



DISSERTATION

Titel der Dissertation

**“Allergen-induced regulatory properties of the
CD4⁺CD25⁺ subset in cord blood and possible
implications on the post-natal tolerance induction”**

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Verfasserin:	Mag. Elisabeth Mayer
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Betreuer:	Univ.-Prof. Dr. Ibrahim Elmadfa

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"The most exciting phrase to hear in science,
the one that heralds new discoveries,
is not 'Eureka!' ('I found it!'), but rather 'hmm....that's funny... '"

- *Isaac Asimov* -

"In der Wissenschaft beginnt alles Neue nicht damit,
dass jemand Eureka! ('Ich hab es entdeckt!') ruft,
sondern eher 'Hm ..., ist ja komisch...'"

- *Isaac Asimov* -

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Abbreviations

AA	Amino acid
Ab	Antibody
α -CD3	anti-CD3
AD	Atopic dermatitis
ADA	Adenosine deaminase
Ag	Antigen
AIRE	autoimmune regulator gene
APC	Antigen presenting cells
APC	Allophycocyanin
Ara h x	Arachis hypogaea allergen
Bet v x	Betula verrucosa allergen
BLG	β -lactoglobulin
BLG [LF]	LPS-free β -lactoglobulin
Bq	Becquerel
BSA	Bovine serum albumin
CBMC	Cord blood mononuclear cell
CD	Cluster of differentiation
cDNA	Complementary DNA
CFSE	5-(-6)-Carboxyfluorescein-diacetat-succinimidyl-ester
Ci	Curie
CMP	Cow's milk protein
Cpm	Counts per minute
CT	Cycle threshold
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen-4
DC	Dendritic cell
dCT	Delta cycle threshold
dest.	Destillata (<i>lat.</i>); distilled
DMSO	Dimethylsulfoxide
DNA	Desoxyribonucleinacid
EDTA	Ethylendiamintetraacetat
EHF	Extensively hydrolyzed formula
ELISA	Enzyme-linked immunosorbend assay
ERK	Extracellular-signal-regulated-kinase
EU	Endotoxin unit
F	Fraction
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FITC	Fluorescein-isothiocyanat
g	Acceleration of gravity = 9.81 m/s ²

GALT	Gut associated lymphoid tissue
GI	Gastrointestinal tract
GM-CSF	Granulocyte-macrophage colony-stimulating-factor
Gy	Gray
HEK293	Human embryonic kidney cells 293
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ISAAC	International study of asthma and allergies in childhood
kDa	Kilodalton
LAG3	Lymphocyte-activation gene 3
LPS	Lipopolysaccharide
LPS UP	Lipopolysaccharide ultra pure
mAb	Monoclonal antibody
MACS	Magnetic activated cell sorting
MHC I/II	Major histocompatibility complex
Min.	Minute
mRNA	Messenger RNA
M_{lim}	Molecular mass exclusion of polysaccharide
MFI	Mean fluorescence intensity
M_w	Molecular weight
NK cell	Natural killer cell
NK T cell	Natural killer T cell
OAS	Oral allergy syndrome
OD	Optical density
PARSIFAL	Prevention of allergy - risk factors for sensitization related to farming and anthroposophic lifestyle
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PE	Phycerythrin
PFA	Paraformaldehyd
PHA	Phytohaemagglutinin
PHWF	Partially hydrolyzed whey formula
RAST	Radioallergosorbent test
RNA	Ribonucleic acid
ROR	Retinoic acid receptor-related orphan receptor
rpm	Rounds per minute
RPMI	Roswell park memorial institute (medium)
RT-PCR	Reverse transkriptase PCR
SD	Standard deviation

Abbreviations

SEM	Standard error of the mean
sIgE	Specific IgE
SPT	Skin prick test
TCR	T cell receptor
Teff	Effector T cell
TEMED	Tetramethylethylenediamid
TGF- β	β -transforming growth factor
Th	T helper cell
TLR	Toll-like receptor
TNF- α	Tumour necrosis factor- α
Tr1 cell	T regulatory 1 cell
Treg	Regulatory T cell
Tris	2-Amino-2-hydroxymethyl-propan-1,3-diol
TRITC	Tetramethyl-rhodamin-isothiocyanat
TT	Tetanus toxoid
TWEEN 20	Polyoxyethylensorbitanmonolaurate
UCC	Ultra culture complete medium

1 INTRODUCTION AND AIMS

The postnatal period is known to be crucial for tolerance development after birth. However the underlying mechanisms of the process remain unclear. Former avoidance strategies are under debate and epidemiological studies describe an inverse association between the level of environmental endotoxin exposure within the first months of life and the prevalence of allergic disease in children.

Cord blood derived mononuclear cells, and in specific T cells, respond to ubiquitous environmental antigens by proliferation upon antigen exposure. This has been mainly attributed to trans-placental priming. Thornton and Upham et al. suggested that proliferative reactivity in cord blood does not refer to the conventional memory T cell population present in cord blood. In addition this type of response was characterized by an antigen-driven apoptosis exceeding those detected in peripheral blood mononuclear cells (PBMCs) from adult individuals on the one hand and the generation of CD4⁺CD25⁺ regulatory cells on the other hand. More recent publications suggest that antigen-specific response in cord blood differ markedly from those occurring later in life and may be less specific than originally suggested. Thus allergen-specific proliferative response in cord blood may reflect a functional immature state of the regulatory T cell (Treg) compartment. To date it remains unclear whether these “heightened” responses can be explained by endotoxin contamination detected in commercially available allergen preparations, by transplacental priming, by the recent thymic emigrants or whether this increased proliferation may relate to diminished, immature functional properties of the regulatory T cell compartment.

Cord blood was used as a model system for a naïve, human system, unexperienced to antigens. Peripheral blood derived mononuclear cells of adult donors were used as control to delineate Tregs in an adult system as well. Endotoxin contaminations in allergen preparations (in particular beta-lactoglobulin (BLG), the major milk allergen) have been proved to affect antigen-specific proliferative response in the murine system. Impact of such potential confounders remains to be described for the naïve human system. The properties of the regulatory T cells in cord blood in the presence of endotoxins and allergens are poorly defined and will be assessed in detail in this thesis.

The aim of this thesis is to define the nature of allergen-specific T cell responses in cord blood *in vitro* and to delineate differences in adults. Special emphasis will be put on nutritional allergens from milk (β -lactoglobulin (BLG)) and peanut (Ara h 1), which represent the most important group of allergens within the first years of life. The recombinant birch pollen allergen rBet v1 will serve representative for inhalational and recombinant allergens.

Moreover the control mechanisms will be of interest to investigate differences in the regulatory T cell subset in cord and peripheral blood at a phenotypic (Treg surface (e.g. CD25 and CD127) and intracellular (e.g. FoxP3) markers and a functional level (inhibition assays).

2 LITERATURE REVIEW

2.1 Allergy

In 1906 the Viennese pediatrician Clemens von Pirquet conceived the term “αλλεργία” (allergy) from the Greek *άλλο* (*allos*; changed or altered state) and *έργο* (*ergon*; reaction or reactivity)³. Allergy is defined as “the immune system’s hypersensitivity to a foreign substance. Typically harmless substances, such as pollen, food, animal dander, stings, mould or drugs lead to a switch in the reactivity of an individual. Upon re-stimulation with this antigen the immune system reacts as if the substance was harmful whereas no response occurs in non-allergic people”^{3,4} (Figure 1).

The prevalence of allergies has risen extensively in the past two decades in industrial nations⁵ and asthma has become the most common chronic disease⁶ making allergy a significant public health burden. However, recent studies could show a plateau or abatement in these prevalence rates^{7,8}.

In Austria per contra, in contrast to other European countries, Schernhammer et al (2008) reported a significant increase in the prevalence rates of allergy such as asthma, allergic rhinitis, and eczema in schoolchildren (ISAAC study)⁹. Additionally, the prevalence of allergic sensitization also increased significantly from 1996 to 2006 in a Swedish cohort¹⁰ as well as in Greenland¹¹ and Ghana¹¹.

The underlying cause has not been clarified yet and although it is known that the development of allergies are influenced by genetic, developmental, and environmental factors the influence of allergens and lipopolysaccharide on neonatal tolerance induction remains to be answered.

Atopy:

“an exaggerated tendency to mount IgE responses to a wide variety of common environmental allergens¹”.

Allergy:

“an enhanced or inappropriate immune response initiated by exposures to antigens such as mould spores, pollen, or certain drugs and foods together with an increased IgE level.

It may cause symptoms such as allergic conjunctivitis, pruritus, urticaria, runny nose, and asthma¹.”

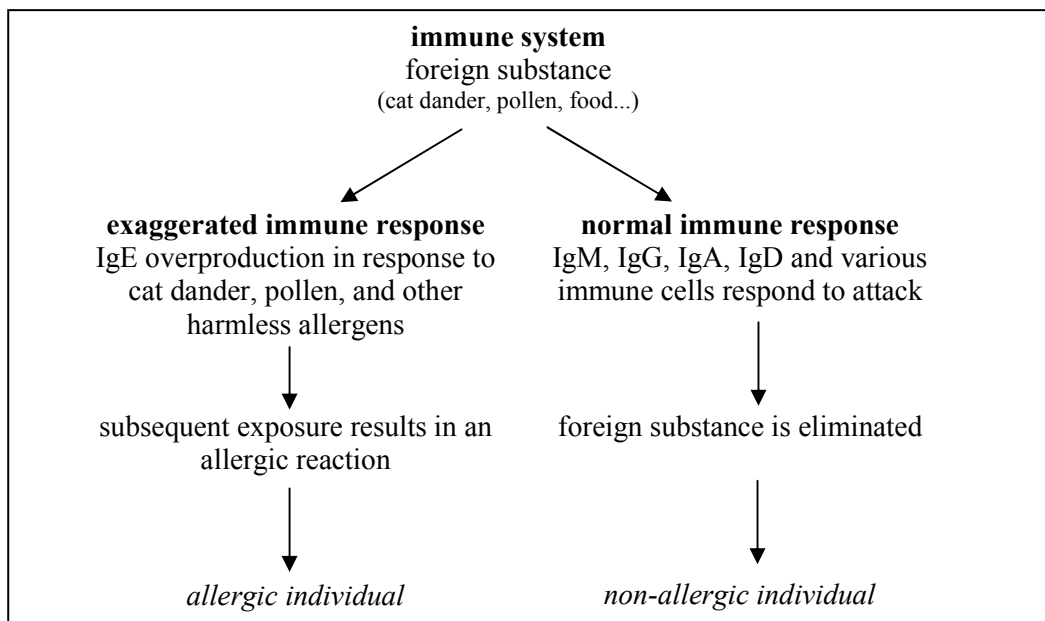


Figure 1 A simplified schema for the immune system's response to become allergic or non-allergic¹

The naïve immune system responds to foreign, harmless substances (cat dander, pollen, food...) with an exaggerated immune response, for instance IgE overproduction, as a preliminary stage to allergy. In case of a “normal” immune response the foreign substance is eliminated without an allergic reaction.

2.1.1 Immunological Sensitization

An immunological sensitization always precedes allergy, although not every sensitization necessarily succeeds in clinically manifest allergy. The underlying mechanisms leading to immunological sensitization are very complex and not yet completely understood.

Sensitization most likely occurs via the inhalational or gastrointestinal route. After entering the mucosa antigen presenting cells (APC) incorporate the protein, process it very efficiently and present peptide epitopes on the major histocompatibility complex (MHC) class II for T helper (Th) cells. This may form the initial event to induce the differentiation of a naïve CD4⁺ T cell with the respective T cell receptor (TCR) direction towards Th2. These CD4⁺ T helper cells with the characteristic Th2-type cytokine production (interleukin (IL)-4, IL-13 and IL-5) are the first step towards

sensitization¹². IL-4 and IL-13 lead to an isotype switch from IgG to IgE production in B lymphocytes and IL-5 recruits and activates eosinophils¹. Antigens that selectively evoke Th2 cells that drive an IgE response are known as allergens. Presence of very low antigen doses favour the activation of Th2 cells over T helper 1 (Th1) cells. Additionally, peptides that bind strongly to the TCR are more likely to stimulate a Th1-like response. On the other hand, peptides that interact weakly with the TCR provoke a Th2 response. Since allergens commonly cross the mucosal barrier in low doses, a Th2 response is more likely¹.

The IgE antibody binds with its Fc part to the high affinity receptor (FcεRI) which is located on mast cells, basophile granulocytes, and activated eosinophils. IgE production can be further amplified as these cells produce IL-4 and CD40 ligand upon activation. Cells are thereby “sensitized” and can survive for a long time due to the stability of the IgE-receptor-complex. Re-exposure leads to cross linking of IgE molecules and the release of inflammatory mediators (e.g. histamine – responsible for a prompt increase in the local blood flow and vessel permeability, mast-cell chymase, tryptase, and serine esterases) that cause the allergic reaction (Figure 2). The likelihood for an increased IgE production is influenced by hereditary factors. Due to a predisposition to develop Th2 type responses, atopic individuals are more prone to develop allergies.

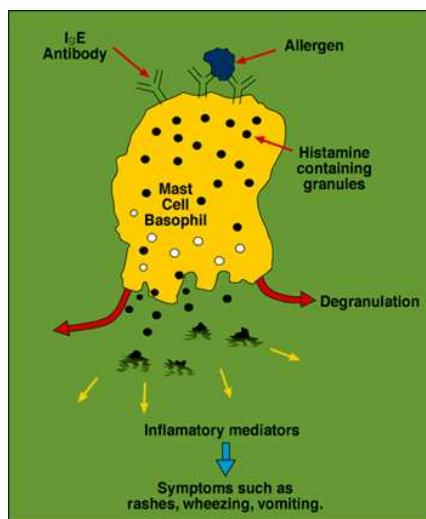


Figure 2 An IgE induced mast cell¹³

IgE binds to the FcεR on mast cells and basophils and therefore leads to a mediator release which causes allergic symptoms such as rashes, wheezing and even vomiting.

2.1.2 Food Allergy

Food allergies have significantly increased over the last few decades¹⁴. Speaking of which, it is necessary to differentiate between food allergy and food intolerance. Food allergy derives from failing to establish and/or maintain oral tolerance in infancy¹⁵ whereas food intolerance is not immune-mediated and represents a negative reaction to foods. This is caused by the lack of enzymes or chemicals to digest food (e.g. lactase deficiency in lactose intolerance).

Allergies can be classified into an IgE, non-IgE or a mixed response. Clinical symptoms range from milder IgE-mediated responses like rhinoconjunctivitis, gastrointestinal symptoms, urticaria, and exacerbation of atopic dermatitis to life threatening conditions like angioedema, asthma, or even anaphylaxis.

Mast-cell degranulation, as first-line defence after allergen encounter of allergic patients, follows a more persistent inflammation the late phase response. This phase is characterized by the recruitment of Th2 cells, basophils as well as eosinophils and are crucial for the severity of the allergic response. Lipid mediators (e.g. leukotrienes) synthesized by mast cells are able to cause smooth muscle contraction, mucus secretion, an increased vascular permeability and importantly also the activation of leukocytes, which are responsible for the late-phase response. On account of these mechanisms diarrhoea and vomiting are likely to occur because of the smooth muscle contraction and transepithelial fluid loss. In the dermis urticaria can result from activation of connective tissue mast cells¹. In case of allergens that are rapidly absorbed from the gut or directly enter the bloodstream, activation of the connective tissue mast cells that are associated with all blood vessels, can cause the life-threatening systemic anaphylaxis. The increase of the vascular permeability can cause loss of blood pressure, difficulties in breathing because of airways constrict, and most fatal suffocation by swelling of the epiglottis: the anaphylactic shock.

Food allergy is one of the primary causes for anaphylactic shock medicated in emergency departments, in children. Approximately 30,000 anaphylactic shock reactions are reported in the United States every year, including 200 deaths. The anaphylactic shock can be controlled by an immediate injection of epinephrine, which instantly relaxes the smooth muscle and inhibits cardiovascular effects.

Food allergy is fairly common in childhood and data from the 90's suggests that approximately 6-8% of infants would be affected in the first three years of life ¹⁶. However this data had to be adjusted to a lower prevalence in the recent years. The perceived, self-reported prevalence is always more frequent than allergies evaluated through objective measures objective measures and may vary from 3.24% to up to 34.9% surveyed by Madsen in 2005 ¹⁷. This can be explained by that in most cases no difference is made between food allergy and food intolerance. In a meta-analysis of 2007 the prevalence of food allergy varied from 1.2% to 17% for milk, 0.2% to 7% for egg, 0% to 2% for peanuts and fish, 0% to 10% for shellfish, and 3% to 35% for any other food ¹⁸. In a randomized telephone survey conducted in 10 European countries (Austria, Belgium, Denmark, Finland, Germany, Greece, Italy, Poland, Slovenia and Switzerland) parents were interviewed with a standard questionnaire about their children's allergies. 8,825 children could be reached resulting in a parentally perceived food allergy prevalence of 4.7%. Still, great differences between the countries could be observed and the prevalence ranged from 1.7% in Austria to 11.7% in Finland. Medical treatment was only reported for 75.7% of the allergic children and so an actual 3.55% food allergy was calculated ¹⁹.

Cow's milk and hen's egg are the most frequent food allergens in infants and the corresponding allergic diseases often resolve spontaneously. In adolescence and adulthood peanut and tree nuts are the most prominent food allergens. They are taken later in life, are more likely to persist and are more severe as even trace amounts can cause an anaphylactic shock ²⁰. Thus, peanut allergy is a significant public health problem, especially in schools, when children are unintentionally exposed to peanuts ¹. The Multicenter Allergy Study (MAS) recorded specific IgE levels of 216 children to a panel of allergens in yearly intervals. During the first six years of life, sensitization to food allergens decreased from 10% (at 1 year) to 3% (at 6 years) ²¹.

Oral tolerance is most likely to develop to food proteins with epitopes that are grouped in a conformational structure (as in milk, egg, wheat, and soy). Vice versa it is also more unlikely to outgrow food allergy when the epitopes are arranged in linear binding epitopes (as in nuts, seeds, and fish) ²². Several methods are used to verify a suspected food allergy: patient's history, the measurement of specific immunoglobulin E antibodies (sIgE) via RAST (radioallergosorbent test), the newer ImmunoCAP, skin

prick testing (SPT), and the atopy patch test. The golden standard to determine food allergy still is the double-blind, placebo-controlled food challenge (DBPCFC) ²³.

Food allergen-specific CD4⁺ T lymphocytes synthesize high levels of IL-4 and IL-13 which are known to initiate IgE synthesis and to be involved in determining the site of allergic inflammation ²⁴. Regulatory T cells in particular, such as TGF- β producing Th3 cells, IL-10-producing Tr1 cells as well as natural killer T (NKT) cells are involved in oral tolerance induction ²⁵. Natural Tregs have become of the focus of increased interest as they may be involved in the prevention of sensitization to allergens. Feeding experiments with mice and guinea pigs showed that repeated allergen application induced activation of functional Tregs and therefore prevented anaphylaxis ²⁶⁻²⁸. At present no definitive treatment for food allergy is available ²⁹. Two ways of treating food allergy are used in clinical practice: desensitization on the one hand and blockade of the effector pathways on the other hand ¹.

Specific immunotherapies (SIT) such as subcutaneous immunotherapy (SCIT) and the non-invasive sublingual immunotherapy (SLIT) are acknowledged methods to induce tolerance. However not for food allergies. The underlying mechanism why SIT does not work for food allergies is still an enigma. The efficacy is well documented only for grass pollen- and mite-allergies as reviewed in Cox et al (2006) ³⁰. SLIT at an early stage could decrease the risk by 3.8 fold of getting asthma as documented in a multicenter study ³¹. For food allergy SIT is not recommended because of an unacceptable high rate of adverse systemic reactions ³². Moreover, daily allergen application is mandatory, because cessation of application impairs the tolerance ³³. Additionally, in some patients not even the highest dilution of the allergen is tolerated so that not all patients completed the immunosensitization. However increased allergen doses, compared to the beginning, are tolerated in most studies ³⁴. Based on existing data the underlying mechanisms to induce tolerance may differ for SCIT or SLIT.

It could be shown in mice that a continuous feeding of an allergen induces suppression of Th1 and Th2 type responses ³⁵. Moreover, a significant decrease of specific IgE together with an increase of specific IgG4 after 6 and 18 months, respectively, was observed ³⁴. Oral immunotherapy appears to be a promising approach in treating food allergy, however new, optimized protocols with fewer side effects have to be established.

2.1.2.1 Cow's Milk Allergy

Cow's milk allergy (CMA) is very common in infancy. Five to 15% of infants show symptoms of CMA³⁶. IgE specific to milk, is together with egg, the earliest serological marker for atopic immunoreactivity in infancy²¹. Cow's milk contains 3.3% total protein. Milk protein has two main components which can be distinguished according to their chemical composition and physical properties. Caseins contain phosphorus and comprise about 83% of the milk proteins. Whey (serum), unlike caseins, remains in solution at pH 4.6 and comprises the remaining 17%. The major cow's milk allergens, all proteins, are α -casein, β -casein, κ -casein, and the whey proteins β -lactoglobulin (BLG) and α -lactalbumin³⁷ (Table 1). As BLG can be detected in breast milk, sensitization is possible even in the first months of life³⁸. Proteins from cow's milk and hen's egg are the main cause for anaphylactic shock in infants^{39,40}.

CMA's commonly resolved within the first five years of life and consequently forms an interesting model for the development of oral tolerance.

Component	% Total	% Casein	% Whey
Caseins	83		
Alpha s1 casein	36	44	
Alpha s2 casein	9	11	
Whey	17		
Beta-lactoglobulin	10		58
Alpha-lactalbumin	2		13

Table 1 Components of milk protein⁴¹

The major cow's milk proteins are listed in this table.

2.1.2.1.1 β -lactoglobulin

Beta-lactoglobulin (BLG) is the most allergenic and most abundant protein in whey. It comprises 10% of the total milk protein and 55–65% of the total whey protein⁴². It encloses 162 amino acids with approximately 18,300 Da and has been studied to a great extent over the few last years⁴³ (Figure 3).

At pH values in the range of 5.1 to 8.0, which also includes the pH of milk (pH 6.7), β -lactoglobulin exists as a dimer. Below pH of 3.0 and above pH of 8.0, β -lactoglobulin

exists in its monomeric form. Between a pH 3.1 and 5.1, at high protein contents and low temperatures, it even forms octamers ⁴⁴.



Figure 3 β -lactoglobulin ⁴⁴

The 3D structure of β -lactoglobulin from the protein data bank (PDB) entry.

Beta-lactoglobulin is synthesized in the mammary gland and even though it has a well-defined structure, its role is still an enigma. Although various roles have been proposed, including the phosphate metabolism in the mammary gland ⁴⁵ or in the transfer of passive immunity to the newborn ⁴⁶, no function has been definitively ascribed to this protein. In general, milk from ruminants like cattle, sheep, goats, and deer and of some non-ruminants, such as pigs, horses, dolphins, kangaroos, cats, and whales, contains β -lactoglobulin. Humans on the other hand, as well as rodents lack this protein. This may explain why the allergenicity of β -lactoglobulin is so high. As β -casein and β -lactoglobulin from bovine and equine milk share a higher degree of homology with human milk, breastfed children have a chance of being sensitized to β -lactoglobulin even if the mother's diet is completely milk free ⁴⁷.

2.1.2.2 Peanut Allergy

Peanut (*Arachis hypogea*) is botanically classified to the family of pulses (Leguminosae). Allergies are often severe and typically permanent. Recent studies from North America and the United Kingdom (UK) place peanut allergy rates among children near or slightly over 1% ^{48, 49}. Compared to milk allergy, only 20% of young children lose their allergy by school age ⁵⁰. Grundy et al (2002) reported a doubling in peanut allergy and a triplication in peanut sensitization during the last years ⁴⁹.

2.1.2.2.1 Peanut Allergens

Peanut is a prominent food in the world due to its high nutrition and low cost. However, it is also the food item that is most likely to cause an anaphylactic shock as even trace amounts of peanut are sufficient to cause an allergic reaction ^{20, 51}. Most

peanut allergens are heat-stable, water-stable reservoir proteins with sizes ranging from 7,000 up to 65,000 Da. To date, nine different peanut allergens have been identified. The allergens were numbered consecutively from Ara h 1 to Ara h 9 according to the find ⁵². Ara h 1 and Ara h 2 are considered to be the major allergens because more than 95% of peanut-allergic patients recognize them ⁵³.

In the USA, about 1.3% of adults have a peanut or a tree nut allergy ⁵⁴ and 100% of all children at two years of age have already consumed peanut-containing food ⁵⁵.

It seems that peanut avoidance is the only evidence-based treatment for peanut allergy. Early reports of “desensitizing” patients with oral or subcutaneous administration were poorly controlled and therefore not compelling ^{56, 57}. However, newer placebo controlled studies ³⁴, one now being conducted by a group in the UK led by Hugh Sampson, could confirm the effect of immunotherapy ⁵⁸.

2.1.3 Inhalational Allergy

Worldwide 10-20 million people suffer from pollen allergies. Approximately one-third exhibits allergic reactions to tree pollen⁵⁹. In the temperate climate zone, Bet v 1, a major birch pollen allergen, accounts for most cases of tree pollinosis. Approximately 20% of the people worldwide suffer from hay fever caused by pollen from trees (e.g. birch, alder...), shrubbery (e.g. haze...), grass, grain (e.g. rye...) and herbs (e.g. mugwort...). Allergic patients suffer from symptoms like watery, itchy eyes, coryza, sneezing, plugged nose, coughing, dyspnoea, asthma, gastrointestinal symptoms, eczema, headache, migraine, insomnia, fatigue, depression, and in 30-40% of all cases bronchitis or asthma follow⁶⁰.

2.1.3.1 Birch Pollen Allergens

In Europe birch pollen allergy ranks among the main causes of allergy from spring to early summer⁶¹. Bet v 1 (*Betula verrucosa*), the major birch pollen allergen and one of the most frequent environmental allergens, has a molecular mass of 17,000 Da⁶². About 60% of birch pollen allergic patients react exclusively to this allergen and about 96% that are allergic to tree pollen also display IgE antibodies to Bet v 1⁶³. Recombinant Bet v 1, as the major allergen, is used for immunotherapy for birch pollen allergy. Other birch pollen allergens known and classified are Bet v 2, Bet v 3, and Bet v 4. Birch pollen is responsible for cross-reactive food allergies. Between 50% and 93% of birch pollen-allergic individuals develop cross-reactions to e.g. apples, hazelnut, carrots, and celery. This is due to cross-reactive IgE antibodies to Bet v 1^{64, 65}. In case of birch pollen allergy, Mal d 1 (*Malus domestica*), the major apple allergen, bears the highest cross reactivity. IgE antibodies of most Bet v 1 allergic patients can bind Mal d 1 regardless of clinical reactivity with apples⁶⁴. Next to the humoral cross-reactivity between pollen and food allergens, cross-reactivity at the T cell level has been postulated as well^{66, 67}. Mal d 1 shares 64% of its amino acid sequence with Bet v 1 and cross-inhibition experiments demonstrated the presence of common IgE epitopes⁶⁸. Dau c 1 (*Daucus carota*) from carrot and Api g 1 (*Apium graveolens*) from celery also have a high cross-reactivity potential for birch allergic people.

Recently, Bet v 1-specific T cells were discovered in allergic patients, shedding more light in the processes happening at the cellular level. Those specific cells mostly secrete Th2-type cytokines like IL-5, some IL-10, but no IFN- γ upon allergen contact. Surprisingly, also healthy, non-allergic, individuals possess Bet v 1-specific T cells, but with fundamental differences. These cells secrete Th1-type cytokines like IFN- γ , and IL-10 in response to allergen contact ⁶⁹. Moreover, they express markers for regulatory T cells such as CTLA-4, GITR, and FoxP3.

A very recent paper by Mobs et al (2010) ⁷⁰ could show an increase in Bet v 1-specific, IL-10 secreting T regulatory 1 (Tr1) cells combined with a decreased ration of IL-5 producing Th2/Tr1 cells upon specific birch pollen immunotherapy. Interestingly, the number of CD4⁺CD25⁺CD127^{low} regulatory T cells did not change. Moreover the allergen-specific IgE and IgA levels remained the same whereas the allergen-specific IgG levels increased ⁷⁰. Another recent study by Grindebacke et al (2009) could show elevated IL-10 levels of SIT-treated patients as well as a stable expression of FoxP3 and CD25^{high} Tregs compared to allergic controls throughout the study ⁷¹. Interestingly, both studies seem to favour suppression due to an increase of Tr1 cells and not due to a reduction or suppression of Th2 cells ⁷⁰⁻⁷². The therapeutic approach favours an allergy vaccine that should epitomize protective Th1/regulatory T cell responses ⁶⁹.

2.2 T Cells

2.2.1 T Helper Cell Subsets

T helper cells are a subgroup of lymphocytes that mature in the thymus and express special surface markers (e.g. CD3 and CD4). The subsets are distinguished by their specialized cytokine profile and protect the body from many types of pathogens⁷³. Th cells per se do not possess phagocytic or cytolytic activity but are crucial for activating and directing other immune cells, such as macrophages, mast cells, eosinophils, neutrophils, natural killer (NK) cells, and B cells⁷⁴. When naïve, CD4 positive ($CD4^+$) T cells only produce IL-2 and they pass a transient, pre-activation state (Th0) to become either Th1, Th2, Th9, Th17 or T follicular helper (Tfh) cells, depending on the co-stimulatory and cytokine signals they receive (Figure 4).

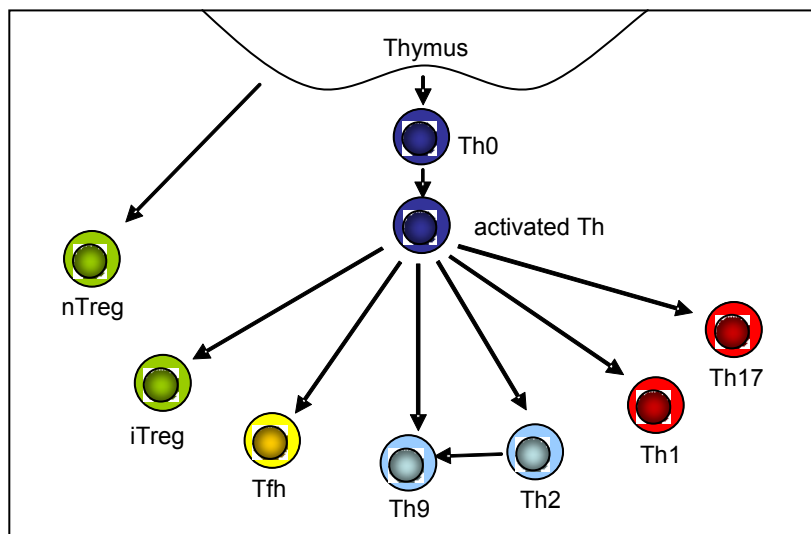


Figure 4 T helper cell subsets

Precursor T cells derive from hematopoietic stem cells in the bone marrow and have to pass various differentiation stages until maturing into functional T cells. Major Th-cell subsets include Th1, Th2, and Th17, and also recently discovered Th9 and Tfh cells. Regulatory T cells can either derive directly from the thymus (nTregs) or can be induced upon stimulation from Th cells (iTregs).

2.2.1.1 Th1 Cells

T helper 1 (Th1) cells are characterized by their production of IL-2, TNF- α , TNF- β and IFN- γ . They are involved in the cellular immune response and host defence fighting intracellular pathogens and viruses, as well as cancer cells. IFN- γ , for example, activates the lysosome activity in macrophages to destroy microorganisms. They express the transcription factors T-bet and STAT-4 and have been linked to many chronic auto-inflammatory disorders, such as multiple sclerosis, diabetes, and rheumatoid arthritis ¹.

2.2.1.2 Th2 Cells

T helper 2 (Th2) cells can be distinguished by their production of mainly IL-4, IL-5, IL-6, IL-10, and IL-13 ¹. They are involved in the humoral immune response and host defence fighting extracellular organisms by up-regulating antibody production. Th2 cells with the transcription factors GATA-3 and STAT-6 are responsible for tolerance to xenografts or the foetus during pregnancy and play a role in allergic responses. IL-4 is responsible for antibody production and IL-5 on the other hand stimulates eosinophil response, both are directed toward extracellular parasites ¹.

2.2.1.2.1 Th1/Th2 Balance

The Th1/Th2 paradigm is a oversimplification, insufficient to explain such a Gordian phenomenon, but will be explained here for completeness. The homeostasis between Th1 and Th2 cells, distinguished by their different cytokine production, was a model system for explaining immune regulation that arose in 1986 by the observation that T helper cells in mice express different cytokine patterns ⁷⁵. This hypothesis was modified for humans with Th1- and Th2 cells ^{76, 77} and suggests that those cell types counter-regulate each other.

A homeostasis between Th1 and Th2 cells indicates a healthy immune system, whereas an excess of Th-specific cytokines leads to either an inflammation or allergic disease, respectively.

However, in spite of all the research focussing on the Th1 or Th2 cytokines and on their potential to modify the immune response, many other dynamic factors influence the Th1 and Th2 cell maturation such as nature of the antigen, antigen dose, direct cell-

to-cell interaction with APCs, and the cytokine receptors available on naïve cells (as reviewed in Kidd et al ⁷⁸).

2.2.1.3 Th17 Cells

For almost two decades the Th1/Th2 model was used to explain immuno-regulation and the interaction between innate and adaptive immunity and for allergy this schema is still in use. However the fact that IL-17 is a product of activated CD4⁺ T cells has been known for over a decade. Only recently it was discovered with the unique characteristics of preferentially producing IL-17, therefore called Th17 cells ^{79, 80}. This previously unknown branch of T helper cells - the Th17 lineage was defined in 2005 by Harrington et al ⁸⁰. This additional cell subset mediates autoimmune diseases and is characterized by a production of IL-17A, IL-17F, IL-21, IL-22, and IL-26 ^{80, 81}.

They are located in the skin and the gastrointestinal tract, as well as in the interface between the external and the internal environment to fight bacteria. They do this by recruiting e.g. neutrophils to get rid of the intruders by secreting inflammatory cytokines like IL-6, IL-8, IL-13, IL-23, GM-CSF, CXCL5, and metalloproteinases.

Nevertheless, inflammation is a “double-edged sword”. IL-17 is overproduced in autoimmune diseases like inflammatory bowel diseases, multiple sclerosis, rheumatoid arthritis, and psoriasis (reviewed in Chen et al ⁸²). As a consequence, activation of these cells leads to the recruitment of granulocytes and maintenance of chronic tissue inflammation ⁸³. Cytokines such as IL-1, IL-6, TGF- β and IL-23 lead to a Th17 differentiation. The transcription factor retinoic acid receptor-related orphan receptor γ t (ROR γ t) regulates Th17 differentiation as shown in table 2.

2.2.1.4 Th9 Cells

Th9 cells, a distinct Th subpopulation, have first been described by Veldhoen et al (2008) only recently ⁸⁴. Th9 cells can either differentiate from Th2 cells in the presence of TGF- β , a cytokine that is also crucial for differentiating Th17 cells, or directly from naïve CD4⁺ T cells together with IL-4 and TGF- β . Although the IL-9 production has always been ascribed to Th2 cells, recent publications suggest that the lately discovered

Th9 cells are the main source for this cytokine ⁸⁴. These cells may be involved in asthma and tissue inflammation ⁸⁵ and are known to fight extracellular parasites. The main transcription factors are early GATA-3 and STAT-6, identical to Th2 cells (Table 2). Clear evidence for the existence of this lineage in humans remains lacking.

2.2.1.5 Tfh Cells

Follicular helper T (Tfh) cells are a recently (2009) discovered T cell subset that is characterized by providing help for B cell responses ⁸⁶ and displays one of the most important subsets of effector T cells in the lymphoid tissue ⁸⁷. They can be discriminated from other T helper subsets phenotypically by a high CXCR5 together with a low CCR7 expression that aid their homing to B cell follicles ⁸⁶, location/migration as well as their function in helping B cells. The main transcription factor for Tfh cells is Bcl-6, known to inhibit GATA-3 and therefore Th2 cell differentiation ⁸⁸. Tfh cells produce IL-21, which can stimulate B cell differentiation into Ab-forming cells through IL-21R ⁸⁷ (Figure 4; Table 2).

Type	Cytokine Stimulus	Master Transcription Factor	Effector Cytokines	Effector Functions	Pathological Effects
Th1	IL-12	T-bet	IFN- γ	Immunity to intracellular pathogens	Autoimmunity; cell-mediated allergies
Th2	IL-4	GATA-3	IL-4	Immunity to extracellular pathogens	Asthma and IgE-mediated allergies
Th17	TGF- β + IL-6	ROR γ	IL-17 IL-22	Immunity to extracellular bacterial and fungal infection	Autoimmune diseases
Treg	-	FoxP3	TGF- β IL-10	Regulation, Tolerance	Cancer?
Treg	TGF- β - IL-6	FoxP3	TGF- β IL-10	Regulation, Tolerance	Cancer?
Th9	TGF- β IL-4	GATA-3 (early)	IL-9	Immunity to parasites	Unknown
Tfh	IL-21 IL-6	Bcl-6	IL-21	T cell help for B cells	Unknown

Table 2 Th1, Th2, Th17, Tregs, Th9, and Tfh: an overview modified after Kimball et al.⁸⁹

It can be presumed that current T helper subsets will be renamed after their cytokine profile or their function in the coming years. Additionally, new T helper subsets may be discovered as this field is focus of great scientific interest.

2.2.2 Recent Thymic Emigrants, a Subset of Naïve T cells

As T cells originate in the bone marrow, mature in the thymus - undergoing the positive and negative selection - and only then migrate to the periphery, the thymus is mandatory for T cell development. Naïve T cells (Th0 cells) are already fully developed cells that have not yet encountered antigen in the periphery. These antigen-inexperienced T cells express CD45RA, CD62L, CD27, CD28, and CCR7 but lack expression of CD45RO, CD11a, CD95, CXCR3, and CCR4⁹⁰, and activation markers such as CD25, CD69 and CD44. In this naïve state, T cells are characterized by quiescence and hypoproliferation as well as the crucial requirement for IL-7 together with self-peptide/MHC molecules for homeostatic survival⁹¹.

