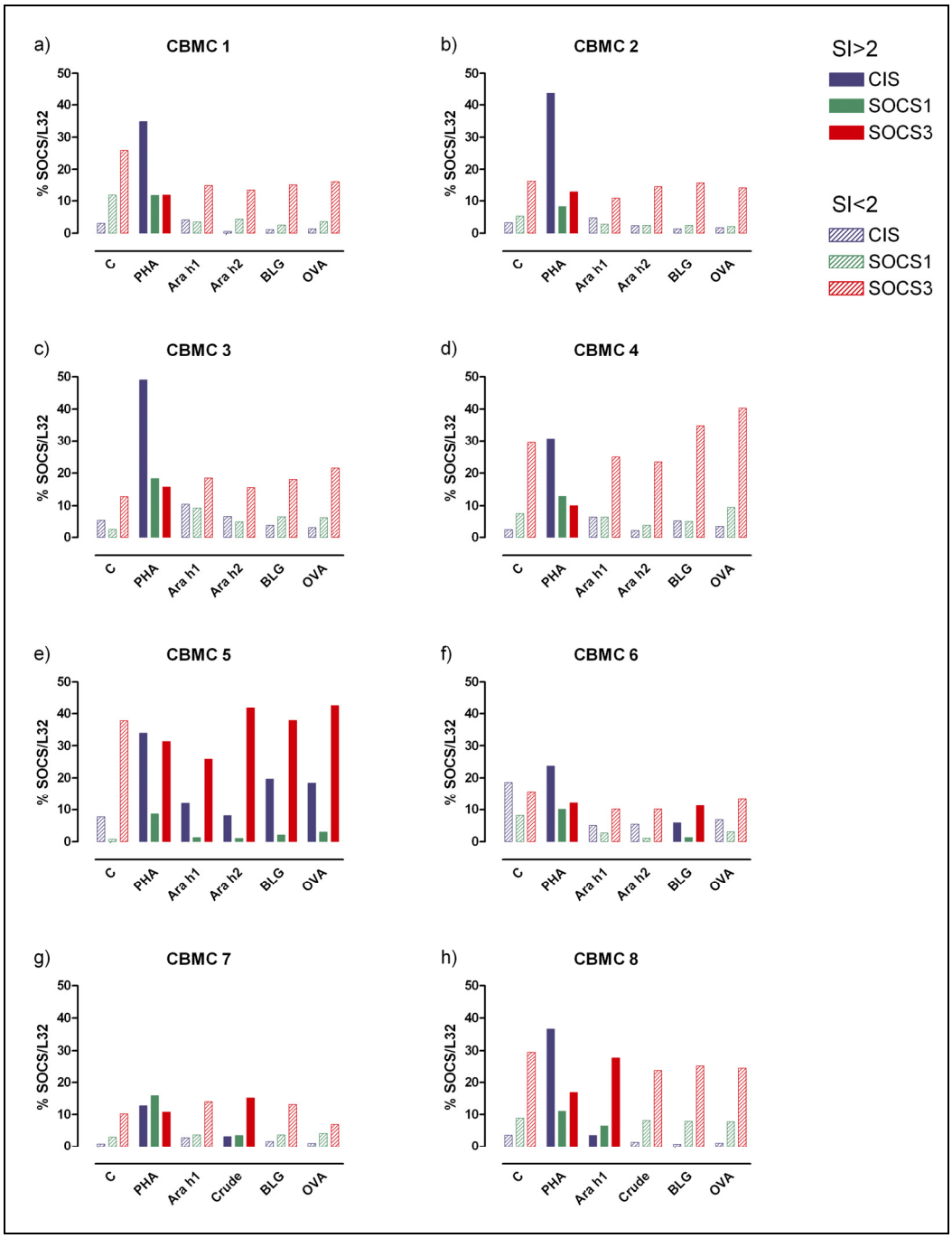


Fig. 4-7: **Relation between proliferation and SOCS expression in response to food allergens.**
PHA- and allergen-induced CIS (blue), SOCS1 (green) and SOCS3 (red) expression of the CBMCs of 8 individuals are displayed in connection to the proliferative response to the same allergens (SI>2: filled, SI<2: hatched). CBMCs were co-cultured with Ara h1, Ara h2 (*a-f*), crude peanut extract (*g-h*) [50µg/mL], BLG and OVA [100µg/mL] and the mitogen PHA (1%). Medium alone served as negative control. Total RNA of the CBMCs was prepared after 24 hours of incubation according to the protocol. The CIS, SOCS1 and SOCS3 gene expression was measured by ribonuclease protection assay (RPA). Data are presented relative to the housekeeping gene L32. Each graph represents the SOCS expression pattern of one individual.

Abbreviations: see *Fig. 4-6*; SI: stimulation index

4.2 Bacteria-induced immune response in cord blood

4.2.1 Cytokine production

4.2.1.1 Kinetics and dose dependency

Kinetics were assessed for each cytokine (day 1, day 2 and day 3). To analyze Th1-type (IL-12, INFγ), Th2-type (IL-5) and regulatory cytokine production (IL-10), the respective protein was measured in the cell free supernatants of CBMCs after stimulation with the three bacterial strains (Lactococcus lactis (LL), Lactobacillus plantarum (LP), Escherichia coli (EC)) and the superantigen staphylococcal enterotoxin A (SEA), which was described to induce a more Th2-type response (Pochard, Gosset et al. 2002). IL-4 levels were below the detection limit, and therefore not included. Dose dependency for each bacterial strain was assessed by using 3 concentrations of bacteria, ranging from 10^5 CFU/mL to 10^7 CFU/mL, corresponding to a bacteria/cell ratio from 1/10 to 10/1.

IL-10 and IL-12 expression reached their highest level at day 1 irrespective of the bacterial strain applied and remained at this plateau at day 2 and day 3 (*Fig. 4-9 and 4-11*). In contrast, both IL-5 and IFNγ expression were detected at low levels at day 1, while the peak was reached at day 2 in CBMCs stimulated with EC and SEA, and at day 3 with lactic acid bacteria (*Fig 4-8 and 4-10*).
Kinetics of SEA induced cytokine production were comparable to those of EC, excepting IL-12 expression, which was found to be barely above the detection limit at any time point measured.

A trend to dose dependency with higher bacterial concentrations inducing increased cytokine expression was observed. Higher quantities of LL, LP and EC induced elevated production of all cytokines measured (*Fig. 4-8 to 4-10)*. LP was the most potent cytokine inducer within the group of bacteria supplied, eliciting the strongest IL-5 (*Fig. 4-8d-f*), IL-12 (*Fig. 4-10d-f*) and IFNγ (*Fig. 4-11d-f*) responses at 10^5 and 10^6 CFU/mL. LP, applied at a concentration of 10^7 CFU/mL, led to a decreased

cumulative cytokine amount in the supernatant as compared to 10^6 CFU/mL (*Fig. 4-8 to 4-10d-f*). Interestingly, IFNγ expression did not experience a decline at this concentration (*Fig. 4-11d-f*).

Concerning IL-5 production, LL and LP induced elevated cytokine levels starting at day 2 (*Fig. 4-8b and e*), showing dose-dependent IL-5 production at day 3 only (*Fig. 4-8c and f*). Solely EC elicited an IL-5 response at day one (*Fig. 4-8g*), while significant dose dependency was observed at day 3 (*Fig. 4-8i*).

Higher concentrations of LL and LP caused enhanced IL-10 levels from day 1 to day 3 (*Fig. 4-9*), while EC elicited significantly stronger IL-10 responses in low dosage compared to the lactic acid bacteria at day 1 (*Fig. 4-9g*), showing dose dependency at day 2 and 3 only (*Fig. 4-9h and i*).

IL-12 production increased with ascending doses of LL and LP from day 1 to day 3 (*Fig. 4-10*). IL-12 levels induced by EC were low at all days, compared to the other bacterial strains (*Fig. 4-10g-i*).

We observed an augmentation of LL- and LP-provoked IFNγ production as well as dose dependency of the IFNγ level from day 1 to day 3 (*Fig. 4-11*). The lowest concentration of EC didn't lead to an IFNγ level higher than the negative control at all days, while 10^6 CFU/mL and 10^7 CFU/mL of EC caused increased cytokine levels from day1 to day 3 (*Fig. 4-11g-i*), ascending with the dose of bacteria used at day 2 and 3 (*Fig. 4-11h and i*).

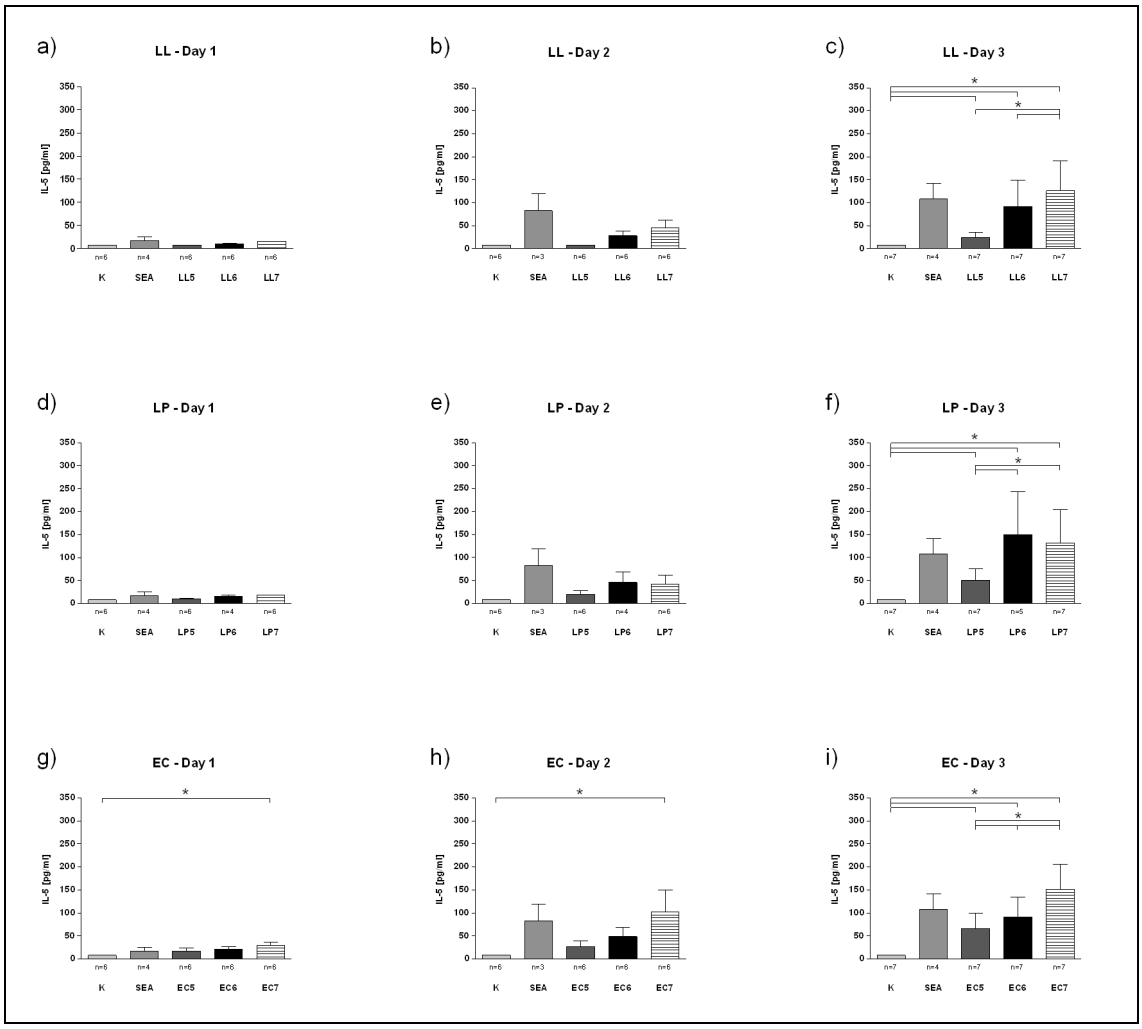


Fig. 4-8: **IL-5 production: Kinetics and dose dependency over the course of 3 days.**
CBMCs were co-cultured with LL, LP, EC at 3 different concentrations (10^5, 10^6 and 10^7 CFU/mL) and the superantigen SEA [2µg/mL]. Medium alone served as negative control. After 24 hours (day 1), 48 hours (day 2) and 72 hours (day 3), total IL-5 in the cell free supernatant was quantified using sandwich ELISA. Significance was determined by Wilcoxon signed-rank test (*$p<0.05$). Subgroups with $n<5$ were excluded from statistical evaluation. For reasons of simplicity, significances between SEA and the bacteria are not presented in these graphs.

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; LL: Lactococcus lactis; LP: Lactobacillus plantarum; EC: Escherichia coli; SEA: Staphylococcus enterotoxin A; IL-5: Interleukin 5; CFU/mL: colony forming units per mL
The limit for detection was 8pg/mL.

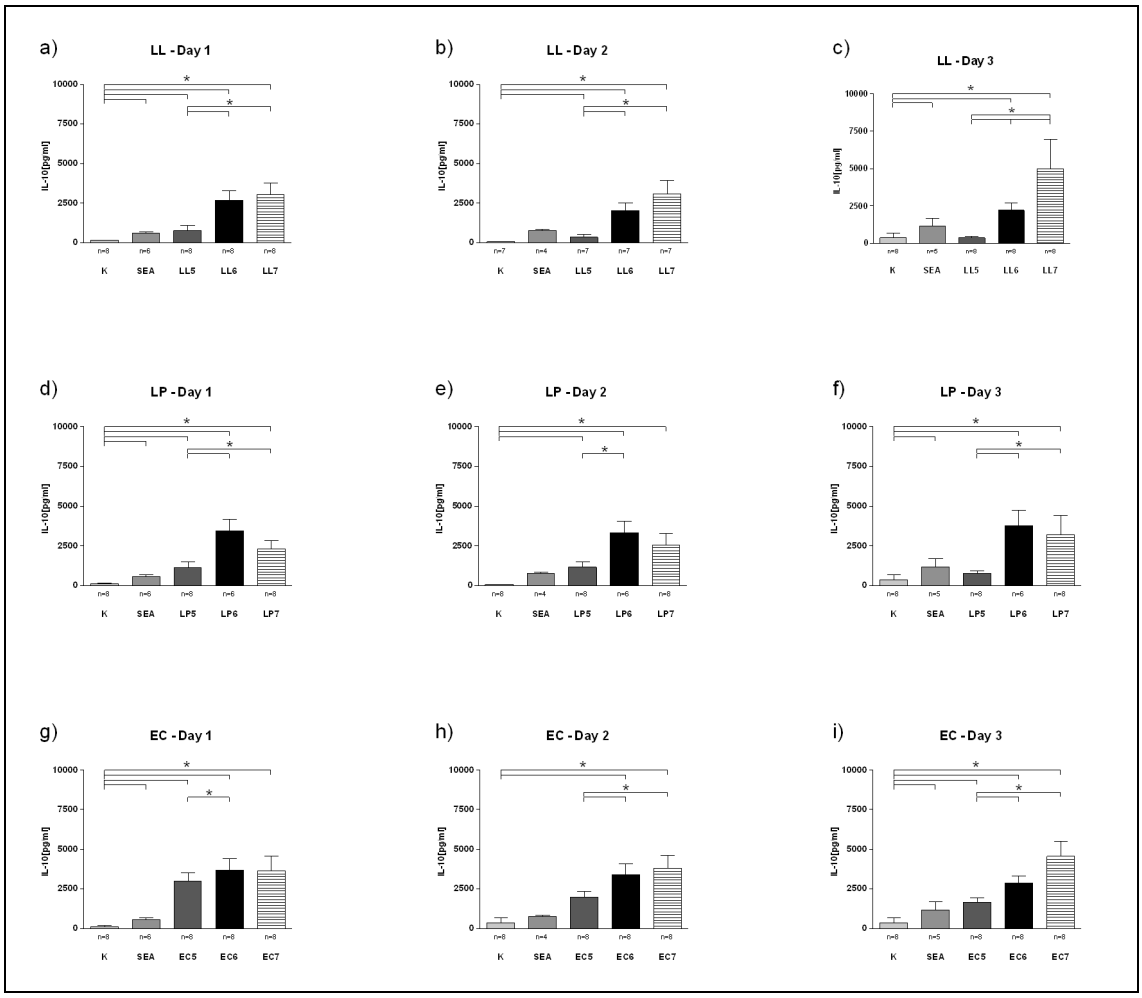


***Fig. 4-9*: IL-10 production: Kinetics and dose dependency over the course of 3 days.**
CBMCs were co-cultured with LL, LP, EC at 3 different concentrations (10^5, 10^6 and 10^7 CFU/mL) and the superantigen SEA [2µg/mL]. Medium alone served as negative control. After 24 hours (day 1), 48 hours (day 2) and 72 hours (day 3) total IL-10 in the cell free supernatant was quantified using sandwich ELISA. Significance was determined by Wilcoxon signed-rank test (*$p<0.05$). Subgroups with $n<5$ were excluded from statistical evaluation. For reasons of simplicity, significances between SEA and the bacteria are not presented in these graphs.

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; LL: Lactococcus lactis; LP: Lactobacillus plantarum; EC: Escherichia coli; SEA: Staphylococcus enterotoxin A; IL-10: Interleukin 10; CFU/mL: colony forming units per mL
The limit for detection was 63pg/mL.

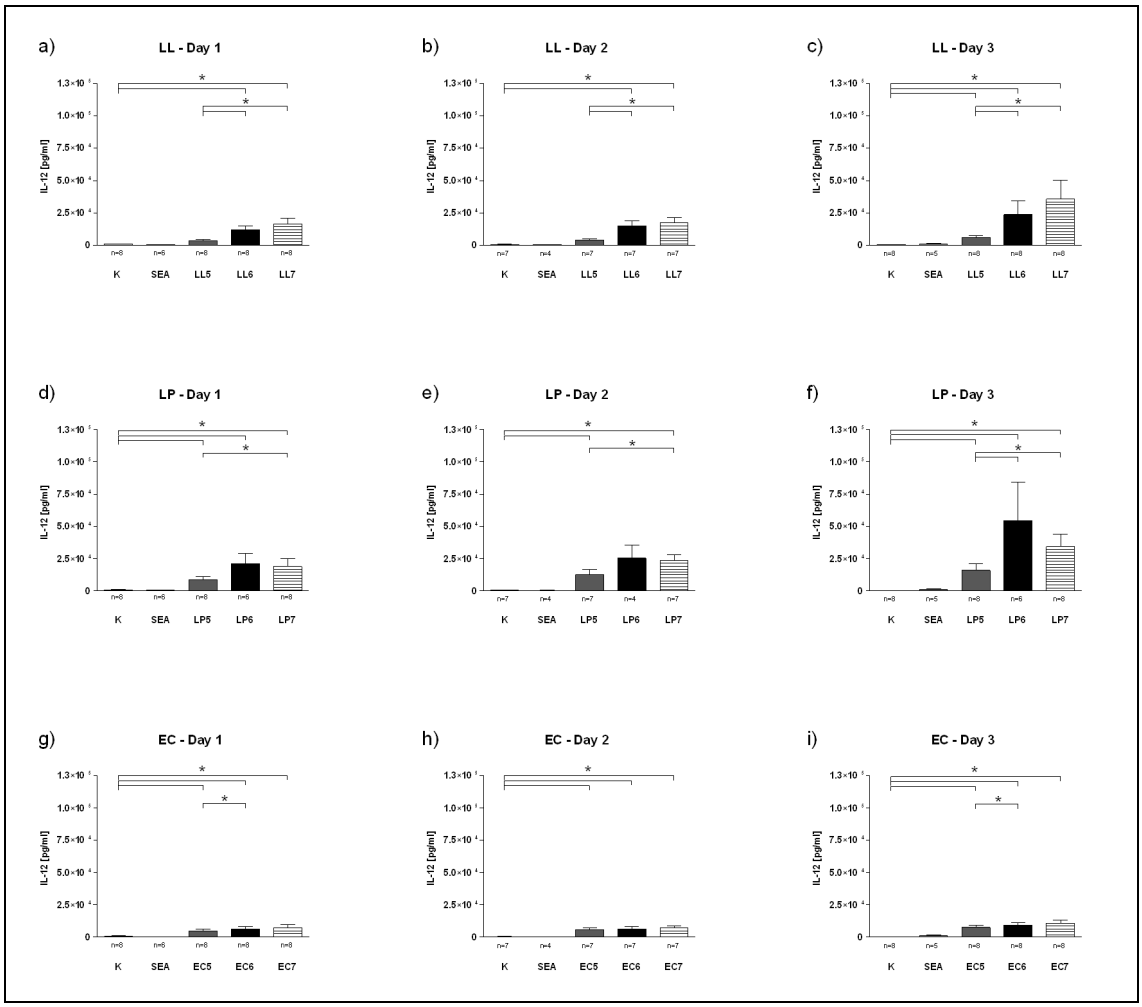


***Fig. 4-10*: IL-12 production: Kinetics and dose dependency over the course of 3 days.**
CBMCs were co-cultured with LL, LP, EC at 3 different concentrations (10^5, 10^6 and 10^7 CFU/mL) and the superantigen SEA [2µg/mL]. Medium alone served as negative control. After 24 hours (day 1), 48 hours (day 2) and 72 hours (day 3) total IL-12 in the cell free supernatant was quantified using sandwich ELISA. Significance was determined by Wilcoxon signed-rank test (*$p<0.05$). Subgroups with $n<5$ were excluded from statistical evaluation. For reasons of simplicity, significances between SEA and the bacteria are not presented in these graphs.

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; LL: Lactococcus lactis; LP: Lactobacillus plantarum; EC: Escherichia coli; SEA: Staphylococcus enterotoxin A; IL-12: Interleukin 12; CFU/mL: colony forming units per mL
The limit for detection was 313pg/mL.