A subgroup of naïve T cells, recent thymic emigrants (RTE), are characterized by their high content of T cell receptor excision cycles (TRECs), which are stable DNA epitopes that are formed during TCR rearrangement. They do not replicate during mitosis⁹² and so they dilute out after every cell division⁹³. Therefore the TREC content is a very good marker for RTE⁹⁴. Apart from the TREC content, CD31 (PECAM-1; platelet endothelial cell adhesion molecule) is an excellent marker for RTE to distinguish CD31⁺ ^{thymic}naïve (RTE) and CD31⁻ ^{central}naïve CD4⁺ T cells in the peripheral blood of healthy humans⁹⁵. In cord blood and neonates CD31⁺ ^{thymic}naïve RTE dominate with a percentage of 90% to 95% of the CD4⁺CD45RA⁺ T cell pool whereas in adults the majority of the naïve T cell pool lacks CD31 expression (CD31⁻ ^{central}naïve)⁹⁶. RTE decrease during ageing and this correlates with the decline in TRECs⁹⁷. Interestingly, the TREC amount decreases 50 to 100 fold⁹⁸ whereas the naïve T cell amount (CD45RA⁺) decreases only by a factor of 2 or 3 with age⁹⁹. This can be explained by a considerable expansion of naïve T cells in the periphery (Figure 5) whereas the RTE dilute out during age. Furthermore, RTE may differentiate into ^{central}naïve CD4⁺ T cells upon contact with self-peptides and MHC. Both populations however, ^{thymic}naïve and ^{central}naïve T cells, can differentiate to memory/effector CD45RO⁺ T cells upon antigen exposure¹⁰⁰.

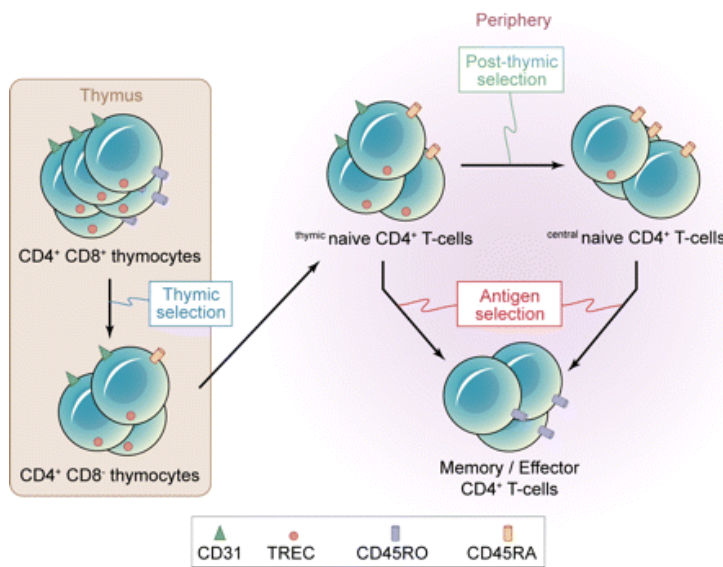


Figure 5 Naïve human CD4⁺ T cells¹⁰⁰

T cells undergo selection and maturation in the thymus. RTEs emigrate directly from the thymus and express CD31 (thymic) and a high content of TRECs (TREC^{hi}). Upon contact with self-peptides and MHC^{thymic} naïve T cells proliferate and differentiate into central naïve T cells (central naïve T cells). They do not display CD31 on their surface and also lack TRECs (TREC^{lo}). central Naïve CD4⁺ T cells originate

from the RTE in a post-thymic selection and are crucial for the maintenance of the naïve CD4⁺ T cell pool. On foreign antigen exposure, both subsets can differentiate directly into memory/effector CD4⁺ T cells.

2.2.3 Regulatory T Cells

Several mechanisms of immune tolerance have been postulated to protect self tissues. One mechanism for maintaining “self-tolerance” is eliminating self-reactive T cells during the negative selection in the thymus. The immune system itself has peripheral mechanisms to cope with cells that escape central tolerance and cells that police the immune system.

In the early 1970s the concept of regulatory T cells was described first by Gershon et al ¹⁰¹. Then, maybe also due to insufficient technologies neither the cell type nor the soluble factors could be defined. Hence, despite important studies, extensive scepticism arose about the existence of these cells ¹⁰². The concept of suppressive T cells left centre stage of immunologic research for decades.

25 years later, in 1995 Sakaguchi et al were first to find and characterize a subpopulation of CD4⁺ T cells with regulatory function in a mouse model ¹⁰³. The elimination of the suppressor cells, then newly named regulatory T cells (Tregs), led to autoimmune diseases, like autoimmune gastritis or thyroiditis. Co-transfer of those newly discovered regulatory T cells prevented the disease ¹⁰³. Three years later Thornton et al (1998) could further demonstrate that the suppressive activity of CD4⁺CD25⁺ T cells persists in *in vitro* testing ¹⁰⁴. Eventually Jonuleit and Enk (2001) could isolate a regulatory CD4⁺ T cells from peripheral blood ¹⁰⁵ and characterize them as CD4⁺ T cells expressing CD25 (the IL-2 receptor (IL-2R) α -chain), CD45RO and the cytotoxic T-lymphocyte-associated molecule-4 (CTLA-4).

Since then, research in the field of regulatory T cells exploded. Ever since researchers found out that Tregs play an important role in the pathogenesis of cancer ¹⁰⁶⁻¹⁰⁸, autoimmune disease ^{106, 109}, transplantation immunology ¹¹⁰, pathogenesis of atherosclerosis ¹¹¹, allergy and asthma ¹¹², and immune tolerance in pregnancy ^{108, 113}.

The terminology positive (+), negative (-), high (^{high}), intermediate (^{int}) and low (^{low}) comes from the field of fluorescence activated cell sorting (FACS). It characterizes the expression of the protein, either on the surface or intracellularly. CD25⁺ e.g. means that all cells express CD25 on their surface, whereas CD25⁻ indicates that those cells lack CD25. CD25⁺ again can be subclassified for their CD25 expression in CD25^{high}, CD25^{int} and CD25^{low}. However the distinction between high, intermediate and low is not strictly objective but depends on the researcher's judgement and can vary slightly.

The mechanism and the potential clinical application of regulatory T cells in the treatment of many diseases are widely but also controversially discussed as an overload of Tregs may support the survival of cancer cells as well ¹¹⁴.

CD4⁺CD25⁺ Treg cells are typified by hypoproliferation, little IL-2 but no IL-4 production. In specific, Tregs stand out by their suppressive capacity. They are able to suppress T cell proliferation, and cytokine production. In addition they are capable of suppressing the function of B cells ¹¹⁵, natural killer T (NKT) cells ¹¹⁶, natural killer (NK) cells ¹¹⁷, and dendritic cells (DCs) ¹¹⁸. After Treg removal, proliferation of peripheral blood mononuclear cells (PBMCs) from healthy individuals to various stimuli is enhanced ^{119, 120}.

Besides CD25, several other surface markers are known to be expressed on regulatory T cells (intracellular CTLA-4^{high}, surface CTLA-4^{low}, HLA-DR^{high}, GITR⁺, CD27⁺, CD28⁺, CD38⁺, CD39⁺, CD45RB^{low}, CD45RA^{low}, CD45RO^{high}, CD5⁺, CD62L^{high}, CD73⁺, LAG3, CD122^{high}, CD127^{low}, BUN^{ny}, CD103⁺, CD130⁻, OX40^{high}, LFA-3^{int}, CCR4⁺, CCR7⁺, CCR8⁺, TNFR2⁺).

Recently, GARP (LRRC32) ¹²¹ and latency-associated peptide (LAP) ¹²² have been implicated to be preferentially expressed in Tregs activated through the TCR. GARP expression on the surface was postulated to mediate suppressive activity and to result in a Treg phenotype of increased FoxP3, CD25 and CD62L expression, a decreased IFN- γ and IL-2 production as well as an impaired proliferative capacity ¹²³⁻¹²⁵. On the other hand, silencing GARP in Tregs could diminish their suppressive activity ¹²³. Moreover, GARP was highly expressed in the placenta ¹²⁶ which could be crucial for a “tolerant” environment and protect the fetus against rejection.

LAP, the latency-associated peptide, was identified to be an excellent marker to identify the TGF- β producing CD4⁺CD25⁺ regulatory T cells ¹²⁷. These cells secrete TGF- β , and express surface TGF- β as well as surface receptors for TGF- β . Moreover, LAP⁺ Tregs possess elevated FoxP3 levels and Treg-associated molecules (e.g. CTLA-4 and glucocorticoid-induced tumour necrosis factor receptor (GITR)-related gene). Most importantly, cells that express LAP show an increased suppressive function via a cell contact and soluble factor dependent mechanism ¹²⁷.

Runx (runt-related transcription factor) factors are expressed on hematopoietic stem cells and three different types (RUNX 1 - RUNX 3) are known to date. They are crucial

for hematopoiesis¹²⁸ and the differentiation and function of peripheral T cells¹²⁹ as well as thymic T cell development¹³⁰. Runx proteins only possess transcriptional activity when forming heterodimers with CBF β ¹³¹ which stabilizes the Runx-DNA interaction and prevents degradation¹³². RUNX 1 (AML1, CBF α 2 or PEBP2 α B) and RUNX 3 (AML2, CBF α 3 or PEBP2 α C) are crucial in repressing CD4 expression¹³³. An over expression of RUNX 1 in mature CD4⁺ T cells promotes a differentiation into Th1 cells, whereas a differentiation into Th2 cells is inhibited by GATA-3 expression¹³⁴. The transcription factors T-bet together with RUNX 3 activate IFN- γ and silence IL-4 in Th1 cells¹³⁵.

Very recently, RUNX 1 and RUNX 3 have been postulated to play a vital role for regulatory T cells. The transcription factors Runx and CBF β are required for maintenance of FoxP3 mRNA and protein expression in Treg cells¹³².

It has been shown that RUNX 1 can bind to the IL-2 and IFN- γ promoters and therefore up-regulate IL-2 and IFN- γ production, respectively. When FoxP3 binds to RUNX 1 however, IL-2 and IFN- γ production is repressed and on the other hand genes encoding CD25 and CTLA-4 are activated¹³⁶. Knockdown of RUNX 1 and RUNX 3 (with siRNA) leads to a decreased FoxP3 expression indicating that those proteins are indispensable for *in vivo* regulatory T cell function (suppressive activity and optimal expression of FoxP3)^{132, 137, 138}. Moreover, RUNX 3 was found in direct proximity in human tonsil regulatory T cells, making RUNX 1 and RUNX 3 crucial molecules for Treg function¹³⁸.

However, none of these markers seem to be exclusively expressed on regulatory T cells. Eight years after Sakaguchi et al (1995)¹⁰³ had discovered the regulatory T cells, the gene forkhead box P3 (*foxp3*, Scurfin), that codes for a transcription factor, gained increased interest as it is specifically expressed in CD4⁺CD25⁺ regulatory T cells¹³⁹. Studies could demonstrate that T cells from Foxp3 transgenic mice and naïve T cells that were transfected with *foxp3* acquired CD4⁺CD25⁺ regulatory T cell features, like expression of Treg markers (CD25, CTLA-4, CD103, and GITR), decreased IL-2, IFN- γ and IL-4 production and suppression of effector CD4⁺ cells¹³⁹. In mice there is an exemplary correlation between the Foxp3 and CD25 expression. In humans, the situation is considerably more complex. Almost all CD4⁺CD25^{hi} cells are FoxP3⁺, although there is a variable percentage of CD25^{int} cells that express considerable, but lower, amounts of FoxP3. Unfortunately, FoxP3 expression is not exclusively specific to Tregs¹⁴⁰ nevertheless it is currently the best Treg marker we have¹⁴¹.

Next to FoxP3 the Treg specific demethylated region (TSDR) can give higher information about a transient or “real” FoxP3 expression. Although FoxP3 is the best marker we have, CD25⁺ T cells may transiently express FoxP3 upon activation. Using epigenetic analysis the transient FoxP3 expression can be ruled out. Within the analysis a defined *FOXP3* region is checked for

IPEX

IPEX, a fatal, recessive disorder characterized by immune dysregulation, polyendocrinopathy, enteropathy and X-linked inheritance and a mutation in *FOXP3*. It is characterized by insulin-dependent diabetes mellitus (IDDM), infections, enteropathy, thrombocytopenia and anemia, other endocrinopathy, eczema and cachexia².

WAS

The Wiskott-Aldrich syndrome is a lifethreatening immunodeficiency caused by mutation in the WAS protein. It is characterized by thrombocytopenia with small platelets, eczema, recurrent infections caused by immunodeficiency and increased incidence of autoimmune manifestations and malignancies.

APS type 2

The autoimmune polyglandular syndrome type 1 is caused by the loss of central tolerance due to mutations in autoimmune regulator (AIRE), whereas APS type 2 apart from an association with polymorphisms in CTLA-4 and an HLA-extended haplotype is of unknown pathogenesis. It is characterized by multiple endocrine diseases initiated by an autoimmune process.

ALPS

The Autoimmune lymphoproliferative syndrome is associated with diffuse autoimmune manifestations.

ALPS type Ia patients have mutations in TNFRSF6, which encodes CD95; ALPS type Ib patients have mutations in TNFSF6, which encodes CD95 ligand; ALPS type II patients have mutations in CASP10, which encodes caspase-10.

demethylation¹⁴². “Real” Tregs possess a demethylated FOXP3 locus whereas the transient expressed FoxP3 cells have this region methylated.

Isolation and identification of Tregs therefore is problematic. As FoxP3 only appears intracellularly it cannot be used for isolation with the common sorting techniques. CD25 expression (5-10% of CD4⁺ cells) is widely used for regulatory T cell isolation^{105, 143}. Only cells with the highest levels of CD25 expression (~2% of CD4⁺ cells) are postulated to carry suppressive effects¹⁴⁴ as CD25 is also expressed on activated T cells. Moreover almost all CD25^{high}FoxP3⁺ cells express low levels of the IL-7 receptor (CD127). However CD127 is down regulated in the early course of T cell activation. Only 40% of the CD127^{low} population are FoxP3⁺ and even the CD4⁺CD25⁺CD127^{low} cells are only 85-90% FoxP3⁺¹⁴⁵. From the recent point of view, expression of CD127^{low} in combination with CD25^{high}, allows the most reliable sorting to gain regulatory T cells (CD4⁺CD25⁺CD127^{high}) and to discriminate them from effector cells *ex* and *in vivo*¹⁴¹.

Some groups^{139, 146} have demonstrated that mice that miss or have mutations in the *foxp3* gene (scurfy mice) suffer from a systemic autoimmune disease associated with the absence of Tregs, emphasizing that Foxp3 is a master switch gene for Tregs¹⁴⁷. This species of mouse is characterized by the scurfiness of its skin from which the name is derived. The defect results in death of affected males at about 3-4 weeks of age¹⁴⁸. In humans mutations in FoxP3 cause an immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome¹⁴⁹. Loss of FoxP3 expression leads to immune disorders and a reduction of regulatory T cells.

Wiskott-Aldrich syndrome (WAS)¹⁵⁰ and autoimmune polyglandular syndrome type 2 (APS type 2)¹⁵¹ are further examples for autoimmune diseases that are caused by genetic defects and also affect the Treg suppressive function¹⁵². Another genetic defect, autoimmune lymphoproliferative syndrome (ALPS)¹⁵³, is characterized by a T cell expansion together with a decline in the number of Tregs. Although the underlying mechanisms still remain unclear, hope lies in the prospect that one day these genetic diseases could be treated with regulatory T cells.

2.2.3.1 Natural Regulatory T Cells

Natural regulatory T cells originate in the thymus and are CD4⁺ T cells that express CD25 and FoxP3¹⁵⁴. Tolerance towards “self” is “educated” in exactly this period and is an alternative mechanism to elimination of autoreactive T cells in the thymus. For Treg development a T cell receptor (TCR) with high affinity for a self peptide is a precondition¹⁵⁵. Because of the necessity for high affinity it can be assured that only a minimal percentage becomes regulatory. The remaining cells however are very sensitive for special antigens.

Natural Tregs are not only committed to self-tolerance but also of immune responses to non-self (such as pathogenic or communal microbes¹⁵⁶ or allogenic organ transplants¹⁵⁷ or quasi-self (such as autologous tumour cells¹⁵⁸). Another important role of natural Tregs is to create and maintain tolerance toward the foetus expressing alloantigens during pregnancy, thereby preventing abortion¹¹³.

2.2.3.2 Inducible Regulatory T Cells

Although Tregs are relatively short-lived they are present throughout life at a steady state¹⁵⁹. As thymic function declines with age and the number of Tregs stays constant it is unlikely that all CD4⁺CD25⁺FoxP3⁺ Tregs are generated in the thymus alone but are also induced in the periphery¹⁵⁹ (Figure 6). They derive in the periphery from the memory T cell pool or from naïve T cells that are CD4⁺CD25⁻ by following adequate stimulation, especially in a particular cytokine milieu, e.g. TGF-β, IL-10 and IL-4¹⁶⁰.

Once differentiated, iTregs, like nTregs, express CD25 and FoxP3. In humans, FoxP3 can be induced in CD4⁺CD25⁻FoxP3⁻ cells by T cell receptor (TCR) stimulation together with TGF-β¹⁶¹. In mice, Foxp3 does not seem to be induced under the same stimulation conditions. The discrimination of nTregs and iTregs, is not always easy¹⁶² (Table 3).

Yamagiva et al (2001) were the first to demonstrate that naïve human CD4⁺ T cells can be induced by TGF-β to develop contact-dependent suppressor activity that is mediated by TGF-β or IL-10¹⁶³.

T regulatory 1 (Tr1) cells differentiate from T cells together with IL-10 and antigens or when immature DCs present their antigens to T cells. They were first

characterized for their role in preventing autoimmune colitis¹⁶⁴. IL-10 is their marker cytokine, although they produce TGF- β and IFN- γ as well^{162, 165}. It is presumed that Tr1 cells are derived *in vivo* from antigen-specific anerg T cells. The repeated exposition to a specific antigen is crucial for this process. They express CD132 rather than CD25 and also may be FoxP3^{low}¹⁶⁶.

Th3 cells were first identified because of their role in oral tolerance due to their TGF- β production¹⁶⁷. There is a recent report that suggests that children who develop egg allergy in the early years of life have reduced Treg function in cord blood¹⁶⁸.

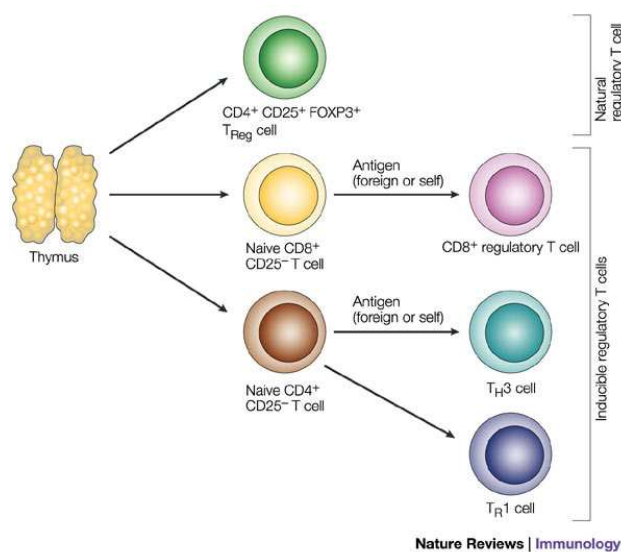


Figure 6 Model for nTreg and iTreg induction

Natural Tregs directly migrate from the thymus. Th3 and Tr1 as well as CD8⁺ induced regulatory T cells can be induced from naïve CD4⁺CD25⁻ or CD8⁺CD25⁻, respectively, in the periphery.^{156, 169}

2.2.3.3 Oral Tolerance and Tregs

Oral tolerance is defined as “specific suppression of cellular and/or humoral immune responses to an antigen by prior administration of the antigen by the oral route”¹⁷⁰. Therefore the effects are not restricted to the local immunity within the gut¹⁷¹. Low antigen doses favour active suppression, whereas higher doses favour clonal anergy¹⁷¹. Oral tolerance effector mechanisms do not only include apoptosis or anergy of antigen-specific T cells in the gut but additionally active suppression of antigen-specific Tregs¹⁷². Long-lasting oral tolerance can be explained by memory cells with antigen-specific regulatory functions¹⁷³.

Already 24-48 h after oral antigen administration, antigen-specific Tregs could be observed in the Payer’s patches, and mesenteric lymph nodes¹⁷⁴, suggesting that the

APCs that cause the tolerogenic response primarily exist in the draining lymphoid organs as well as the gut associated lymphoid tissue (GALT). The question remains whether the T cells that originate in the gut are solely responsible for the immune regulation or whether tolerogens and/or DCs enter the lymph node isolated and generate Tregs *in situ* ¹⁷⁰.

On the current level of knowledge, various regulatory T cells are involved in tolerance induction like Tr1, Th3, nTregs, and CD4⁺LAP⁺ T cells ¹⁷¹. TGF- β , mainly produced by Th3 cells, plays a central role in oral tolerance induction and was first described by Faria et al ¹⁷⁵. Tr1 cells by secreting IL-10 also enhance the TGF- β production by Th3 cells. A compelling connection has been reported between TGF- β and FoxP3, that TGF- β induces FoxP3 expression in effector T cells (CD4⁺CD25⁺) to become inhibitory functional ¹⁷⁶. On the other hand FoxP3 can also down regulate Smad7 expression which in return blocks TGF- β signalling thus making T cells ¹⁷⁷. Moreover TGF- β also plays a role in differentiation of Th1 and Th2 cells as it blocks the subtype specific transcription factors T-bet and GATA-3 ¹⁷⁸. CD4⁺LAP⁺ T cells suppress cells in a TGF- β -dependent manner and possess a membrane-bound form of TGF- β ¹⁷⁹. They resemble Th3 cells due to their cytokine secretion profile, except that they also produce mentionable amounts of IFN- γ ¹⁸⁰.

2.2.3.4 Additional Regulatory Populations

A subgroup of regulatory T cells, only mentioned for completeness, are certain γ/δ TCR-expressing CD4⁻CD8⁻ T cells and CD8⁺CD28⁻ T cells. Even a subgroup of NK cells were found to possess regulatory properties as they could suppress autoimmunity ¹⁸¹. Very recently B cells with regulatory function (Bregs) have been identified that are able to suppress in an IL-10 dependent manner ¹⁸². Further subtypes may be discovered in the future.

Cell Type	Treg subset	Regulatory mechanisms	Phenotype	Target cells	Function
CD4⁺ Tregs	nTregs (thymic-derived)	Cell-cell contact-dependent, cytokines	CD4 ⁺ CD25 ⁺ FoxP3 ⁺	T cells, APCs	Suppression of autoimmunity; inhibition of allograft rejection and immune responses induced by microbial infection; mediation of UV-induced immunosuppression
	nTregs (peripheral-induced)		CD4 ⁺ CD25 ⁺ FoxP3 ⁺		
	CD4 ⁺ CD25 ⁻ Tregs	cytokines	CD4 ⁺ CD25 ⁻ FoxP3 ⁺	T/B cells, APCs	Suppression of autoimmunity
	Tr1 cells	IL-10	CD4 ⁺ CD25 ^{-/+} FoxP3 ⁻ IL-10 ^{hi}	T cells	Suppression of autoimmunity
	Th3 cells	TGF-β	CD4 ⁺ CD25 ^{-/+} FoxP3 ⁺ TGF-β ^{hi}	T cells	Suppression of autoimmunity
	TGF-β IL-10 double positive		TGF-β ⁺ IL-10 ⁺ CD4 ⁺ CD25 ⁻ FoxP3 ⁻	T cells	Suppression of autoimmunity
NK Tregs	NKTregs	IL-4, IL-10, TGF-β, cytotoxicity	CD3 ⁻ CD56 ^{bright}	T cells, APCs, tumour cells	Elimination of tumours and pathogens; suppression of autoimmunity; mediation of UV-induced suppression of protective tumour immunity
CD8⁺ Tregs	IL-10 producing CD8 T cells	Cell contact-dependent, cytotoxicity, cytokines	CD8 ⁺ IL10 ⁺	T cells	Suppression of autoimmunity; regulation of peripheral TCR repertoire
	T suppressor cells	Induction of ILT3/ILT4 in DCs	CD8 ⁺ CD28 ⁻	DCs/ APCs	Regulation of autoimmunity

Table 3 Natural and induced regulatory T cells¹⁸³

2.2.3.5 Mechanisms of Suppression

CD4⁺CD25⁺FoxP3⁺ natural regulatory T cells are able to inhibit different cell types and cytokine production as explained in the chapter “natural Tregs 1.2.3.1”. The mode of suppression of Tregs, however, is not completely understood. *In vitro* experiments indicate that cell-cell contact dependent mechanisms are crucial for suppression of conventional T cells. Low amounts of antigen are required for Tregs due to the high T cell receptor affinity. After the initial antigen dependent activation Tregs seem to be stimulation-independent in their mechanism and are capable of suppression even in the absence of the appropriate antigen¹⁸⁴. Conventional T cells with different antigen-specificities can be suppressed in vicinity to the Tregs. This mechanism is known as “bystander suppression”.

One way of suppression is the interaction between the CTLA-4 or the lymphocyte-activation gene 3 (LAG3; CD223) with CD80 and CD86 on the surface of APCs which inhibits T cell activation¹⁸⁵. CTLA-4 induces the tryptophan-catabolic enzyme indoleamine 2,3-dioxygenase (IDO) in DCs¹⁸⁶. The enzyme IDO catalyzes the essential amino acid tryptophan into kynurenine, which has immunosuppressive effects (Figure 7).

Tregs can kill target cells cell-cell contact dependent via different pathways¹⁸⁷. For instance, Tregs use the same pathway (granzyme/perforin) to induce cell contact-mediated suppression as cytotoxic T lymphocytes and natural killer cells for killing virally infected cells and tumour cells. The release of perforin, granzyme A and B can kill T cells and monocytes through a cell contact-mediated mechanism¹⁸⁸. Another pathway for suppressing alloreactive responses of CD4⁺CD25⁻ T cells is the programmed death (PD)-1/PD-1 ligand (PD-L1) pathway. A blockade of this pathway restores the CD4⁺CD25⁻ T cell proliferation by inhibiting the Treg function¹⁸⁷. IL-2 is of great importance for Tregs as the addition of IL-2 abolishes Treg anergy but the suppressive function maintains. Neutralization of IL-2 on the other side abolishes suppression¹⁸⁹.

IL-2 is important for all Tregs but in contrast to nTregs, Tr1 and Th3 cells suppress in an independent cell-cell-contact manner and through the secretion of immunosuppressive cytokines IL-10 and TGF- β , respectively¹⁹⁰.

Recent studies imply a suppressive function of adenosine produced by Tregs¹⁹¹. Adenosine is a signaling molecule that binds to various adenosine receptors (A1, A2a, A2b and A3). Ligation of adenosine to the receptors triggers immunoinhibitory potential through cytoplasmic cAMP¹⁹² as well as inhibiting proliferation and cytokine production¹⁹³. Tissue derived adenosine did not only play a role in regulating T cell function but also in the adenosinergic pathway to the functional activity of human Tregs. Expression of CD39 and CD73 together with low levels of CD26 and adenosine deaminase (ADA) are characteristic markers for Tregs. One factor responsible for suppressive activity in Tregs can be explained by the ability of Tregs to hydrolyze ATP and therefore generate high pericellular concentrations of adenosine¹⁹⁴.

Nevertheless, not all aspects of suppression can be explained with the known mechanisms. Most probably it is a combination of miscellaneous mechanisms depending on the environment and also the cells present. There is a great chance however that still unknown key mechanisms will soon be discovered¹⁹⁵.

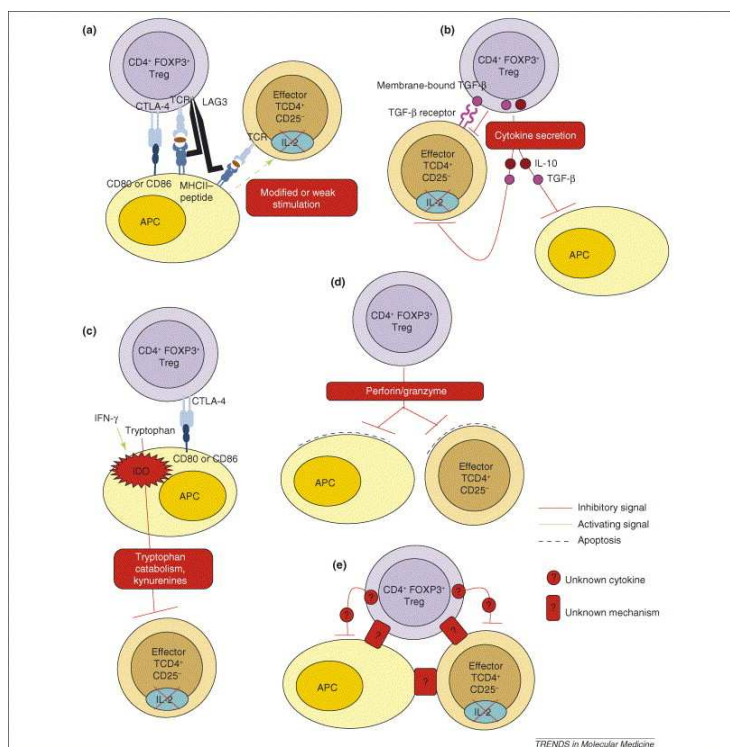


Figure 7 Various mechanisms of suppression
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Various suppressive mechanisms of Treg are known by now like weak stimulation with CTLA-4 and LAG3 interaction with the co-stimulatory molecules on APCs (a), immunosuppressive IL-10 and TGF-β secretion (b), tryptophan catabolism induction with CTLA-4 (c), cytotoxicity (d) and maybe an unknown key mechanism¹⁹⁵.

2.2.3.6 Regulatory T Cells in Pregnancy

Regulatory T cells are a necessary component of maternal immune tolerance of paternal alloantigens present on the trophoblast. The type of antigen that is recognized by Tregs in pregnancy however is not known.

They might recognize maternal self antigens on semi-allogenic trophoblasts and then suppress alloreactive effector cells by "bystander suppression". CD4⁺CD25^{high}FoxP3⁺ Tregs could suppress effector T cells to allogenic dendritic cells ¹⁹⁷ whereas the possibility of direct alloantigen recognition by Tregs could not be excluded ¹⁹⁸. This observation could be confirmed by transferring CD4⁺CD25⁺ Tregs into abortion-prone mice. Abortion could only be prevented when Tregs directly originate from normal pregnant mice indicating that Tregs previously need to be exposed to paternal alloantigens to exert regulatory activity ¹¹³. Anti-CD25 antibody addition to pregnant mice before mating resulted in depletion of Tregs and foetus rejection ^{198, 199}

The proportion of CD4⁺CD25⁺ Tregs significantly increased in the spleen in pregnant mice uterine-draining lymph nodes and peripheral blood, starting from the early post-implantation period. During early pregnancy an increase in circulating Tregs was observed. The highest level however was monitored in the second trimester and then the level declines *post partum*. CD4⁺CD25^{high} Tregs were increased in peripheral blood of women having a normal pregnancy compared to women with spontaneous abortion and non-pregnant subjects ²⁰⁰.

2.3 *Antigen-Presenting Cells*

As their name implies, antigen-presenting cells (APCs) process and present antigen material on their cell surface for T helper cells. During the last years, APCs, DCs in specific, have gained interest because they play a key role in immunobiology of tolerance and autoimmunity as well as anti-cancer responses with a possible use in tumour vaccines. They play a pivotal role in $CD4^+$ and $CD8^+$ T cell activation²⁰¹.

Antigens that are re-expressed on the cell surface interact with Th cells which have profound immune-regulatory effects, after they have been activated²⁰². Functionally DCs are the most potent APCs and are essential for the induction of the first immune response and initiating the T cell immunity²⁰³. They migrate to secondary lymphoid compartments (e.g. lymph nodes (LNs)) to present antigen-peptide-complexes to naïve $CD4^+$ T cells and $CD8^+$ cytotoxic T cells. Subsequently, naïve $CD4^+$ T cells differentiate into memory Th cells (reviewed in Satthaporn et al (2001)²⁰⁴).

Various subtypes of DCs differ in their surface markers (e.g. expression of CD11c integrin). Depending on the DC subpopulation, different cytokines skew T cells into a Th1 or a Th2 immune response¹⁰⁵. Jonuleit et al (2000) demonstrated that repetitive stimulation from naïve $CD4^+$ T cells with allogous, immature DCs induce Tregs²⁰⁵.

Immature APCs are the predominant DCs especially in the neonatal period. Immature and defective APCs have reduced co-stimulatory and a pro-Th1 cytokine signals²⁰⁶. Infants that have a prolonged functional immaturity in APC function are more prone to develop a Th2-mediated allergic disease²⁰⁷. IL-12 produced by APCs provides activating signals for neonatal cells. A weaker pro-Th1 signal also favours a Th2 response²⁰⁸. The findings of Langrish (2002)²⁰⁹ concur with other studies, which have shown that cord blood derived DCs have decreased functional activity as well as a different and decreased phenotypic expression of surface markers²¹⁰. Moreover, not only T cell proliferation (both adult and cord blood) is less effective in the presence of cord blood derived DCs²¹⁰ but they also fail to direct a Th1 polarization²⁰⁹. Thus, APCs have an important role in postnatal development of immune competence, particularly in maturation of Th1 responses.

2.4 Cord Blood

2.4.1 Cord Blood and Allergy

Some evidence exists that T helper cells from neonates are primed *in utero*. First, allergen-specific IgE was found in cord blood and amniotic fluid, so it is either transferred through the foetal membranes from the maternal circulation or it is produced by the foetus itself²¹¹. Second, many studies have demonstrated that T cell proliferate and produce cytokines upon stimulation with allergens²¹²⁻²²⁰ suggesting that the cells already had contact with the stimulus. CBMCs exerted a generally higher proliferation against birch pollen when mothers were exposed to this allergen at a certain period of gestation²²¹. No effect of seasonal exposure to pollens on proliferation on CBMCs could be shown however in a larger observation group²²².

Based on the fact that neonatal PBMCs proliferated upon stimulation with allergens, *in utero* sensitization was taken for granted. It was assumed that this proliferative response reflected immunological memory induced by intrauterine exposure to dietary or environmental allergens via diaplacental transfer. However, CBMCs also proliferate in the presence of the keyhole limpet antigen, to which natural *in utero* exposure is extremely unlikely. Therefore lymphoproliferation in cord blood may be more unspecific than originally suggested²²³.

The foetus' immune system is strongly skewed towards Th2 cytokines, especially when the mother is atopic²²⁴. Because of that allergen priming *in utero* was thought to be a key factor for predisposition to allergy. Our group showed that allergens can diaplacentally cross the maternal-foetal barrier. Beta-lactoglobulin for example could even be detected within the first minutes of perfusion in the cotyledon²²². Transplacental allergen transfer is coupled with the actively transmitted immunoglobulin G (IgG)²²⁵ and children with asthma displayed higher IgG4 levels than the asthmatic adults²²⁶. Another hypothesis states that the foetus gets in contact with the allergens via the amniotic fluid because ovalbumin and the major allergen of house dust mite, Der p 1 (*Dermatophagoides pteronyssinus*) have been found in amniotic fluid²²⁷.

In the 80's elevated IgE-levels in cord blood were thought to be a marker for atopic disease²²⁸. However, intensive research demonstrated that the IgE-level alone was not

sensitive enough for an allergy prediction. A study by Magnusson et al (1988), for example, screened 190 newborns and found a specificity of 93% - the sensitivity however only reached 68.4% ²²⁹. A recent, Danish study found matching allergen-specific IgE against inhalant allergens in 14% of the screened cord blood samples. The allergen-specific IgE disappeared at the age of 6 months indicating that the specific IgE was either related to contamination by the mothers' blood or was transferred from the mother to the foetus and not produced from the foetus due to intrauterine sensitization ²³⁰.

Supernatants of stimulated CBMCs mainly contain Th2 cytokines ²¹⁴ therefore many researchers focused on cytokine production as a predictive marker for allergy. Some groups concluded that a decreased cytokine production (especially IFN- γ) at birth leads to an atopic illness later on ^{213, 219}, other research groups could not find that connection ²³¹. Taken together, no reliable and safe marker in newborns exists for predicting allergic diseases.

2.4.2 Differences between CBMCs and PBMCs

The foetal and the adult immune systems differ in phenotypic and functional characteristics^{232, 233}. Cord blood can be distinguished from peripheral blood due to different response to allergens, a lower cytokine production, a diminished cytotoxicity and due to differential surface markers²³⁴. Umbilical cord blood T cells are mainly naïve antigen-inexperienced cells, and greater than 90% of CD3⁺ T cells express a CD45RA⁺ naïve-phenotype. Adult CD4⁺ T cells have a predominantly CD45RO⁺ memory-phenotype²³⁵. Cord blood derived T cells differ from naïve cells in adult blood. They do not produce IL-2 upon stimulation whereas the PB derived naïve T cells do²³⁶. Thus the cord blood derived CD4⁺CD45RA⁺ subset may be more naïve than the adult cells. However other studies ascribed this effect to the numerous immature dendritic cells in cord blood as they can induce T cell tolerance²³⁷. The immature DCs may capture antigens but are said to be unable to process and present them to T cells and therefore induce T cell anergy as well as Tregs²³⁸.

Cord blood and thymic derived naïve T cells (CD4⁺CD45RA⁺) express a number of characteristics that distinguish them from naïve T cells that are present later in life. They have more similarities to RTEs²³⁹. Neonatal T cells in general also express CD38²⁴⁰, the IL-7R α which makes them highly responsive to IL-7 and harbour a high expression level of TRECs. The TREC content indicates that those cells are more akin to RTEs than to the naïve T cells from adult individuals²⁴¹. In adults, CD38 is not expressed by all CD4⁺ T cells and is mainly expressed by CD45RO⁺ T cells²⁴².

Interestingly, it was shown that even upon removal of the low numbers of memory cells from cord blood still 50% of the donors were responsive to the allergen²¹².

PBMCs from the mother secrete a significantly stronger Th1 response (higher IL-12 levels) than neonatal PBMCs. There was a positive correlation when maternal and infants were matched for house dust mite (HDM) and a negative correlation when matched for neonatal Th2 response (IL-13)²⁰⁸. Prescott et al (2003) indicated that the changed allergen-specific cytokine responses may be associated with differences in the function of the APCs in early life and later allergy²⁰⁸.

In general one can say that the cytokine profile of cord blood T cells is skewed towards a Th2 phenotype: higher IL-4, IL-5 and IL-13 levels which are involved in the

induction of the IgE synthesis together with reduced IFN- γ levels compared to adult T cells. A reduced IFN- γ level appears to be associated with the subsequent development of atopic eczema²⁴³, allergic rhinitis, or asthma²¹⁹. This cytokine pattern may play an important role in allergy development. Evidence is increasing that the aberrant cytokine responses of CBMCs rather relate to different regulatory properties and immaturity of the APC²⁴⁴.