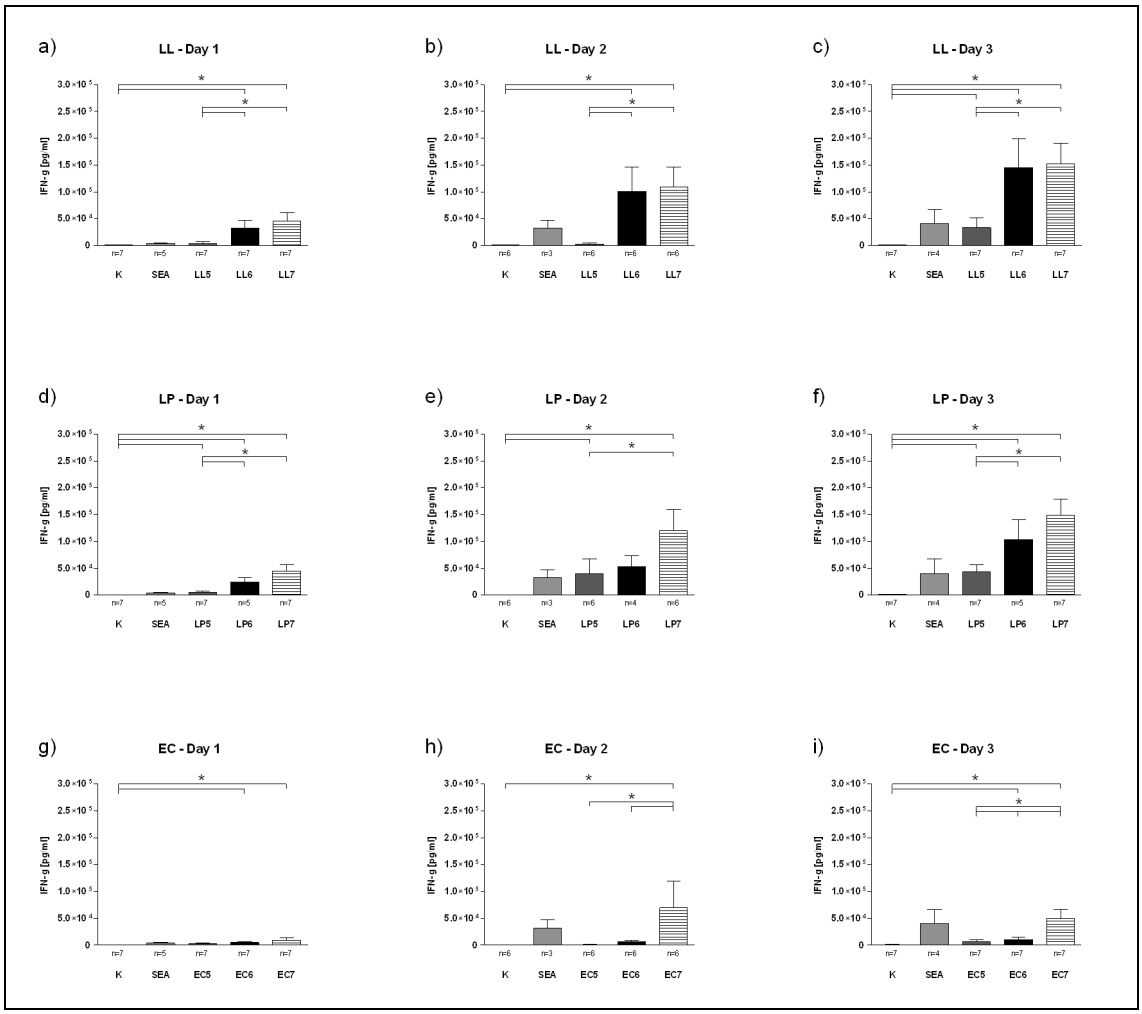


***Fig. 4-11*: IFNγ production: Kinetics and dose dependency over the course of 3 days.**
CBMCs were co-cultured with LL, LP, EC at 3 different concentrations (10^5, 10^6 and 10^7 CFU/mL) and the superantigen SEA [2µg/mL]. Medium alone served as negative control. After 24 hours (day 1), 48 hours (day 2) and 72 hours (day 3), total IFNγ in the cell free supernatant was quantified using sandwich ELISA. Significance was determined by Wilcoxon signed-rank test (*$p<0.05$). Subgroups with $n<5$ were excluded from statistical evaluation. For reasons of simplicity, significances between SEA and the bacteria are not presented in these graphs.

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; LL: Lactococcus lactis; LP: Lactobacillus plantarum; EC: Escherichia coli; SEA: Staphylococcus enterotoxin A; IFNγ: Interferon gamma; CFU/mL: colony forming units per mL
The limit for detection was 470pg/mL.

4.2.1.2 Strain specific differences

It is known that different bacterial strains do have different effects on cytokine expression in vivo. To evaluate differences between LL, LP, EC and SEA, expression of Th1-type (IL-12, INF-γ), Th2-type (IL-5) and regulatory cytokines (IL-10) induced by these bacterial strains were compared. Based on the kinetics the highest (10^5 CFU/mL) and the lowest (10^7 CFU/mL) concentration of bacteria was expressed and discussed at day 1, day 2 and day 3.

Generally, differentiated strain specific Th1/Th2-type cytokine expression patterns were observed at different concentrations.
Regarding IL-5 and IL-10, strain specific differences were observed at the lowest concentration (10^5 CFU/mL) (*Fig. 4-12*), while at 10^7 CFU/mL cytokine expression induced by all bacterial strains was on a similar level (*Fig. 4-13*). Reversely, no differences with regard to the applied strain were found in the expression of IL-12 at the lowest concentration, excepting LP which induced significantly higher IL-12 levels in response to all bacterial strains (*Fig. 4-12c, g and k*). Similarly, IFNγ production showed strain specific differences only with regard to LP at day 2 and day 3 at the lowest bacterial concentration (*Fig. 4-12h and l*). 10^7 CFU/mL of bacteria applied, we observed differences in both IL-12 and IFNγ production with regard to all strains tested (*Fig. 4-13*).

EC and SEA elicited higher amounts of the Th2-type cytokine IL-5 and the anti-inflammatory cytokine IL-10 compared to LL and LP (*Fig. 4-12 and 4-13*).
The highest concentration of EC applied (10^7 CFU/mL), IL-5 production was significantly enhanced compared to the two other strains (LL and LP) at day 1 (*Fig. 4-13a*). This was also observable as a trend at all other timepoints and concentrations but did not reach significance (*Fig. 4-12 and 4-13a, e and i*). SEA was found to be comparable to EC in the highest concentration, but no significances could be determined ($n<5$) (*Fig. 4-13a, e and i*). Nevertheless, SEA was found to be a potent inducer of IL-5, being surpassed only by the highest concentration of bacteria at day 3 (*Fig. 4-13i*).

IL-10 production was significantly increased by EC at all concentrations and timepoints, compared to LL and LP (*Fig. 4-12 and 4-13b, f and j*). Importantly, SEA induced a significantly lower IL-10 production than EC (*Fig. 4-12 and 4-13b, f and j*).

On the other hand, expression of Th1 cytokines IL-12 and IFNγ was increased in CBMCs co-cultured with lactic acid bacteria, compared to EC and SEA (*Fig. 4-12 and 4-13*). Generally, LP was found to be a more potent inducer of cytokine expression than LL, as demonstrated in the lowest concentration (10^5 CFU/mL), where both IL-12 and IFNγ expression was significantly higher in cells treated with LP compared to LL (*Fig. 4-12*).

Higher IL-12 expression elicited by lactic acid bacteria compared to EC was observed as early as at day 1 at 10^7 CFU/mL (*Fig. 4-13c*), while at 10^5 CFU/mL only LP reached significance (*Fig. 4-12c, g and k*). SEA induced significantly decreased IL-12 production, compared to all concentrations of all bacterial strains (*Fig. 4-12 and 4-13c, g and k*).

A significantly increased production of IFNγ was observed at 10^7 CFU/mL as well (*Fig. 4-13d, h and l*), while at the lowest concentration only LP reached significance (*Fig. 4-12d,h and l*). SEA induced a lower IFNγ production than LL and LP at 10^7 CFU/mL only, reaching significance at day 1 (LL and LP) (*Fig. 4-13d*) and day 3 (LP, and EC) (*Fig. 4-13l*).

At 10^5 CFU/mL, cytokine expression elicited by LL and LP peaked at day 3 and day 2, respectively (*Fig. 4-12h and l*), while at 10^7 CFU/mL both LL and LP reached significance at day 1, compared to EC (*Fig. 4-13d*).

Ratios of Th1 (IL-12, IFNγ) and Th2 (IL-5) cytokine expression were calculated in order to highlight changes that are not reflected by absolute cytokine amounts in the supernatant.

The IL-12/IL-5 ratios induced by both lactic acid bacterial strains were shown to be significantly elevated compared to EC at day 1 (*Fig. 4-15a*) and day 3 (*Fig. 4-15b*) if high bacterial loads (10^7 CFU/mL) were added. At the lowest concentration

(10^5 CFU/mL) only the most potent bacterium, LP, showed a significantly higher ratio than EC at day 1 (*Fig. 4-14a*).

A similar trend is reflected by the IFNγ/IL-5 ratio. A trend to an elevated ratio of LL and LP compared to EC was shown at 10^7 CFU/mL (*Fig. 4-17*), reaching significance at 24 hours (*Fig. 4-17a*). At the lowest concentration (10^5 CFU/mL), this trend could only be observed at day 3, not reaching significance (*Fig. 4-16b*), while at day 1 the IFNγ/IL-5 ratio was found to be very low in general (*Fig. 4-16a*).

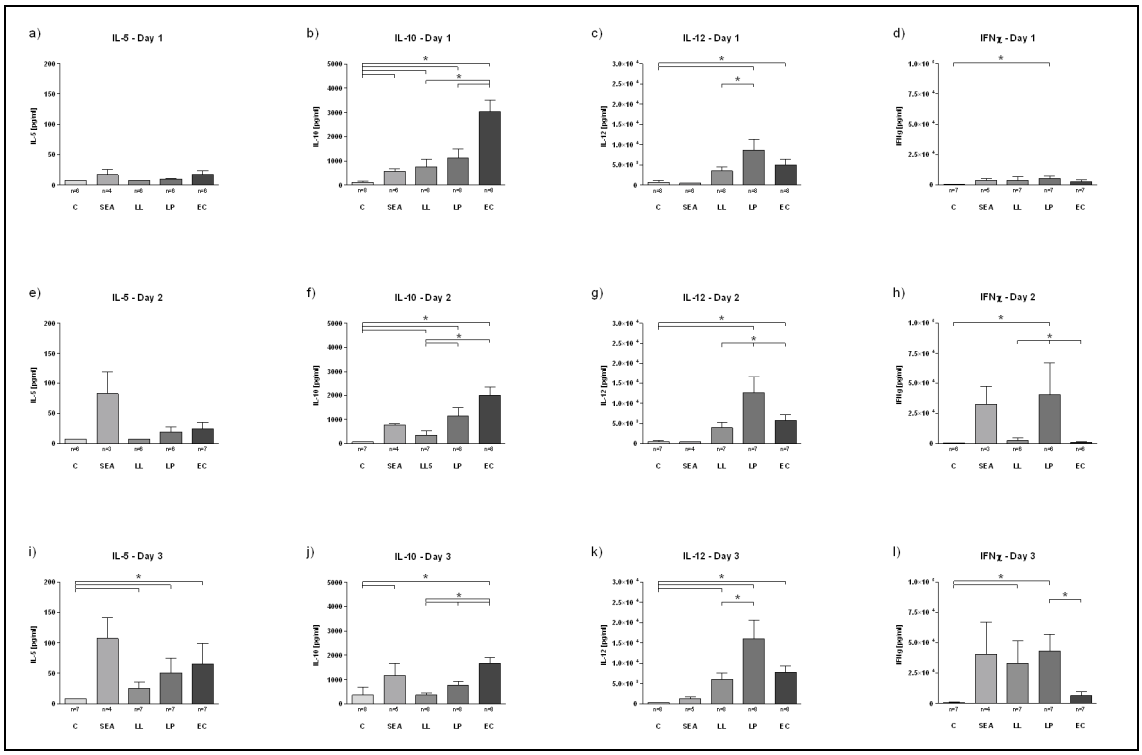


Fig. 4-12: **Strain specific differences of cytokine patterns in the presence of 10^5 CFU/mL of LL, LP and EC over the course of 3 days.**
CBMCs were co-cultured with 10^5 CFU/mL of LL, LP and EC and the superantigen SEA [2µg/mL]. Medium alone served as negative control. Total expression of IL-5, IL-10, IL-12 and IFNγ was quantified in the cell free supernatant using sandwich ELISA after 24 (day 1), 48 (day 2) and 72 (day 3) hours. Significance was determined by Wilcoxon signed-rank test (*$p<0.05$). Subgroups with $n<5$ were excluded from statistical evaluation.

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; LL: Lactococcus lactis; LP: Lactobacillus plantarum; EC: Escherichia coli; SEA: Staphylococcus enterotoxin A; IFNγ: Interferon gamma; IL-5: Interleukin 5; IL-10: Interleukin 10; IL-12: Interleukin 12

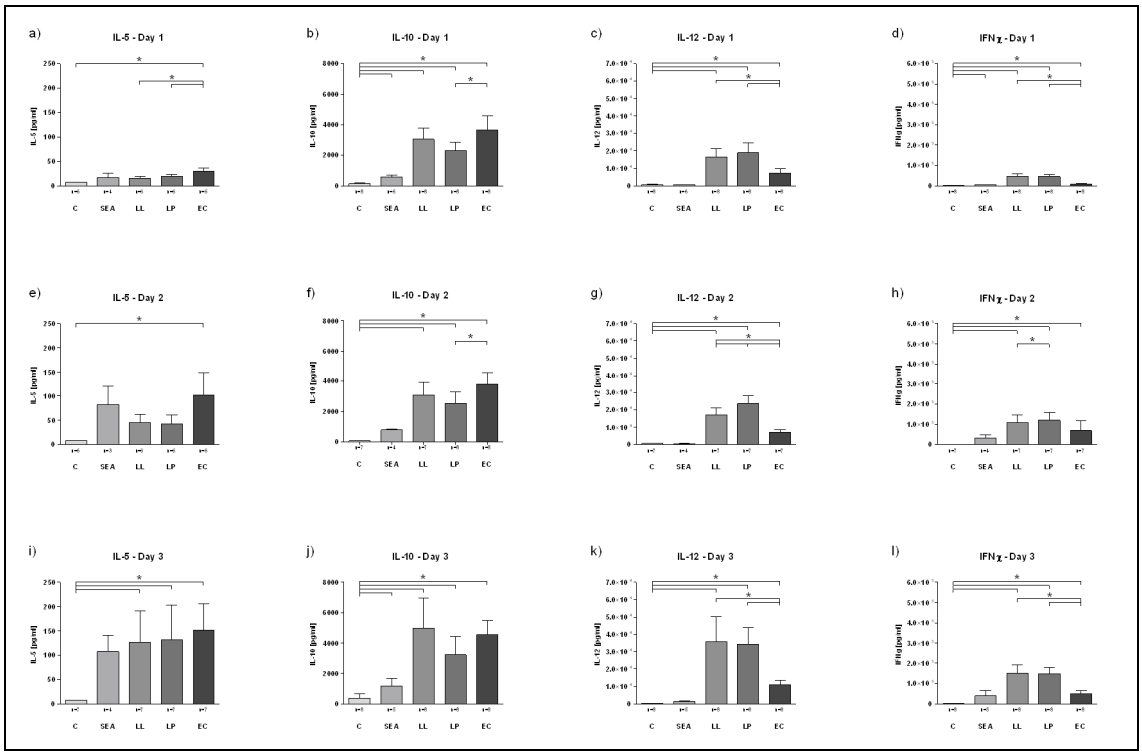


Fig. 4-13: **Strain specific differences of cytokine patterns in the presence of 10^7 CFU/mL of LL, LP and EC over the course of 3 days.**
CBMCs were co-cultured with 10^7 CFU/mL of LL, LP and EC and the superantigen SEA [2µg/mL]. Medium alone served as negative control. Total expression of IL-5, IL-10, IL-12 and IFNγ was quantified in the cell free supernatant using sandwich ELISA after 24 (day 1), 48 (day 2) and 72 (day 3) hours. Significance was determined by Wilcoxon signed-rank test (*$p<0.05$). Subgroups with $n<5$ were excluded from statistical evaluation.

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; LL: Lactococcus lactis; LP: Lactobacillus plantarum; EC: Escherichia coli; SEA: Staphylococcus enterotoxin A; IFNγ: Interferon gamma; IL-5: Interleukin 5; IL-10: Interleukin 10; IL-12: Interleukin 12

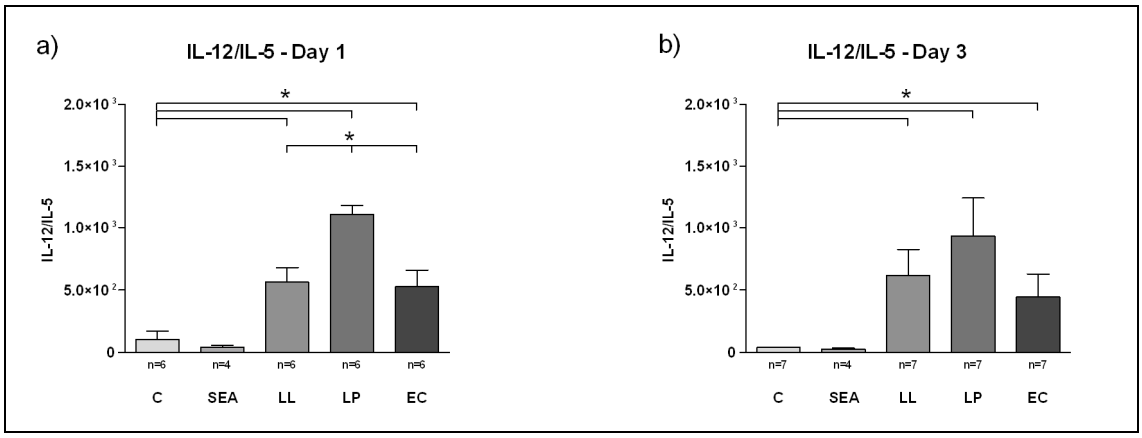


Fig. 4-14: **Ratio of IL-12/IL-5 expression of CBMCs in the presence of 10^5 CFU/mL of LL, LP and EC at day1 and day 3.**
The ratio of the Th1 cytokine IL-12 and the Th2 cytokine IL-5 expressed in CBMCs co-cultured with LL, LP, EC [10^5 CFU/mL] and SEA [2µg/mL] according to the above mentioned protocol was determined. Medium alone served as negative control.
Significance was determined by Wilcoxon signed-rank test (*$p<0.05$). Subgroups with $n<5$ were excluded from statistical evaluation.

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; LL: Lactococcus lactis; LP: Lactobacillus plantarum; EC: Escherichia coli; SEA: Staphylococcus enterotoxin A; IL-12: Interleukin 12; IL-5: Interleukin 5

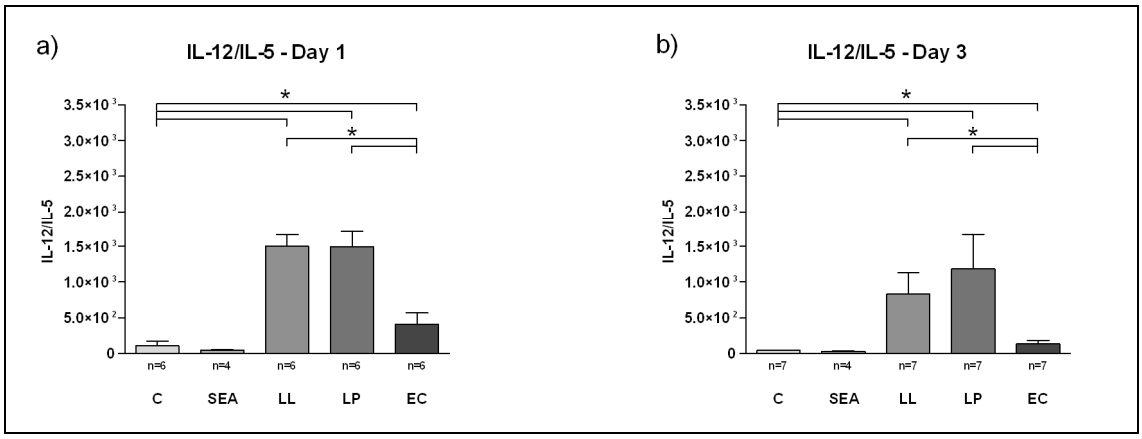


Fig. 4-15: **Ratio of IL-12/IL-5 expression of CBMCs in the presence of 10^7 CFU/mL of LL, LP and EC at day 1 and day 3.**
The ratio of the Th1 cytokine IL-12 and the Th2 cytokine IL-5 expressed in CBMCs co-cultured with LL, LP, EC [10^7 CFU/mL] and SEA [2µg/mL] according to the above mentioned protocol was determined. Medium alone served as negative control.
Significance was determined by Wilcoxon signed-rank test (*$p<0.05$). Subgroups with $n<5$ were excluded from statistical evaluation.

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; LL: Lactococcus lactis; LP: Lactobacillus plantarum; EC: Escherichia coli; SEA: Staphylococcus enterotoxin A; IL-12: Interleukin 12; IL-5: Interleukin 5;

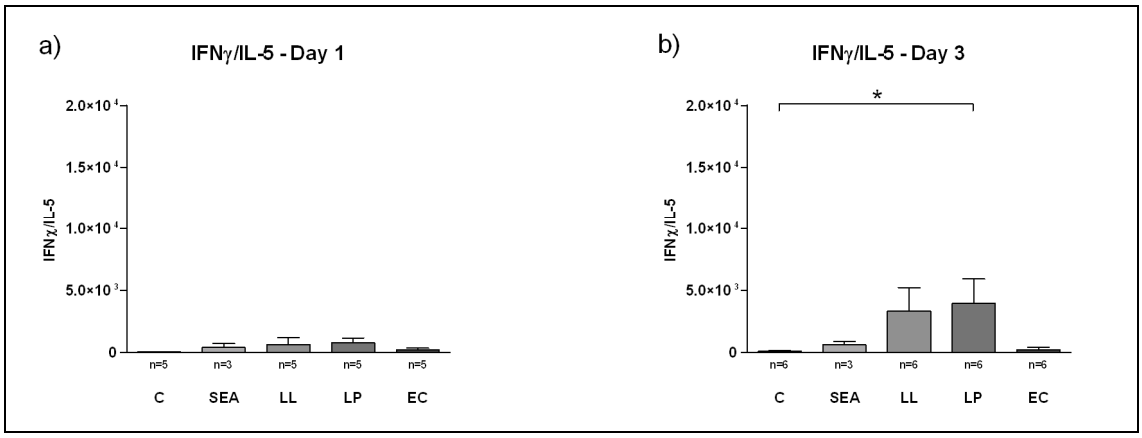


Fig. 4-16: **Ratio of IFNγ/IL-5 expression of CBMCs in the presence of 10^5 CFU/mL of LL, LP and EC at day 1 and day 3.**
The ratio of the Th1 cytokine IFNγ and the Th2 cytokine IL-5 expressed in CBMCs co-cultured with LL, LP, EC [10^5 CFU/mL] and SEA [2µg/mL] according to the above mentioned protocol was determined. Medium alone served as negative control.
Significance was determined by Wilcoxon signed-rank test (*$p<0.05$). Subgroups with $n<5$ were excluded from statistical evaluation.