In cord blood, the frequency of CD4⁺CD25^{high} regulatory T cells is higher at gestational week 25 (12% of CD4⁺ T cells) than at birth. At birth the same frequency as in adult donors was measured²⁴⁵. Naïve Tregs represent 0.2 – 0.8% of total CD4 cells, whereas in cord blood the majority of Tregs belong to the naïve apoptosis-resistant population²⁴⁶. Naïve natural Tregs are more prominent in young adults and decrease with age together with the total naïve CD4⁺ population. In cord blood 7% and in adult blood 0.2-3.3% of total CD4⁺ T cells display this naïve Treg phenotype²⁴⁷. This may be an explanation why cord blood derived Tregs have a reduced regulatory function²⁴⁸⁻²⁵⁰. Cord blood derived regulatory T cells (CD4⁺CD25^{high}) do not control allergen-specific proliferative responses in CB per se as is the case in adult blood cells. However, cord blood derived Tregs can become functional when pre-activated with different stimuli^{122, 251}. Furthermore, the Th17 function in cord blood was impaired as well²⁵². Taken this together, it is suggested that potent immune stimulation early in life may counteract an allergic disease.

2.4.3 The Perinatal Period

The perinatal period delineates the period around the time of birth. “This period commences at 22 completed weeks (154 days) of gestation and ends seven completed days after birth”²⁵³. T cells as well as the cytokine profile play a decisive role in the perinatal immunity.

2.4.3.1 The Prenatal Period

The development of the immune system, especially in the prenatal period, has far-reaching consequences not only for health during early childhood but also throughout life²⁵⁴.

Pregnancy is an immense alteration for the maternal and innate immune system. Nevertheless, the maternal immune system tolerates the foetal tissue during normal pregnancy. As 50% of its genetic material derives from its father, Medawar et al (1953) were first to propose that the growing foetus should be viewed as an allotransplant²⁵⁵. The foetus' susceptibility to rejection by the maternal immune system is very high and similar to transplanted organs. An appropriate regulation is essential for the foetal survival. The maternal immune system tolerates the fetus whereas it still can fight adequately against infections. This “paradox of pregnancy” is a ambiguous topic in immunology as a skewing towards Th2 cytokines helps the survival of the foetus from the critical time point of implantation onwards²⁵⁶ and reduce the risk for preeclampsia²⁵⁷. Pregnant women produce augmented Th2-cytokine and diminished Th1-cytokine levels^{256, 258}. This mechanism is reasonable, because cytotoxic Th1 immune responses (e.g. IFN- γ) would activate natural killer-cells (NK-cells) which would be disadvantageous for maintenance of pregnancy. In animal models IL-2 and IFN- γ doses can induce elevated abortion levels. There is also evidence in humans as well that high concentrations of Th1-cytokines (TNF- α/β , INF- γ and IL-2 levels) can induce labour. Although studies reject the Th2-paradigm as the major mechanism providing foetal allograft survival, women with higher Th1 levels are found to have more abortions²⁵⁹. It is considered that only CD4⁺ T cells are responsible for this cytokine production in pregnancy. Nevertheless, also CD8⁺ cytotoxic T cells, NK cells

and NKT cells ^{260, 261} are responsible for this cytokine shift. Borzychowski et al (2005) could demonstrate that the Th2 shift during pregnancy could be better explained by NK CD56⁺CD3⁻ and CD56⁺CD3⁺ cells instead of T helper cells or cytotoxic T cells ²⁶². They announced that the innate immune system, involving NK and NKT cells, is the predominant cell population of the peripheral blood in pregnancy, which stands in controversy to Wegemann's first hypothesis, who was an advocate of the theory that Th2 cytokines inhibit the Th1 response ²⁶². Shi et al (2007) manifested these findings showing that the percentages of NK cells and NKT cells were increased in peripheral blood and IFN- γ expression was suppressed for women during early pregnancy ²⁶³. On the other hand no difference of IL-4 expression was seen in NK and NKT cells between non-pregnant and pregnant women. Matching other data, no difference of IL-4 and IFN- γ cytokines was detected between pregnant and non-pregnant women in T helper lymphocytes.

Apparently, the initial Th2 (IL-4, IL-5, IL-6, IL-10, and IL-13) cytokine-shift of neonates reforms within the first 2 years of life, as long as no atopy has evolved. These Th1 dampening effects may be amplified by the placenta, through synthesis of high levels of prostaglandin E2 ²⁶⁴, which selectively inhibits INF- γ and local progesterone as well as stimulating IL-4 production ²⁵⁸. Based on both murine and human studies there were several mechanisms proposed to contribute to that tolerance: First of all Rouas-Freiss et al (1997) could show that HLA-G protects cytotrophoblasts against natural killer (NK) cytotoxicity ²⁶⁵. As HLA-G expression is limited to the gestational period, the hypothesis arose that this played an essential role in the immunological tolerance of the foetus by the mother ²⁶⁶. So far, several mechanisms have been proposed for tolerating the foetus by the mother. One known mechanism is that the maternal uterine NK cells protect the foetus by lytic activity. Second, heme oxygenase (HO)-1 expression on decidual and placental tissues ²⁶⁷ was shown to increase trophoblast-induced apoptosis of maternal effector cells and therefore protect the fetus ²⁶⁸. Third, indoleamine 2,3-dioxygenase (IDO) ^{269, 270} also may contribute to tolerance. Fourth, placenta-derived exosome particles present in the maternal circulation could regulate immune activation by mediating activation-induced apoptosis of maternal reactive lymphocytes ²⁷¹. Moreover an adequate Th1/Th2 cytokine cross-talk ^{272, 273} can induce tolerance after birth.

2.4.3.2 The Postnatal Period

Directly after birth, newborns possess a Th2 type cytokine profile^{214, 218} combined with a decreased ability to produce cytokines. The postnatal maturation of the immune system is regulated by contact with microbial exposure. Exposure to bacterial products has a protective effect and promotes a Th1 response²⁷⁴. A deficiency in receptors, like CD14 or Toll like receptors (TLR), that are important for lipopolysaccharide (LPS) interaction with immune cells, could decrease appropriate Th1 and default in Th2 responses²⁷⁵. Allergen-specific Th1/Th2-cytokines²⁷⁶ and especially a reduced IFN- γ production²¹⁴ can be observed in children in a family with a history of atopic disease. Hence the IFN- γ production of T cells plays an important role in developing a Th1 cytokine profile. CMBCs produce higher levels of IL-13 than adult PBMCs and CD8⁺ T cells are found to be the major source²⁷⁷. No cytokine-pattern has been found yet to predict a later sensitization or develop an atopic disease later in life. A diminished IL-12 production by APCs leads to reduced lymphoproliferation, stronger Th1 and weaker Th2 response to allergens. Early life seems to be crucial for a Th1 or Th2 cytokine response as well as a possible development of allergy later on²⁰⁸. This frequently discussed “window of opportunity“ in the postnatal period offers perspectives for allergy prevention. For primary intervention maturation of the immune system via modulation of the innate immune system in particular may provide a potential explanation. The Th2 pattern in the first years of life is most probably the initial phase for the pathogenesis of atopic diseases and is therefore principal for investigations. However, the underlying factors that lead to a Th1 or Th2 response still remain unclear.

2.5 Factors Influencing Immune Deviation

Numberous factors that influence the immune deviation are known. Genetic predisposition and more importantly environmental factors play a major role in the genesis of allergy.

2.5.1 Genetic Predisposition

Twin studies have shown that genetic predisposition comprises the strongest risk factors for asthma and allergy development ^{278, 279}. Various genes on different chromosomes were identified for being responsible for allergic diseases (e.g. coding for a mast cell tryptase on chromosome 5q23-33, 11q13, 14q11.2 and coding for the α -chain of the IL-4 receptor on 16p12) ²⁸⁰.

Takahashi (2005) found a connection between the IL-12RB1-gene and an increased serum level of Th2-cytokines. A variant of the gene that codes for an arginase correlates with an increased risk for atopic dermatitis ²⁸¹. The increasing prevalence of becoming allergic in the last decade however cannot be explained via genetic predisposition. Environmental factors as well as lifestyle and gene-interactions must play a major role as well ²⁸².

2.5.2 Environmental Conditions

The impact of the environment can be monitored in the “natural experiment” of the German reunion in 1989. For forty years, two genetically similar groups had been exposed to different environmental conditions ²⁸³. In the industrial zone of Eastern Germany high concentrations of sulphur dioxide and pollutants were present ²⁰³ and the prevalence of atopic eczema was significantly higher than in Western Germany ²⁸³.

2.5.2.1 Inhalational Substances

2.5.2.1.1 Smoke

In utero exposure to smoke can cause long-lasting effects. Organic functions, such as respiration, can be profoundly affected. Maternal smoking is a relevant factor for atopic diseases *in utero* and during the first years of life (reviewed by Pauly et al ²⁸⁴). One smoking person per household doubles the risk for wheezing and other asthma-related symptoms. Neonates and children that are exposed to tobacco smoke have a significantly increased prevalence to developing asthma ²⁸⁵. However, this could only be seen with cigarette smoking and, very interestingly, only for children with allergic predisposition from a longitudinal analysis from birth to 10 years ²⁸⁶.

2.5.2.1.2 Chemicals and Pollutants

Sulfur dioxide ²⁸⁷, nitrogen oxide ²⁸⁸, diesel particles ²⁸⁹, and ozone ²⁹⁰ can also result in allergic diseases and asthma. Children that are exposed to air pollutants during fetal development and early life acquire health problems such as abnormal development (low birth weight, decreased lung growth) preterm birth, intrauterine growth restriction, childhood asthma, increased rates of respiratory tract infections, as well as behavioral problems, and neurocognitive decrements ²⁹¹.

2.5.2.2 Nutrition

Some studies link the increased prevalence in childhood allergy to an impaired nutrient supply (reviewed in Enke et al ²⁹²). A well-developed placenta as well as an excellent foetal nutrition is an important prerequisite for a functional immune system. An increase in atopy is attributed to a decreased intake of fruits and vegetables containing a large amount of antioxidants ²⁹³ and n-3 polyunsaturated fatty acid (PUFA), rich in fish ²⁹⁴ intake as well as an increased n-6 PUFA (rich in vegetable oil, margarine) intake ²⁹⁴. A decreased antioxidant intake could result in a lower lung antioxidant defence and an increased airway susceptibility to oxidative damage that may result in asthma and airway inflammation ²⁹⁵. Various metabolic processes and gene expression of vital proteins like certain cytokines and enzymes demand fatty acids and their derivatives for early immune development and immune maturation. An increased

n-6 linoleic acid intake leads to elevated arachidonic acid and prostaglandin E₂ (PGE₂) production which could lead to a Th2 sensitization, asthma, and atopic disease²⁹⁴. N-3 as well as n-6 PUFAs affect the cell membrane fluidity, cell signalling, and gene transcription and therefore modulate T cell function²⁹⁵.

An inverse correlation has been found between dietary vitamin E and C intake and asthma²⁹⁶ and atopy²⁹⁷ as it is positively correlated with the ventilatory function of the lungs in children and adults²⁹⁸. Vitamin E as a strong antioxidant prevents membrane injury but has non-antioxidative effects on immune function as well. Vitamin A is important for lung and normal epithelial development in the respiratory tract²⁹⁵. As much as for vitamins E and C also vitamin A and carotenoids are essential for the ventilatory function, and respiratory symptoms²⁹⁹.

Folate (its natural occurring form) and folic acid play a major role in preventing neural-tube defect in newborns³⁰⁰ and supplementation for young women of childbearing potential is recommended. Folic acid is also proven to have beneficial effects on coronary heart disease, colon cancer as well as dementia³⁰¹. Interestingly, some new studies suggest that folic acid supplementation during early pregnancy is not merely beneficial but increases the risk of asthma wheezing, and respiratory disease³⁰².

However, only isolated substances, such as vitamins and fatty acids could be correlated with a positive effect on the immune system. Neither fruit syrup nor orange juice, or honey at 1 month appeared to be a significant risk factor for atopic sensitization. Only sterilized water was shown to be a significant risk factor for asthma³⁰³.

However, interventional studies resulted in disappointing results for the dietary hypothesis²⁹⁵. Foetal and numerous factors in early life influence the development of asthma and atopic disease, therefore focus was shifted on these factors for preventing allergy in early childhood.

In summary, a balanced diet is a precondition to give birth to a healthy child. Malnutrition in every form generates several problems. Those cannot be ascribed to single substances as the whole metabolism is too complex to draw causal conclusions.

2.5.2.2.1 Infant Feeding

2.5.2.2.1.1 Breastfeeding

Breastfeeding is the preferable method of infant feeding. Exclusive breast-feeding for at least 4 months (preferably 6 months) seems to be advantageous to at least delay atopic dermatitis and milk allergy³⁰⁴. Some studies could show that breastfeeding alone in the first 6 months of life reduced allergies and asthma³⁰⁵ and decreased the risk of atopy³⁰⁶. Possible explanations for a protective effect of breastfeeding are the special composition of human milk promoting an intestinal environment rich in bifidum bacteria that induce a Th1 shift. The avoidance of cow's milk protein is another factor for the beneficial effects of breastfeeding. In a detailed analysis of systematic reviews from the US Agency of Healthcare Research and Quality (AHRQ) in 2007³⁰⁷ a possible protective effect of breastfeeding on allergy and eczema is suggested³⁰⁸ but as all studies included observational studies no direct conclusion can be drawn.

Not only allergy protection is one of the major advantages for breastfeeding. A significant lower infection rate (e.g. diarrhea³⁰⁹, otitis media³¹⁰, haemophilus influenza³¹¹, respiratory infections³¹² together with an enhanced vaccine response³¹³), an enhanced protection against illnesses (e.g. AIDS³¹⁴, multiple sclerosis³¹⁵, cryptorchidism³¹⁶, infant survival³¹⁷) and also an enhanced development and intelligence (³¹⁸has been observed in breastfed children.

Therefore, breastfeeding is strongly recommended for its multiple benefits on child health.

2.5.2.2.1.2 Hydrolyzed Formula Feeding

To eliminate the allergenicity of the cow's milk proteins, protein hydrolysate formula is commercially available to reduce the antigenic load as well as the risk for sensitization. A hypoallergenic formula should be well tolerated by at least 90% of the milk-allergic patients³¹⁹. One can distinguish between an elemental formula with free amino-acids that can be tolerated by all individuals, extensively hydrolyzed formula (EHFs) and partially hydrolyzed whey formula (PHWFs). 95% of all cow's milk allergic people tolerate EHF, whereas PHWF can cause allergic reactions in 30-50% of milk allergic individuals and are therefore not considered to be hypoallergenic³¹⁹. A

meta-analysis of 15 randomized controlled trials revealed that PHWFs is effective in allergy prevention for children at high risk for allergy. For already allergic individuals only elemental formula and EHF are an optimal substitute³²⁰

2.5.2.2.2 *Mother's Diet*

The type of the mother's diet can affect the development of allergy-related diseases. In a Cochrane study an "antigen-free diet" for women at high risk of giving birth to an atopic child does not seem to have a strong protective effect on the incidence of atopic eczema or asthma during the first 18 months of life³²¹.

Intake of fish oil during pregnancy seemed to be beneficial for asthma whereas consumption of processed fish, a source of trans-fatty acids, increased the risk of asthma in children. The type of fish ingested can be important because of the different pattern of fatty acids³²² and intake of fish is recommended during pregnancy. Dunstan et al (2003) found a reduced risk for infant allergy after maternal polyunsaturated fatty acid (PUFA) supplementation³²³. The mechanism could be explained by a reduced neonatal oxidative stress, reduced production of inflammatory leucotriene B4 (LTB4), and an altered T cell function³²⁴.

2.5.2.2.3 *Obesity*

Obesity is associated with an increased prevalence of asthma, especially in women, and asthma also seems to be more severe in the obese. Obesity goes along with low-grade systemic inflammation, as well as with increased levels of inflammatory cytokines, adipokines, and acute phase proteins, including leptin, IL-6, TNF- α , and C reactive protein³²⁵. Taylor et al (2008) could also demonstrate a significant association between asthma severity and obesity³²⁶. The underlying mechanisms are not completely understood but could be related to a impaired function of regulatory T cells³²⁷.

Independent of exercise, a Mediterranean diet (higher intake of monounsaturated to saturated fats; rich in vegetables, fruits, grains and pulses, and a moderate dairy consumption) had a slight protective effect for current severe asthma in schoolchildren³²⁸. They concluded that obese people can perceive their asthma as more severe than normal-weight people. Calorie restriction resulting in weight loss, independent of specific food and nutrient intake, seems to improve asthma best³²⁵.

Not surprisingly, children that have untreated asthma are less fit and have a higher rate of body fat and higher obesity³²⁹. However, to date, there is little data about obesity and asthma and most study outcomes are from badly designed studies. For that obesity and asthma remains controversial and no causal correlation could be established. Most likely physical factors, such as higher mobility and an increased quality of life are involved in this process.

It has also been suggested that induction of Treg development might be a useful tool for asthma treatment³³⁰. However, increasing Treg might also increase the cancer risk by impairing host anti-tumour immune response. Thus the safest way to improve asthma in obese patients is as hard as weight reduction.

2.5.3 Hygiene Hypothesis

The hygiene hypothesis arose from many different observations, starting in Switzerland, and was first published by Strachan et al in 1989³³¹. One major observation was that children growing up in rural areas (e.g. a farm) are by far less likely to become allergic later in life than compared to children growing up in urban areas. An interesting observation was that the risk for allergy is more prevalent in western industrialized countries than in developing nations and immigrants adapt the risk of the community to which they move³³². Furthermore, smaller and wealthier families bear a higher risk for developing atopic dermatitis (AD)³³³. Moreover cleanliness and frequency of washing is linked to an increase in AD³³⁴. Taken together, these observations link westernized lifestyle to AD and allergy.

The hypothesis states that a lacking microbial burden in context with allergens leads to an increased susceptibility to develop allergic diseases. Children growing up on a farm have a significantly lower prevalence of allergic diseases like allergic rhinitis or allergic asthma as compared to children growing up in urban areas. Regular presence in the stable, drinking non-pasteurized milk, presence of older siblings, frequent contact with endotoxins within the first year were defined as independent protective factors^{335, 336}. Improved hygienic conditions in western or developed countries result in less infection-driven or microbial illnesses. Immunologically, this can be explained by the imbalance of Th1 and Th2 cells with a Th2 shift. This Th2 shift that predominates from birth through critical childhood periods results in a higher incidence of atopy and asthma. A more recent explanation is the impact of regulatory T cells that become more regulatory in the presence of farm dust and other stimuli.

2.5.3.1 Anthroposophic Lifestyle

Anthroposophy is based upon the writings of the philosopher Rudolf Steiner (1861-1925). Together with the popular interest in the hygiene hypothesis anthroposophic lifestyle and its impact on allergy development have gained interest. It includes a restrictive use of antibiotics, fever-reducing agents and (certain) vaccination. Atopic dermatitis (AD) was 70% less common in children that were raised according to the

anthroposophic lifestyle compared to schoolchildren from non-anthroposophic background ³³⁷. The “Prevention of Allergy-Risk Factors for Sensitization Related to Farming and Anthroposophic Lifestyle” (PARSIFAL) study focused on more than 6,600 children aged between 5 to 13 years conducted in five European countries (Austria, Germany, Netherlands, Sweden, and Switzerland). Certain features of the anthroposophic lifestyle, such as restrictive use of antibiotics and antipyretics, were associated with a reduced risk of allergic disease in children ³³⁸. Blood samples were taken and the children’s infection history, diet, animal contact, environmental exposures, anthroposophic lifestyle factors as well as symptoms and diagnoses of allergic illnesses were interviewed in a parental questionnaire. Children with an anthroposophic background had a significantly reduced risk for atopic eczema, rhinoconjunctivitis, and atopic sensitization ³³⁸. This anthroposophic lifestyle phenomenon can be explained by more childhood infections in this group as well as a reduced antibiotic use and by different eating habits, like eating more fermented vegetables rich in *Lactobacillus*. Taken together these facts affect the immune system in an allergen preventive way ³³⁹. Mechanisms will be explained in more detail in the following chapters.

2.5.3.2 Childhood Infections

Childhood infections, such as chickenpox, measles, mumps, whooping cough, diarrheal disease, and pneumonia are associated with an allergy-related illness ³⁴⁰. Factors influencing allergic diseases are age, severity, type of virus, location and timing of the infection together with the interplay with allergens ²¹⁶.

A protective effect could be found for certain types of pathogens ³⁴¹. Illi et al (2001) found a negative association between atopic eczema, asthma, episodic wheezing in children that had several viral infections of the herpes type and a runny nose before the age of 3 years ³⁴². McKeever et al (2001) ³³³ could affirm the data of Strachnan et al (1989) ³³¹ that the presence of older siblings seems to be protective against hay fever suggesting that this effect may be directed by infections. A measles infection in childhood seems to be protective against the risk of IgE-mediated eczema ³⁴³. Floistrup et al (2006) could show that children who received the measles-mumps-rubella (MMR)

vaccine are at higher risk of having rhinoconjunctivitis³³⁸. On the other hand, wheezing up to the age of 7 correlated with diminished respiratory tract infections in the first three years of life. The topic remains controversial however because several other studies could not find former mentioned beneficial effects³⁴⁴. McKeever et al (2004) for example, who screened 29,238 children in an observational cohort study, could indeed find a correlation between MMR, diphtheria, polio, pertussis, and tetanus (DPPT) vaccination and the incidence of asthma and eczema. Importantly, this correlation was biased by the fact that those children, where the correlation was found, were also those who barely requested care from a general practitioner. They concluded that the observed correlation was more likely to be caused by a study bias than a biological effect³⁴⁴. Additionally, a population-based cohort study by Schmitt et al (2009) could not find any evidence that infections per se significantly increased the risk for atopic dermatitis³⁴⁵.

An evidence-based relationship between vaccination and the development of allergic diseases are hard to fulfill as most children are vaccinated and do not develop an allergic disease. On the other hand, unvaccinated children are rare and therefore a very atypical group with a generally different lifestyle – thus difficult to compare.

Moreover in this context it needs to be mentioned that the positive effects of vaccination are outweighing the side-effects by far. Many diseases (e.g. poliomyelitis, tuberculosis, etc.) have been eradicated from most countries all over the world because of the area-wide vaccination³⁴⁶.

2.5.3.3 Antibiotics

The frequent administration of antibiotics during the first year of life leads to alteration in the intestinal flora and because of that to a Th1/Th2-imbalance that induces a higher incidence of allergic diseases³⁴⁷. A two-fold increased risk of asthma could be reported in the first year of life after antibiotic use³⁴⁸. A 1.5 times higher risk of having asthma at the age 7 was reported for children who had received more than four courses of antibiotics when compared to those who had not received any antibiotics³⁴⁹. Controversial to that, Celedon et al (2004) could not find any association for asthma until the age of five years in children receiving multiple courses of antibiotics³⁵⁰.

The discrepant findings can be explained by a different population studied. Kozyrskyj et al (2007) enrolled half of the cohort from rural areas whereas Celedon et al (2004) drew the data from an urban population^{349, 350}. The type of antibiotics used in rural children may also lead to an increased risk for asthma. Broad-spectrum cephalosporins leads to the significant suppression of *Lactobacilli* or *Bifidobacteria*, combined with an increase of *C. difficile*³⁵¹ which is considered to increase the risk of developing allergy.

The epidemiologic evidence is conflicting. Four cross-sectional studies^{352, 353} found a positive correlation between the early use of antibiotics and the development of atopic dermatitis, three longitudinal studies^{347, 354, 355}, and a case-control study³⁵⁶ did not find any association between antibiotic exposure in infancy and subsequent incident atopic dermatitis. Recent findings suggest that broad-spectrum antibiotics (cephalosporines or macrolides) increase the risk for atopic dermatitis. Therefore antibiotics with a narrow spectrum, like penicillin, should be administered as first choice antibiotics if indicated³⁴⁵.

2.5.3.4 The Intestinal Flora

The intestine of the newborn is sterile at birth. The colonization of commensal bacterial flora takes place immediately after birth. This process is assumed to play a critical role in immune deviation.

Children in agriculturally dominated countries have a special microbiota composition in the gut. Their gut comprises higher levels of anaerobic bacteria, like *Lactobacilli* or *Bifidobacteria* and lower levels of *Clostridium difficile*³⁵⁷. The same pattern of gut microbiota is seen in non-allergic infants who discern them from allergic infants³⁵⁸.

The gastrointestinal microflora promotes potential anti-allergenic processes. Two pathways are assumed to induce a predominant Th2 cytokine pattern. The first, works through bacterial derived LPS resulting in stimulation of the immune system with Th1 dominated properties³⁵⁹. Simultaneously for the second pathway, the generation of TGF- β is essential for the suppression of Th2-mediated allergic inflammation and induction of oral tolerance³⁶⁰.

The bacterial composition in the gut is crucial for immunological tolerance and consequently may impact allergy development³⁶¹. This theory is supported by the study of Kalliomaki et al (2001) that could demonstrate a 50% decrease of the clinical phenotype of atopic dermatitis after oral administration of *Lactobacillus GG*³⁶¹. *Lactobacilli* are able to inhibit antigen-induced IgE production and can change the interleukin pattern. Majamaa et al (1997) found out that infant milk formula supplemented with *Lactobacilli* is advantageous for infants that suffer from milk allergy and atopic dermatitis as it can reduce allergy symptoms as well as intestinal inflammation³⁶². On a T cell level the induction of Tregs by different probiotic strains is discussed controversially. Some studies could prove that the number and the potency of Tregs increased after probiotic implementation^{363, 364} whereas others could not show this effect³⁶⁵.

However, probiotics alone cannot cure everything³⁶⁶. Only few of the potential health benefits could be confirmed in randomized, controlled trials - especially in the paediatric population. There is convincing evidence for using probiotics in treatment for acute viral diarrheal disease, antibiotic associated diarrhoea and pouchitis. The role in irritable bowel syndrome, necrotising enterocolitis, inflammatory bowel disease and atopy remains controversial and needs to be backed up with evidence³⁶⁷. However, only a small number of probiotic strains have been tested and many candidates are in the pipeline. Hence, the effects of different probiotic microorganisms are not equivalent; therefore results cannot be generalized³⁶⁸.

2.5.3.4.1 Microflora Hypothesis and Regulatory T Cells

The explanations in the former chapter are most likely inaccurate due to oversimplification. Direct examination could indicate that Th1 cells were able to reduce mucus production and airway eosinophilia but failed to inhibit Th2 cell-induced airway hyperreactivity³⁶⁹. Hence other mechanisms than the Th1 and Th2 shift are needed for the switch between atopic and non-atopic phenotypes. Consequently regulatory T cells became the main focus of research^{207, 370}. Tregs that can be induced via different kinds of microbial stimulation can control an immune response through IL-10 and TGF- β production³⁴⁰. The "microflora hypothesis" postulates that the microbial flora in the gastrointestinal tract (GI) tract is indispensable for immunological tolerance with

Tregs³⁷¹. Allergens can pass through an impaired barrier of the epithelium (also in the GI) shown by inflammatory skin infections early in life. Antibiotic application disrupts the intestinal microflora and can subsequently increase the inflammation by preventing immunologic tolerance via regulatory T cells³⁷². Chronic stimulation of the gut-associated lymphoid tissue (GALT) through microbial antigens accounts for developing a Th1- as well as a regulatory T cell dominated cytokine milieu. This can prevent the clinical expression of allergic disease.

2.5.3.5 Endotoxins

Lipopolysaccharide (LPS) is a structural component of gram-negative bacteria that is released when bacteria are lysed and unlike bacteria is thermo-stable.

LPS consists of a hydrophilic polysaccharide chain and a lipophilic lipid moiety (Figure 8). The polysaccharide chain varies between different bacteria and endotoxins and has an approximate size of 10 kDa up to 1000 kDa. It is associated with immunogenicity. The lipid moiety (lipid A) is responsible for the toxic effect. Hence, LPS mediates proinflammatory and toxic functions that lead to severe infections like sepsis and septic shock. In human volunteers endotoxin injection produces fever, shaking chills³⁷³, a reduced blood pressure together with blood and lung inflammatory reaction in which different immune cells, such as monocytes, macrophages, B cells, polymorphic nuclear- and endothelial vascular cells are involved³⁷⁴.

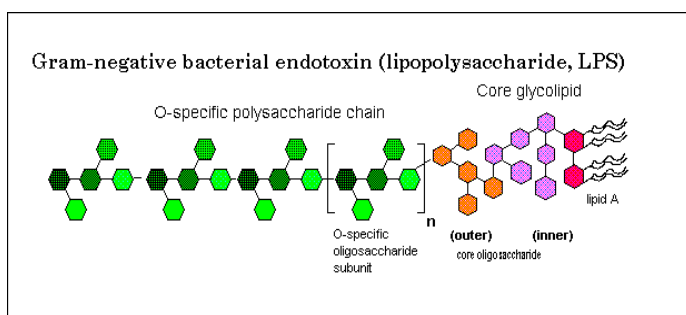


Figure 8 Three part construction of LPS

LPS consists of 3 parts: a variable chain with repeating oligosaccharides, a conserved region with hetero oligosaccharides and lipid A

LPS binds to the lipid binding protein (LBP) in the serum. This complex is transferred to CD14, a protein abundantly expressed on the cell membrane, and Toll-like receptor-4 (TLR-4). TLR-4 and CD14 are expressed on macrophages and dendritic

cells and are indispensable for secreting pro-inflammatory cytokines that usually are beneficial and effective but may also become excessive in their effects and lead to an "endotoxic shock" ³⁷⁵.

LPS induces different proinflammatory cytokines (IL-10, IFN- γ , and TNF- α) by activating transcription factors like NF κ B or IRF-3 ³⁷⁶. These cytokines explain the protective effects of pets and farm environment against allergic disease ³⁷⁷. Children living in a household with two or more dogs, together with a high level of indoor endotoxins (e.g. house dust) were a third less likely to develop wheezing than infants brought up without dogs ³⁷⁸. Pets in early childhood seem to be protective against allergen sensitization or asthma in children ³⁷⁹. The presence of animals (e.g. pigs) even in rural areas is associated with a lower likelihood of allergy ^{380, 381}.

A recent mouse-study conducted by Conrad et al (2009) could show a direct involvement of the bacterium *Acinetobacter lwoffii* F78, as a model organism for maternal bacterial exposure and the functional maternal TLR signaling inducing an asthma protection later on ³⁸².

2.5.3.6 Peptidoglycan

Peptidoglycan is a cell wall component of all bacteria (except some bacteria such as *Mycoplasma* species, *Planctomyces*, *Rickettsia* and *Chlamidiae*). Unlike endotoxins which are only present in gram-negative bacteria, peptidoglycan is a good marker for microbial exposure ³⁸³.

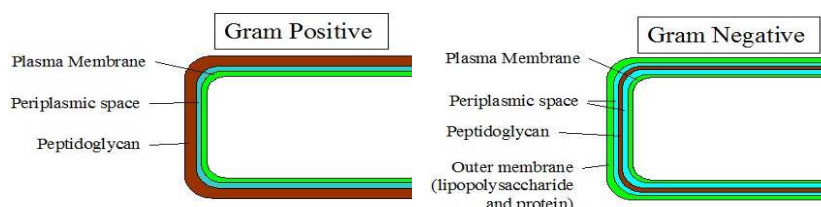


Figure 9 Cell wall of gram positive and gram negative bacteria ³⁸⁵

Gram positive bacteria possess a thicker cell wall with higher amounts of peptidoglycan compared to gram negative bacteria. Gram negative bacteria have an extra outer membrane carrying additional LPS.

N-acetyl-muramic acid (2-acetamido-3-O-[(R)-1-carboxy-ethyl]-2-deoxy-D-glucose), a peptidoglycan, is only present in bacteria and not found elsewhere in nature³⁸⁴. As gram-positive bacteria possess a thicker cell wall compared to gram-negative bacteria, peptidoglycan is more abundant in gram-positive bacteria (Figure 9 and 10). In contrast to endotoxin, which acts via TLR-4, peptidoglycan acts via TLR-2 and therefore causes a different cellular innate immune response compared to endotoxin³⁸⁶. This is concordant with the fact that TLR-2 genes are more strongly expressed in farm children³⁸⁷. Peptidoglycan induces IL-6, TNF- α , and IL-1 in monocytes, although a higher dose is required to get the same load compared to endotoxin³⁸⁸. Exposition to muramic acid, measured in mattress dust, was inversely related to wheezing and asthma, whereas no association with atopy could be observed³⁸⁶. This is in contrast to the hygiene hypothesis as bacterial exposure mediates a Th1 immune response with a reduced apoptotic response.

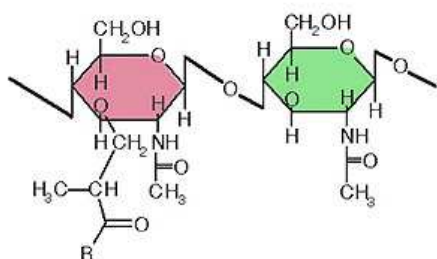


Figure 10 N-acetylmuramic acid-N-acetylglucosamine

Peptidoglycan is composed of two sugars, N-acetyl glucosamine and N-acetyl muramic acid cross-linked by amino acid bridges.