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; LL: Lactococcus lactis; LP: Lactobacillus plantarum; EC: Escherichia coli; SEA: Staphylococcus enterotoxin A; IFNγ: Interferon gamma; IL-5: Interleukin 5

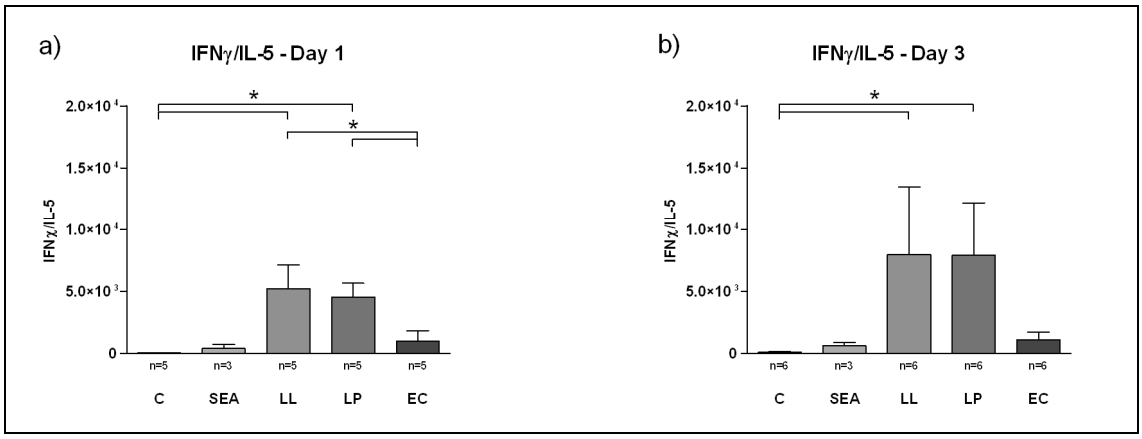


Fig. 4-17: **Ratio of IFNγ/IL-5 expression of CBMCs in the presence of 10^7 CFU/mL of LL, LP and EC at day 1 and day 3.**
The ratio of the Th1 cytokine IFNγ and the Th2 cytokine IL-5 expressed in CBMCs co-cultured with LL, LP, EC [10^7 CFU/mL] and SEA [2µg/mL] according to the above mentioned protocol was determined. Medium alone served as negative control.
Significance was determined by Wilcoxon signed-rank test (*$p<0.05$). Subgroups with $n<5$ were excluded from statistical evaluation.

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; LL: Lactococcus lactis; LP: Lactobacillus plantarum; EC: Escherichia coli; SEA: Staphylococcus enterotoxin A; IFNγ: Interferon gamma; IL-5: Interleukin 5

4.2.2 SOCS mRNA expression

4.2.2.1 Kinetics over 24 hours

To analyze Th1-type and Th2-type responses which occur before the expression of cytokines in CBMCs, expression of suppressors of cytokine signaling (SOCS), which were reported to be encoded by immediate early genes, was analyzed. CBMCs were co-cultured with LL, LP, EC and SEA for 1, 2, 16 and 24 hours. SOCS mRNA expression was semi-quantified relative to the housekeeping gene L32 utilizing the ribonuclease protection assay (RPA) for CIS, SOCS1 and SOCS3.

Expression of SOCS1 and SOCS3 was detectable in unstimulated cells at 0 hours (*Fig. 4-18*), and CIS, SOCS1 and SOCS3 expression was visible and evaluable by RPA at 1, 2, 16 and 24 hours in CBMCs stimulated with the three bacterial strains and SEA (*Fig. 4-18;* LP shown).

In order to assess the optimal time point for each SOCS to be measured, CIS, SOCS1 and SOCS3 expression at different time points was evaluated and relative SOCS expression was diagrammed as ratio to the negative control (*Fig. 4-20*). Since SOCS are encoded by immediate early genes, an initial dataset was investigated to assess an eventual up-regulation in the first hour. No SOCS up-regulation was observed in the 0 to 30 minutes timeframe, excepting a slight increase in SOCS3 mRNA in cells co-cultured with LP and EC (*data not shown*). At 1 hour, only E. coli induced a distinct increase in relative SOCS1 and SOCS3 expression (*Fig. 4-20h and i*), while no increased SOCS expression was induced by LAB and SEA (*Fig. 4-20*). A slight increase in relative SOCS1 and SOCS3 expression was observed at the 2 hour time point in CBMCs co-cultured with LP (*Fig. 4-20e and f*).

Consequently, CIS, SOCS1 and SOCS3 mRNA was quantified at 1, 2, 4 (n=3) 16 and 24 hours. Although SOCS1 and SOCS3 expression in unstimulated CBMCs decreased over the course of 24 hours (*Fig. 4-18*), ratios of stimulated cells (LAB, E. coli and SEA) to the negative control reached their respective peaks at 24 hours (*Fig. 4-20*).

On closer observation, LL didn't induce an elevated CIS or SOCS1 response compared to hour 0 at any timepoint (*Fig. 4-20a and b*), while SOCS3 expression was increased at 24 hours only (*Fig. 4-20c*). With LP, EC and SEA, an increase in CIS expression occurred at 16 hours (*Fig. 4-20d, g and j*), while a raise of SOCS1 and SOCS3 mRNA expression over the basal level appeared not until 24 hours (*Fig. 4-20*). Additionally, E. coli induced an early CIS, SOCS1 and SOCS3 response at the 1 hour timepoint, and an earlier raise of SOCS1 expression at 16 hours, compared to the other stimulants (*Fig. 4-20g-i*).

Because of these observations and the timepoints featured in our cytokine assay (24, 48 and 72 hours), 24 hours was chosen as the readout timepoint for further experiments concerning strain specifity.

4.2.2.2 Strain specific differences

In order to highlight differences between the 3 strains of bacteria, significances were calculated comparing the SOCS expression of LL, LP, EC and SEA.

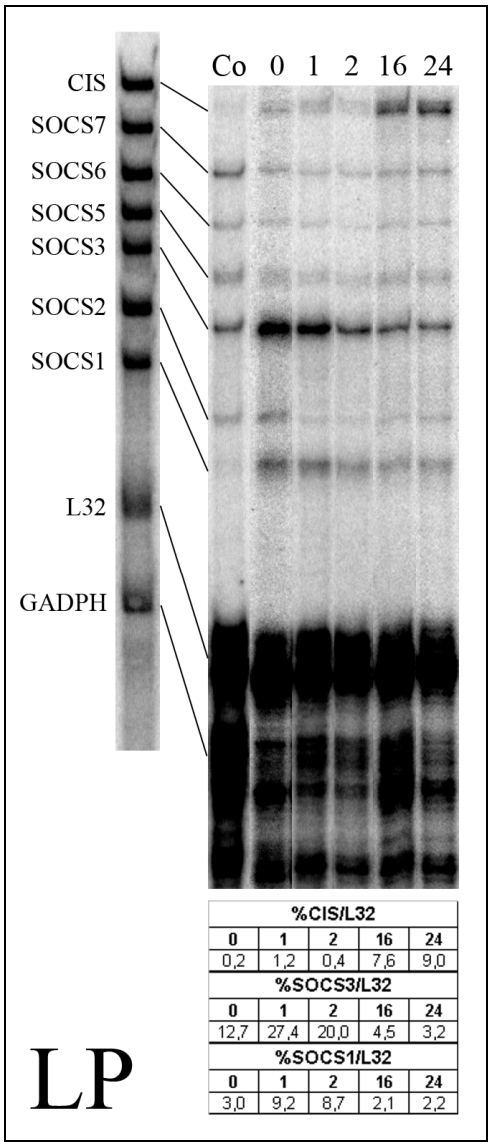
Significant differences between the control and CBMCs co-cultured with bacteria and SEA were not observed at 16 hours (*Fig. 4-21g-i*), excepting a significant increase in CIS expression induced by SEA, while at 24 hours strain specific differences could be evaluated (*Fig. 4-21j-l*).

LL elicited no significant increase in SOCS expression compared to the control at any time point, with the exception of SOCS3 at 2 and 24 hours (*Fig. 4-21f and l*). Additionally, LP and EC were shown to significantly increase expression of CIS, SOCS1 and SOCS3 compared to LL at 24 hours (*Fig. 4-21j-l*). A trend to increased expression of SOCS1 and SOCS3 was observed in CBMCs co-cultured with LP compared to LL from 2 to 16 hours, reaching significance at the 2 hour time point only (*Fig. 4-21e*). LP was a significantly more potent SOCS inducer then the other gram-positive germ, LL (*Fig. 4-19*).

EC induced the highest SOCS3 response compared to LL, LP and SEA at 24 hours (*Fig. 4-21l*), showing a significantly increased SOCS3 expression compared to LL already at the 1 hour time point (*Fig. 4-21c*). In our preliminary experiment, we observed elevated SOCS3 expression induced by E. coli after 30 minutes (*data not shown*).
SEA was demonstrated to elicit significantly higher CIS and SOCS1 expression than the 3 bacterial strains at 24 hours (*Fig. 4-21j and k*). Importantly, SOCS3 expression induced by SEA was comparable to LL and LP, and significantly decreased compared to E. coli at 24 hours (*Fig. 4-21l*).

Ratios of Th1 (SOCS1) and Th2 SOCS (CIS, SOCS3) expression at the 24 hour time point were calculated in order to highlight potential differences between the investigated bacterial strains and SEA not reflected by absolute SOCS expression defined by RPA. Data are presented in the log2 scale.
Significant differences between CBMCs co-cultured with the different bacteria were found at 24 hours only, while the tendency of SOCS1/SOCS3 increase in CBMCs co-cultured with SEA could be already observed at 16 hours (*data not shown*).
At 24 hours, the SOCS1/SOCS3 ratio in cells stimulated with LP was significantly increased compared to LL and EC (*Fig. 4-22a*). The trend of decreased Th1-type SOCS expression elicited by LL compared to EC observed in the SOCS1/CIS ratio should be treated with caution because of very low values (*Fig. 4-22b*).
The effect of SEA on the SOCS1/SOCS3 as well as the SOCS1/CIS ratio was a significant increase compared to all bacteria and the control (*Fig. 4-22*).

In summary, relative SOCS expressions induced by LAB, E.coli and SEA peaked at 24 hours. E. coli was observed to induce elevated SOCS expression much earlier, at 30 min (SOCS3) and 1 hour (CIS and SOCS1), compared to 16-24 hours in CBMCs stimulated with LAB and SEA. At 24 hours, LL elicited a significantly lower SOCS response than LP and EC, while LP induced a significantly increased SOCS1/SOCS3 ratio compared to LL and EC. SEA led to increased CIS and SOCS1 but not SOCS3 values, compared to all bacterial strains.



%CIS/L32				
0	1	2	16	24
0,2	1,2	0,4	7,6	9,0
%SOCS3/L32				
0	1	2	16	24
12,7	27,4	20,0	4,5	3,2
%SOCS1/L32				
0	1	2	16	24
3,0	9,2	8,7	2,1	2,2

Fig. 4-18: **RNase protection assay of SOCS expression in CBMCs co-cultured with LP over the course of 24 hours.**
CBMCs were co-cultured with LP (10^4 CFU/mL). Total RNA of the CBMCs was prepared after 1, 2, 4 and 16 hours of incubation according to the protocol. SOCS gene expression was measured by ribonuclease protection assay (RPA). SOCS expressions are shown above the housekeeping genes L32 and GAPDH.

Abbreviations: CBMC: cord blood mononuclear cell; LP: Lactobacillus plantarum; CIS: cytokine-inducible SH2 domain-containing protein; SOCS1 to 7: suppressors of cytokine signaling 1 to 7; L32: ribosomal protein L32; GAPDH: glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene)

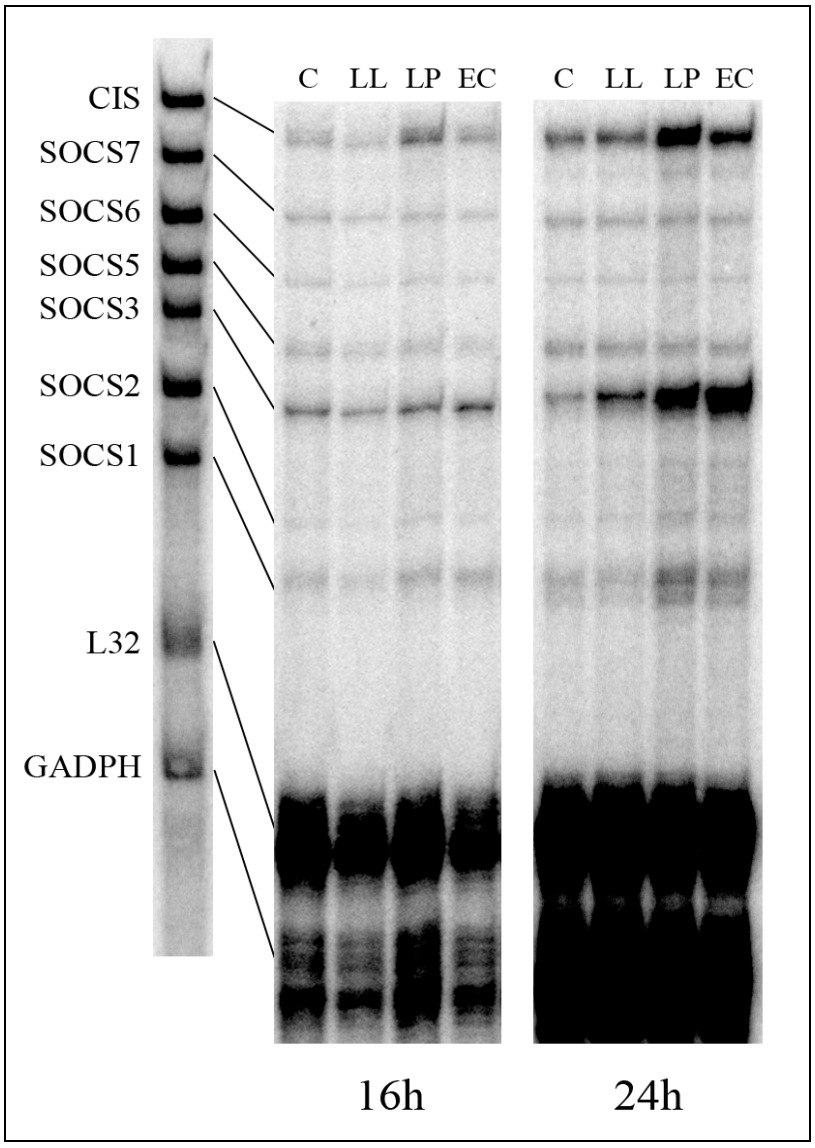


Fig. 4-19: **RNAse protection assay illustrating strain specific differences in the SOCS expression of CBMCs at 16 and 24 hours.**
CBMCs were co-cultured with 10^4 CFU/mL of the bacteria LL, LP and EC. Medium alone served as negative control. SOCS gene expression after 16 and 24 hours of incubation was determined according to the above mentioned protocol. SOCS expressions are shown above the housekeeping genes L32 and GAPDH.

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; LL: Lactococcus lactis; LP: Lactobacillus plantarum; EC: Escherichia coli; CIS: cytokine-inducible SH2 domain-containing protein; SOCS1 to 7: suppressors of cytokine signaling 1 to 7; L32: ribosomal protein L32; GAPDH: glyceraldehyde-3-phosphate dehydrogenase (housekeeping gene)

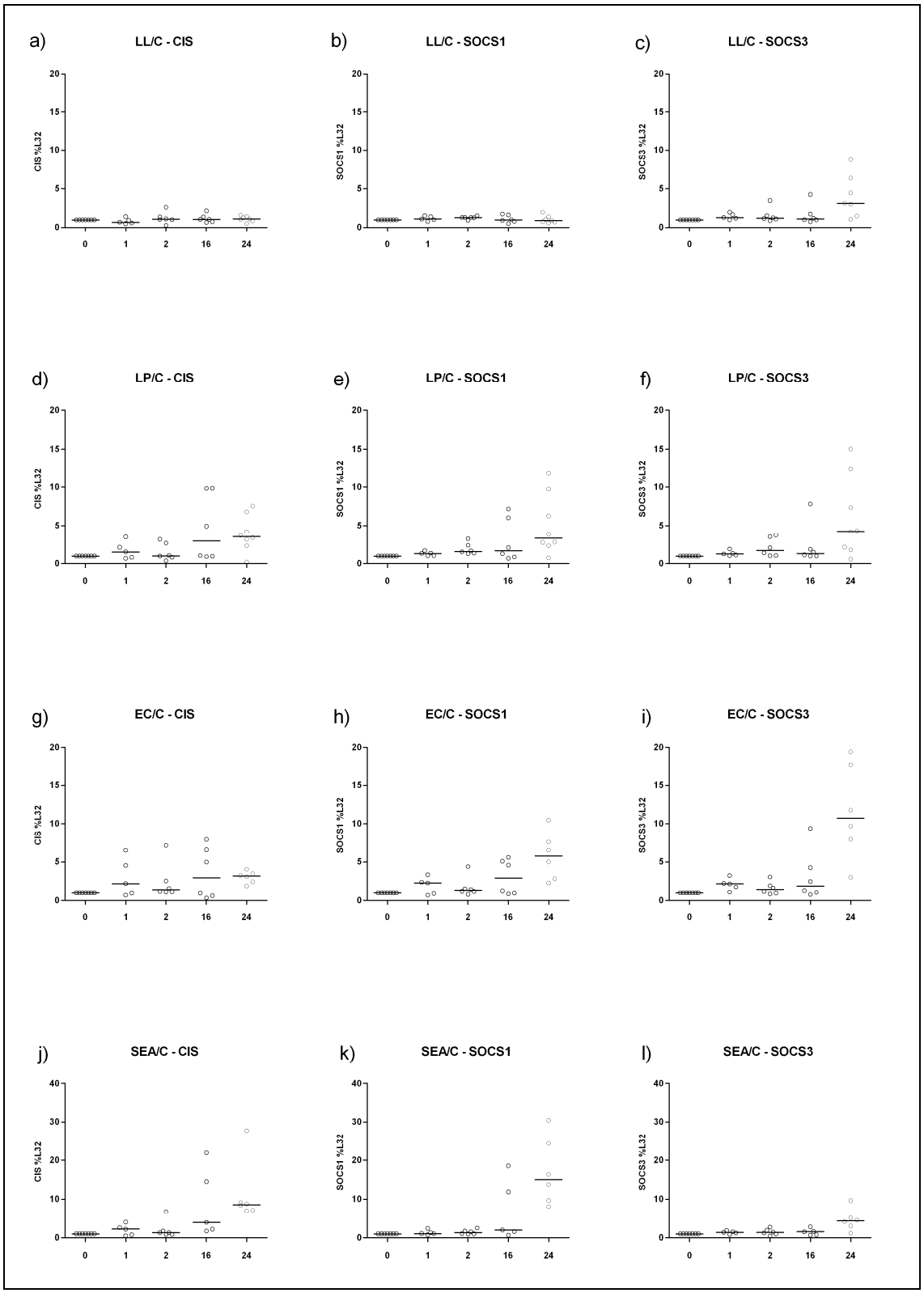


Fig. 4-20: **Kinetics of SOCS expression of CBMCs incubated with LL, LP, EC and SEA over the course of 24 hours, diagrammed as ratios to the negative control.**

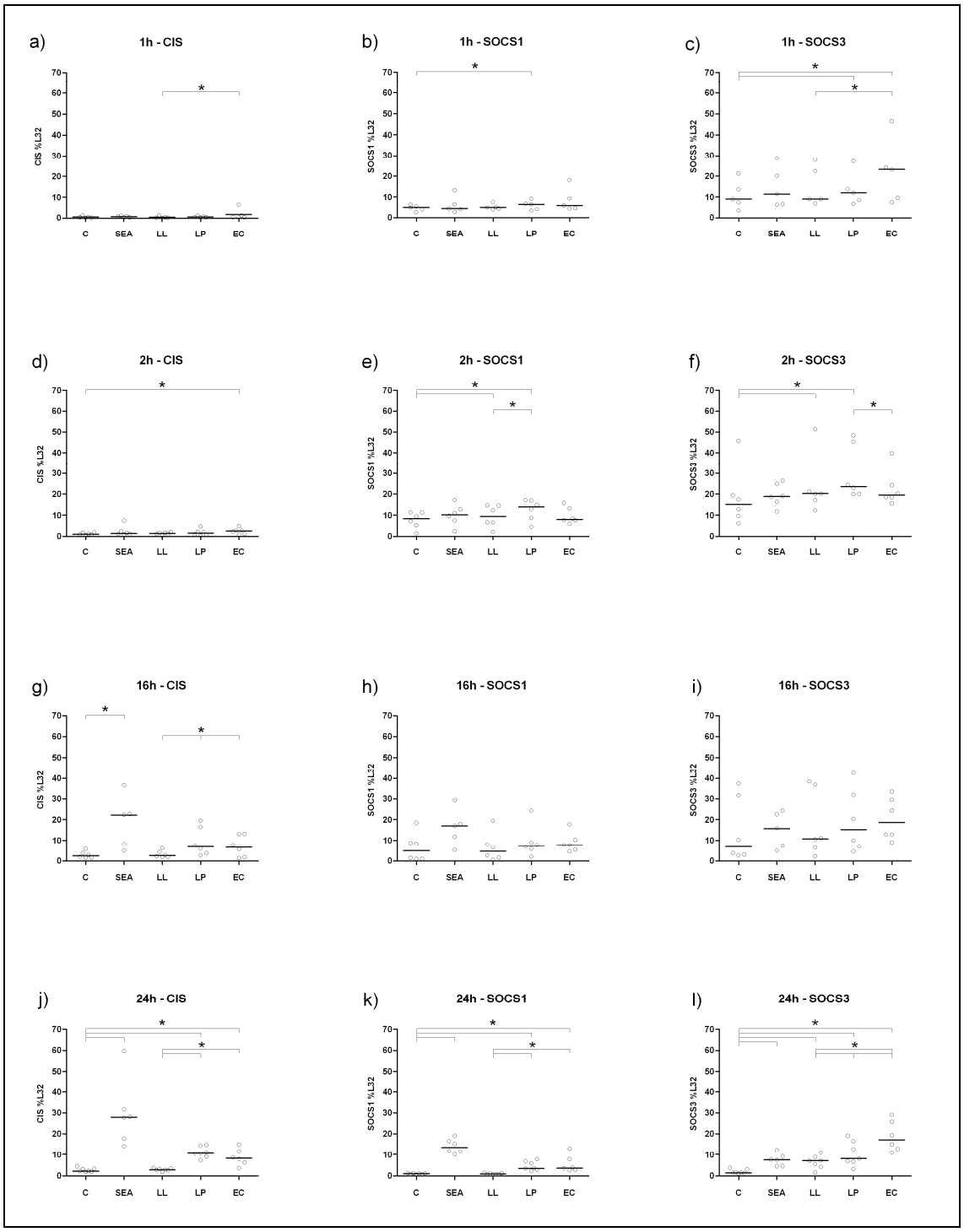


Fig. 4-21: **Strain specific differences in the SOCS expression of CBMCs over the course of 24 hours.**

Fig. 4-20: **Kinetics of SOCS expression of CBMCs incubated with LL, LP, EC and SEA over the course of 24 hours, diagrammed as ratios to the negative control.**
CBMCs were co-cultured with LL, LP, EC (10^4 CFU/mL) and the superantigen SEA [2µg/mL]. Medium alone served as negative control Total RNA of the CBMCs was prepared after 1, 2, 16 and 24 hours of incubation according to the protocol. The CIS, SOCS1 and SOCS3 gene expression was measured by ribonuclease protection assay (RPA). Data are presented relative to the housekeeping gene L32 as the median of the separate patients.