3 MATERIALS AND METHODS

3.1 Materials

3.1.1 Expendable Items

Dish	Company
Cellstar® Test tubes, sterile, 50 mL	<i>Greiner, Austria</i>
Cellulose acetate syringe filter, sterile, 0,20 µm pore size	<i>Iwaki, Tokyo, Japan</i>
Culture Flask 25 cm ² (0.2 µm seal cap)	<i>Becton Dickinson; San José, CA, USA</i>
Culture plates:	
24-well flat-bottom	<i>Becton Dickinson; San José, CA, USA</i>
48-well flat bottom	<i>Becton Dickinson; San José, CA, USA</i>
96-well round-bottom	<i>Iwaki, Tokyo, Japan,</i>
96-well flat-bottom	<i>Costar-Corporation, Cambridge, MA, USA</i>
ELISA plates	<i>Costar-Corporation, Cambridge, MA, USA</i>
Falcon™ FACS tubes, 5 mL	<i>Becton Dickinson, San José, CA, USA</i>
MACS-columns	
LD-column	
LS-column	<i>Miltenyi Biotec, Bergisch Gladbach, Germany</i>
MS-column	
MACS-tubes 13mL	<i>Miltenyi Biotec, Bergisch Gladbach, Germany</i>
Nalgene® MF75™ Sterile Bottle-Top Filters, 0,20 µm pore size	<i>Nalge Nunc International, Rochester, NY, USA</i>
PCR heat sealing film	<i>Eppendorf, Hamburg, Germany</i>
PCR plate, ep.twintec 96-well skirted	<i>Eppendorf, Hamburg, Germany</i>
PCR tips	<i>Eppendorf, Hamburg, Germany</i>
PCR tubes (thin-walled, clear)	<i>Eppendorf, Hamburg, Germany</i>
Pipette tips	<i>Eppendorf, Hamburg, Germany</i>
Polypropylen Tubes; 1,4 mL	<i>Micronic Systems, Lelystad, Holland</i>
Sodium heparin tubes, Vacuette®	<i>Greiner, Austria</i>

Sterilin, sterile, 30 mL	<i>No label</i>
Stripette® serological pipetts	<i>Costar-Corporation, Cambridge, MA, USA</i>
Wallac filter mate	<i>Perkin Elmer, Boston, MA, USA</i>
Wallac sample bag	<i>Perkin Elmer, Boston, MA, USA</i>

Table 4 Expendable items

3.1.2 Additives and Kits

Additive	Company
3.1.2.1 Cell culture	
[³ H]-thymidine (37MBq)	<i>Amersham-Pharmacia, Freiburg</i>
Anti-TLR-2	<i>eBioscience, San Diego, CA, USA</i>
Anti-TLR-4	<i>eBioscience, San Diego, CA, USA</i>
Anti-IgG2	<i>eBioscience, San Diego, CA, USA</i>
Brefeldin A	<i>Sigma–Aldrich, St. Louis, MO, USA</i>
BSA	<i>Sigma–Aldrich, St. Louis, MO, USA</i>
CFSE (5 mM in DMSO)	<i>Sigma–Aldrich, St. Louis, MO, USA</i>
Cytometric bead array (Flex set)	<i>Becton Dickinson, San José, CA, USA</i>
DMEM (Eagle’s medium)	<i>PAA laboratories GmbH, Pasching, Austria</i>
DMSO	<i>Merck, Darmstadt, Germany</i>
Dynalbeads® CD3/CD28 T Cell Expander	<i>Invitrogen, Dynal®, Carlsbad, CA, USA</i>
EDTA	<i>Sigma–Aldrich, St. Louis, MO, USA</i>
IL-8 ELISA	<i>BenderMedSystems, Vienna, Austria</i>
IL-13 ELISA	<i>BenderMedSystems, Vienna, Austria</i>
FCS	<i>Gibco, Life Technologies Inc., Rockville, MD</i>
Ficoll-Paque™ PLUS	<i>Amersham-Pharmacia, Freiburg</i>
FOXP3 staining set, PE, anti-human	<i>eBioscience, San Diego, CA, USA</i>
Gentamycine-sulfate	<i>Sigma–Aldrich, St. Louis, MO, USA</i>
IgG1 (heavy chain) anti-mouse	<i>Sigma–Aldrich, St. Louis, MO, USA</i>
IntraPrep	<i>BeckmanCoulter, Fullerton, CA, USA</i>
Ionomycin	<i>Sigma–Aldrich, St. Louis, MO, USA</i>
KCl	<i>Merck, Darmstadt, Germany</i>

KHCO ₃	<i>Merck, Darmstadt, Germany</i>
KH ₂ PO ₄	<i>Merck, Darmstadt, Germany</i>
L-glutamine	<i>Sigma–Aldrich, St. Louis, MO, USA</i>
Limulus assay (LAL)	<i>Cambrex Bio Science, Walkersville, USA</i>
Magnet for Dynalbeads® MPC-15	<i>Dynal, Oslo, Norwegen</i>
NaCl	<i>Fluka Chemica, Buchs, Switzerland</i>
Na ₂ HPO ₃ x 2H ₂ O	<i>Merck, Darmstadt, Germany</i>
NH ₄ Cl	<i>Merck, Darmstadt, Germany</i>
Paraformaldehyde	<i>Merck, Darmstadt, Germany</i>
Phorbol myristate acetate	<i>Sigma–Aldrich, St. Louis, MO, USA</i>
Puromycin	<i>Sigma–Aldrich, St. Louis, MO, USA</i>
RPMI 1640 ohne L-Glutamin	<i>Sigma–Aldrich, St. Louis, MO, USA</i>
Saponin	<i>Sigma–Aldrich, St. Louis, MO, USA</i>
Scintillation solution for β-Counter	<i>Rotiscint eco, Roth</i>
TMB-Substrate	<i>Becton Dickinson; San José, CA, USA</i>
Trypane blue solution 0.4%	<i>Sigma–Aldrich, St. Louis, MO, USA</i>
Tuerk Solution, 100 mL	<i>Fluka Chemica, Buchs, Switzerland</i>
Tween 20, 500 mL	<i>Sigma–Aldrich, St. Louis, MO, USA,</i>
Ultra culture medium (serum-free)	<i>Bio Whittaker, Walkersville, MD, USA</i>

3.1.2.2 MACS

CD4 ⁺ T cell Isolation Kit II, human	<i>Miltenyi Biotec, Bergisch Gladbach, Germany</i>
CD4 ⁺ CD25 ⁺ regulatory T cell Isolation Kit	<i>Miltenyi Biotec, Bergisch Gladbach, Germany</i>
CD25 ⁺ MicroBeads	<i>Miltenyi Biotec, Bergisch Gladbach, Germany</i>

3.1.2.3 PCR

Ethanol abs.	<i>Carl Roth, Karlsruhe, Germany</i>
GenElute™ Mammalian Total RNA Miniprep Kit	<i>Sigma–Aldrich, St. Louis, MO, USA</i>
iScript cDNA synthesis kit	<i>Bio-Rad Laboratories, Hercules, CA, USA</i>
Nuclease free water (not DEPC-treated)	<i>Ambion Inc, Austin, TX, USA</i>
iQ SYBR Green Supermix	<i>Bio-Rad Laboratories, Hercules, CA, USA</i>
RNaseZap® RNase Decontamination Solution	<i>Ambion Inc, Austin, TX, USA</i>
QuantiTect Primer Assays	<i>Qiagen, GeneGlobe Valencia, CA, USA</i>

Table 5 Additives and Kits

3.1.3 Laboratory Units

Unit	Company
BetaCounter Wallac 1450-024 MicroBeta	<i>Perkin Elmer, Boston, MA, USA</i>
C1000, Thermal cycler	<i>Bio-Rad Laboratories, Hercules, CA, USA</i>
Centrifuges	
Biofuge fresco	<i>Heraeus Instruments, Hanau, Germany</i>
LMC-3000	<i>HVD Life Sciences, Vienna, Austria</i>
4K15	<i>Sigma–Aldrich, St. Louis, MO, USA</i>
CO2-Incubator, CB210	<i>Binder, Tuttlingen, Germany</i>
¹³⁷ Cs-γ-irradiator	
ELISA-Reader	<i>Anthos 2010, Salzburg, Austria</i>
ELISA-Software	<i>ADAP Software, HVD</i>
FACSCalibur	<i>Becton Dickinson; San José, CA, USA</i>
FACSCanto	<i>Becton Dickinson; San José, CA, USA</i>
FACS Sorter Aria	<i>Becton Dickinson; San José, CA, USA</i>
Harvester	<i>Perkin Elmer, Boston, MA, USA</i>
Heat Sealer	<i>Eppendorf, Hamburg, Germany</i>
NanoDrop™ ND-1000	<i>NanoDrop Technologies, Wilmington, DE, USA,</i>
pH-Meter	<i>WTW, inoLab®, Weilheim, Germany</i>
Pipettes	<i>Eppendorf, research; Hamburg, Germany</i>
Precision scale, AT261 DeltaRange®	<i>Mettler Toledo, Vienna, Austria</i>
RT-PCR, CFX96 Optical Reaction Module	<i>Bio-Rad Laboratories, Hercules, CA, USA</i>
RT-PCR, Mastercycler® ep realplex 4S	<i>Eppendorf, Hamburg, Germany</i>
Scale, digital	<i>Mettler, Vienna, Austria</i>
Sterile bench, biosafe2	<i>Ehret, Emmendingen, Germany</i>
Vortex	<i>HVD Life sciences, Vienna, Austria,</i>
	<i>Invitrogen, Molecular Probes, Carlsbad, CA,</i>
Qubit™ fluorometer	<i>USA</i>

Table 6 Laboratory units

3.1.4 Allergens and Other Stimuli

Special emphasis was put on the major milk allergen β -lactoglobulin (BLG) and the major peanut allergen Ara h 1 which are among the most important food allergens in childhood. The milk-derived allergen (BLG) is a prominent food antigen often used as a model food allergen which leads to a high frequency of proliferative responses in cord blood and other cell types such as PBMCs, lymphocytes of the lamina propria and Peyer's patches of mononuclear cells^{216, 220, 389-392}. To investigate potential LPS contamination and the impact thereof following BLG preparations were used: (BLG 3908, BLG 3908 (purified), BLG L-0130, BLG L-0130 (purified) (all Sigma Aldrich) and BLG (LPS-free [LF]; generated by Brix et al³⁹³). Purification procedure was performed using endotoxin removal columns (Pierce) with trace amounts of LPS remaining present (named as purified). Because of that, and for the fact that the protein content was decreased a purified BLG fraction, manufactured by Brix et al³⁹³ served as the internal LPS-free control (BLG [LF]). In short, whey was separated and size-exclusion chromatography was performed with the Superdex 75 column (Hiload 26/60, AP Biotech, Uppsala, Sweden) using 150 mmol/L ammonium acetate at the pH of 6.0. The two collected BLG peaks were pooled, dialyzed (Spectra/Por, with a cut-off of 12 to 14 kDa) in water (MilliQ, Millipore), and lyophilized. The endotoxin content of this LPS-free BLG 100 times lower than the un-purified sample and 15 times lower than the purified (Pierce column) BLG (Table 10).

Size-exclusion chromatography of commercially available BLG (Sigma Aldrich, L-0130) was performed using a Superdex 75 column (Hiload 26/60, AP biotech, Sweden) as described in detail by Brix et al³⁹³. Briefly BLG was dissolved in ammonium acetate (0.15 M, pH 6.0), and 5 mL [20 mg/mL] was applied to the column. Elution was carried out using the same buffer at 2.5 mL/min. Fractions of 10 mL were collected and frozen. Before inclusion in cell cultures, 2 x 1 mL from each fraction was vacuum-dried, and twice evaporated with distilled water to remove any remnants of eluting buffer.

The recombinant Bet v 1 was used representative for inhalational allergens and purchased from Biomay, Vienna, Austria.

Plate-bound anti-CD3 (UCTH1 clone, Dako), a polyclonal TCR dependent T cell activator, was used for proliferation and inhibition assays and tetanus toxoid (TT) was used only in peripheral blood proliferation assays representative for a recall-antigen.

For LPS proliferation LPS from *Escherichia coli* serotype 055:B50 and *Salmonella minnesota* strain R595 were used. The highly purified LPS *S. minnesota* strain R595 “ultra pure” was used for TLR-inhibition experiments.

Stimulus		Abbreviation	Source	Preparation
Anti-human T cell CD3		aCD3 (UCTH1 clone)	DAKO, Golstrup, Denmark	Product No. M0835
Birch	Betula verrucosa	rBet v 1	Biomay, Vienna, Austria	Lot #22 Isoform Bet v 1.0101
Egg	Ovalbumin	OVA	Sigma–Aldrich, St. Louis, MO, USA	Product No. A5503
Human Interleukin-2		rIL-2	Roche Applied Science, IN, USA	Product No. 1 147 528
Lipopolysaccharide		LPS E.coli	Sigma–Aldrich, St. Louis, MO, USA	<i>Escherichia coli</i> serotype 055:B50
		LPS S. minnesota	LuBio Science, Lucerne, Switzerland	<i>Salmonella. minnesota</i> strain R595
		LPS S. minnesota (Ultra pure)	LuBio Science, Lucerne, Switzerland	PURE <i>Salmonella.</i> <i>minnesota</i> strain R595
Milk		BLG 3908	Sigma–Aldrich, St. Louis, MO, USA	Product No. 3908
		BLG L-0130		Product No L-0130
			Susanne Brix (BioCentrum- DTU, Technical University of Denmark, Lyngby, Denmark)	Protein obtained from raw milk. Purification and analysis as described in Brix et al (2003) ³⁹³

Peanut	<i>Arachis hypogea</i>	Ara h 1	<i>Clare Mills (IFR Norwich)</i>	Allergen obtained from whole Virginia red variety peanuts. Procedure described by ^{394, 395}
Phytohem-agglutinin	M Form	PHA	<i>Gibco Invitrogen Corporation, NJ, USA</i>	Product No. 10576-015
Tetanus toxoid	<i>Clostridium tetani</i>	TT	<i>Calbiochem, La Jolla, CA, USA</i>	Product No. 582231

Table 7 Stimuli used

3.1.5 Antibodies

3.1.5.1 Monoclonal antibodies for flow cytometry

Specificity	Fluorescent labelling	Origin	Ig-Isotype	Clone	Dilution	Company
CD 3	PerCP	Mouse	IgG1	SK7	1:10	BD
CD 3	APC	Mouse	IgG1	SK7	1:100	BD
CD 4	FITC	Mouse	IgG1	SK3	1:10	BD
CD 4	PE	Mouse	IgG1	SK3	1:10	BD
CD 4	FITC	Mouse	IgG2a	VIT4	1:10	Miltenyi Biotech
CD 8	PerCP	Mouse	IgG1	SK1	1:10	BD
CD 8	PE	Mouse	IgG1	SK1	1:10	BD
CD 25	APC	Mouse	IgG1	2A3	1:100	BD
CD 25	PE	Mouse	IgG2b	4E3	1:10	Miltenyi Biotech
CD31	PE	Mouse	IgG1	AC128	1:10	Miltenyi Biotech
CD45RA	PE	Mouse	IgG2b	HI100	1:10	BD
CD45RB	PE	Mouse	IgG1	MT4 (6B6)	1:10	BD
CD45RO	PE	Mouse	IgG2a	UCHL-1	1:10	BD
CD45RO	PE-Cy5	Mouse	IgG2a	UCHL-1	1:10	Miltenyi Biotech
CD45RO	APC	Mouse	IgG2a	UCHL1	1:100	BD
CD62L	APC	Mouse	IgG	145.15.3	1:10	Miltenyi Biotech
CD69	PE	Mouse	IgG1	L78	1:10	BD
CTLA4	PE	Mouse	IgG2a	BNI3	1:10	BD
HLA-DR	PE	Mouse	IgG2a	L243 (G46-6)	1:10	BD
CD103	PE	Mouse	IgG1	Ber-ACT8	1:10	BD
CD127	PE	Rat	IgG2b	SB/199	1:10	BD
CD127	APC	Mouse	IgG1	40131	1:100	R&D
FOXP3	PE	Rat	IgG2a	PCH101	1:10	eBioscience
GARP	PE	hybridoma tissue culture supernatant	IgG2b	Plato-1	1:1000	Alexis Corporation

Table 8 Monoclonal antibodies for flow cytometry

3.2 Medium and Cells

3.2.1 Media and Buffers

Buffer/medium	Abbreviation	Ingredients	Storage
Ammonium-Chloride buffer	Ery-Lys	NH ₄ Cl [155mM] 46 g KHCO ₃ [10mM] 500,6 mg EDTA [0,1mM] 14,6 mg	4° C
CFSE buffer	-	500 mL PBS 500 mg BSA	4° C
Eagle's medium	DMEM	10% foetal bovine serum, 100 mg/L gentamycine-sulfate 5 µg/mL puromycin	4° C
ELISA antibody diluent buffer	-	1% BSA PBS	4° C
ELISA biotinylated diluent buffer	-	1% BSA PBS	4° C
ELISA HRP-streptavidin diluent buffer	-	1% BSA 0,1% Tween 20 PBS	4° C
ELISA Saturation buffer	-	5% BSA PBS	4° C
ELISA Wash buffer	-	0.05% Tween 20 PBS	4° C
Freezing medium	-	25 mL RPMI 1640 5 mL DMSO 20 mL heat inactivated FCS 170 mg/L gentamycine-sulphate	4° C
MACS buffer	-	500 mL PBS (pH=7,3) 2,5 g BSA 0,3684 g EDTA	4° C
Phosphate buffered saline	PBS	8 g NaCl 0,2 g KCl 1,8 g Na ₂ HPO ₄ 2H ₂ O 0,24 g KH ₂ PO ₄ A.d. ad 1000 mL	4° C

RPMI 1640 complete medium	-	RPMI 1640	4° C
		2mM L-glutamine	
		85 mg/L gentamycine-sulphate	
		10% heat inactivated FCS	
Saponin-Buffer	-	10g Saponin	4° C
Stock-Solution 10%		23,8g HEPES	
		100 mL PBS	
Serum-free Ultra culture complete medium	UCC	UC-medium	4° C
		2mM L-glutamine	
		170 mg/L gentamycine-sulphate	

Table 9 Media and Buffers

Experiments were performed using sterile buffers and media listed in table 9. All media and buffers were sterile-filtered with bottle top filters (BD, San José, CA) or syringe filters if less than 20 mL was needed (e.g. freezing medium). The pH value was always adjusted between 7.2 and 7.4. For washing procedure and counting cells sterile PBS (pH 7.2-7.4) was used if not otherwise noted. If fetal calf serum (FCS) was used it has before been inactivated 30 min. at 56°C and then stored at -20° C.

3.2.2 Cord Blood and Patient Characteristics

Human umbilical cord blood was collected at the Medical University of Vienna at the Department of gynaecology. The protocol was approved by the local Ethics Committee of the Medical University of Vienna.

Cord blood from randomly chosen full term healthy infants was obtained by venopuncture of the umbilical vein immediately after delivery and placed in sterile sodium heparin tubes (VACUETTE®, Greiner).

As a homogenous population of healthy neonates was required, following exclusion criteria needed to be met to prevent potential T cell influence:

- < 37 week of gestation and body weight <2500g)³⁹⁶;
- Neonates with infections, assisted respiration, metabolic problems, organ failure, chromosome aberrations³⁹⁶;
- Chronical illness of the mother (autoimmune diseases like diabetes mellitus, rheuma, morbus crohn, colitis ulcerosa etc.; chronical inflammation (e.g. HIV³⁹⁷, chronical hepatitis³⁹⁸);
- and/or drug addiction³⁹⁹.

Individuals with no history of food allergy and no sensitization (no specific IgE measured with CAP/Phadia, skin prick testing (SPT) negative (wheal diameter < 3 mm) with a mean age of 30 years were used as controls.

3.3 Methods

3.3.1 Cell Isolation Techniques

3.3.1.1 Separation of Cells via Density-Gradient Centrifugation

Density gradient centrifugation is an efficient way to separate blood cells. During centrifugation, cells are preserved on an area matching their own density. Mononuclear cells, which are mainly lymphocytes, monocytes, and granulocytes with a density between 1.04 and 1.14 g/mL are concentrated in the interphase after centrifugation.

Human plasma	~1.03 g/mL
Monocytes and Lymphocytes:	~1.08 g/mL
Neutrophils:	~1.12 g/mL
Neutrophils and Eosinophils:	~1.13 g/mL
Eosinophils:	~1.14 g/mL

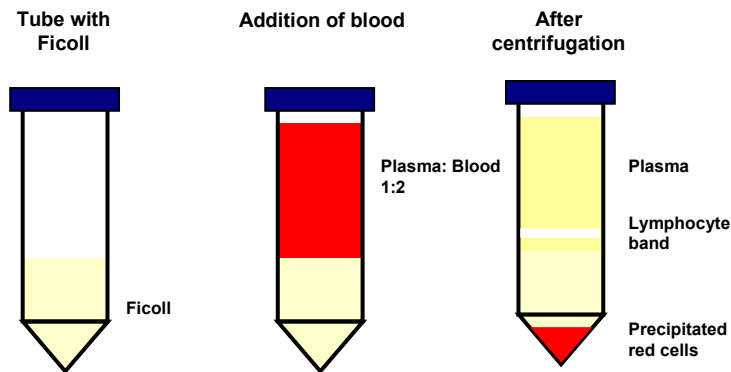


Figure 11 Separation of cells via Ficoll

Ficoll was used for separating mononuclear cells from whole blood.

Heparinized blood was diluted 1:2 with PBS, and was drawn over Ficoll-Paque™ PLUS (Pharmacia, Uppsala, Sweden), a neutral, highly branched, hydrophilic sucrose polymer (400,000 Da) with a specific density of 1.077 g/mL at 10°C. After centrifugation at 300 g (1250 rpm) for 30 minutes with no brakes at room temperature (Sigma centrifuge) the light thrombocytes remain in the plasma phase and granulocytes and erythrocytes sediment through the Ficoll (Figure 11). Lymphocytes and monocytes are kept in the interphase in a fleecy band that was recovered with a 5 mL stripette (Eppendorf). Cells were washed three times with PBS by centrifugation to separate

them from Ficoll and plasma. Where erythrocyte lysis was needed isotonic ammonium-chloride buffer was applied for 5-7 minutes. Cells were always freshly isolated and immediately used, if not otherwise noted. For inhibition assays after priming, CBMCs and $CD4^+CD25^-$ T cells needed to be frozen. They were frozen immediately in 1 mL ice-cold freezing-media in cryotubes which contained the cryoprotectant DMSO.

For thawing cryotubes were held in 37° C water while gently shaking. Immediately when only a small ice cube remained cells were transferred into culture media (10x volume) and centrifuged at 300 g for 10 minutes. Afterwards the supernatant was decanted and cells were cultured in fresh medium.

Cells were counted in a Buerker-Tuerk chamber (Figure 12 (a)). An aliquot (10 μ L) of the cell suspension was mixed with the same volume of either Tuerk solution (for freshly isolated cells) or Trypan blue (for thawed cells). Using the Tuerk dye, nuclei are colored in purple and round cells were counted. When Trypan blue was used the blue-coloured cells were left out and not counted as dead cells appear blue with this dye because when dead cells lose their potential to actively discharge the dye.

The chamber comprises of 9 squares (Figure 12 (b)). Each square has a defined area of 1 mm² with 0.1 mm sample depth hence a defined volume of 0.1 μ L can be calculated. Five squares were counted in the Buerker-Tuerk chamber for receiving a representative mean and the total cell count per 1 μ L is determined using the following equation:

$$= \frac{\text{cells counted}}{\text{counted area (mm}^2\text{) * sample depth (mm) * dilution}}$$

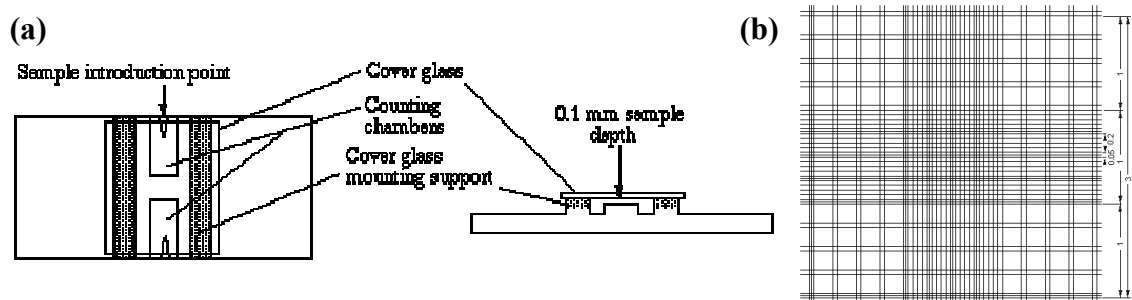


Figure 12 Buerker-Tuerk chamber

All mononuclear cells were counted with this Buerker-Tuerk chamber. 9 squares, each with 1 mm² area and 0.1 mm sample depth comprise this chamber and five squares were counted to get a representative result.

3.3.1.2 MACS Separation

Magnetic cell sorting (MACS®, Miltenyi Biotec) is a technique that uses monoclonal antibodies coupled to magnetical microbeads to isolate cell fractions. Magnetically labeled cells can be separated over ferreous columns. For CD4⁺CD25⁺ Treg isolation, a two step isolation procedure was used (CD4⁺CD25⁺ regulatory T cell isolation kit, Miltenyi Biotec). As a first step, non-CD4⁺ cells (monocytes, B cells, NK cells, cytotoxic T cells, dendritic cells, normocytes, thrombocytes and basophile granulocytes) were indirectly labeled with biotin-conjugated antibodies and then magnetically labeled with anti-Biotin beads. CD4⁺ cells were negatively depleted with a MACS-LD-column and obtained in the flow-through. In a second step, CD4⁺ cells were directly labeled with CD25-microbeads and then positively depleted by a MACS-MS-column. The last step was repeated 1-2 times to gain a higher purity. After the magnetic field was removed, cells were flushed with 1 mL MACS buffer to get the positively selected (labeled) CD4⁺CD25⁺ Tregs (Figure 13).

To isolate the effector T cells from the same cell population CD4⁺CD25⁻-cells were negatively selected from the first flow-through from the MS-column (Figure 13). The enriched CD4⁺CD25⁻ T cells were washed, centrifuged and negatively selected with a LS column to deplete remaining CD25⁺ cells.

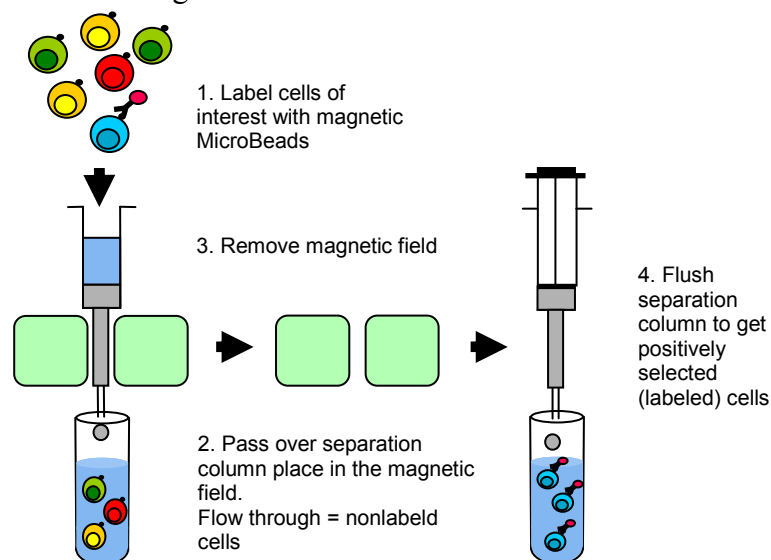


Figure 13 Model for MACS separation

At the first step, cells of interest are labeled with specific microbeads (e.g. CD25). Cells are separated over a magnetic column and retrieved after the magnetic field is removed.

3.3.2 Proliferation Assays

For the proliferation assay CBMCs or PBMCs were seeded at a density of 10^5 cells/200 μ L if not otherwise noted. For all experiments serum free media was applied (UCC; Ultra culture medium; Bio Whittaker, Walkersville, MD).

Initially, LPS dependent proliferation was assessed in cord blood to define the time point with maximal response. Consequently proliferation assays were performed after 72h (LPS-response) and 7 days (allergen-specific proliferation) at a humidified atmosphere with 5% CO₂ at 37°C as previously described^{217, 218}. Recombinant IL-2 [25 IU/mL] served as a positive control and medium alone with cells was used as negative control. Cells were pulsed with [³H]-thymidine ([³H]TdR; 0.5 μ Ci [0.0185MBq]) per well for the last 16 h of the culture. Thereafter cells were directly harvested or frozen at -20°C to stop the incorporation. The incorporated radioactivity (cpm, counts per minute) was measured by liquid scintillation. Proliferation was expressed as stimulation index (SI) of a geometric mean of triplicates. The SI is determined using the following equation:

$$SI = \frac{\text{cpm in stimulated culture}}{\text{cpm in unstimulated culture}}$$

According to literature an SI > 2 was considered positive²¹⁷.

For LPS proliferation, CBMC were isolated and co-cultured with LPS in limiting dilution. At the two defined read-out time points (72h (day 3) and day 7), proliferation and TNF- α production was measured (Proliferation n=8; TNF- α measurement n=5; standard error of the mean (SEM)).

For antigen-specific proliferation assays, dose response experiments applying allergen concentrations from 0.1-100 μ g/mL and the corresponding LPS-content were assessed to define the explicit relevance of different amounts of contamination present.

For the tetanus toxoid proliferation assay, tetanus toxoid (TT) was chosen as a model antigen known to induce a positive proliferative response in vaccinated individuals. TT was initially proven (via certificate and via internal limulus control) to contain endotoxin below the limit of relevance (Table 10). A sub-maximal dose of 1 μ g/mL was

spiked with increasing levels of endotoxin corresponding to the amount of contamination present in BLG.

3.3.3 LPS Measurement via Limulus Amoebocyte Lysate

The limulus amoebocyte lysate (LAL) method bases on the fact that the blood of the horseshoe crab, *Limulus polyphemus*, coagulates upon endotoxin exposure. The toxin of gram-negative bacteria can be quantified with this LAL test (Cambrex, 50647U). The sample and the LAL is mixed and incubated at 37°C for 10 minutes. Subsequently a substrate solution is mixed with the LAL-sample and incubated at 37°C. Concentration is determined spectrophotometrically at 405-410 nm (limit of detection: 0.035 EU/mL).

3.3.4 Cytokine Measurement

Cytokine measurement was performed either via ELISA (BenderMedSystems, Vienna, Austria) or cytometric bead array (Flex set, Becton Dickinson, San José, CA, USA). CBMCs (5×10^5 /mL UCC) were cultured in 96-well round-bottom plates in the presence of the respective antigen in a dose dependent manner in triplicates. For monocytes, in case of TLR-inhibition experiments, 48-well flat-bottom wells were used. Medium with cells alone served as negative control. Cell-free supernatants were harvested at the respective time points and immediately frozen at -80° Celsius.

IL-1 β , IL-5, IL-10, TNF- α and INF- γ levels were measured via cytometric bead array (CBA; Flex Kit, Becton Dickinson, San José, CA, USA) using a FACS Canto (Becton Dickinson, San José, CA, USA) according to the instructions provided by the manufacturer. IL-13 was measured via ELISA (limit of detection [1.56 pg/mL]; BenderMedSystems, Vienna, Austria).

3.3.5 TLR Assays

3.3.5.1 TLR Inhibition Assays

Cord blood derived monocytes were isolated via adherence in 48-well flat-bottom plates. TLR-inhibitory antibody (anti-TLR-4 [1 µg/mL]) and the respective isotype control (IgG2a [1 µg/mL]) were added (all from eBioscience, San Diego, CA, USA) for 1 hour. Samples or positive control (LPS) were added and cell free supernatant removed after 6 hours of incubation and stored at -80°C. TNF-α served as read-out parameter and was measured via sandwich-ELISA (limit of detection [8 pg/mL]; BenderMedSystems, Vienna, Austria).

3.3.5.2 TLR-2 and TLR-4 Transfected Human Embryonic Kidney Cells (HEK293)^A

Human embryonic kidney cells (HEK293) were stably transfected with an expression vector for human CD14 (293-hCD14), CD14 and TLR-2 (293-hCD14/TLR-2) and CD14 and TLR-4 (293-hCD14/TLR-4) (a kind gift of Dr. M. Yazdanbakhsh, Leiden University Medical Center, The Netherlands). Cells were cultured in standard Dulbecco's modified Eagle's medium (DMEM; PAA laboratories GmbH, Pasching, Austria) for 24h. For stimulation experiments, 4×10^4 cells/well were placed in 96-well flat-bottom plates (Costar) in complete medium and co-incubated with the respective antigen at 37°C in 5% CO₂. Cell-free supernatants for cytokine measurements were collected. Untreated cells served as controls. For stimulation of 293-hCD14/TLR-4 cells, MD-2 (a protein imperative for the responsiveness to LPS) was added to the culture medium. The concentration of IL-8 in the supernatant was used as the read-out parameter and measured via ELISA (Endogene) according to the manufacturer's instructions.

^A This experiment was performed by Irma Schabussova, Medical University of Vienna

3.3.6 Flow Cytometry

The analysis and the phenotypical characterization of lymphocytes is one of the most important techniques in immunological research and diagnostics. With fluorescence activated cell sorting (FACS-analysis) different cell populations and cell types can be identified. Different types of lymphocytes can be recognized due to their surface antigens that are usually defined through their binding to specific monoclonal antibodies. When those antibodies are marked with a fluorescent dye (fluorochrome), the expression of the corresponding protein on cells is analyzed with the FACS. Different parameters of one cell can be detected by using this method. Hence, it is possible to extrapolate the gathered information of each individual cell.

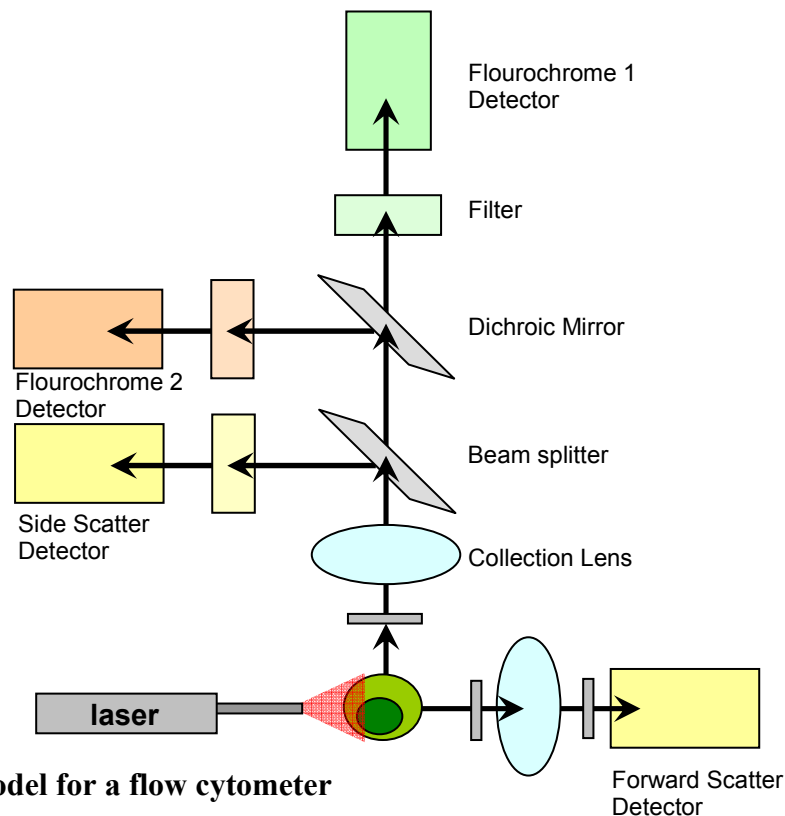


Figure 14 Model for a flow cytometer

Using extra- and intracellular staining cellular activation (transcription factors (e.g. FoxP3) and cytokine production etc. can be analyzed. A laser beam with a single wavelength aims on a focused flow of fluid. The FSC (Forward Scatter, FSC; 2° - 15° from laser) correlates with the cell volume. SSC (Side Scatter, SSC, 90°) depends on the granularity of the particle. Dead cells are normally smaller and also possess more granularity than live cells. Therefore a differentiation between dead and alive cells is

possible. Flow cytometry is an excellent method for counting, examining and sorting cells. Each cell scatters the light in a specific way. Fluorescent antibodies attached to the cell surface emit light of a specific wave length which can be detected and differentiated through analysis with special software.

Acquisition of cells was performed on a FACS-Calibur four-colour cytometer and subsequent analysis of the measured parameters was done with the Cell Quest software (both Becton Dickinson, San José, CA). A total of 10^5 cells were analyzed in case of intracellular staining and a minimum of 2×10^4 for surface staining.

For both surface and intracellular staining, human mononuclear cells and the standard protocol were used. For intracellular staining a permeabilization/fixation kit (IntraPrep; BeckmanCoulter, USA) or saponine detergent as an alternative protocol, which reversibly forms holes in cell membranes, were used. Fluorescent antibodies as listed in table 8 and the respective isotype control were utilized for all experiments.

3.3.6.1 CFSE

5(6)-Carboxyfluorescein N-succinimidyl ester (CFSE) is a fluorescent cell staining dye to visualize and qualify division of cells. CFDA-SE (Carboxyfluorescein diacetate, succinimidyl ester), the pre-form, enters the cell cytoplasm by diffusion and is cleaved by intracellular esterase and converted to CFSE. CFSE is not cell permeable and irreversibly binds to free proteins (Figure 15).



Figure 15 CFSE

CFDA-SE enters the cell membranes and intracellular esterase converts it to CFSE. CFSE binds to proteins which results in a stable intracellular fluorescence that is equally distributed between two daughter cells after division.

Whenever a stained cell divides, CFSE labelling is partitioned equally between the two daughter cells¹⁴⁸(Figure 16 (a, b).

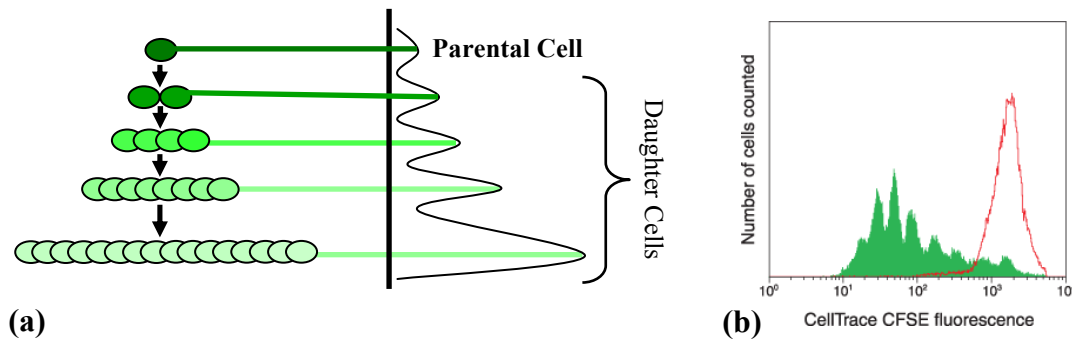


Figure 16 Division of CFSE labeled cells

The highly fluorescent, CFSE stained parental cell gives half of its fluorescent dye to the daughter cells (a) which can be visualized in a flow cytometer. Figure (b) shows a CFSE histogram. Here, the highly fluorescent parental cells are seen on the green vertex on the right, whereas all the peaks to the left show one divided daughter population (green). The red line represents the CFSE stained cells that have not undergone proliferation.

The two-fold decrement in cellular fluorescence intensity can be analyzed with flow cytometry using excitation at 488 nm. CFSE labelling is a standard technique to assess proliferation similar to [^3H]-thymidine incorporation but providing a higher information content. With CFSE labelling one can distinguish whether an entire population divides once or one fourth of a population divides three times which cannot be distinguished with [^3H]-thymidine incorporation alone. This is a big advantage compared to the [^3H]-thymidine incorporation assay which allows only measurement of proliferation within a specific time frame.

Prior to use a 1 mM CFSE stock solution (Sigma-Aldrich, 21888) was prepared in 1000 μL pre-warmed PBS/0.1% BSA. The fresh isolated lymphocytes were resuspended in pre-warmed PBS/0.1% BSA at a final concentration of 10^7 cells/mL. Via titration an optimal working concentration of 5 μM was defined. The staining (10 min. gently shaking at room temperature) was quenched by the addition of 5 volumes of ice-cold PBS/0.1% BSA to the cells and then pelleted. Cells were washed three times in fresh media. Afterwards cells were placed in appropriate well plates for further experiments and analyzed in a FACS Calibur at 488 nm.

3.3.6.2 FACS Sorting

Fluorescence activated cell sorting (FACS) uses the technique of flow cytometry as explained before. The main difference here is that cells cannot only be analyzed but also sorted due to different fluorescent surface markers. As the information from the scatter and the fluorescence the computer can process the information and hence determine which cells are to be separated. A very high purity (>99%) can be achieved with that method (Figure 17).

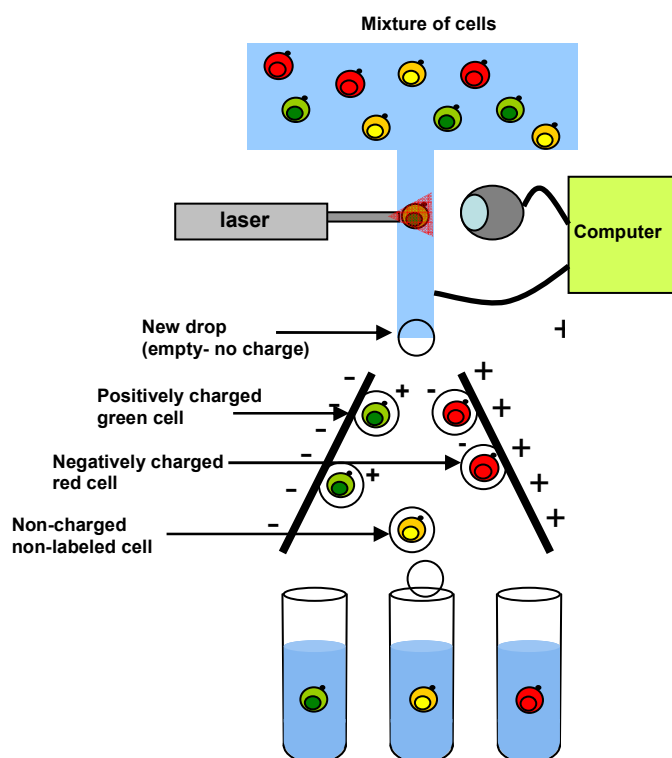


Figure 17 FACS sorting scheme

The required cells are fluorescently labeled with specific fluorescent antibodies (shown in red or green). As not every cell expresses the epitope some are not labeled in either colour (yellow). Those cells are discarded due to their electrical charge in the waste tube.

3.3.6.2.1 Sorting according to CD25 and CD127 Expression

CD4⁺ T cells were purified via negative isolation applying MACS sorting. Cells were labeled with CD4, CD127, CD3 and CD25.

After staining and washing cells were FACS sorted and 4 different fractions were gained as shown in figure 18.

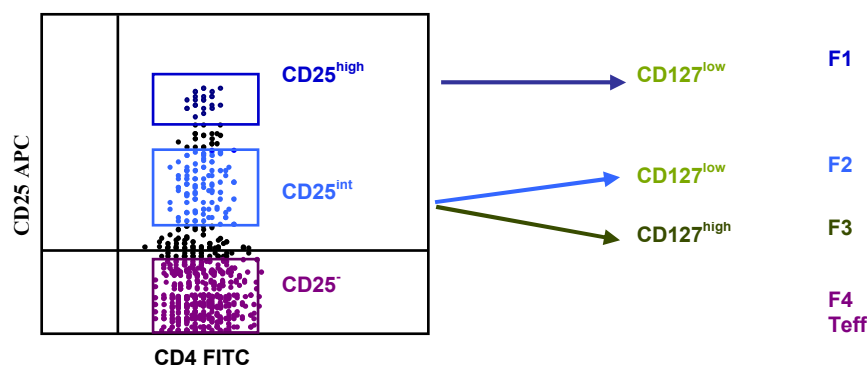


Figure 18 Scheme of fractions that were FACS sorted according to their CD25 and CD127 expression

F1	CD4 ⁺ CD25 ^{high} CD127 ^{low}
F2	CD4 ⁺ CD25 ^{int} CD127 ^{low}
F3	CD4 ⁺ CD25 ^{int} CD127 ^{high}
F4, Teff	CD4 ⁺ CD25 ⁻ (effector T cells)

3.3.6.2.2 Sorting According to CFSE Intensity and CD25 Expression

To distinguish which cell population gains inhibitory potential after the priming procedure FACS sorting due to CFSE intensity was applied.