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; LL: Lactococcus lactis; LP: Lactobacillus plantarum; EC: Escherichia coli; SEA: Staphylococcus enterotoxin A; CIS: cytokine-inducible SH2 domain-containing protein; SOCS1 and 3: suppressors of cytokine signaling 1 and 3; L32: ribosomal protein L32

Fig. 4-21: **Strain specific differences in the SOCS expression of CBMCs over the course of 24 hours.**
CBMCs were co-cultured with 10^4 CFU/mL of the bacteria LL, LP, EC and the superantigen SEA [2µg/mL]. Medium alone served as negative control. CIS, SOCS1 and SOCS3 gene expression after 1, 2, 4, 16 and 24 hours of incubation was determined according to the above mentioned protocol. Data are presented relative to the housekeeping gene L32 as the median of the separate patients.
Significance was determined by Wilcoxon signed-rank test ($*p<0.05$). Subgroups with $n<5$ were excluded from statistical evaluation. For reasons of simplicity, significances between SEA and the bacteria are not presented in these graphs.

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; LL: Lactococcus lactis; LP: Lactobacillus plantarum; EC: Escherichia coli; SEA: Staphylococcus enterotoxin A; CIS: cytokine-inducible SH2 domain-containing protein; SOCS1 and 3: suppressors of cytokine signaling 1 and 3; L32: ribosomal protein L32

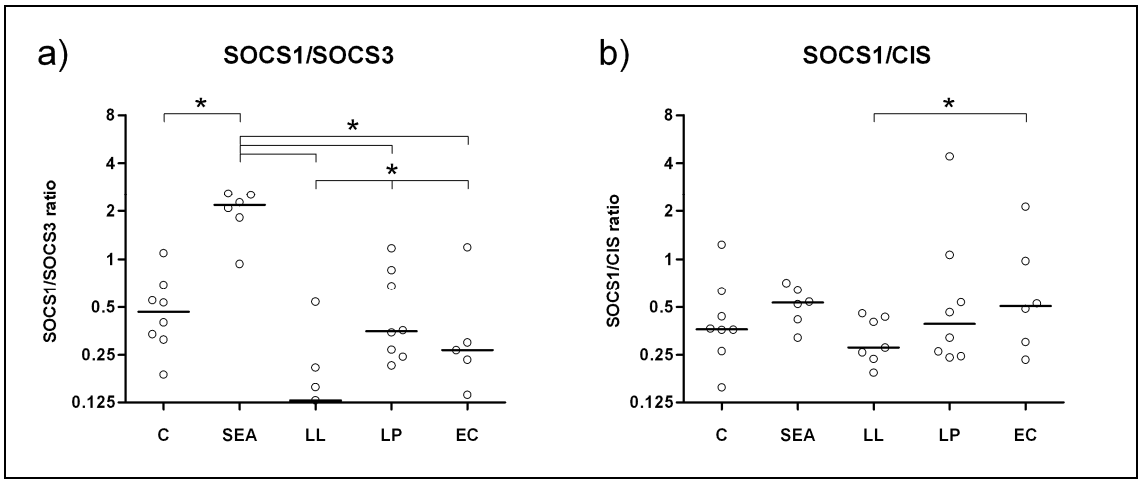


Fig. 4-22: **Ratio of SOCS1/SOCS3 and SOCS1/CIS expression of CBMCs induced by LL, LP and EC.**
CBMCs were co-cultured with LL, LP, EC [10^4 CFU/mL] and the superantigen SEA [2µg/mL]. Medium alone served as negative control. CIS, SOCS1 and SOCS3 gene expression after 2, 16 and 24 hours of incubation was determined according to the above mentioned protocol. The ratios were calculated from SOCS expressions relative to the housekeeping gene L32. Data are presented as the median of the separate patients in a log2 scale.
Significance was determined by Wilcoxon signed-rank test (*$p<0.05$).

Abbreviations: CBMC: cord blood mononuclear cell; C: control with medium alone; LL: Lactococcus lactis; LP: Lactobacillus plantarum; EC: Escherichia coli; SEA: Staphylococcus enterotoxin A; CIS: cytokine-inducible SH2 domain-containing protein; SOCS1 and 3: suppressors of cytokine signaling 1 and 3; L32: ribosomal protein L32

4.3 References

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Pochard, P., P. Gosset, et al. (2002). "Lactic acid bacteria inhibit TH2 cytokine production by mononuclear cells from allergic patients." J Allergy Clin Immunol **110**(4): 617-23.

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5 Discussion

The establishment of type I allergy later in life may be supported in the perinatal period by environmental factors. These to date poorly understood mechanisms interfere with the sequential switch from a Th2 skewed pregnancy into a balanced Th1/Th2 milieu within the first years of life. Apart from genetic predisposition, reduced microbial exposure ("western life style") has been linked to the development of allergy. In a broader context, the colonization of the gut in terms of composition has been shown to confer with the prevalence of allergy. This association has been transferred into preventive approaches via the application of probiotics in the perinatal period, with varied outcomes (Kalliomaki, Salminen et al. 2001; Kalliomaki, Salminen et al. 2003; Isolauri 2004; Brouwer, Wolt-Plompen et al. 2006; Taylor, Dunstan et al. 2007).
We herein tried to define allergen and strain specific differences in a cord blood in vitro model simulating the immune system at birth. Importantly, we did not exclusively check for cytokine expression but also for key regulators in cytokine signaling and consequently cell activation, namely the suppressors of cytokine signaling (SOCS).

Food allergens are known to induce proliferation in a relatively high number of cord blood samples, despite of no direct linkage to the amount of allergen exposure (Prescott, Macaubas et al. 1998; Edelbauer, Loibichler et al. 2004). Although more than 90% of T cells in cord blood are naïve, CBMCs have been demonstrated to show proliferative activity to allergens without the aid of memory cells (Devereux, Seaton et al. 2001), utilizing a "broad range" response with reactivity to much more regions of an allergen than adult cells (Thornton, Upham et al. 2004). Neonates with an immediate family history of atopy tended to show higher allergen-specific CBMC proliferative responses than low-risk neonates (Prescott, Macaubas et al. 1999). Accordingly, modified levels of Th2 cytokines in CBMCs of term babies have been correlated to atopy in several studies. While elevated levels of the Th2 cytokines IL-4 and IL-13 next to reduced IFNγ and IL-12 levels were detected by several groups in neonates at risk of atopy (Gabrielsson, Soderlund et al. 2001; Lange, Ngoumou et al. 2003), Prescott et al. demonstrated lower levels of IL-4, IL-6, IL-10 and IL-13 in high-risk children at birth compared to low-risk children, with low IFNγ levels in both high-risk and low-risk

groups (Prescott, Macaubas et al. 1999). Investigations concerning proliferation and cytokine expression of CBMCs in response to food allergens (BLG and OVA) have been undertaken (Prescott, Macaubas et al. 1998), but mostly to uncover differences between neonates with high and low risks of atopy. Although connections between atopy and SOCS proteins do exist (Seki, Inoue et al. 2003; Harada, Nakashima et al. 2007), SOCS expression of CBMCs in response to food allergens has, to our knowledge, not been investigated yet.

We observed positive proliferation (SI>2) in a similar range for BLG (36,8%), OVA (31,6%) and Ara h1 (31,6%). A proliferation frequency of approximately 40% in CBMCs co-cultured with either BLG or OVA has been documented (Prescott, Macaubas et al. 1998) and confirms that T cells are activated via the TCR, not the innate immune system (via LPS contamination). The frequency of cord blood samples proliferating to the second peanut allergen, Ara h2, was substantially lower (13,3%). Interestingly, in the literature, Ara h2 is described as a much more potent peanut allergen compared to Ara h1, concerning the induction of allergic symptoms, IgE cross-linking and cell activation in peanut-allergic patients (Koppelman, Wensing et al. 2004; Palmer, Dibbern et al. 2005). However, it seems that in CBMCs, Ara h1 elicits a stronger proliferative response than Ara h2, possibly due to more epitopes being recognized by the “broad range” response utilized by the naïve T cells in cord blood. Individuals who show positive proliferation to Ara h2 also show positive proliferation to Ara h1, but to BLG and OVA as well. Crude peanut extract induced a proliferation frequency of 80%. This might be due to a greater variety of epitopes present, but may also relate to impurity and residual LPS despite of purification attempts. To exclude this possible confounder, crude extract was excluded from further experiments (Eiwegger, Mayer et al. in press).

Examining allergen concentrations ranging from 0,5-50µg/mL and 1-100µg/mL (Ara h1/Ara h2 and BLG/OVA, respectively), no clear dose dependency was observed. The pattern of reactivity of the CBMCs to certain concentrations of allergens seems almost random. The small number of patients (n=19) does not allow to draw conclusions concerning this variance. Since most of the literature relates to the highest allergen concentrations applied, we decided to use the highest allergen concentration for

semi-quantification of cytokine and SOCS mRNA expression and subsequent relation to proliferation.

According to the literature, cytokine mRNA expression is lower in CBMCs than in PBMCs. While LPS- and PHA-stimulated cytokine expression was evaluable with the ribonuclease protection assay (RPA), allergen-stimulated expression proved to be under the detection limit of this method. SOCS background expression, on the other hand, was demonstrated to be above the limit of detection of the used method.

Concerning SOCS expression, we demonstrated that Ara h1 induces significantly increased levels of CIS compared to the other allergens. Elevated CIS levels have been shown to promote T cell receptor mediated proliferation (Li, Chen et al. 2000). This could at least partly explain the increased CIS levels induced by Ara h1 compared to Ara h2, as CBMCs co-cultured with Ara h1 showed positive proliferation almost 3 times as often as Ara h2. No allergen-specific differences in SOCS1 and SOCS3 expression were observed, apart from a slight increase of SOCS1 expression in CBMCs co-cultured with OVA, compared to BLG. These allergen-specific differences were highlighted by the SOCS1/CIS ratio, where Ara h1 induced a significantly lower SOCS1/CIS ratio than the other allergens and the negative control.

PHA induced increased CIS and SOCS1 expression, but decreased SOCS3 expression, compared to the allergens. Aside from the general, strong T cell response via cross-linkage of the TCR, PHA is known to be a potent Th1-inducer. This might explain the low SOCS3 expression; SOCS3 is reported to be selectively expressed in Th2-cells. SOCS1, connected with a Th1-type response, is strongly induced by PHA, compared to the allergens. On the other hand, CIS expression is linked to Th2-type development as well. Elevated expression of CIS can be explained by the selective induction of CIS in T cells after TCR stimulation (Li, Chen et al. 2000). We found these observations mirrored in the SOCS1/CIS ratio, with PHA inducing a lower ratio than the allergens and the negative control, and, in the opposite, in the SOCS1/SOCS3 ratio. These ratios point out that the Th1/Th2-type classification plays a secondary role in the SOCS1/CIS ratio, where the above mentioned factors play a major role. Although SOCS1 and SOCS3 have been connected to Th1 and Th2 polarized differentiation (Egwuagu, Yu et al. 2002), both SOCS are equally induced by a range of cytokines, most importantly

IFNγ and IL-6. Furthermore, allergen-induced SOCS1 and SOCS3 expression at 24 hours did correlate, while the expression of CIS showed no positive correlation with either SOCS1 or SOCS3 expression. These findings point to induction by the same cytokines rather than a Th1/Th2-type activation of SOCS mRNA expression.

We found no relation between positive proliferation and SOCS expression induced by a certain allergen, either in inter- or intra-individual comparison. While the proliferative properties and cytokine profiles of CBMCs differed individually, no distinction between the influence of proliferation positive and negative allergens on the CIS, SOCS1 and SOCS3 expression of CBMCs could be established. While connections between proliferation and the regulatory SOCS proteins are apparent (Yu, Mahdi et al. 2004), in our setup SOCS mRNA expression was not sufficiently activated to show any differences between allergen-stimulated positive and negative cells. The main reasons are the low proliferation frequency and the low cytokine levels induced by food allergens via the TCR in general, as opposed to cytokine induction via the innate immune system.