CBMCs were stained with CFSE according the protocol described above and cultured with BLG L-0130, BLG [LF] and Ara h 1 (all [50 µg/mL]) in 25 cm² culture flasks (Falcon, BD, San José). At day 6 cells were CD4 (untouched) MACS-sorted and stained for CD3 (PerCP) and CD25 (APC). The fluorescent dye PE was not used due to possible overlapping into the CFSE channel. We sorted the cells according to their CFSE and CD25 expression presented in figure 19.

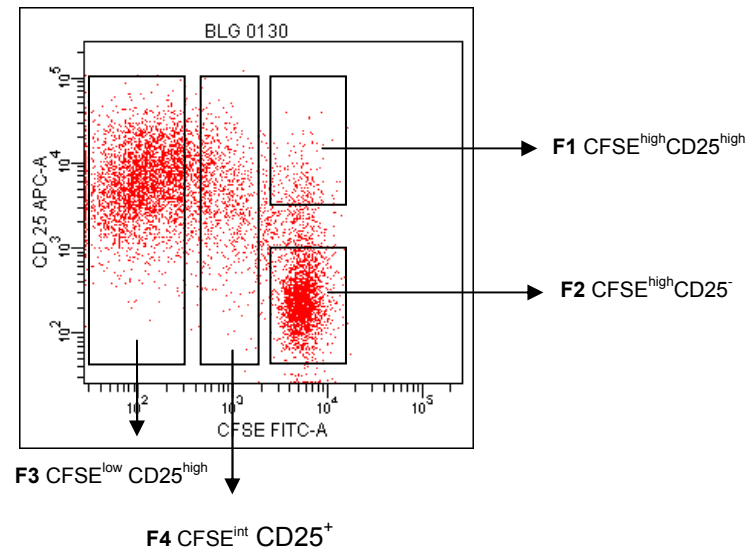


Figure 19 Scheme of fractions that were sorted due to their CFSE and CD25 expression

Subsequently inhibition assays were performed with autologous irradiated CBMCs and $CD4^{+}CD25^{-}$ T cells in the presence of α -CD3, BLG L-0130, BLG [LF], Ara h 1, and without stimulus (control). In case of limited cell counts only TCR crosslinking was performed (α -CD3).

3.3.7 Treg Inhibition Assay

The regulatory potential of cellular fractions can be assessed by adding an increasing numbers of cells (2.5×10^3 – 2.5×10^4 cells/well) to effector T cells defined as $CD4^+CD25^-$ (Teff; 2.5×10^4 cells/well). Cell subsets alone (either only Tregs or Teff) as well as combined cells were stimulated with 250 ng/mL plate-bound anti-CD3 (UCHL1 clone, Dako) or the respective antigen in the presence of autologous, irradiated (3,000 rad) CBMCs or PBMCs (5×10^4 cells/well), respectively. Proliferation was assessed with [^3H]-thymidine incorporation assay on day 3 and 7. Inhibition is expressed as relative proliferation compared to the $CD4^+CD25^-$ cells.

3.3.8 Priming Assay

The $CD4^+CD25^-$ T cell subset was MACS-sorted as explained above and frozen. CBMCs were first stimulated with BLG L-0130, BLG [LF] or Ara h 1 (all [50 μ g/mL]) in a 25 cm² culture flask (BD Falcon) for 6 days. On day 6, the “allergen-primed” $CD4^+CD25^+$ Tregs were isolated via magnetic cell sorting (MACS) and inhibition assays were performed with the newly generated Tregs, together with the antigen-inexperienced effector T cells ($CD4^+CD25^-$) (Figure 20).

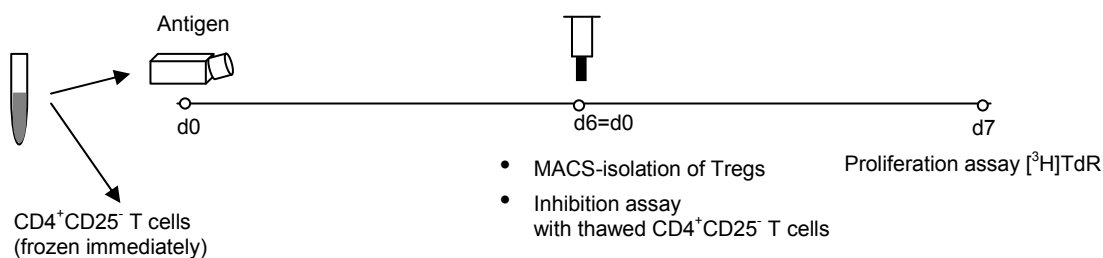


Figure 20 Scheme of priming CBMCs

Cord blood derived mononuclear cells were co-cultured with the antigen. At day six $CD4^+CD25^+$ T cells were MACS-sorted and an inhibition assay was performed with the $CD4^+CD25^-$ (that had been frozen on day 0) with stimulation with anti-CD3, BLG L-0130 or BLG [LF].

3.3.9 RNA Isolation

RNA was isolated using the “GenElute Mammalian Total RNA Miniprep Kit” (Sigma Aldrich) according to the manufacturer’s instructions.

The RNA content was measured with the NanoDropTM (ND-1000 spectrophotometer; (260/280 and 260/230 nm ratios)). NanoDrop delivers the most accurate result available with remarkable reproducibility together with only 1.5 µL sample needed. Especially for RNA measurement for Tregs this is crucial, as only very little RNA is extracted. Moreover no cuvettes and capillaries are needed.

3.3.10 cDNA Synthesis

The RNA was reverse-transcribed applying “iScript cDNA synthesis kit” from BioRad according to the manufacturer’s instructions using oligo dT primers. For all experiments in this thesis 25 ng RNA was applied and reverse transcribed with the thermal cycler C1000 (BioRad).

3.3.11 Semi-Quantitative Real-Time RT-PCR

3.3.11.1 PCR Overview

Polymerase chain reaction (PCR) allows exponential amplification of short (100 to 600 bases) DNA sequences. PCR requires a pair of primers (~20 nucleotides) that is complementary to a defined sequence on each of the two strands of the DNA. DNA polymerase extends the primers by copying the designated sequence. As the same primers can be reused to amplify the DNA, theoretically a logarithmic amplification is the result. Quantitative RT-PCR can be sub-classified into three phases: Denaturation, Annealing and Extension. In the first phase, double stranded cDNA is separated at about 90° C. In the annealing phase (50 to 60°C) specific primers can bind to the sample and in the expansion phase polymerases lead to an exponential amplification.

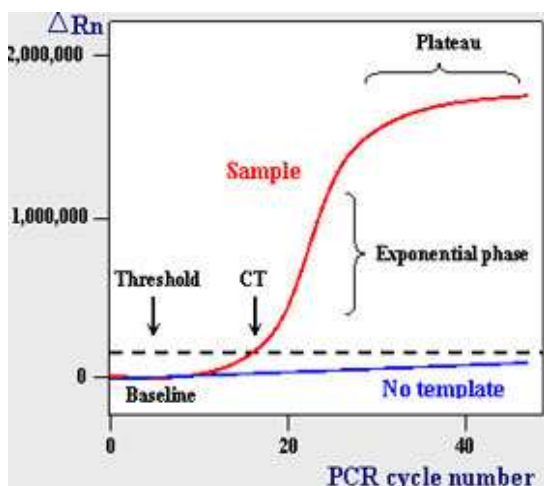


Figure 21 Diagram of single a PCR cycle in a Real- time PCR machine ⁴⁰⁰

The X-axis shows the PCR cycle number and in the the copy number is plotted on Y-axis. The dotted threshold line is user-defined and used to determine the cycle threshold (Ct). The curve has a sigmoidal course with an exponential phase and a plateau phase. A negative control, the blue line, is performed with RNase free water alone.

A negative control (RNase free water alone without template) and a positive control (a housekeeping gene, e.g. 18S) are always applied to have a blank value and a reference gene, respectively.

SYBR[®] Green with its high fluorescence in the presence of double stranded DNA is used in this experimental setting. For validation, the samples are analyzed at the melting temperature. This is the temperature at which a DNA double helix dissociates into single strands to check for contamination, mispriming or primer-dimer artefacts (Figure 22).

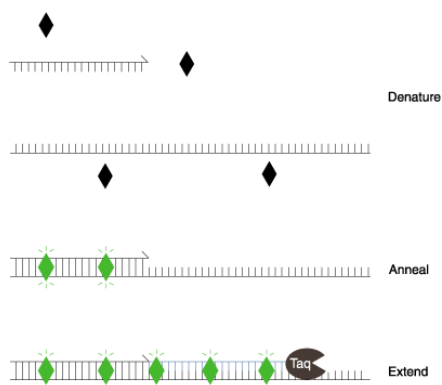


Figure 22 Schematic Real-Time PCR with SYBR[®] Green ⁴⁰¹

Schematic representation of real-time PCR (Denaturation - Annealing - Extension) with SYBR Green dye. SYBR Green dye (black rhomb) becomes fluorescent (green rhomb) upon binding to double-stranded DNA, providing a direct method to quantify PCR products.

3.3.11.2 PCR Methodology Used

The iQ SYBR Green Supermix (BioRad) together with QuantiTect primers were used. Primers (Qiagen) were dissolved in 1.1 mL nuclease-free water prior to use. After sealing each plate with a microseal “B” Heat sealing film (BioRad), a two step

temperature program was run on the RT-PCR machine CFX96 and recorded via the included software CFX manager vs. 1.1 (both BioRad).

Thermal protocol used:

95°C	2 min. Initial template denaturation (1x)	} 40x
95°C	15 sec. Cycled template denaturation	
60°C	1 min. Annealing/extension	

The stage at which the fluorescence crosses the threshold is called the cycle threshold (Ct). The lower the Ct value, the more copies are present in the specific sample. The mRNA expression levels were calculated using the [DELTA] Ct calculation. The relative expression of a target gene (e.g. *FOXP3*) was calculated based on the Ct deviation of this target gene versus the housekeeping gene: (Delta Ct sample = Ct gene (e.g. *FOXP3*) – Ct housekeeping gene (e.g. 18S).

To compare expression of genes, the sample with the lowest amount of the respective gene was set as internal standard to 1, and relative expression was calculated as follows

$$2^{-(\Delta Ct \text{ sample of interest} - \Delta Ct \text{ lowest sample})}$$

After 40 cycles the melting curve is checked using the following thermal protocol: 60°C-90°C for 20 seconds (Figure 23).

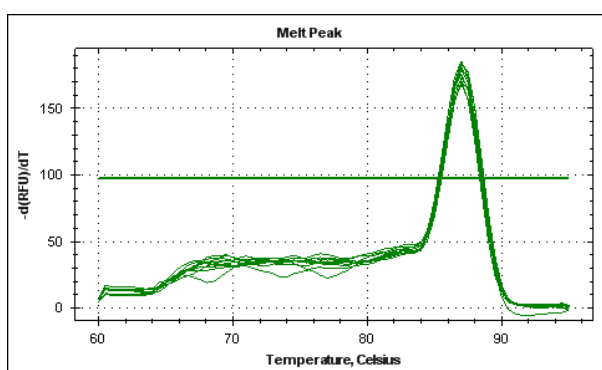


Figure 23 Melting curve for the housekeeping gene 18S

The melting temperature of the amplicon occurs at 87°C. No contaminating products are present in this reaction. Contaminated DNA or primer dimers would show up as additional peaks.

3.3.12 Expanding Tregs with CD3/CD28 Dynalbeads®

Dynalbeads® are used to expand T cells since they provide both TCR signal and the co-stimulatory signal. When rIL-2 is present Tregs can expand properly. Dynalbead® CD3/CD28 T Cell Expander are 4.5 µm, super paramagnetic polystyrene beads coated with monoclonal antibodies against the CD3 and CD28 cell surface molecules of human T cells. Both antibodies are coupled to the same Dynalbead®, mimicking *in vivo* stimulation by antigen presenting cells.

Tregs (CD4⁺CD25⁺ T cells) were isolated via MACS (Miltenyi Biotech) as described above. A CD4⁺CD25⁻ and a CD3-cell depleted fraction were stored in liquid nitrogen. Those two fractions served as effector T cells and APCs, respectively, for the inhibition assay after the expansion.

10⁵ Treg cells were expanded according to the manufacturer's protocol (Figure 24) using a cell: bead ratio of 1:4 in the presence of 500U/mL rIL-2 in UCC medium. Cells were held under strict control - medium /w 500U/mL rIL-2 was replenished and cells were split when needed.

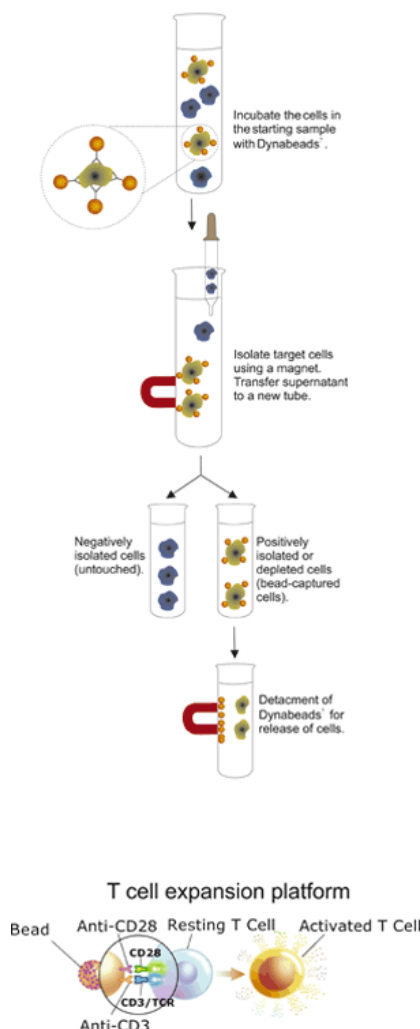


Figure 24 Schematic Dynalbead® expansion

On day eight the Dynalbeads® were removed by a special Magnet (Dylan®) and re-stimulated on day 15-18. Expanded Tregs were subjected to functional assays to evaluate their potential to inhibit mentioned activation using [³H]-thymidine incorporation assay.

3.3.13 Statistics

For statistical evaluation non-parametrical tests were applied (in case of matched pairs Wilcoxon signed rank test otherwise Mann Whitney U-Test). In some cases t-tests (student t-test or matched pairs t-test) were performed if Gaussian distribution was given. For all analysis SPSS for WINDOWS Release 17.0.1 (SPSS Inc, Chicago, USA) or GraphPad Prism 4 for Windows (GraphPad Software, San Diego, CA, USA) was used. A *p* value of less than 0.05 was considered statistically significant and marked with an asterisk (*). P values of less than 0.01 and 0.001 were considered highly significant and marked with two (**) and three (***) asterisks, respectively.

4 RESULTS

The thesis aims at showing the effect of endotoxin on allergen-specific responses in cord blood compared to adult blood. Moreover the effect of endotoxin and allergens on the generation of regulatory T cells and on neonatal tolerance induction has been observed.

During the last years several studies could show a high rate of proliferation of various cell types (e.g. lymphocytes of the lamina propria, mononuclear cells of the peyer's patches, PBMCs, CBMCs,...) upon BLG ^{216-218, 403}. This observation has raised increased interest for this effect in cord blood. Several explanations for this phenomenon exist. Firstly, the transplacental priming, where cells from the mother diffuse to the fetus and thence induce proliferation ^{404, 405}. Secondly, thymic emigrants which represent a considerable fraction in cord blood proliferate in response to antigens with limited specificity before they become apoptotic ²⁴⁹. Thirdly, high amounts of endotoxins are present in commercially available BLG preparations ³⁹³ as well as in other allergens. This may contribute to the increased frequency of allergen-specific proliferation of cord blood derived T cells despite of limited exposure *in utero*.

4.1 The Impact of Endotoxin Contamination on Allergen-Specific Responses in Cord Blood and Peripheral Blood

4.1.1 LPS-Content in Allergens

Initially, the LPS content of different allergen preparations/reagents used was measured via limulus amoebocyte lysate (LAL) assay. One endotoxin unit (EU), an entity to declare the LPS content, is a standardized unit of biological activity measured with the LAL test and calibrated to a reference endotoxin: 1 EU = 0.1 ng LPS.

Reagents	LPS-content (EU/mL)	LPS-content (EU/well)
BLG 3908 [100 µg]	515	103
BLG 3908 (purified) [100µg]	52.8	10.56
BLG L-0130 [100 µg]	118	23.6
BLG L-0130 (purified) [100 µg]	16	3.3
BLG [LF] [100 µg]	0.95	0.19
Ara h 1 [50 µg]	<0.05	<0.05
UCC	0.7	0.14
TT [1 µg]	0.55	0.11
rBet v 1 [100 µg]	1.94 (according to the producer)	0.388

Table 10 LPS-content (EU/mL) of various reagents

Different reagents were tested for their endotoxin content with the LAL test. Removal with polymyxin B columns reduced the LPS content to a tenth (BLG purified). The generated BLG (BLG [LF]), which was intended to be free of LPS contamination served as our LPS-free control.

In the experimental settings different BLG formulations were utilized. BLG 3908 and BLG L-0130 are commercially available BLG preparations purchased from Sigma-

Aldrich. Conventional endotoxin removal strategies like polymyxin columns were able to reduce the endotoxin amount to a tenth (BLG 3908 and BLG L-0130 from 515 EU to 52.8 EU and from 118 EU to 16 EU, respectively). However, there was still a considerable amount of endotoxin present (Table 10; BLG 3908 (purified) and BLG L-0130 (purified)).

Polymyxin B, a polycationic antibiotic, binds to the lipid A domain of the LPS and therefore neutralizes LPS and its biological and toxic effects^{406, 407}. This has been proven to be the most effective adsorption technique to deplete LPS, however significant protein loss during passage through the column has been noted (e.g. 20% for BSA (M_w 6.9×10^4)) even more pronounced when using a larger pore size (molecular mass exclusion of polysaccharide, $M_{lim} > 2 \times 10^6$; 78% for BSA)⁴⁰⁸.

A BLG preparation, virtually devoid of LPS was generated by Susanne Brix and Vibeke Barkhold (University of Copenhagen) and published in 2003. It has been purified from raw milk as explained in detail in Brix et al (2003)³⁹³ and in the methods section. This highly purified BLG was a generous gift and was used as LPS-free control. Upon re-evaluation it contained trace amounts of LPS ($50 \mu\text{g BLG}/200\mu\text{L} \sim 0.095 \text{ EU/well} = \sim 0.01\text{ng}$). According to literature which affirms that a level below 1 EU has to be considered LPS-free [LF]⁴⁰⁸. Based on various tests, we are confident that these low amounts do not induce biological effects and impact the outcome.

The purified major peanut allergen Ara h 1 was chosen as an alternative food allergen containing endotoxin below the measurable threshold measured by LAL assay (threshold 0.035 EU).

Recombinant birch allergen, rBet v 1, served as a representative for both inhalational allergen and recombinantly expressed protein. According to the producer the LPS content was in a similar low range as our BLG [LF] (0.95 EU/mL; table 10).

4.1.2 Defining Kinetics and Thresholds for LPS Sensing in CB

Initially proliferation experiments were performed to define suitable read-out time points reflecting the peak of LPS-specific and allergen-specific responses (day 1- day 7; d1-d7) in cord blood using CBMCs. Inferentially two read-out time points were set. Day 3 (d3) was chosen to serve representative for endotoxin-dependent proliferation and day 7 (d7) for allergen-specific responses.

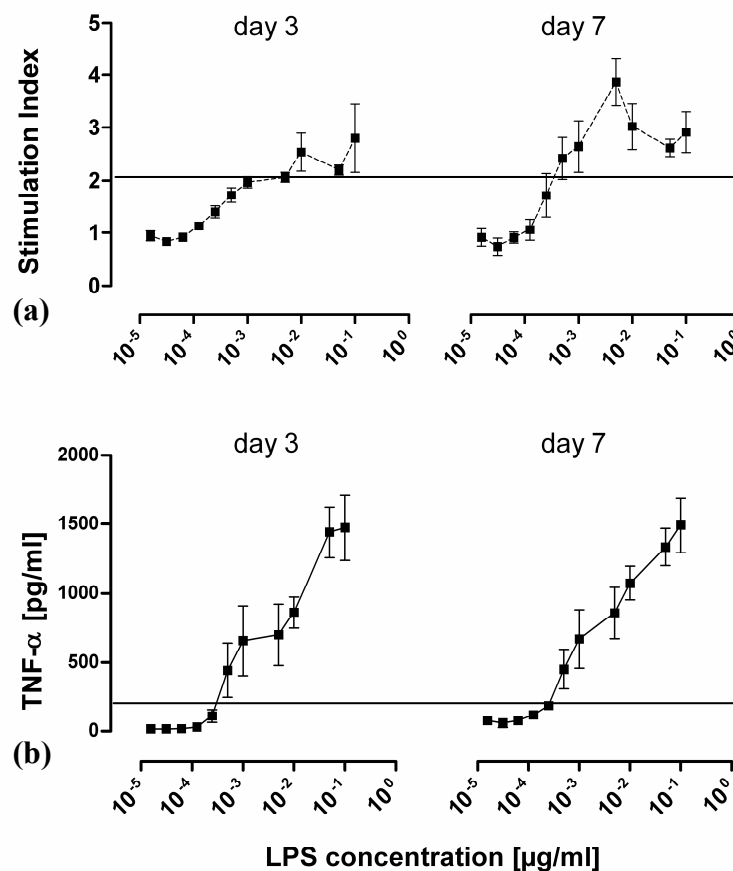


Figure 25 Defining a threshold of CBMCs to LPS at a proliferation and a cytokine level

CBMCs were isolated and co-cultured with LPS in limiting dilution. At two defined read-out points (72h (day 3) and day 7) proliferation (a) and TNF-α production (b) was measured with [^{3} H]-thymidine incorporation assay and ELISA, respectively.

(Proliferation n=8; TNF-α measurement n=5; Mean ± SEM). A stimulation index >2 was considered positive.

Limiting dilution of LPS revealed a critical amount of approximately 1 ng LPS (~10 EU) to induce a positive proliferation response. This threshold has been defined by a stimulation index >2 measured by [³H]-thymidine assay. However, it became apparent that a 10-fold lower concentration affects the proliferation outcome too (Figure 25 (a)).

TNF- α , an important proinflammatory cytokine, was used as a read-out parameter for LPS induction. At a level of 0.07 ng LPS (~0.7 EU) TNF- α was measured via ELISA and therefore this amount was set as critical amount to induce cytokine production being relevant for a cellular system measured in cord blood (Figure 25 (b)).

4.1.3 Allergen- and LPS-Specific Proliferation of Cord Blood Mononuclear Cells

Dose response experiments applying allergen concentrations from 0.1-50 $\mu\text{g/mL}$ and the corresponding LPS-content were assessed to define the explicit relevance of different amounts of contamination present. The relevance of LPS in BLG preparations used is shown in figure 26.

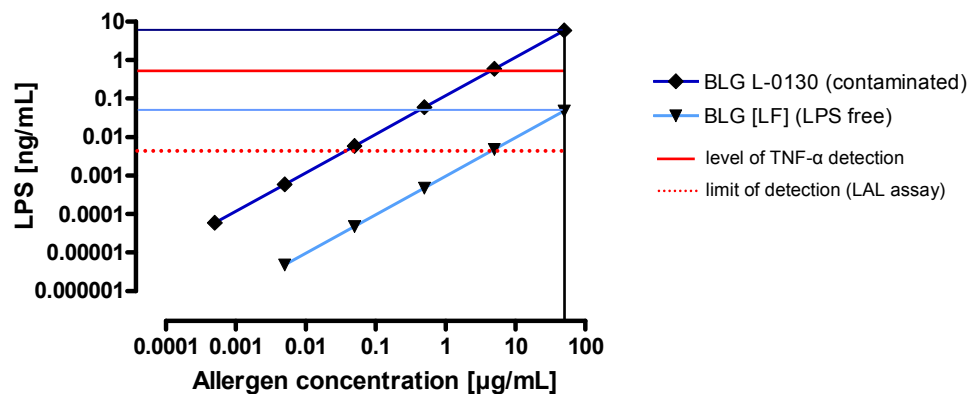


Figure 26 Relevance of LPS contamination in BLG preparations used

The LPS contamination of BLG [LF] is below the level of TNF- α detection (red permanent line; 0.7 ng/mL). The limit of detection for the limulus assay is at 0.035 ng/mL and very sensitive. Hence, BLG [LF] can be used equivalent to LPS-free BLG as even the highest concentration does not induce TNF- α production and only 5 $\mu\text{g/mL}$ allergen can be detected via limulus assay.

BLG L-0130 (“contaminated”, un-purified allergen) induced a proliferative response on CBMCs as opposed to purified BLG (BLG [LF]) at day 3 and day 7. This effect disappeared at the highest applied concentration at day 7 (Figure 27 (a)). By adding an identical LPS-amount that was present in the un-purified BLG L-0130 to the purified allergen (BLG [LF]), the proliferative response could be restored and the proliferation profile was similar to the LPS contamination alone. A significant difference between contaminated allergen (BLG L-0130) and LPS spiked purified allergen (BLG [LF]) could only be observed between the lowest concentrations (Figure 27 (a)).

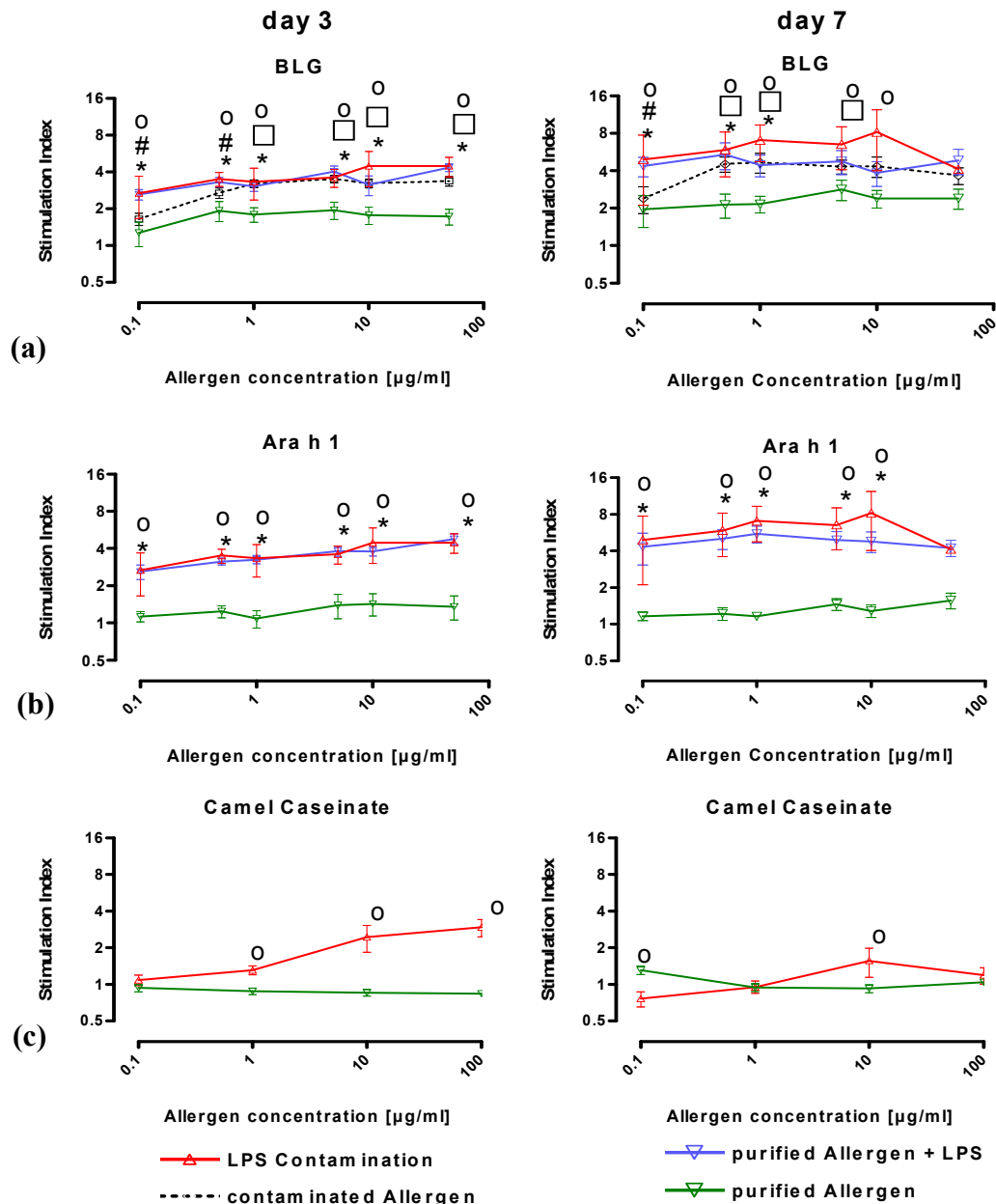


Figure 27 The impact of endotoxin contamination on allergen-specific proliferation of cord blood mononuclear cells

Cord blood-derived mononuclear cells were co-cultured with different food allergens (BLG, Ara h 1) either in its contaminated form (BLG L-0130) (a), its purified form (BLG [LF]; Ara h 1(b)) or with LPS matching the degree of contamination present in the respective contaminated allergen [BLG L-0130]. The calculated amount of endotoxin contamination present served as internal control (contamination) (BLG: $n=13$; Ara h 1: $n=7$; camel caseinate (c): $n=3$; mean $\pm$ SEM)

* $p<0.05$ purified allergen+LPS vs purified allergen; # $p<0.05$ contaminated allergen vs purified allergen+LPS; ° $p<0.05$ contaminated allergen vs purified allergen; □ $p<0.05$ LPS contamination vs purified allergen).

The exact same pattern was observed using Ara h 1 (Figure 27 (b)). Here the LPS dependent effect is even more striking. The LPS-spiked pure Ara h 1 showed an almost identical proliferative pattern when compared to LPS contamination alone. A significant difference could be observed between spiked allergen and contamination alone when compared to the purified Ara h 1. Again, the significant differences disappeared only at the highest concentration.

Camel caseinate was chosen as irrelevant allergen since exposure is extremely unlikely. Camel caseinate itself did not induce a proliferation (SI >2) neither on day 3 nor on day 7. However, spiking with LPS significantly affected the proliferation on day 3 and on day 7. A positive stimulation index could be observed at day 3 at the highest concentration (Figure 27 (c)).

4.1.4 Cytokine Induction by Different BLG Preparations in CB

As a next step various cytokines (IL-1 β , IL-5, IL-10, IL12p70, IFN- γ , TNF- α) were measured in different BLG preparations.

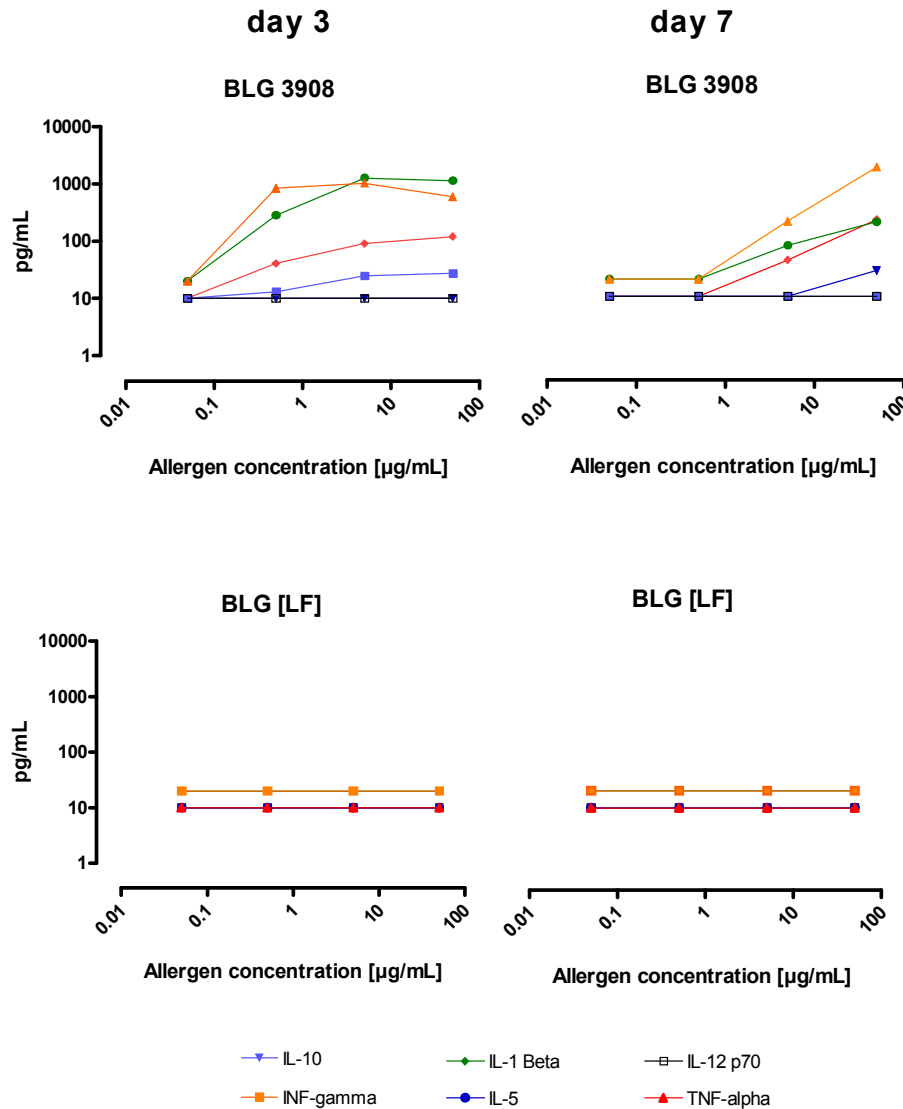


Figure 28 Cytokine pattern of CB after stimulation with different BLG preparations

The cytokine pattern was determined in CBMCs in the presence of different BLG preparations (BLG 3908, BLG [LF]) and measured via cytometric bead array technology (Flex kit; BD) at day 3 and day 7. The graph represents data from one out of three independent experiments.

Supernatants of CBMCs stimulated with contaminated (BLG 3908) and purified BLG (BLG [LF]) were harvested on day 3 and day 7. Cytokine production was measured with cytometric bead array (Flex kit, BD) at both read-out time points.

Contaminated BLG (3908) led to an increased cytokine production; particularly of IFN- γ , IL-1 β , and TNF- α on day 3 and day 7. In contrast, purified BLG [LF] did not induce cytokine production neither on day 3 nor on day 7 (Figure 28) indicating that only the contamination but not the allergen per se had a significant impact on cytokine production.

4.1.5 Proliferative Responses are Significantly Higher in Cord Blood than in Peripheral Blood from Adult Donors

To evaluate the relevance of these findings for adult blood, PBMCs of healthy adult donors without a clinically relevant allergy to milk proteins (IgE, and skin prick test negative) were stimulated with BLG and LPS.

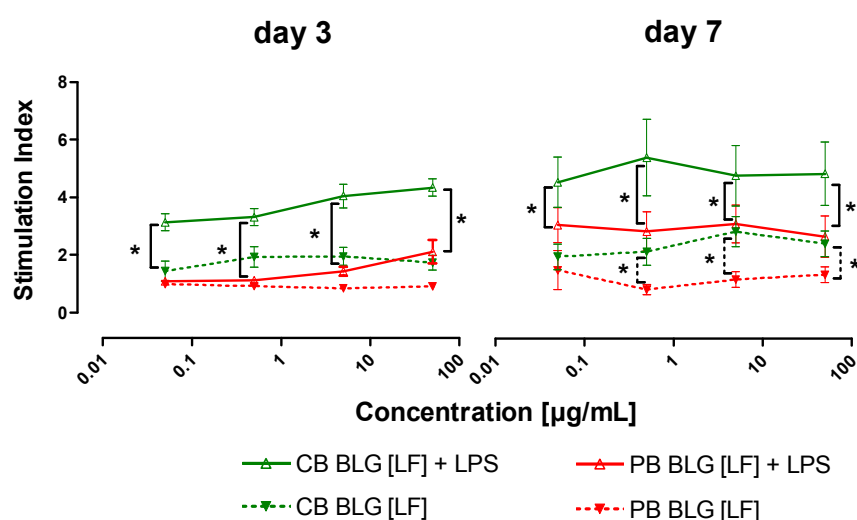


Figure 29 Proliferative responses to allergen and allergen plus endotoxin are significantly higher in cord blood than in peripheral blood from adult donors

Proliferative responses to allergen and allergen plus endotoxin are significantly higher in cord blood (green) than in peripheral blood (red) from adult donors. The impact of endotoxin contamination is significantly stronger in CB (green) as compared with PB from adult donors (CB: n=17; PB: n=10).

Graph indicates the mean $\pm$ SEM.

Purified allergen (BLG [LF]), and BLG [LF] + LPS were used. BLG [LF] + LPS emulates the contamination present in the BLG L-0130 preparation.

In all experiments, CBMCs showed a significantly higher proliferative response to purified as well as spiked allergen. Again, a distinct influence of endotoxin on proliferation was detectable in cord and peripheral adult blood (Figure 29). However, any effect of allergen alone remained detectable at day 7.

4.1.6 rBet v 1, Representative for Inhalational Allergens, Affected the Proliferative Response of PBMCs and together with LPS also CBMCs

In order to evaluate the relevance of endotoxin contamination in response to an inhalational allergen, the recombinant major birch pollen allergen rBet v 1 was chosen.

CBMCs and PBMCs of non-allergic donors (IgE- and skin prick test-negative) were stimulated with titrated concentrations of rBet v 1 in the range of 0.1–50 $\mu\text{g/mL}$. No proliferative response was detected in CBMCs (Figure 30 (a), green line) whereas rBet v 1 induced proliferation in PBMCs from three out of six non-allergic donors (Figure 30 (a), red line). Based on dose response experiments a concentration of 10 $\mu\text{g/mL}$ was chosen for proliferation assays to investigate the impact of LPS. rBet v 1 was spiked with 10 fold increasing concentrations of LPS starting from 20 pg/mL up to 20 ng/mL. While CBMCs responded already in the presence of 200 pg LPS/mL, PBMCs only displayed a trend towards an impact of LPS within that range that did not reach statistical significance.