Another question addressed in the present study was how lactic acid bacteria and E. coli influence the Th1/Th2 cytokine balance in cord blood mononuclear cells from randomly selected term newborns.

There has been a lot of speculation about the anti-allergic effects of lactic acid bacteria (Isolauri, Arvola et al. 2000; Kalliomaki, Salminen et al. 2003; Rosenfeldt, Benfeldt et al. 2003). A Th1 skewing influence on the Th1/Th2 cytokine balance has been investigated in PBMCs (Pochard, Gosset et al. 2002; Repa, Grangette et al. 2003), and in CBMCs (Karlsson, Hessle et al. 2002). Karlsson et al. investigated the influence of different gram-positive and gram-negative strains, including LP and E.coli, on the production of IL-6, IL-10, IL-12 and TNFα.

To gain further information about the effects of LAB on CBMCs, reactions of mononuclear cells to the two LAB strains (LL and LP) were compared to reactions to E.coli and the superantigen SEA (staphylococcal enterotoxin A). SOCS are directly induced by gram-positive bacteria and LPS via Toll-like receptors (Stoiber, Kovarik et al. 1999; Dennis, Jefferson et al. 2006) as well as by certain cytokines, including IFNγ

and IL-10. To our knowledge, an influence of LAB on SOCS has not been investigated yet.

We demonstrated that in CBMCs, IL-10 and IL-12 are expressed in full quantity on day 1 (Bacteria and SEA), levelling off over the course of 3 days, whereas IL-5 and IFNγ reach their peak at day 2 (EC, SEA) to 3 (LAB), depending on the stimulant. An earlier expression of IL-10 and IL-12 was expected, as these cytokines are directly expressed by macrophages and naïve T cells, respectively, while cells secreting IL-5 and IFNγ require activation by cytokines previously expressed by macrophages and T cells. Different expression patterns of these cytokines have been documented in the cell free supernatant of PBMCs. Hessle et al. reported that IL-10 and IL-12 expression by PBMCs stimulated with gram-positive and gram-negative bacteria was maximal after 24 hours (Hessle, Andersson et al. 2000). IL-5 and IFNγ expression has been shown to peak at 48 hours in PBMCs stimulated with SEA (Pochard, Gosset et al. 2002). We confirmed these observations in CBMCs. Importantly, we demonstrated that LAB induce peak expression of IL-5 and IFNγ at 72 hours, while the response to E. coli is similar to SEA (48 hours). Possibly, these different kinetics are caused by activation of the innate immune system via specific TLRs by gram-positive and gram-negative bacteria. TLR4 is a key molecule for bacterial LPS (or endotoxin) responsiveness, while LAB interact primarily with TLR2 (peptidoglycan) and possibly the intracellular TLR9 (CpG-DNA). This phenomenon was mirrored in SOCS3 expression, where E. coli induced elevated SOCS3 expression after 30 minutes, as opposed to 1 hour (LP) and 2 hours (LL).

The influence on cytokine expression was dependent on the dose of bacteria used, with higher bacterial doses leading to increased expression of IL-5, IL-10, IL-12 and IFNγ. Such a dose-response effect was reported in vitro for gram-positive and gram-negative bacteria in PBMCs (Hessle, Andersson et al. 2000; Pochard, Gosset et al. 2002). Dose dependency for LL and EC was observed in the 10^5-10^7 CFU/mL range, while for LP the highest cytokine levels were observed at 10^6 CFU/mL. We confirmed LP to be the most potent cytokine inducer of the bacteria tested, which was indicated by Repa et al. in a mouse model (Repa, Grangette et al. 2003). The decreased expression of IL-5, IL-10 and IL-12 we observed at the highest concentration of LP might relate to a mild

toxic effect of LP in this dosage, which is not reflected in thymidine incorporation assays (*data not shown*). Interestingly, IFNγ expression did not decrease at the highest concentration of LP. The reason for this is that, unlike the other cytokines tested, IFNγ accumulates in the supernatant because of its comparatively longer half-life.

We demonstrated that lactic acid bacteria (LL and LP) effectively modulate the Th1/Th2 cytokine balance in cord blood. LL and LP induced increased expression of the Th1 cytokines IL-12 and IFNγ, and decreased expression of the anti-inflammatory cytokine IL-10, compared to E. coli and SEA. This is in part consistent with the observations made by Karlsson et al. (Karlsson, Hessle et al. 2002), who reported increased IL-12 and IFNγ levels in response to LP. Concerning IL-10, the same group demonstrated that gram-negative bacteria induce IL-10 more efficiently than gram-positives in adult monocytes (Hessle, Andersson et al. 2000), but not in CBMCs (Karlsson, Hessle et al. 2002). However, it has to be kept in mind that the bacterial concentrations used by this group on both PBMCs and CBMCs were comparatively high ($5x10^7$ CFU/mL). In our study, a significant elevation of IL-10 expression was observed from 24 to 72 hours in response to 10^5 CFU/mL, while at 10^7 CFU/mL significance was barely reached.

Finally, LAB displayed a trend towards lower expression of the Th2 cytokine IL-5 compared to E. coli, and a significantly lower expression compared to SEA. While the ability of LAB to inhibit Th2 cytokine production (IL-4 and IL-5) has been observed in PBMCs of atopic adults (Pochard, Gosset et al. 2002), to our knowledge no data exists on the influence of LAB on the IL-5 expression of CBMCs. We demonstrated that IL-5 expression in CBMCs induced by LL and LP is reduced compared to EC and SEA.

In conclusion, these data indicate that LAB have a Th1-skewing influence on CBMCs through elevated induction of IL-12 and IFNγ, as well as decreased IL-5 and IL-10 expression. In contrast, the gram-negative E. coli induces higher levels of IL-5 and IL-10, and lower levels of IL-12 and IFNγ, compared to LAB. These observations are further accentuated by Th1/Th2 cytokine ratios (IL-12/IL-5 and IFNγ/IL-5). The potent cytokine inducer LP showed increased expression of Th1 cytokines at lower concentrations than LL. Nevertheless, both LAB induced a significantly higher Th1/Th2 ratio than EC and SEA. The Th2-skewing properties of the superantigen SEA, which have already been demonstrated in PBMCs (Pochard, Gosset et al. 2002), have been

confirmed in CBMCs, showing a similar but more pronounced cytokine profile (IL-5, IL-12 and IFNγ) than E. coli. Concerning IL-10 expression, SEA induced comparably low IL-10 levels from day 1 to day 3.

SOCS are encoded by immediate early genes. Nevertheless, no distinct SOCS mRNA up-regulation was detected in the first 30 minutes. Relative SOCS expressions reached their respective peaks at 24 hours, with significantly different SOCS levels being induced by the diverse stimulants at this timepoint. CIS expression increased earlier than SOCS1/3 expression, at 16 hours compared to 24 hours. We assume that CIS expression is induced by IL-2, a cytokine which is expressed at 4 hours in cord blood (Han and Hodge 1999), earlier than IL-6, IL-10 and IFNγ which induce SOCS1 and 3.
In CBMCs co-cultured with LP, a slight increase in relative SOCS1 and SOCS3 mRNA expression was observed at hour 2. This up-regulation may be attributed to an early innate immune reaction via peptidoglycan (TLR2) and possibly CpG-DNA (TLR9) (Dalpke, Opper et al. 2001; Dalpke, Eckerle et al. 2003; Dennis, Jefferson et al. 2006).
E. coli induced a relative up-regulation of all SOCS at the 1 hour timepoint, showing an increase in SOCS3 and CIS levels compared to the LAB. This early CIS, SOCS1 and SOCS3 expression points to direct activation of macrophages and dendritic cells by LPS via TLR4 (Dalpke, Eckerle et al. 2003).
At 4 to 16 hours, no significant SOCS up-regulation compared to the negative control was observed. Furthermore, no strain specific SOCS induction was measured until the 24 hour timepoint.
In the literature, SOCS1 has been connected to Th1-type and CIS and SOCS3 to Th2-type differentiation. Aside from the involvement in the regulation of cytokines that balance Th1/Th2 development, increased expression of SOCS1 and SOCS3 has been connected with atopic disease in clinical studies (Federici, Giustizieri et al. 2002; Seki, Inoue et al. 2003; Harada, Nakashima et al. 2007). As SOCS mRNA expression is effectively induced by bacterial compounds (Naka, Fujimoto et al. 2005), and as different bacterial strains have been shown to elicit Th1- or Th2-skewed responses (Karlsson, Hessle et al. 2002; Pochard, Gosset et al. 2002), it was of interest to observe the influence of these bacteria on Th1/Th2 associated SOCS expression. However, in our experimental setting the oversimplified system of Th1/Th2 SOCS1/SOCS3 could

not be reproduced. At the 24 hour timepoint, SOCS expression is influenced by a variety of cytokines induced by the 3 bacterial strains and SEA, Th1/Th2 cytokines as well as Th1/Th2-unrelated cytokines, most importantly IL-10. Pochard et al. demonstrated the inhibition of Th2 cytokines by LAB in an allergen-specific setup (Pochard, Gosset et al. 2002), while in our study the reaction of CBMCs to certain bacteria was tested in a polyclonal approach. Consequently, the Th1/Th2 skewing properties of LAB and E.coli we observed at cytokine level will not influence SOCS expression in the same way they would have done in an antigen specific setting.

Generally, LL induced no elevated CIS and SOCS1 response compared to the control and the lowest SOCS3 response of all bacterial strains. Corresponding to the observations made regarding cytokine expression, LP was found to be more effective than LL, probably due to the potent effect of LP on IL-10 and possibly IFNγ expression at 24 hours. Importantly, CIS and SOCS1 expression levels induced by LP were more alike to EC than to LL, despite the similarities between LL and LP regarding their cell wall components.

IFNγ protein levels have been shown to be low at 24 hours, while IL-10 is already present at its highest concentration. While both SOCS1 and SOCS3 are strongly induced by IFNγ, IL-10 has been shown to abundantly induce SOCS3, while inducing lower levels of SOCS1 (Dennis, Jefferson et al. 2006). This was confirmed by our observations of an elevated SOCS3 expression in cells stimulated for 24 hours with 10^4 CFU/mL E.coli (induces high levels of IL-10) compared to those stimulated with 10^4 CFU/mL LAB (low IL-10 levels). No difference in SOCS1 expression could be observed between cells co-cultured with LAB or E. coli.

SEA induced high levels of CIS and SOCS1, but not SOCS3 at 16 and 24 hours. SEA, as demonstrated in our cytokine experiments, elicits generally high cytokine levels, excepting IL-10. The comparably low IL-10 expression by this superantigen delivers an explanation for the different results of SEA and E.coli. While in the E.coli group high IL-10 levels probably raise SOCS3 expression, in CBMCs co-cultured with SEA the SOCS3 level was found to be comparatively low, possibly due to the lack of IL-10.

Interestingly, the SOCS1/SOCS3 ratio differed significantly between LP and EC. The SOCS1/SOCS3 ratio elicited by SEA was extremely high, owing to low SOCS3 levels due to IL-10 suppression.

In summary, we demonstrated a Th1-skewing impact of LL and LP on the Th1/Th2 cytokine balance in cord blood mononuclear cells, as well as a dose- and strain-dependent effectiveness of LAB. Furthermore, SOCS expression is induced by gram-positive and gram-negative bacteria before cytokines are released. This points to direct activation of the SOCS gene via the TLR in CBMCs. This study highlights the strain specificity of probiotics reflected in distinct cytokine production and SOCS expression patterns.

5.1 References

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