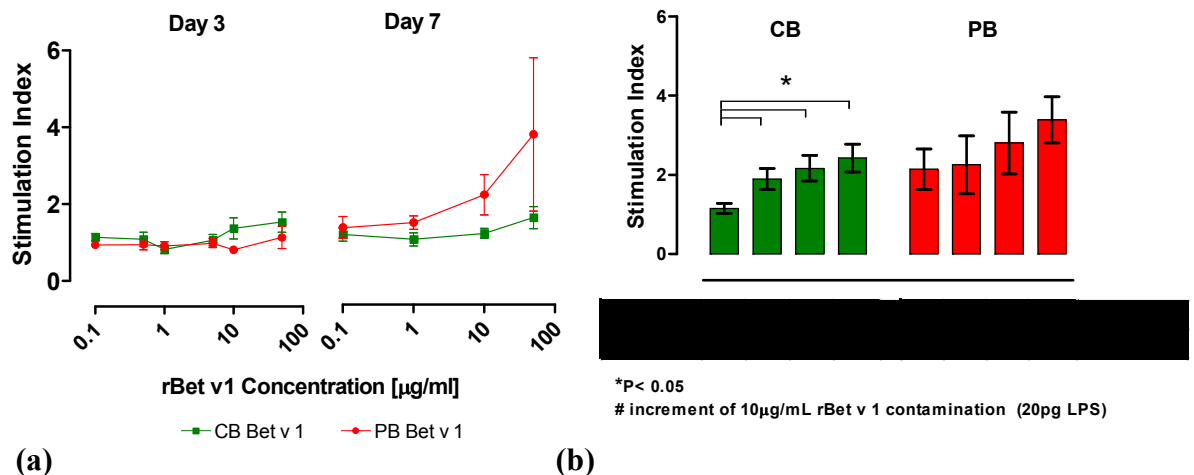


Figure 30 Allergen-specific proliferation of PBMCs and CBMCs to rBet v 1

Cord blood (CB; green) and peripheral blood (PB; red)-derived mononuclear cells were co-cultured for 3 and 7 days with the recombinant Bet v 1 (20 pg endotoxin/10 μg rBet v 1). Three out of six PBMC samples proliferated in the presence of the allergen at a concentration of 10 $\mu\text{g/mL}$. None of the CB-derived samples did (0/6) (a). Contamination at the relevant concentration of 10 $\mu\text{g/mL}$ did not affect proliferation, neither in CB nor in adult blood (b). (n=6 independent experiments CB and PB; results are expressed as mean $\pm$ SEM).

4.1.7 A Recall-antigen, Tetanus Toxoid (TT), together with LPS, Induced Proliferation in PBMCs on Day 7

To investigate the impact of endotoxin contamination on a more Th1 type memory response, tetanus toxoid (TT), a recall-antigen, was chosen as a model antigen. TT was initially proven (via certificate and via internal limulus control) to contain endotoxin below the limit of relevance (Table 10; 0.55 EU/1 μg TT). A sub-maximal dose of 1 $\mu\text{g}/\text{mL}$ TT was spiked with increasing levels (0.35 – 350 ng/mL) of endotoxin corresponding to the amount of contamination present in BLG (3908).

TT + LPS (even at the lowest concentration of 0.35 ng/mL) induced a proliferative response of PBMCs at day seven (red lines) compared to TT alone, that only induced a positive stimulation index (SI) on day 7. The highest amount of endotoxin added to the TT did not additionally increase the proliferative response significantly compared to TT or LPS alone (Figure 31). This is most likely due to the fact that kinetics is modified by the presence of endotoxin. At day 3 (black lines) spiking did not modify proliferation. TT alone could not increase proliferation.

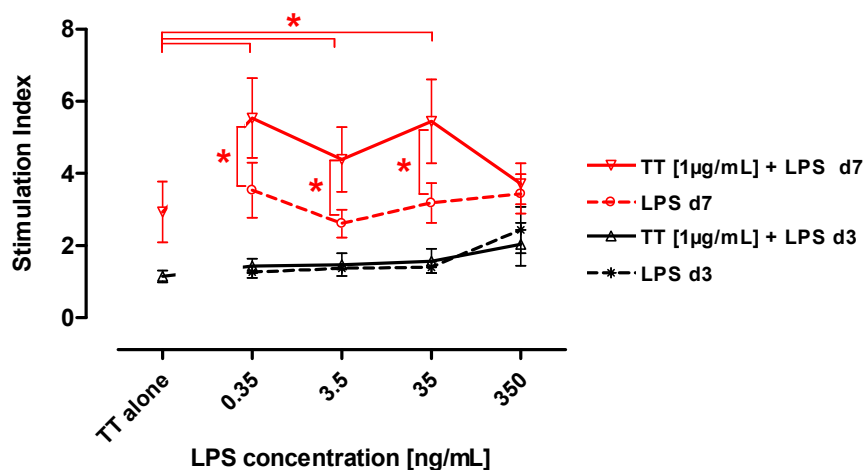


Figure 31 Low level LPS contaminants affect proliferative response to Th1 type recall-antigen TT at day 7 in adult individuals

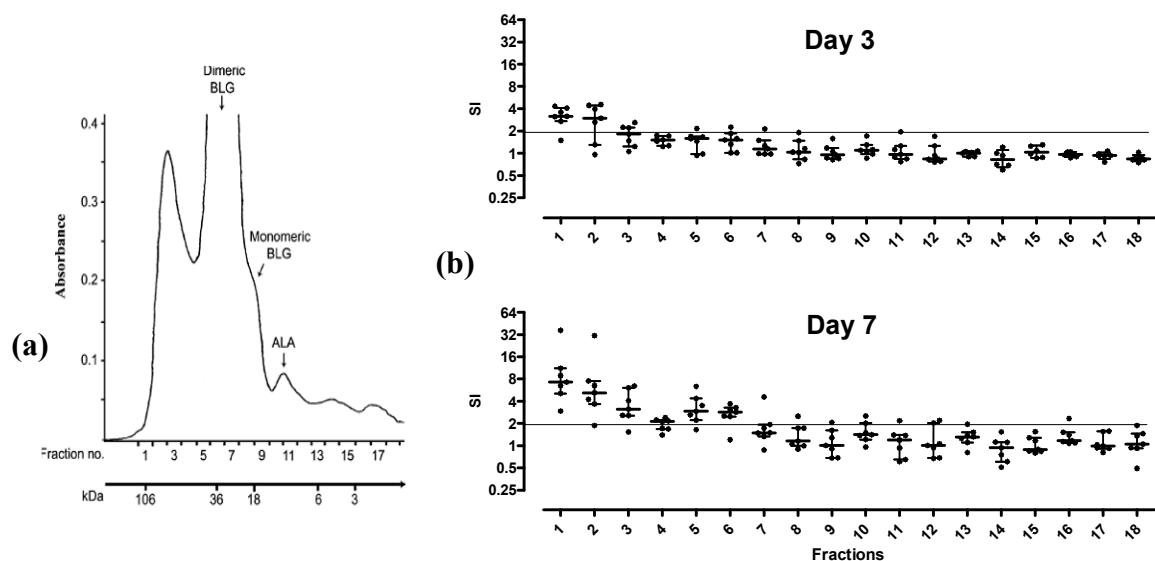
Tetanus toxoid was applied at a constant concentration of 1 $\mu\text{g}/\text{mL}$ and LPS was added according to the amount present in contaminated Sigma BLG (BLG 3908, Sigma). At day 3 (black lines) no effect on the read-out parameters was measurable whereas on day 7 (red lines) significances were detected. Graph indicates the mean and the standard error of the mean (n=6).

4.1.8 Immunomodulatory Properties of Un-purified BLG Relate to High Molecular Weight Fractions

To determine the size of immuno-reactive components within the contaminated BLG sample [BLG L-0130], size exclusion chromatography (SEC) was performed and 18 sub-fractions were gained (Figure 32 (a)). The high molecular weight fractions (fraction 1-3; F1-3; >100kDa) are most likely to represent the endotoxin fraction, whereas the medium weight fraction (Fraction 5-6, F5-6, ~36kDa) most probably corresponds to be the dimeric BLG fraction.

At day 3, only the high molecular weight fraction, represents the endotoxin-fraction (F1, F2), induced a positive proliferative response (Figure 32 (b), upper panel), whereas at day 7 the BLG-corresponding fraction (F5 and F6, representing BLG dimers) also induced clonal expansion (Figure 32 (b), lower panel). This finding is in accordance with our previous experiments.

Extremely low amounts of LPS induce a positive proliferative response (Figure 25 (a)). Assuming a BLG-content of more than 90%, an at least 100-fold higher potency can be attributed to the high molecular weight, endotoxin-contaminated fraction. Purified BLG [LF] and the BLG-corresponding fractions (F5, F6) did not induce significant cytokine production (Figure 32 (c)). In contrast, the contaminated BLG-fraction stimulated cytokine production substantially at very low doses suggesting an innate immunity-driven response as reflected by the LPS-titration results (Figure 25).



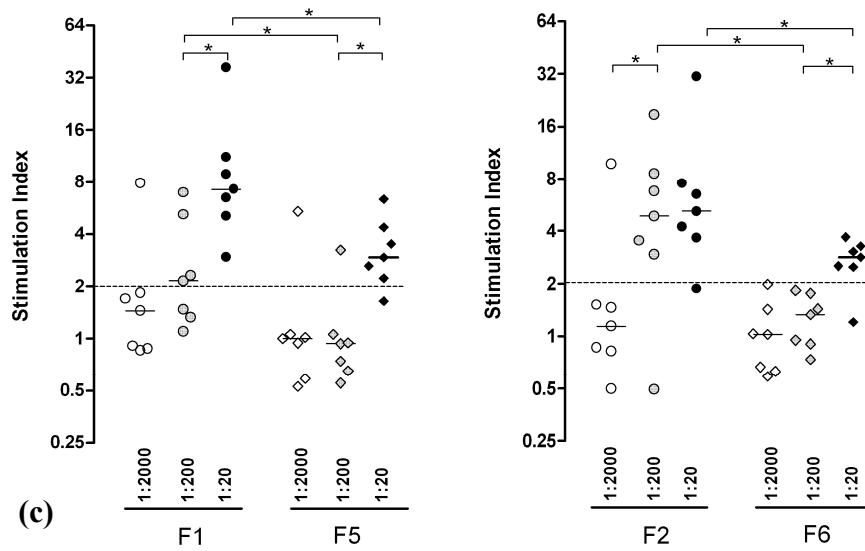


Figure 32 High molecular weight fractions and endotoxin fractions induce proliferation

High molecular weight fragments isolated by size exclusion chromatography (a) led to a BLG-independent proliferative response of CBMCs (Wilcoxon signed rank test (b)). Dose response experiments of high molecular contaminants and dimeric BLG revealed - despite of > 90% purity of the BLG preparation - a more than 10-times higher potency of the contaminants (c). Dots represent data from seven independent experiments ($P < 0.05$; Wilcoxon signed rank test). The fractions 1 and 2 (F1; F2) are representative for the endotoxin contamination whereas fraction 5 and 6 (F5, F6) equal the dimeric BLG. Graph indicates 7 independent experiments ($n=7$).

4.1.9 Un-purified BLG Acts via a Toll-like Receptor 4 Dependent Mechanism

To investigate the involvement of the most prominent receptors for endotoxins (TLR-4) and to delineate whether LPS is mainly responsible for the described effects, an assay using TLR-2 and TLR-4 transfected HEK293 (Human embryonic kidney cells)-cells (generated and kindly provided by the group of M. Yazdanbakhsh; Leiden University Medical Center, The Netherlands) was applied. IL-8 was used as read-out cytokine as it is produced in response to LPS.. TNF- α and LPS were used as a positive control for TLR-4 transfected cells^{409, 410}, whereas TNF- α and Pam3Cys (a TLR-2 ligand) for TLR-2 transfected cells. Medium alone was set as a negative reference value.

The contaminated BLG initiated a robust IL-8 response via a TLR-4 dependent mechanism but had no effect in TLR-2 transfected cells (Figure 33). On the other hand the BLG [LF] induced IL-8 production neither on a TLR-2 nor in the TLR-4 transfected cells (Figure 33).

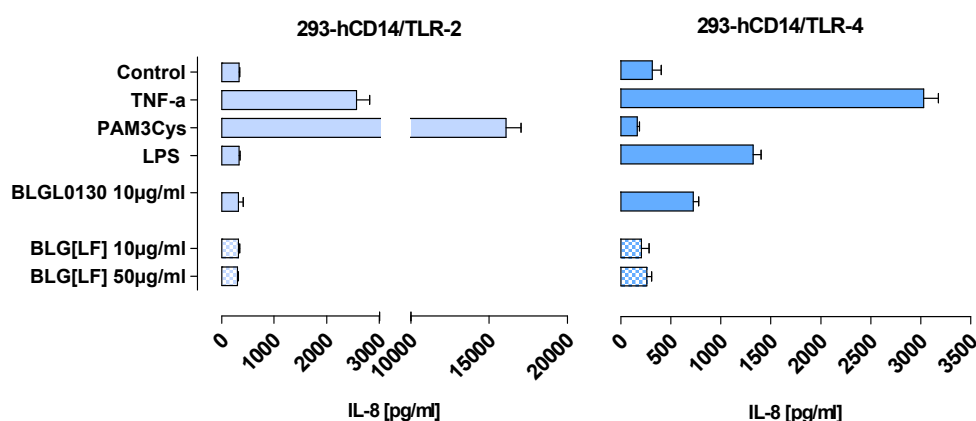


Figure 33 Contaminated BLG leads to a TLR-4 dependent release of IL-8

HEK293 cells, stably transfected with an expression vector for human CD14 and TLR-4 (293-hCD14/TLR-4) or CD14 and TLR-2 (293-hCD14/TLR-2). Concentration of IL-8 was used as the read-out parameter and measured via ELISA (Endogene) (n=4, two independent experiments).

This experiment has been carried out by Irma Schabussova at the Experimental Pathology, Medical University of Vienna.

To further investigate the relevance of TLR-4 in an *in vitro* system with all innate immunity receptors co-expressed, inhibition assays with anti-TLR-4 antibodies were performed on CBMCs. Ultra-pure LPS (LPS UP; *S. minnesota*; stem R595), BLG L-0130, BLG [LF], and BLG sub-fractions generated by size exclusion chromatography were tested. Ultra-pure LPS induced a strong TNF- α production which was significantly inhibited by TLR-4 blockade. The same effect could be observed with BLG L-0130. TNF- α could be strongly induced but was significantly reduced by TLR-4 antibodies. No TNF- α production was induced by the pure BLG [LF]. The high molecular weight fraction (F1; Figure 34) acted via TLR-4, while the putative BLG-dimer (F5) did not induce TNF- α production (Figure 34).

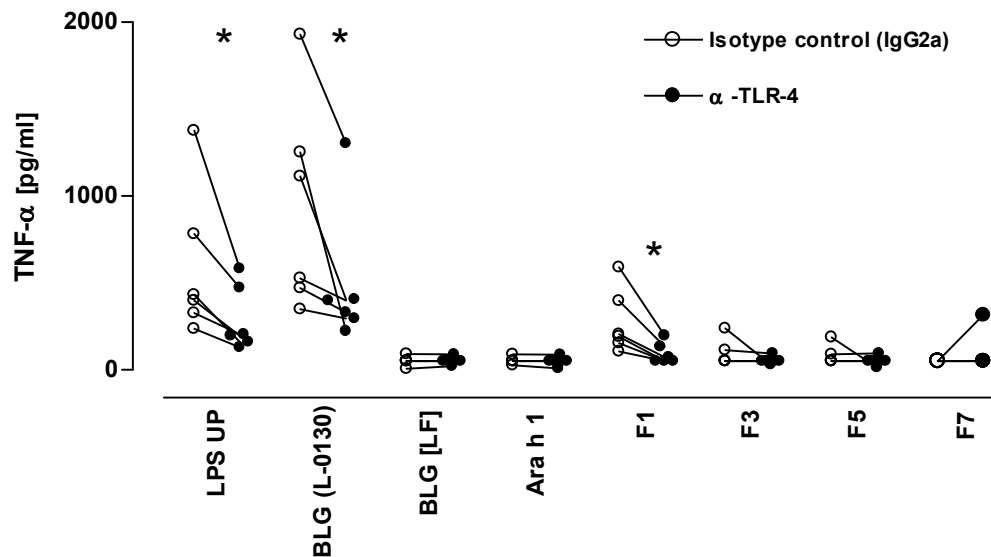


Figure 34 BLG-related TNF- α production is TLR-4 dependent and related to high molecular weight contaminants.

Cord blood derived monocytes were pre-incubated with TLR-inhibitory antibodies or the respective isotype control (α -TLR-4, IgG2a [1 μ g/mL]) for 1 hour. Thereafter allergens (BLG, Ara h 1), from fractionated Sigma BLG (L-0130 no endotoxin removal procedure) or the positive control (LPS ultra pure, *Salmonella minnesota* R 595 [0.1 μ g/mL]) were added. TNF- α production was measured in the supernatant (n=6).

4.2 *Regulatory T cells in the Presence of Allergens and LPS*

4.2.1 Proliferative Responses to BLG are Augmented in Cord Blood Compared to Adult Blood due to Significantly Reduced Inhibitory Capacities of CD4⁺CD25⁺ Cells

A generally higher proliferation level has been observed in CBMCs than in PBMCs from adult donors (Figure 29)^{252, 411}. BLG is considered to induce the highest frequency of responses. At day 7 CFSE stained CBMCs proliferated strongly upon the contaminated BLG L-0130 where the surface marker CD25 was up-regulated. However, the LPS-free BLG [LF] per se did induce a positive proliferative response as well, when compared to the negative control (medium alone, Figure 35).

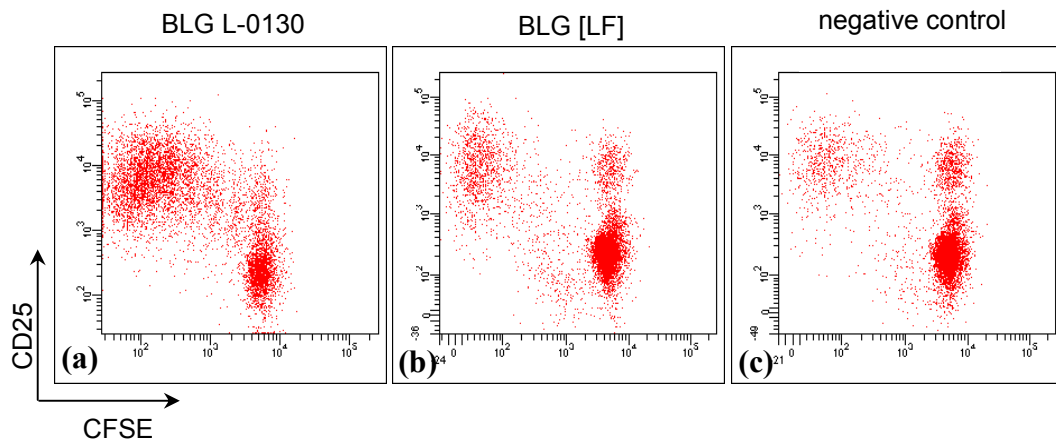


Figure 35 Proliferation and CD25 expression upon stimulation with an un-purified, a purified BLG, and negative control

CFSE stained CBMCs were incubated for 6 days in the presence of BLG L-0130 (un-purified; **(a)**), BLG [LF] **(b)** and with medium alone (negative control; **(c)**). One out of six representative experiments.

To assess whether the reasons for this altered responses in cord blood could be explained by immature regulatory T cells in cord blood, the inhibitory capacity of CB-derived CD4⁺CD25⁺ Tregs were compared to those from adult blood (Figure 36).

Freshly isolated cells PB-derived $CD4^+CD25^+$ regulatory T cells possessed dose dependent inhibitory properties when co-cultured with the effector T cells ($CD4^+CD25^-$) at a ratio of 1:5 (data not shown) and 1:2. This effect could be observed by adding BLG L-0130 ((a) 70.43% $\pm$ 89.38; mean $\pm$ -SD (1:10) and 25.25% $\pm$ 19.71) or plate-bound α -CD3 ((b) 76.86% $\pm$ 51.98 $\pm$ 51.98 (1:10) and 46.15% $\pm$ 24.22 (1:2).

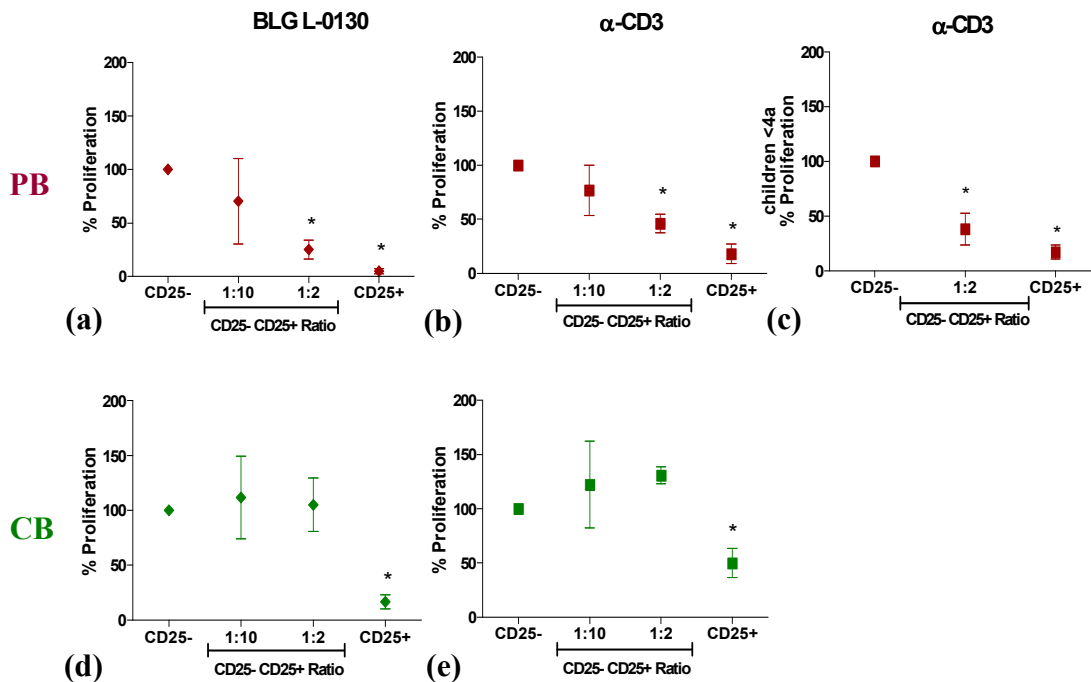


Figure 36: Suppressive potential of $CD4^+CD25^+$ Tregs from CB and PB

Mononuclear cells were freshly obtained. $CD4^+CD25^+$ T cells from CB and PB were isolated via MACS. Inhibition experiments were performed by adding the Treg fraction ($CD4^+CD25^+$ cells) to the $CD4^+CD25^-$ fraction in increasing amounts in presence of irradiated APCs. The PB-derived $CD4^+CD25^+$ fraction possessed a dose dependent inhibitory effect (red) (a-c) while CB-derived $CD4^+CD25^+$ cells did not (green) (d-e). The inhibitory potential is expressed as relative proliferation compared to the $CD4^+CD25^-$ cells alone. Graphs indicate the means of 6 independent experiments $\pm$ SEM for PB and CB and 11 independent experiments $\pm$ SEM for the peripheral blood of children <4a. Wilcoxon sign rank test was applied. P-values of less than 0.05 were considered significant.

CB-derived $CD4^+CD25^+$ regulatory T cells were significantly less potent (Figure 36, BLG L-0130 ((d) 111.98% $\pm$ 84.13; mean $\pm$ -SD (1:10)) and α -CD3 ((e) 122.42% $\pm$ 89.62 (1:10)) - (d) 105.14% $\pm$ 54.49) and ((e) 130.91% $\pm$ 17.39, respectively).

Regulatory T cells alone were hypoproliferative per se. Only a low cell growth in PB and CB could be observed ((a) 5.14% $\pm$ 5.34; (b) 18.15% $\pm$ 25.62; (c) 17.17% $\pm$ 21.46; (d) 16.70% $\pm$ 14.52; (e) 50.03% $\pm$ 29.98). In addition the regulatory properties of 11 children with a mean age of 3.86 years were assessed in the presence of plate-bound α -CD3 the Treg fraction exhibited an inhibitory potency as potent as those from adult cells (38.15% $\pm$ 48.4 at a ratio of 1:2 (c)).

4.2.2 Upon Priming with CD3CD28 Beads, Cord Blood Derived CD4⁺CD25⁺ T Cells become Highly Suppressive

Development of tolerance to a huge number of environmental antigens has to take place within a short period of time after birth. *In vitro* priming assays simulating this procedure were conducted. Initially functional Tregs were generated via *in vitro* Treg differentiation assays to prove the validity of the system and the functionality and possibility of expansion of cord blood derived CD4⁺CD25⁺ cells with conventional Treg generation and expansion protocol.

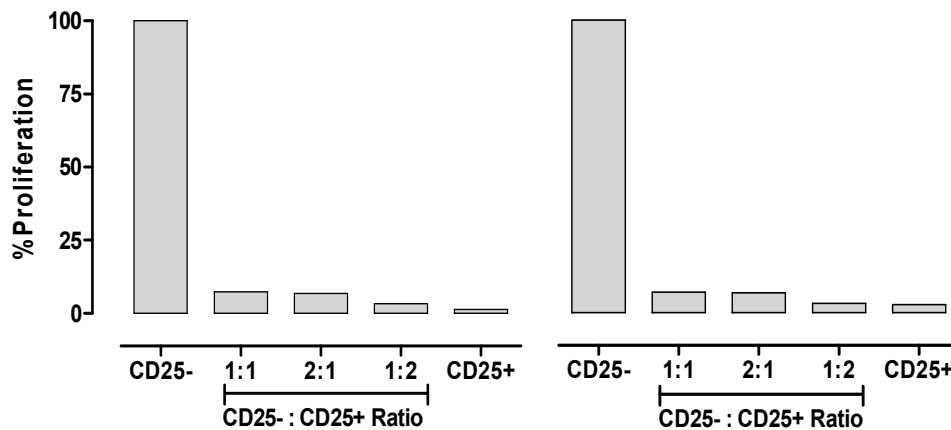


Figure 37 Dynalbead inhibition assay

Cord blood derived Tregs (CD4⁺CD25⁺) were stimulated with CD3CD28 Dynalbeads in the presence of IL-2 for 2 weeks. After this expansion time, this subset became highly suppressive in an inhibition assay. The inhibitory potential is expressed as relative proliferation compared to the effector T cells (CD4⁺CD25⁻) cells. Graphs indicate one out of three independent experiments.

Upon TCR cross-linking in the presence of the co-stimulatory molecules CD3 and CD28 and rIL-2 for 2 weeks this subset expanded and became highly inhibitory (Figure 37). A 92.62% decrease in proliferation was measured at a ratio of 1:2 in the presence of BLG L-0130 and 92.94% in the presence of purified BLG [LF]. The regulatory T cell subset alone was also highly hypo-proliferative and only showed a 1.32% and 2.83% proliferation, compared to the effector T cells.

4.2.3 Expression Profile of CBMCs and to PBMCs on Day 0

Surface (CD25, CD45RA, CD45RO, CD69, CD127) and intracellular marker expression (CTLA-4) for CB (green) and PB (red), was analyzed by flow cytometry at baseline (Figure 38). Percentages indicate mean $\pm$ SD of CD3⁺CD4⁺ cells from 3 or 4 independent experiments.

In cord blood, nearly all cells express the naïve CD45RA marker (93.33% $\pm$ 1.11), whereas in PB cells only 54.39% $\pm$ 3.56; (mean $\pm$ SD) express CD45RA. The memory/effector marker CD45RO on the other hand is expressed on CB in only 1.44% $\pm$ 0.44 of all CD3⁺CD4⁺ cells as opposed to 17.39% $\pm$ 5.25 in PB. CD45RA/RO double positive cells occur in 4.48% $\pm$ 1.61 in CB compared to 24.68% $\pm$ 3.16 in PB.

Observing the CD127^{low}CD25⁺ expression cells were gated on a clearly separable population being the most likely to comprise the Treg compartment. Not an ample difference was observed comparing the percentages of CB and PB (8.11% $\pm$ 2.23 vs 6.49 $\pm$ 0.36, respectively). Notwithstanding, differences in the scatter dot blot could be monitored. In PB an altered cell distribution could be observed in cells expressing CD127^{hi}CD25⁺.

As the protein CTLA-4 is primarily localized in intracellular vesicles, cells had to be permeabilized before staining. The percentages of CTLA-4⁺CD25⁺ T cells however, most likely to represent the Treg subset, did not differ much in percentages (6.59% $\pm$ 3.65 and 5.78% $\pm$ 3.56, respectively) but distribution differences are present.

CD69, a surface molecule, appears to be one of the earliest indicators for leucocyte activation. These unstimulated cells did show a very low percentage of CD69 expression on day 0, neither in CB nor in PB.

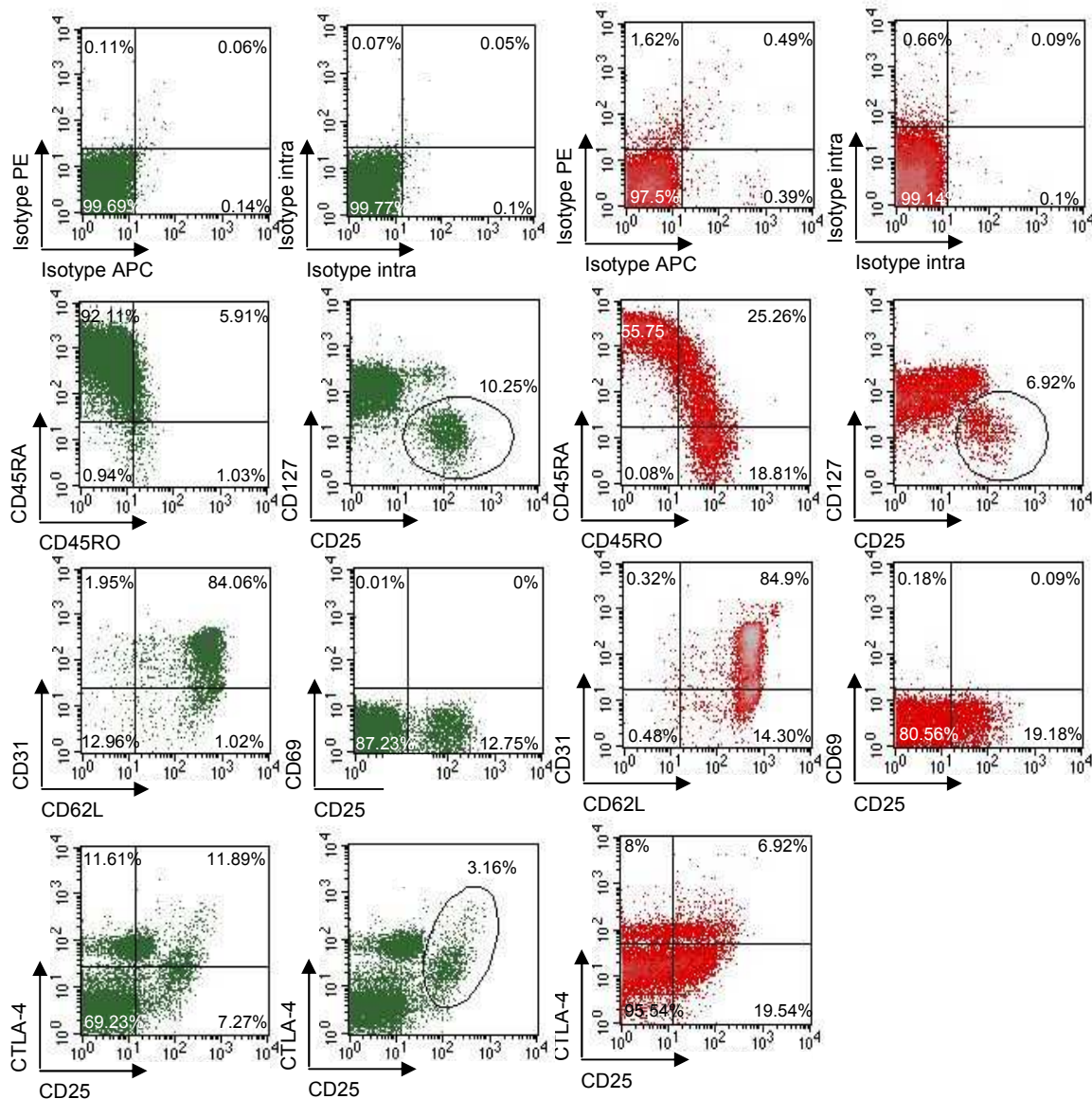


Figure 38 Cytometric analyses of CB and PB derived CD4⁺ T cells

Representative scatter dot plot analyses of CD3CD4 gated lymphocytes. Quadrant markers were set to the isotype control. Surface and intracellular markers were stained and measured via flow cytometry (FACS Calibur, BD, San José). These figures represent one out of 4 independent experiments. Means $\pm$ SD are mentioned above.

4.2.4 Recent Thymic Emigrants in Cord and Peripheral Blood

Recent thymic emigrants are a subpopulation of naïve T cells with a high content of TRECs. As they do not replicate they dilute out during cell division. The RTEs and the homeostatic proliferation of T cells compromise the total naïve T cell pool. For that reason RTE are an excellent tool to quantify the naïve T cell pool of cord and peripheral blood and to check which cells proliferate.

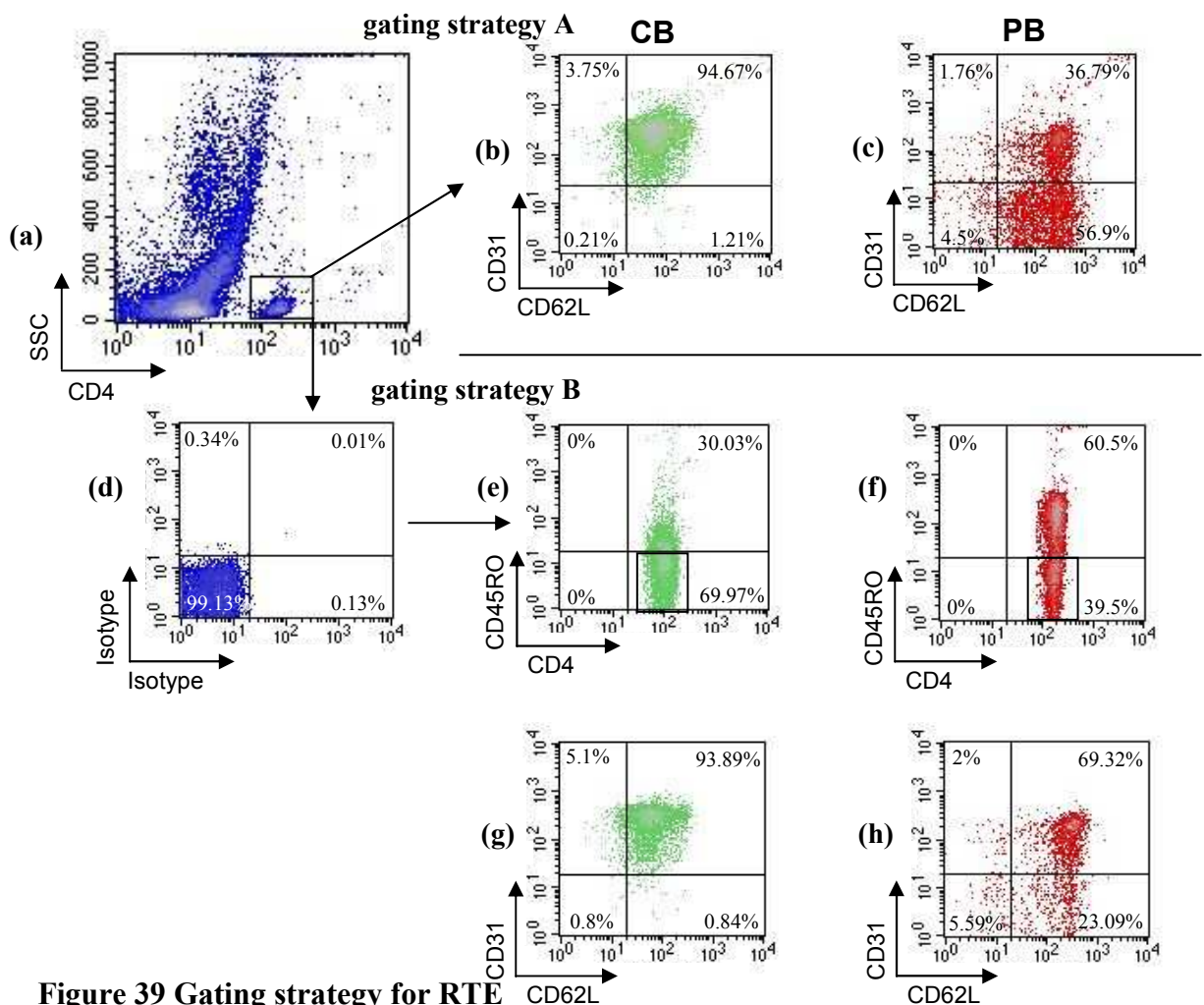


Figure 39 Gating strategy for RTE

Two different gating strategies to identify the percentage of recent thymic emigrants (RTE) were applied. Mononuclear cells were isolated and stained with the CD4⁺ Recent Thymic Emigrant Enumeration Kit (Miltenyi Biotech) that contains antibodies against CD4 FITC, CD31 PE, CD45RO PerCP, and CD62L in APC. Firstly the CD4⁺ cells were gated (a). Within gating strategy A, CD31⁺CD62L⁺ RTE can be easily distinguished out of the CD4⁺ population (b and c). For gating strategy B, naïve cells could be defined by CD45RO⁻ expression (e and f) and RTE could be easily detected (g and h).

In this experiment two different gating strategies were applied (Figure 39) because they differ slightly in their outcome. However, with both gating strategies a significant difference of RTE between CB and PB could be observed. CB (green) has a generally much higher content of RTE (92.19 $\pm$ 6.83 (mean $\pm$ SD) with gating strategy A (Figure 40 (a)) and 83 $\pm$ 2.67 with gating strategy B (Figure 40 (b)) compared to peripheral blood (red; 73.09 $\pm$ 5.54 and 35.26 $\pm$ 6.94, respectively; Figure 40 (a and b)).

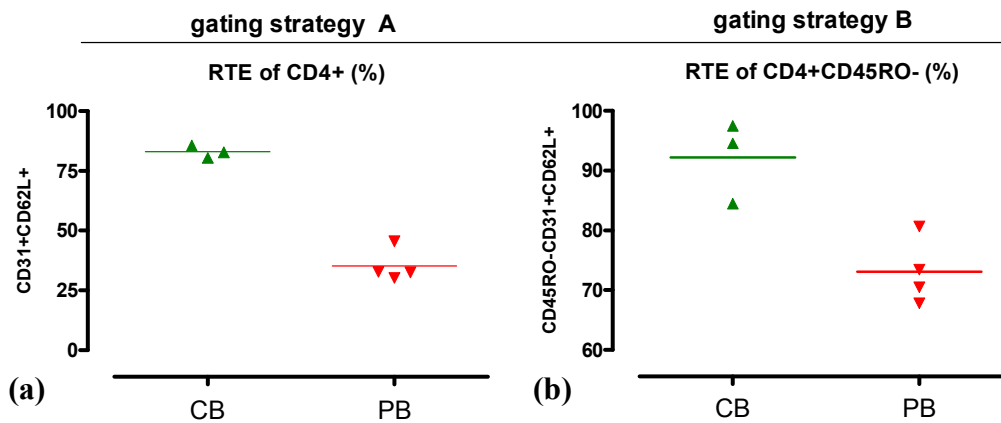


Figure 40 Percentage of RTE in CB and PB

Cells were gated on using the gating strategy A for (a) and B for (b). A significant difference between the CB (green)- and the PB (red)-derived RTE could be seen with both gating strategies (n=3-4).

4.2.5 Insufficient Control of Antigen-Specific Proliferative Responses in Cord Blood by Tregs cannot be Explained by a Lower Frequency of FoxP3⁺CD4⁺CD25^{high} Cells

To investigate whether CD25⁺CD4⁺ T cells in cord blood differ from those in peripheral blood with regard to FoxP3-expression, the most important Treg transcription factor in Tregs. Intracellular FoxP3 staining was performed on CBMCs and PBMCs (Figure 41 (a) for gating strategy and (b) and (c) for FoxP3 expression) and measured via flow cytometry. (The purity of MACS-isolated Tregs was always >95%.)

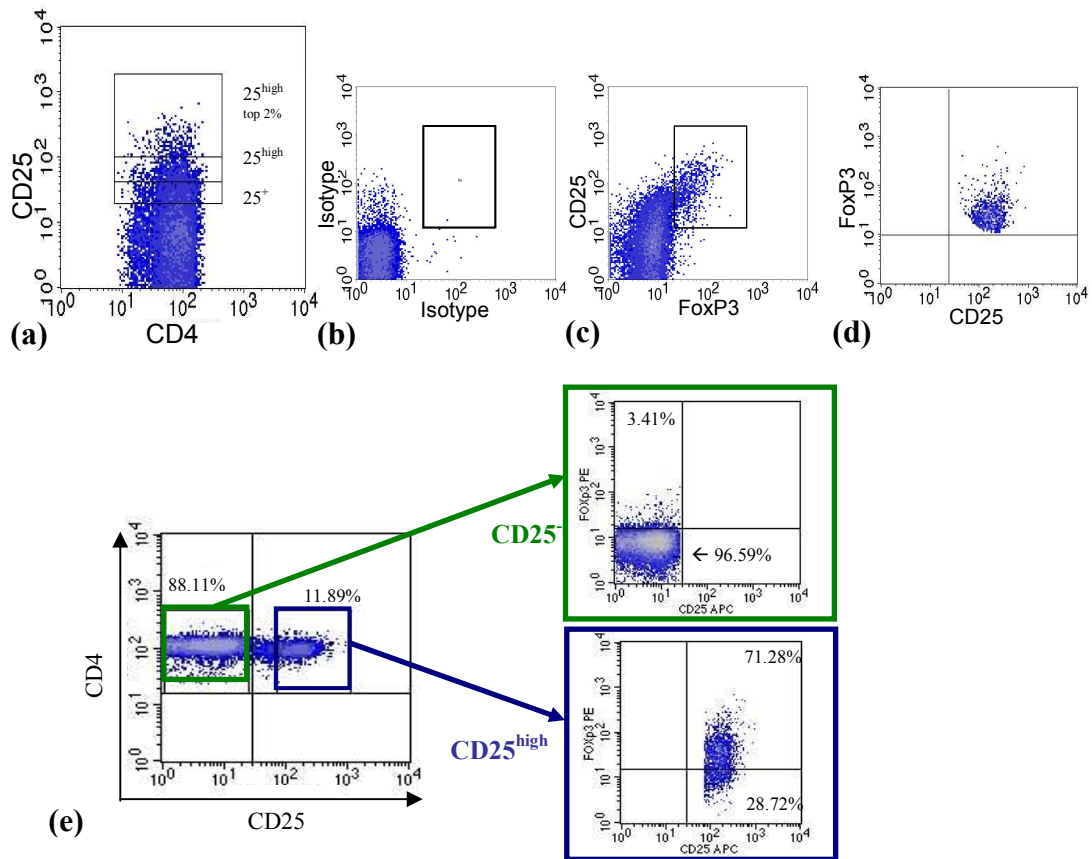


Figure 41: Gating strategy for FoxP3 expression

Characterization of FoxP3 expression: PBMCs stained with anti-CD4 FITC, anti-FoxP3 PE, anti-CD3 PerCP and anti-CD25 APC. A minimum of 50,000 events were collected to display clearly separable subpopulations. In Figure (a), gates for the top 2% of CD25, CD4⁺CD25^{high} cells and CD25⁺ T cells are shown. In Figure (b) and (c) the gating strategy are presented for the isotype control as well as for identifying FoxP3⁺CD25^{high}CD4⁺ T cells, respectively. When back gated, it can be observed that all

CD25⁺CD4⁺ T cells are FoxP3⁺ **(d)** and **(e)**. CD4⁺CD25⁻ T cells do not express FoxP3 (green square) whereas CD4⁺CD25^{high} putative regulatory T cells do so (blue square).

Importantly, when different gating strategies were used, different levels of FoxP3 could be calculated with a differing outcome. Using the gating strategy of Grindebacke et al. (2009) who gated the top 2% of CD4⁺CD25⁺ T cells ⁴¹² (Figure 42 (a and b)), FoxP3 expression in cord blood was decreased (50.02±21.58) compared to adult blood (59.48±34.12) but not significantly (Figure 42 (c); Gating strategy 1; mean±SD).

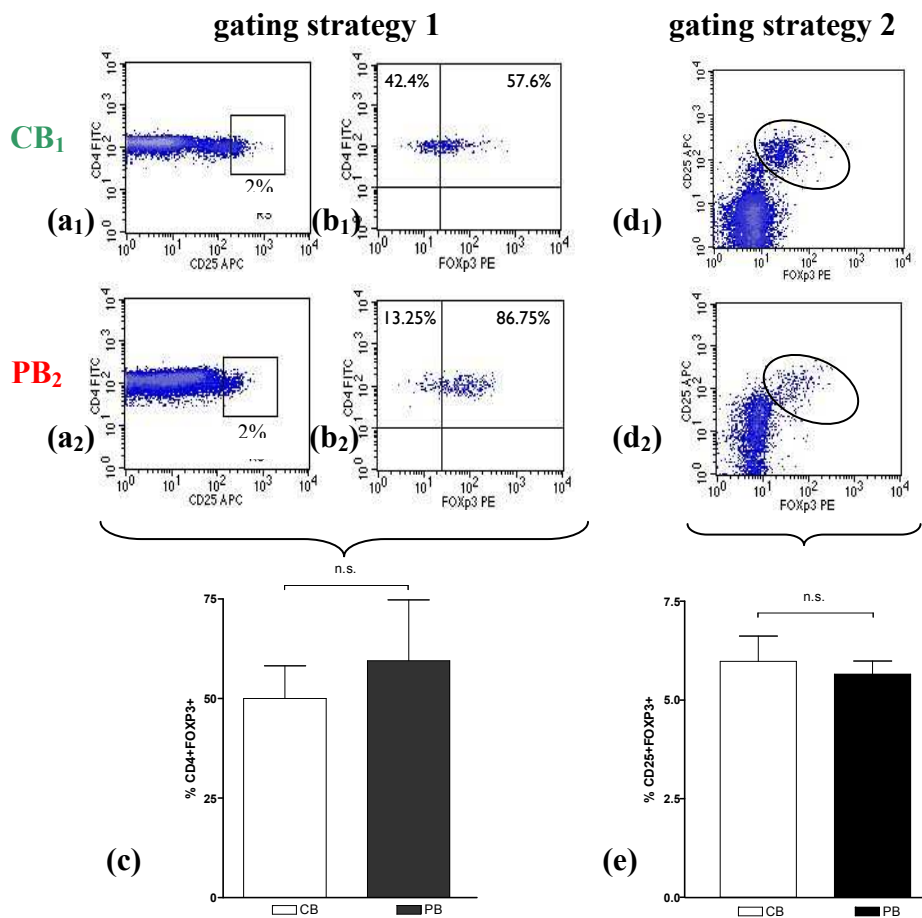


Figure 42: Comparison of two different gating strategies for quantifying FoxP3

For both gating strategies only CD3⁺CD4⁺ T cells were put into account. For gating strategy 1 (Grindebacke et al 2009) the top 2% ((a₁) for CB and (a₂) for PB)) of CD4⁺CD25⁺ T cells were gated and FoxP3⁺ cells ((b₁) for CB and (b₂) for PB)) were visualized (c). For gating strategy 2 ((d₁) for CB and (d₂) for PB)) CD4⁺ T cells that were CD25⁺FoxP3⁺ were acquired (e). The upper panels (a₁, b₁, d₁) represent cord blood and the lower panels (a₂, b₂, d₂) peripheral blood derived FACS scatter.

However, when gating on FoxP3⁺CD25⁺ T cells (Figure 42 (e)), CB-derived cells seem to have a higher FoxP3 level (5.97 $\pm$ 1.7) than PB derived cells (5.65 $\pm$ 0.74) without a statistically significant difference (Gating 2; mean $\pm$ SD).

To check whether pre-exposure to the allergen increases FoxP3 CBMCs were exposed to BLG L-0130. An increase of FoxP3⁺ cells was observed for both gating strategies (1 and 2) for CB (87.23 $\pm$ 13.72 and 7.3 $\pm$ 1.3, respectively) and PB (81.92 $\pm$ 11.29 and 5.97 $\pm$ 1.37, respectively) over time. Statistical significance was only obtained when gating strategy 1 was applied. However, when comparing CB and PB at day 0 and day 6 no statistically significance could be found (Figure 43 (a) and (b)).

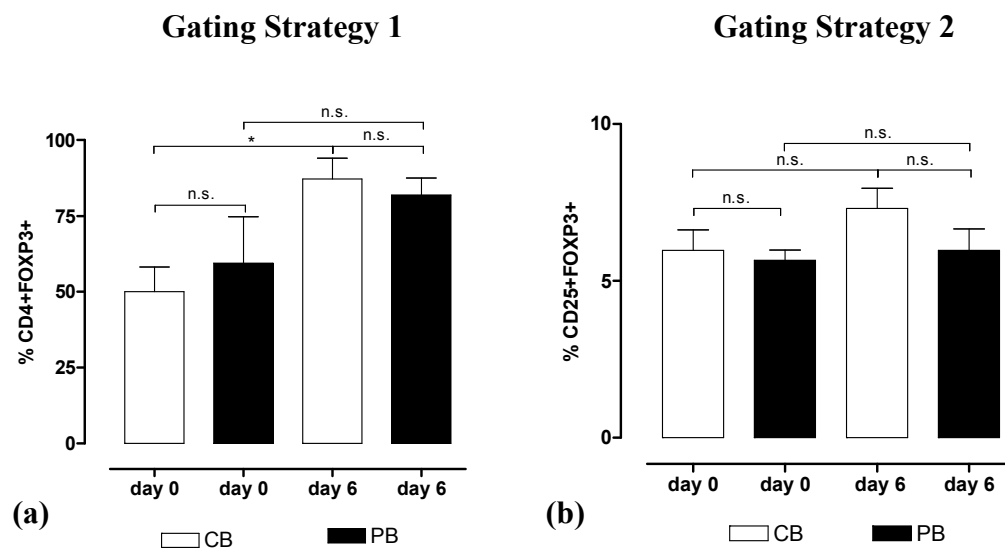


Figure 43: FoxP3 expression in cord compared to peripheral blood

FoxP3 and CD25 expression was measured via flow cytometry at day 0 and day 6, after CBMCs and PBMCs were cultured with BLG L-0130 [50 μ g/mL] or medium alone. Two different gating strategies were applied (Gating strategy 1, Figure 42 (a) and Gating strategy 2, Figure 42 (b)). In cord blood the BLG L-0130 addition leads to a significant increase of FoxP3 expression when compared to day 0 with gating strategy 1 (a) but not with gating strategy 2 (b). In peripheral blood we could not detect any statistically significant increase in FoxP3 expression (a) and (b). No significant difference was detected between CB and PB on day 0 or on day 6 (a) and (b).

Results are representative for 4 (day 6 CB and PB), 5 (day 0 PB), and 7 (day 0 CB) independent experiments and show means $\pm$ SEM.

Moreover, differences, regarding the intensity (MFI; mean fluorescence intensity) of FoxP3 expression, were investigated (Figure 44). No Significant differences could be found (day 0; CB vs PB; 41.06 ± 19.81 vs 57.85 ± 48.18 ; means $\pm$ SD).

Summing up, neither the frequency, nor the density (MFI FoxP3 in $CD4^+CD25^+$ cells of FoxP3 expression) differed significantly between CB and PB on the protein level.

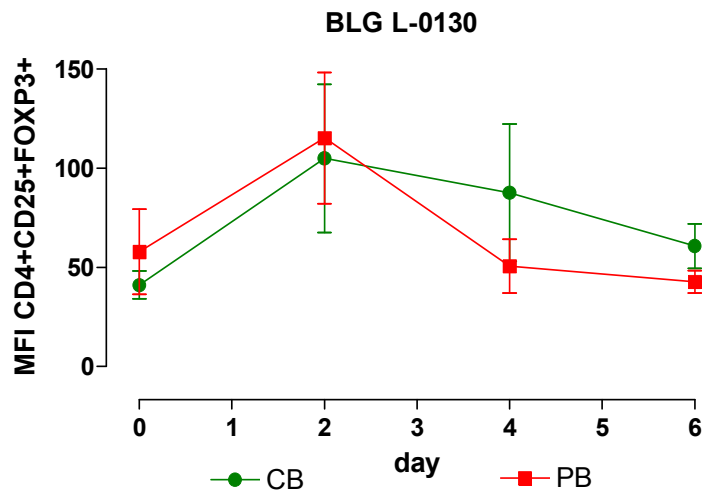


Figure 44 MFI of FoxP3 expression of CB compared to PB

Kinetic experiments were performed. The mean fluorescence intensity of FoxP3 was measured via flow cytometry at day 0, day 2, day 4, and day 6, after CBMCs and PBMCs were cultured with BLG L-0130 [50 μ g/mL]. No significant differences were detected between CB and PB or between different time points. The results are representative for 4 (day 6) to 7 (day 0) independent experiments and show the means $\pm$ SEM.

4.2.6 No indication of Differences of FoxP3, GARP, TGF- β , IL-10, RUNX 1, or RUNX 3 Expression

To investigate potential differences in more detail, FoxP3 and other Treg associated proteins, such as GARP, TGF- β , IL-10, RUNX 1, and RUNX 3, were assessed via quantitative real-time PCR (Figure 45). Cord blood and peripheral blood derived CD4⁺CD25⁻ T effector cells and CD4⁺CD25⁺ regulatory T cells were MACS-sorted. Quantitative RT-PCR was performed to measure the content of the Treg associated genes.

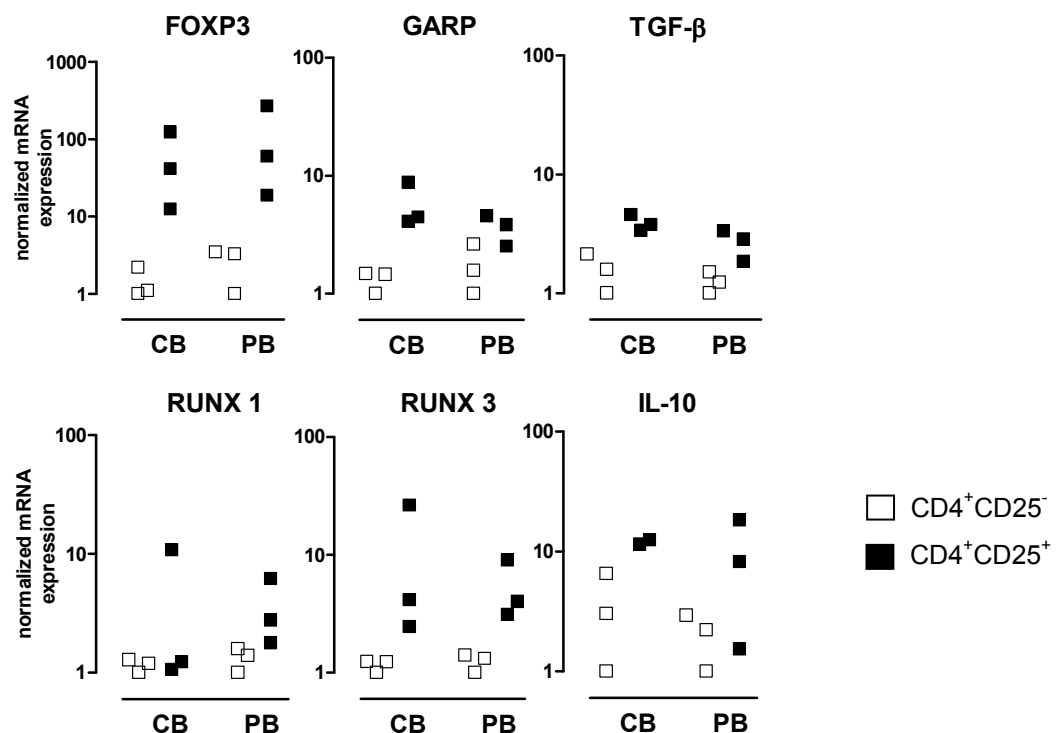


Figure 45 Constitutive FoxP3, GARP, TGF- β , RUNX 1, RUNX 3, and IL-10 expression in cord blood and peripheral blood

T effector cells (Teff; CD4⁺CD25⁻) and T regulatory cells (Tregs; CD4⁺CD25⁺) were isolated via MACS sorting from freshly isolated CBMCs or PBMCs. FoxP3, GARP, TGF- β , IL-10, RUNX 1, and RUNX 3 were measured via qRT-PCR. CB-derived cells are displayed with white (Teff) and black (Tregs) squares, whereas PB-derived cells are illustrated with white (Teff) and black (Tregs) dots. Results are representative for 3 independent experiments showing the normalized expression.

Despite the difference in functionality the putative Treg subset did not differ in their marker expression pattern.

4.2.7 The $CD25^{+}CD127^{low}$ Compartment Harbours a Regulatory T Cell Subset with Limited Net Suppressive Effect

CD127 has been ascribed to be an excellent marker for Treg characterization in general. Recently, the suppressive capacity of $CD4^{+}CD25^{+}CD127^{low/-}$ Tregs in cord blood has been linked to the development of food allergy later on^{213, 249}. The $CD127^{low}$ fraction was purified by means of FACS-sorting from the $CD25^{high}$ and $CD25^{int}$ fraction to check for the importance of CD127 down regulation for Treg function. Three cell fractions ($CD4^{+}CD25^{high}CD127^{low}$, $CD4^{+}CD25^{int}CD127^{low}$ and $CD4^{+}CD25^{int}CD127^{high}$) with a putative suppressive capacity and one cell fraction ($CD4^{+}CD25^{-}$) representing T effector subset were obtained (all fractions had a purity of >99%; Figure 46).

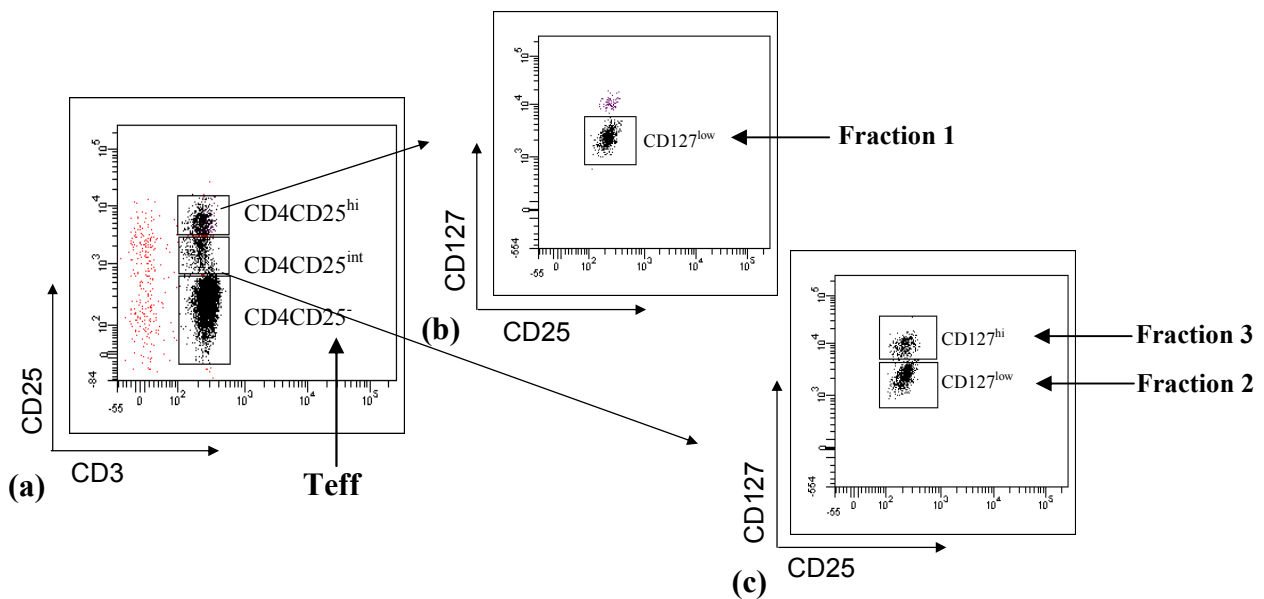


Figure 46: Gating strategy for obtaining $CD4^{+}CD25^{+/-}CD127^{hi/low}$ T cells®

3 subgroups, $CD4^{+}CD25^{hi}$, $CD4^{+}CD25^{int}$ and $CD4^{+}CD25^{-}$ T cells were obtained (a). The $CD4^{+}CD25^{int}$ fraction was further divided into two more fractions, according to their CD127 content (F2 (low), F3 (high)) (c).

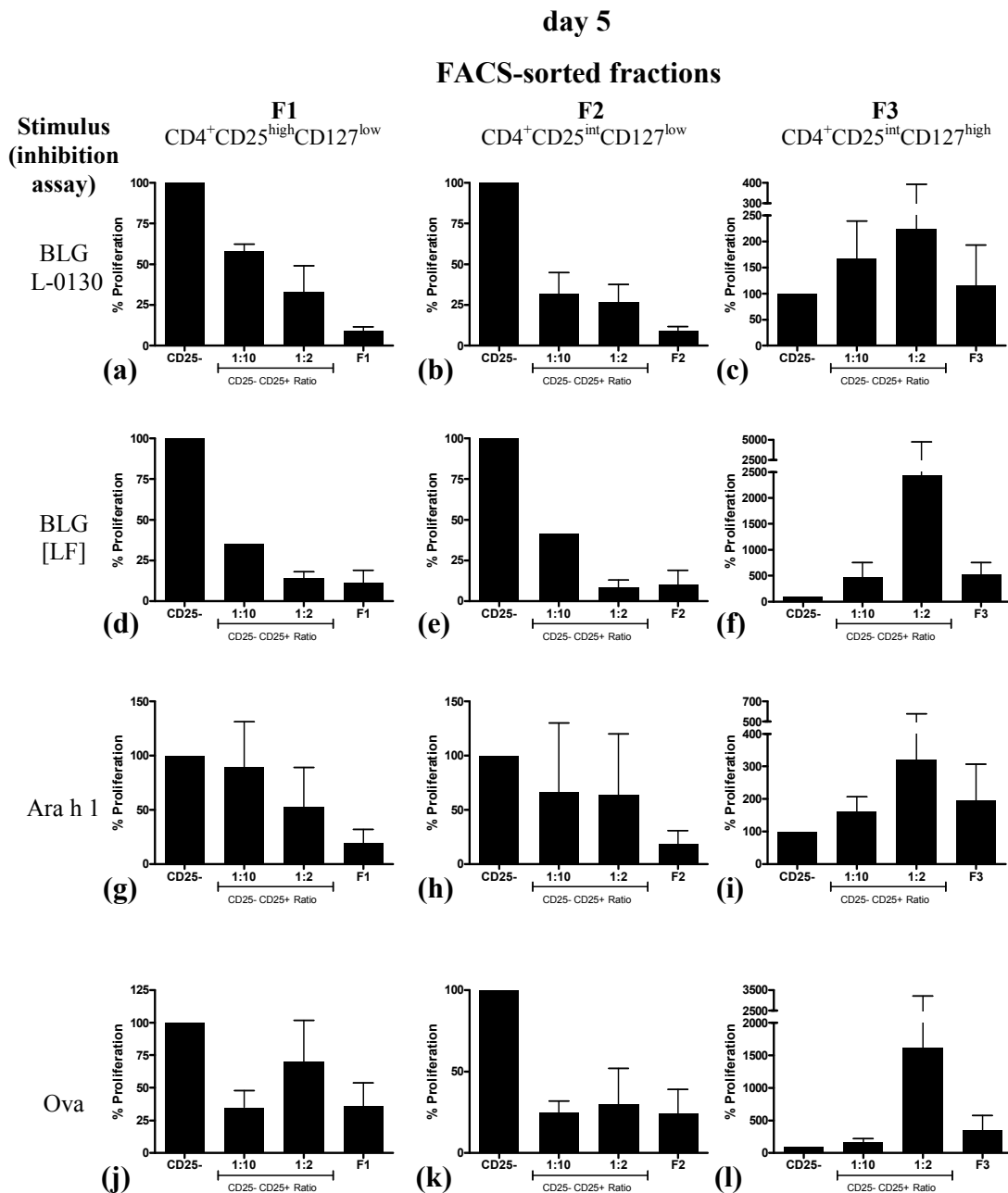


Figure 47: Impact of CD127 on suppressive function of cord blood derived Tregs

Freely isolated CBMCs were FACS sorted, and gated as shown in figure 46. Subsets of CB ($CD4^+CD25^{high}CD127^{low}$ and $CD4^+CD25^{int}CD127^{low}$) expressed inhibitory potential (a,d,g,j) and (b,e,h,k), whereas the $CD4^+CD25^{int}CD127^{high}$ subset even increased proliferation (c,f,i,l). The inhibitory potential is expressed as relative proliferation compared to the effector T cells ($CD4^+CD25^+$) cells alone. Graphs indicate the means of 1-4 (1: for BLG [LF] F1 and F3 1/10; 2 for BLG [LF] F1, F3 1/2 and F alone, all Ara h 1 and all OVA; 3 for BLG L-0130 F1-F3 1/10, BLG [LF] F2 1/10; 4: BLG L-0130 F1-F3 1/2 and F alone, BLG [LF] F2 1/2 and F alone) independent experiments $\pm$ SEM.

Subsets with low CD127 expression ($CD4^+CD25^{high}CD127^{low}$ and $CD4^+CD25^{int}CD127^{low}$) possessed inhibitory potential (Figure 47 (a,d,g,j) and (b,e,h,k)) and very limited proliferative potential (9.25 ± 4.35 and 8.75 ± 6.08 , respectively).

On the other hand, the fraction with high CD127 expression (F3; $CD4^+CD25^{int}CD127^{high}$) even increased proliferation (Figure 47 (c,f,i,l)). At a ratio of 1:10 166 ± 126.81 and at a ratio of 1:2 even 223 ± 341.62). Hence it did not exhibit any suppressive potential at any ratio. Moreover, fraction 3 alone was not hypo-proliferative either ((c); right; 115.75 ± 154.98).

The same trend (suppressive potential of $CD127^{low}$ fractions and proliferative potential of $CD127^{high}$ fractions) can clearly be observed at the fractions stimulated with BLG [LF], Ara h 1, as well as with Ova (Figure 47 d-l).

4.2.8 FoxP3, TGF- β , and IL-10 mRNA Expression is not Substantially Up-regulated in the Regulatory Subsets

Sorted fractions were checked for expression of FoxP3 and regulatory cytokines IL-10 and TGF- β . FoxP3 was enormously increased in all three fractions (F1, F2, F3) compared to the effector T cell subset (a). In all experiments the FoxP3 level for the Teff fraction was below the limit of detection. No difference could be observed between the three fractions (F1-F3), although F3 showed a slightly reduced FoxP3 expression, however with a large standard deviation (160.21 ± 255.37 ; Figure 48 (a)).

The TGF- β mRNA level was not substantially up-regulated in all three fractions. A minor reduction of TGF- β level could be observed in the Teff subset (Figure 48 (b)).

The IL-10 mRNA level in the F1 fraction was the highest (716.26 ± 883.17), although with a high standard deviation. Comparing Teff and F3 with F2, an eminent difference could be observed (15.71 ± 16.24 and 15.68 ± 3.63 , respectively Figure 48 (c)).

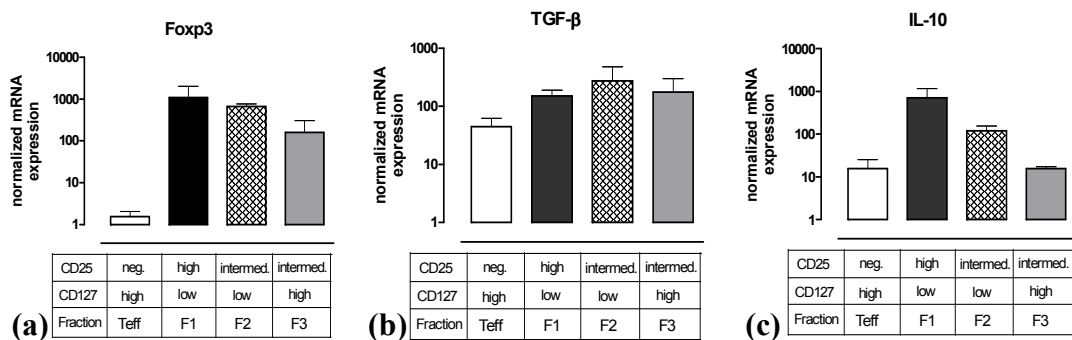


Figure 48: Relative expression of regulatory cytokines

CBMCs were gated on $CD3^+CD4^+$ cells and then FACS sorted according to their CD25 and CD127 expression (see figure 46) and analyzed with real-time PCR. As FoxP3 expression for Teff was below the limit of detection, the deltaCT value was calculated as follows: 40 (cycles used) – cycle of 18S. Results are representative for 3-4 independent experiments showing the normalized expression of means $\pm$ SEM.

4.2.9 Cord blood derived CD4⁺CD25⁺ T cells become Functional Tregs upon Stimulation

Since multiple publications demonstrated the inhibitory properties of the cord blood derived CD4⁺CD25⁺ subset in the context of a pre-activation system, the relevance of allergen exposure on functional activation of Tregs and the impact of endotoxin on the efficiency of this process was investigated. After a six day exposure of CBMCs to BLG in the presence or absence of LPS, CD4⁺CD25⁺ cells were isolated. Their inhibitory potential on α -CD3, BLG L-0130, and BLG [LF] induced proliferation of autologous CD4⁺CD25⁻ cells was monitored on day 3 (Figure 49) and with BLG L-0130 and BLG [LF] on day 7 (Figure 50).

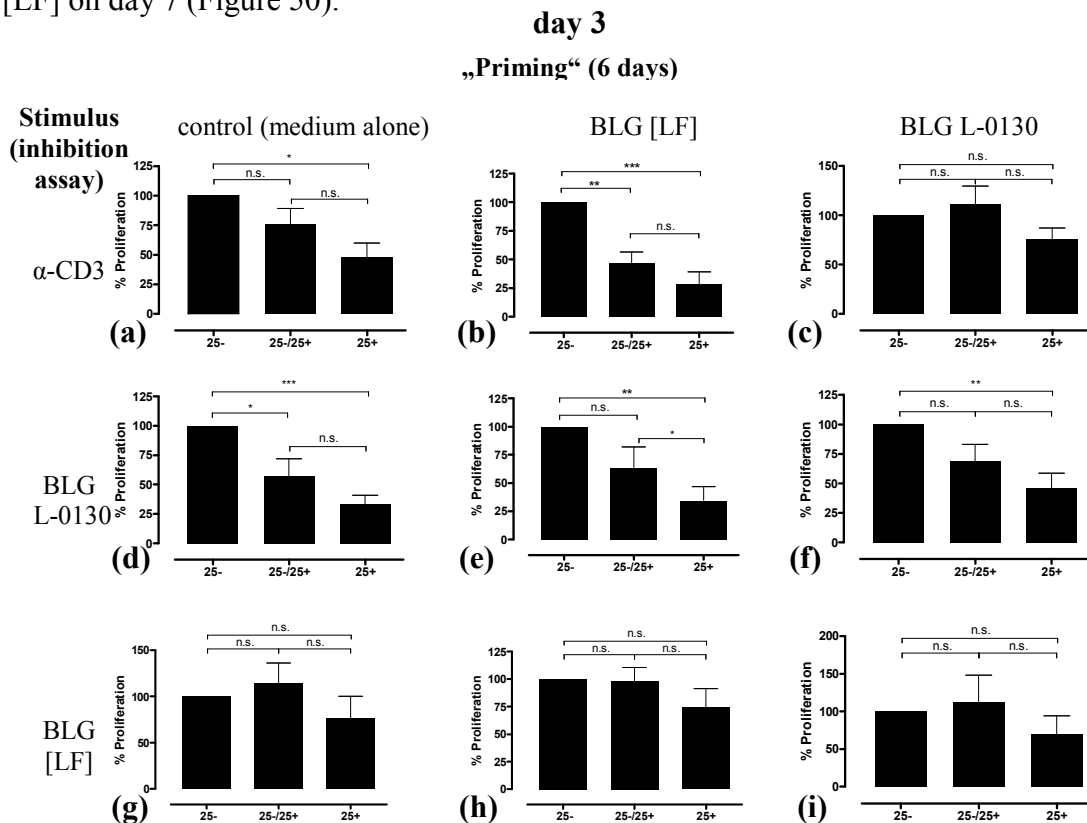


Figure 49 Inhibition assay on day 3

CBMCs were cultured for 6 days in the presence of different stimuli (“primed” with medium alone (a,d,g), BLG [LF] (b,e,h), and BLG L-0130 (c,f,i)) and an inhibition assay was performed with α -CD3 (a-c), BLG L-0130 (d-f), and BLG [LF] (g-h). The inhibitory potential is expressed as relative proliferation compared to the effector T cells (CD4⁺CD25⁻) cells alone. Results are representative for 6-9 independent experiments. T-test was applied ($p < 0.05$ (*); $p < 0.01$ (**); and $p < 0.001$ (***)).

At the read-out time point at day 3, a trend for regulatory T cells to become suppressive could be observed. It worked best upon priming with the purified BLG [LF] for six days then stimulating with α -CD3 (Figure 49 (b) and (e)). On day 3, no suppressive potential of Tregs could be monitored when stimulated with BLG [LF] at no prior priming stimulus (Figure 49 (g-i)).

This suppressive effect gets even more pronounced on day 7, where the CD4⁺CD25⁺ Treg subset, that was not inhibitory on day 0, gained inhibitory potential upon the stimuli BLG L-0130 and BLG [LF] (Figure 50 (a-f)). The trend can be observed in all graphs, however a statistically significant difference could only be observed when using BLG L-0130 (Figure 50 (a-c) with all priming stimuli, and BLG [LF] only when primed with BLG L-0130 (Figure 50 (f)).

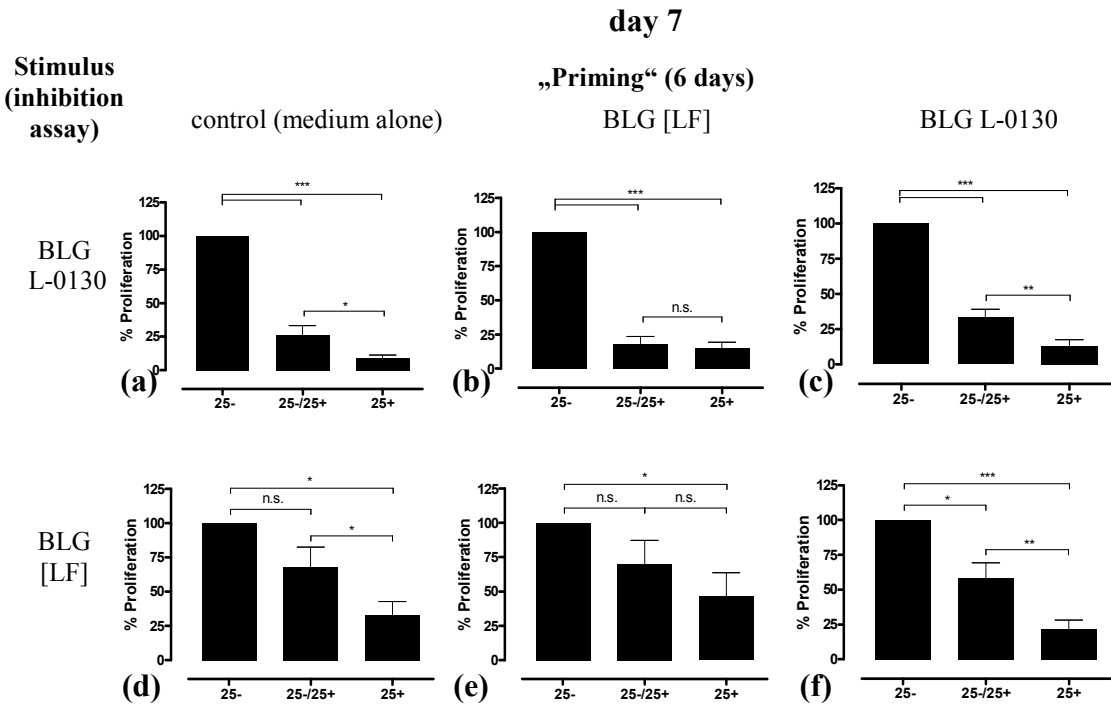


Figure 50 Inhibition assay on day 7

CBMCs were cultured for 6 days in the presence of different stimuli (medium alone **(a,d)**, BLG [LF] **(b,e)**, or BLG L-0130 **(c,f)**). Inhibition assays were performed with and BLG L-0130 **(a-c)**, and BLG [LF] **(d-f)**. Results are representative for 5-6 independent experiments. T-test was applied and SEM is shown ($p < 0.05$ (*); $p < 0.01$ (**); and $p < 0.001$ (***)).

4.2.10 Changes of the Transcription Factors FOXP3, RUNX1, RUNX3 and the Treg Marker GARP Upon Priming

As priming of CBMCs led to suppressive $CD4^+CD25^+$ Tregs (Figure 48 and 49) changes of the transcription factors FOXP3, RUNX 1 and RUNX 3 and the Treg associated surface marker GARP were measured via quantitative real-time PCR and compared to PB-derived Tregs (Figure 51).

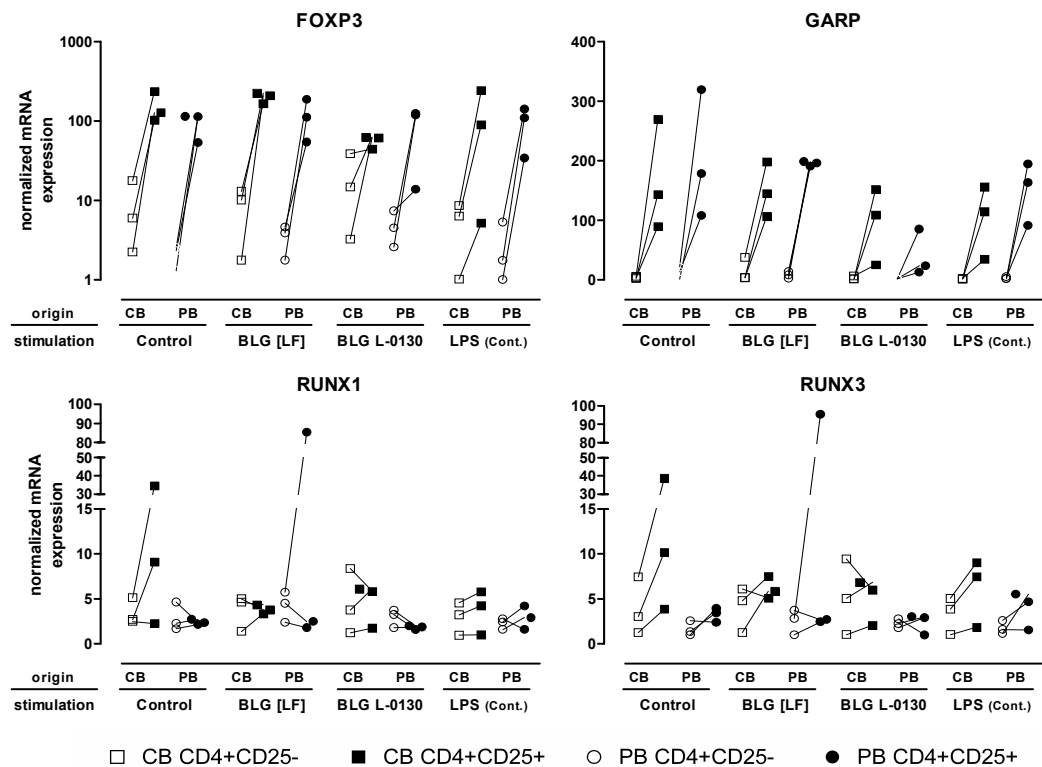


Figure 51 FoxP3, GARP, RUNX 1, and RUNX 3 expression in CB and PB of effector and regulatory T cells after priming for six days

CBMCs or PBMCs were primed for six days with [50 µg/mL] BLG [LF], BLG L-0130, or LPS (the amount present in 50 µg/mL BLG L-0130). After that, T effector cells (Teff; $CD4^+CD25^-$) and T regulatory cells (Tregs; $CD4^+CD25^+$) were isolated via MACS sorting. FoxP3, GARP, RUNX 1; and RUNX 3 were measured via qRT-PCR. CB-derived cells are displayed with white (Teff) and black (Tregs) squares, PB-derived cells are illustrated with white (Teff) and black (Tregs) dots. Results are representative for 3 independent experiments showing the normalized expression.

Cord blood and peripheral blood mononuclear cells were primed for six days with medium alone (control), BLG [LF], BLG L-0130 or LPS (with the amount present in BLG L-0130; at the same concentration).

Subsequently, the $CD4^+CD25^-$ T effector cell subsets and $CD4^+CD25^+$ regulatory T cell subsets were MACS-sorted. QRT-PCR was performed to measure the expression of the Treg associated genes.

Foxp3 and GARP expression was strongly upregulated in Tregs when compared to Teff. There was a trend that medium or BLG [LF] primed displayed the highest FoxP3 and GARP expression was observed.

With RUNX 1 and RUNX 3, the expression pattern is less clear. A trend towards a higher expression of RUNX 1 and RUNX 3 can be monitored in CB compared to PB. Those CB/PB-derived cells with RUNX 1 expression also have a heightened RUNX 3 expression (Figure 51).

No difference in the expression profile could be found between CB and PB, only between the specific subsets.

4.2.11 Phenotypic Characterization of the T Cell Compartment Upon Antigen-Exposure

Flow cytometry was applied for PBMCs (red) and CBMCs (green) after a 7 day culture in the presence of different stimuli (BLG L-0130, BLG [LF], LPS (contamination)) or medium alone (control). FACS scatter of the entity of the CD3 cells (M1; 100%) were assessed for all stimuli. For gaining more information, scatters for the proliferated cells (M2), measured with CFSE staining, are only shown for the stimulus BLG L-0130 as the other stimuli did not provide enough cell counts to show representative FACS scatters.

As shown in previous experiments, CBMCs proliferate stronger upon stimulation than PBMCs (72% vs 25%; BLG L0130 (Figure 56 vs. Figure 52, respectively); 13.56% vs 4%; BLG [LF] (Figure 56 vs. Figure 53); 55.47% vs 19.5%; LPS (Figure 57 vs. Figure 54, respectively) and 4.07% vs 3.68% (Figure 56 vs. Figure 53, respectively); control).

In peripheral blood the markers CD45RO and CD25 were highly upregulated upon stimulation (seen with the stimuli BLG L-0130 and LPS). The same pattern has been observed looking at the markers CD45RB and CD45RO. After proliferation T cells showed an overall expression of ~ 90% that possessed these markers. In cord blood per contra, a different pattern has been observed.

As cord blood cells are mainly CD45RA⁺ on day 0 a shift towards double positive CD45RA CD45RO cells can be observed. About 70% of the T cells express these surface markers upon proliferation. The same arrangement and percentages can be seen when checking the marker CD45RB.

Next to these markers, the activation markers CD69 as well as CD25 have been highly upregulated during the stimulation process. The contaminated BLG L-0130 shares the same activation profile as compared to LPS (contamination). Moreover in PB, comparing the surface pattern of the BLG [LF] and the control less proliferation with a similar pattern can be observed.

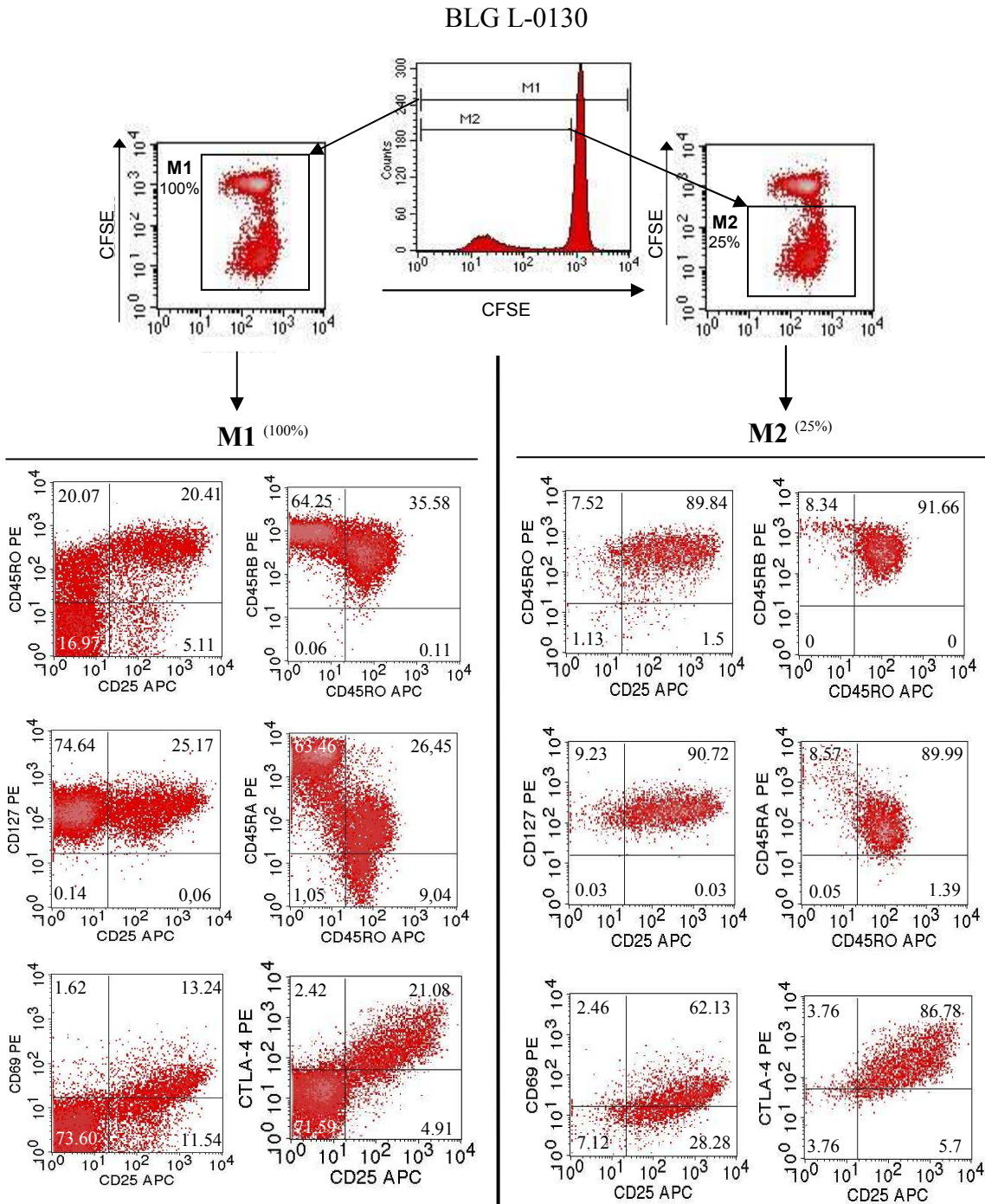


Figure 52 Expression profile of PBMCs stimulated with BLG L-0130 on day 7

PBMCs were stained with CFSE on day 0, then stimulated with BLG L-0130 [50µg/mL] for seven days. The left panel represents all CD3 cells on day seven, the right panel represents the scatters of the according proliferated cells.

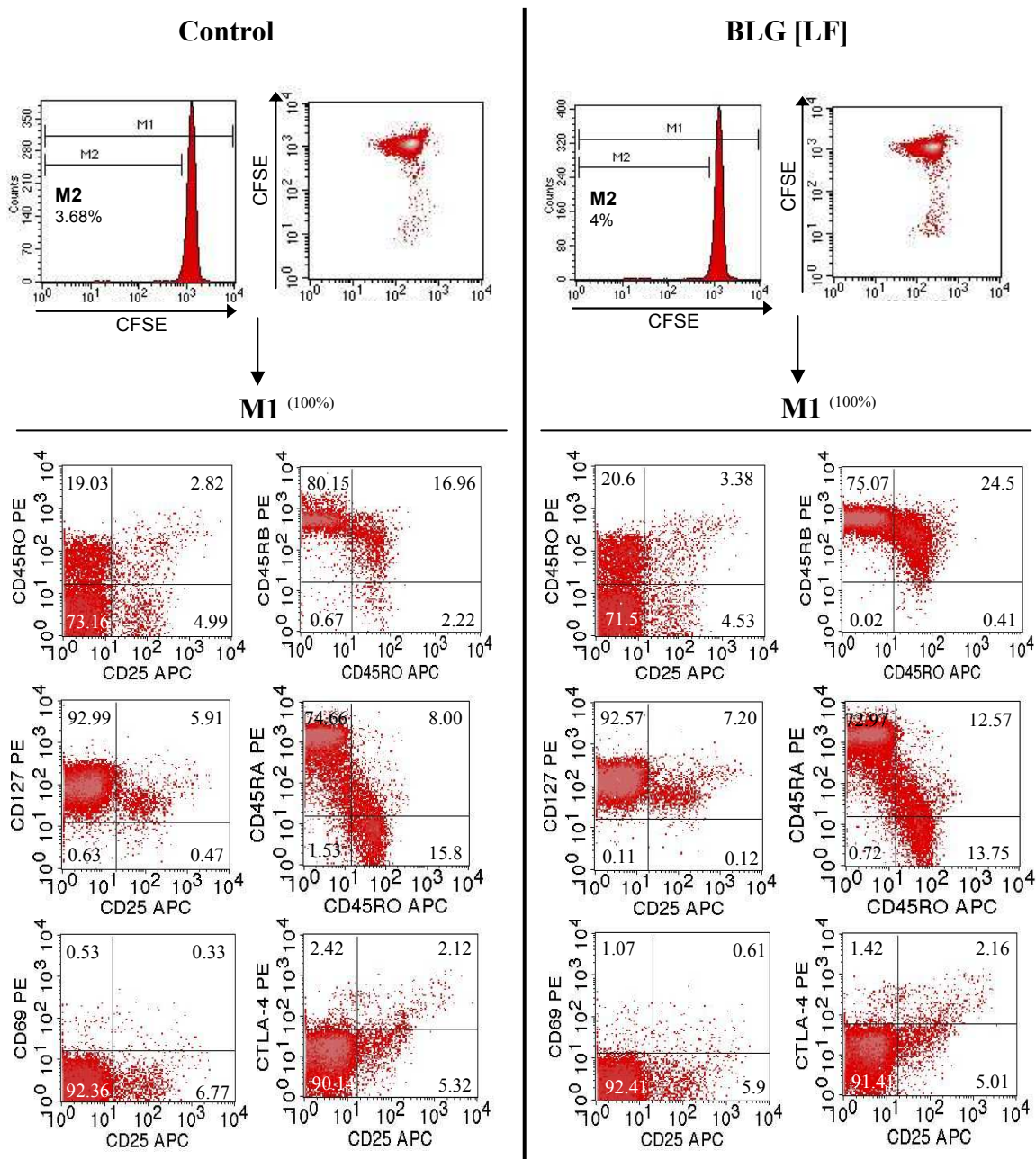


Figure 53 Expression profile of PBMCs alone and stimulated with BLG [LF] on day 7

PBMCs were stained with CFSE on day 0 and then stimulated with BLG [LF] [50µg/mL] for seven days (right panel) or medium alone (control; left panel). The scatters show CD3 cells (M1).

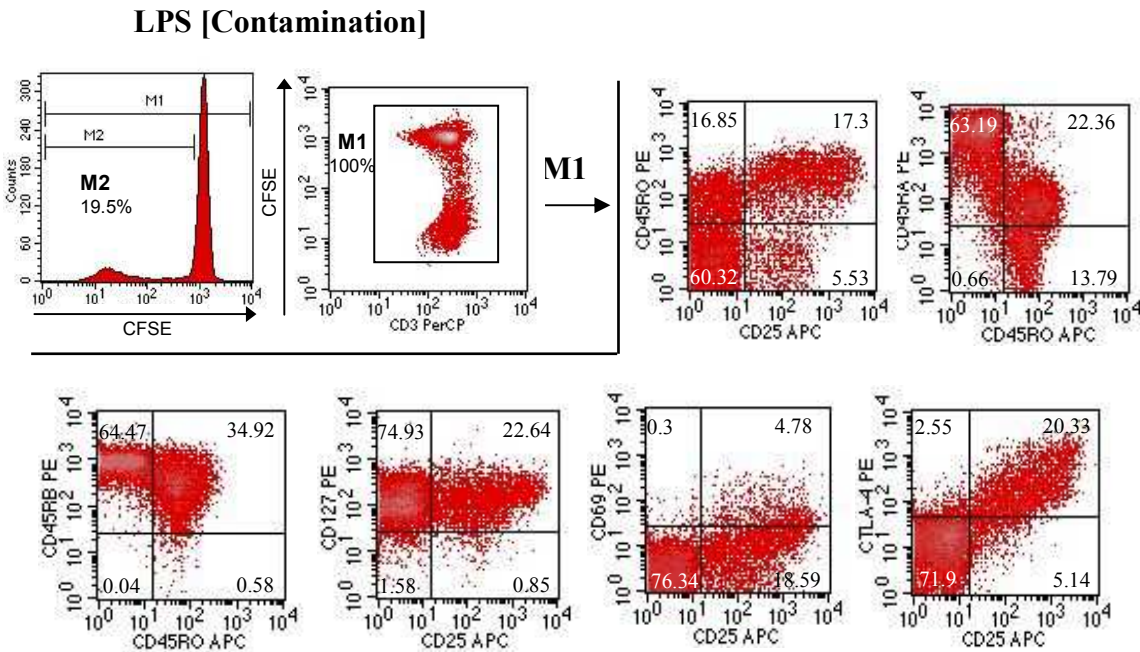


Figure 54 Expression profile of PBMCs stimulated with LPS on day 7

PBMCs were stained with CFSE on day 0 and then stimulated with LPS (the same amount that was present in 50 μ g/mL BLG L-0130) for 7 days. The scatters show CD3 cells (M1).

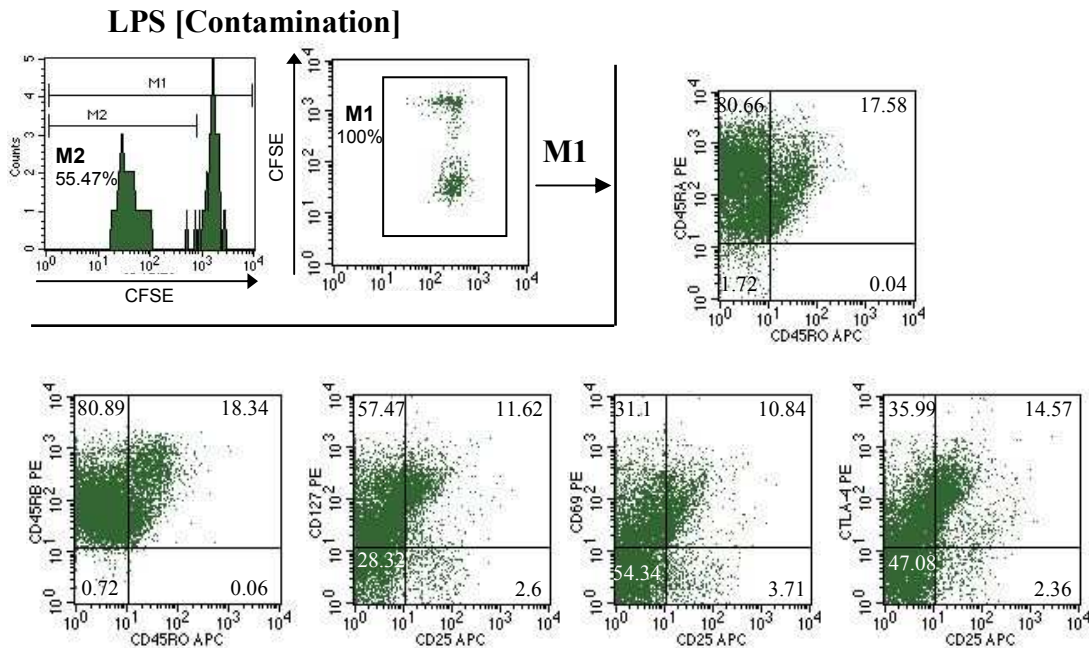


Figure 55 Expression profile of CBMCs stimulated with LPS on day 7

CBMCs were stained with CFSE on day 0 and then stimulated with LPS (the same amount that was present in 50 μ g/mL BLG L-0130) for 7 days. The scatters show CD3 cells (M1).

BLG L-0130

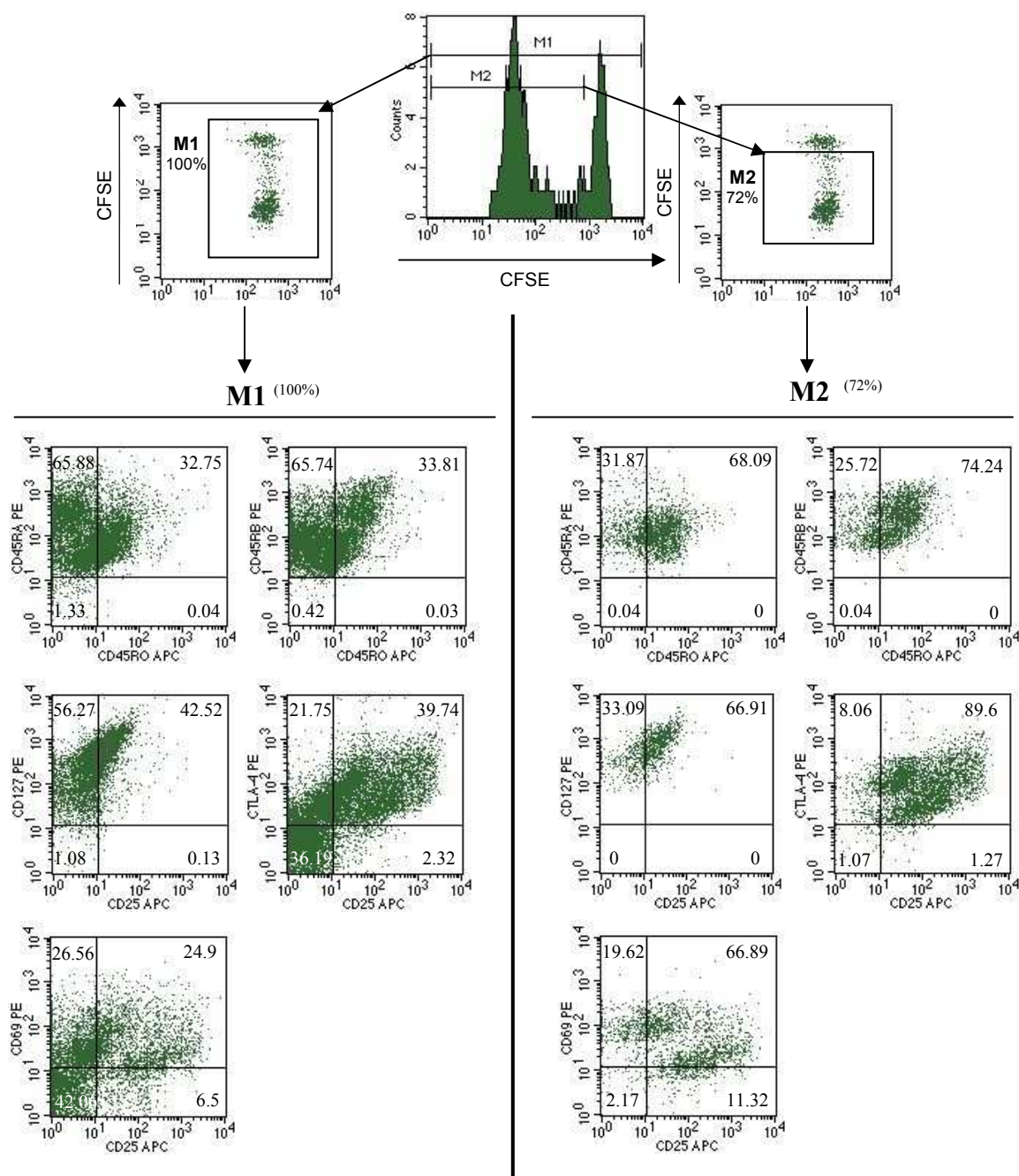


Figure 56 Expression profile of CBMCs stimulated with BLG L-0130 on day 7

were stained with CFSE on day 0, then stimulated with BLG L-0130 [50µg/mL] for seven days. The left panel represents all CD3 cells on day seven, the right panel represents the scatters of the according proliferated, stimulated cells.

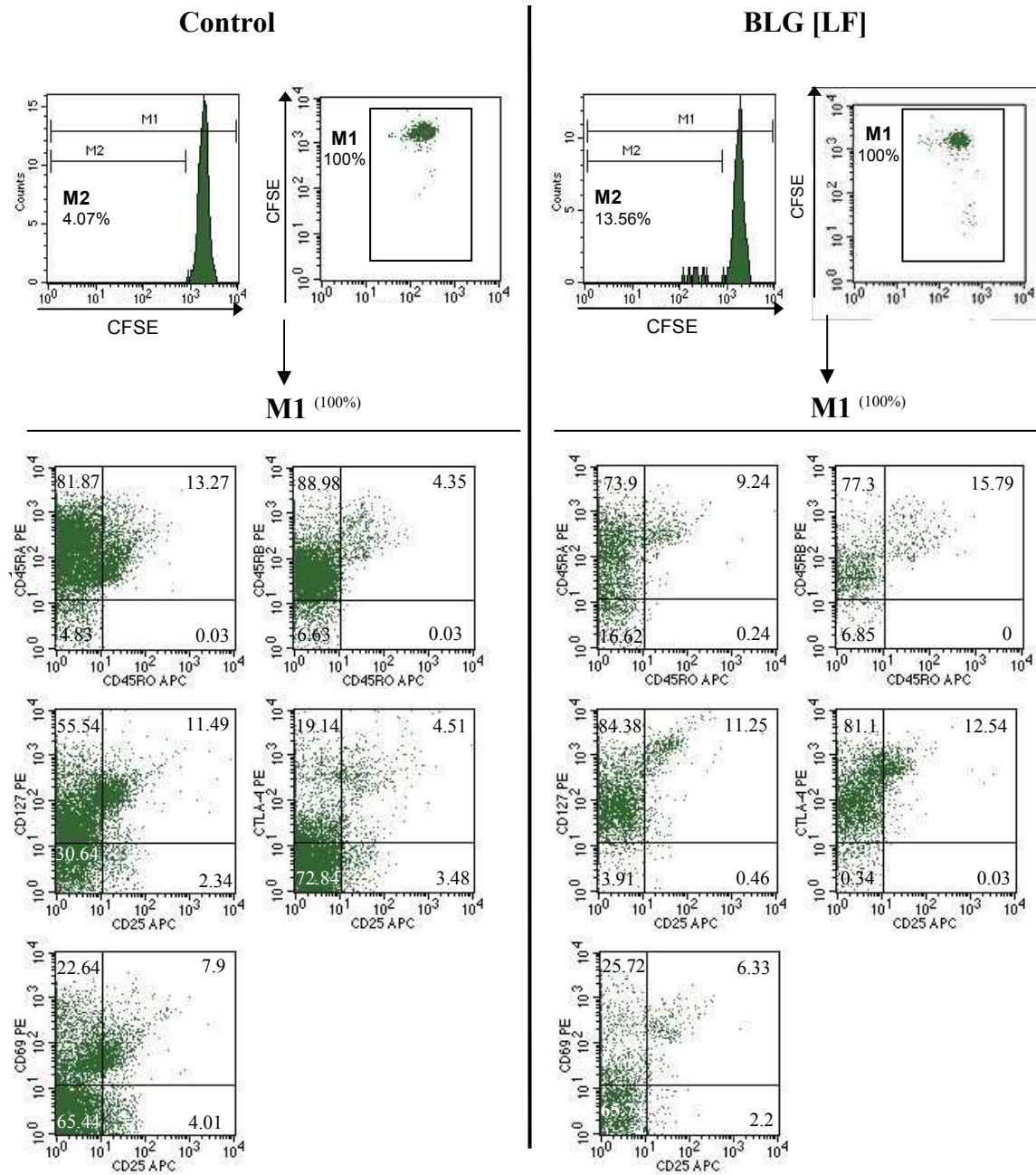


Figure 57 Expression profile of CBMCs alone and stimulated with BLG [LF] on day 7

CBMCs were stained with CFSE on day 0 and then stimulated with BLG [LF] [50µg/mL] for seven days (right panel) or medium alone (control; left panel). The scatters show CD3 cells (M1).

4.2.12 Cord Blood-Derived $CD4^+CD25^+$ T Cells Expand Upon Antigen-Exposure

In order to identify the proliferating cell population $CD4^+CD25^+$ and $CD4^+CD25^-$ were MACS-sorted, CFSE-labeled on day 0 then stimulated with BLG [LF] (middle), or BLG L-0130 (right) (Figure 58 (a and b)).

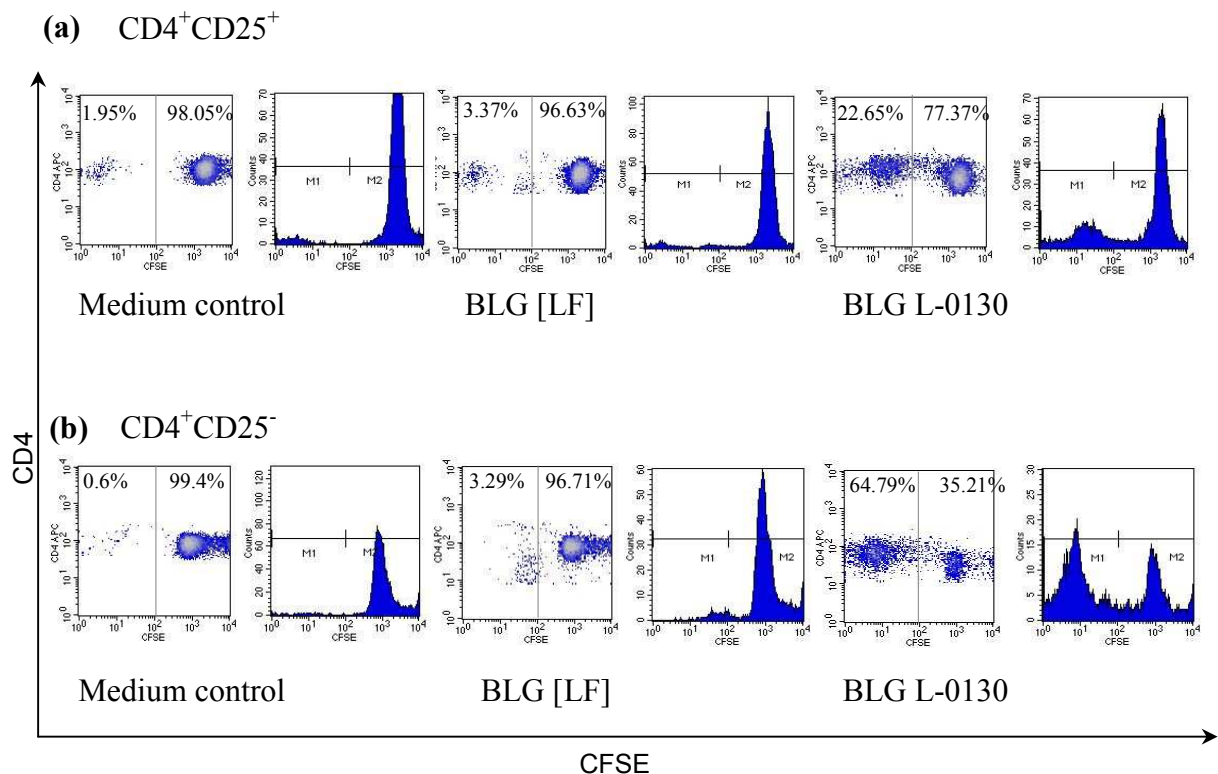


Figure 58 Proliferation of $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ T effector cells

Tregs and Teff were isolated via MACS-sorting on day 0, stained with CFSE and stimulated for 7 days in the presence of medium alone (left), BLG [LF] (middle), and BLG L-0130 (right). Cells are gated on $CD3^+CD4^+$ cells. Proliferation was measured by rating the CFSE distribution. For every stimulus a scatter dot-plot (left) and a histogram (right) is displayed. Data represents one out of four independent experiments.

Cord blood derived $CD4^+CD25^+$ T cells do proliferate upon BLG L-0130 stimulation (22.65%), and to a lesser extent when stimulated with BLG [LF] (3.37%) (Figure 58 (a)). In contrast, $CD4^+CD25^-$ T cells strongly proliferate upon stimulation with BLG L-

0130 (64.79%). BLG [LF] (3.29%) induced proliferation in the $CD4^+CD25^-$ fraction, $CD4$ however was downregulated (Figure 58 (b)).

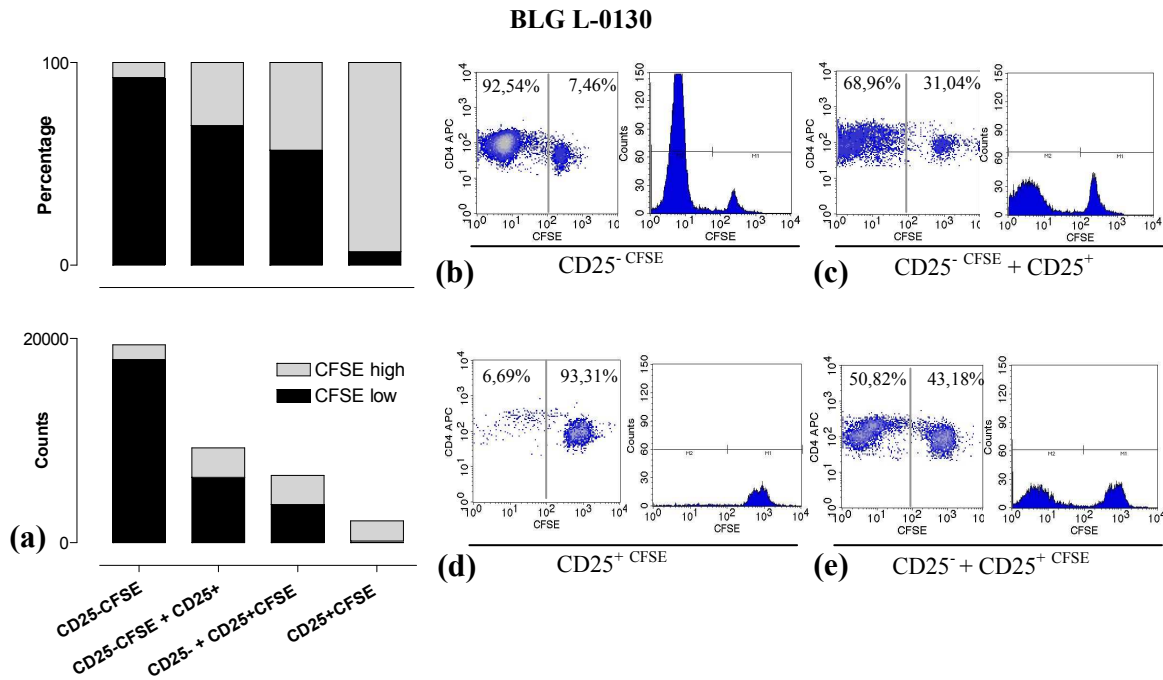


Figure 59 Crossing CFSE stained Tregs and Teff

CBMCs were sorted according to their $CD25$ expression, then stained with CFSE and incubated with BLG L-0130 for seven days. Figure (a) summarizes the figures (b-e), the figure below shows the absolute cell counts. $CD4^+CD25^-$ cells showed the highest proliferation whereas when $CD4^+CD25^+$ cells were added, less proliferation was observed. In figure (b) about 90% of CFSE stained $CD4^+CD25^-$ cells alone proliferated upon this stimulation, whereas only 7% proliferated when CFSE stained $CD4^+CD25^+$ cells proliferated when alone (d). However, when observing CFSE stained $CD4^+CD25^-$ co-cultured with non-CFSE stained $CD4^+CD25^+$ ratio 1:2 at day 0 at a, only about 70% of all cells proliferated (c). When $CD4^+CD25^+$ cells were stained with CFSE and joined with $CD4^+CD25^-$ cells, about 50% proliferated and were CFSE low (e).

As a next step the proliferation pattern of CFSE stained cord blood derived Tregs/Teff cells in combination with the corresponding non-CFSE labeled subset were screened. Again, $CD4^+CD25^-$ cells proliferate strongly upon BLG L-0130 stimulus (more than 90%; upper panel, Figure 59). This is also reflected in total counts. After the 7 days of culture with BLG L-0130 the cell population doubled. However, after addition of Tregs less proliferation was observed (Figure 59 (c,e)).

As mentioned above (Figure 58) again an increased proliferation of Teff cells upon BLG L-0130 was observed (Figure 59 (b)). 90% of CFSE stained $CD4^+CD25^-$ cells alone proliferated upon this stimulation, whereas only 7% proliferated when CFSE stained $CD4^+CD25^+$ alone cells were observed (d). Interestingly, when observing CFSE stained $CD4^+CD25^-$ mixed together with non-CFSE stained $CD4^+CD25^+$ ($CD4^+CD25^-$ CFSE + $CD4^+CD25^+$) at a ratio 1:2 at day 0, only about 70% of all cells proliferated (c). When $CD4^+CD25^+$ cells were stained with CFSE and joined with $CD4^+CD25^-$ cells ($CD4^+CD25^+$ CFSE + $CD4^+CD25^-$), about 50% proliferated and were CFSE low (e).

4.2.13 Functional Assays Demonstrate Suppressive Properties of the Undividing Fraction

In the previous experiments the suppressive potential of “primed” $CD4^+CD25^+$ Tregs has been revealed (Figure 49 and Figure 50). The question arose whether the cells that proliferated or those who did not proliferate become inhibitory during this process.

To distinguish which cell population gains inhibitory potential after priming, cells were sorted according to their CFSE intensity and CD25 expression on day 6 (Figure 60). Three different fractions (Figure 60) were obtained and inhibition assays were performed with fraction 1 (F1; $CFSE^{high}CD25^{high}$), fraction 2 (F2; $CFSE^{high}CD25^-$), and fraction 3 (F3; $CFSE^{int/low}CD25^{high}$), in the presence of thawed, allergen-unexperienced $CD4^+CD25^-$ T effector cells and irradiated CBMCs, which served as APCs. BLG L-0130 (a), and BLG [LF] (b) were used as stimuli for the inhibition assays (Figure 61).

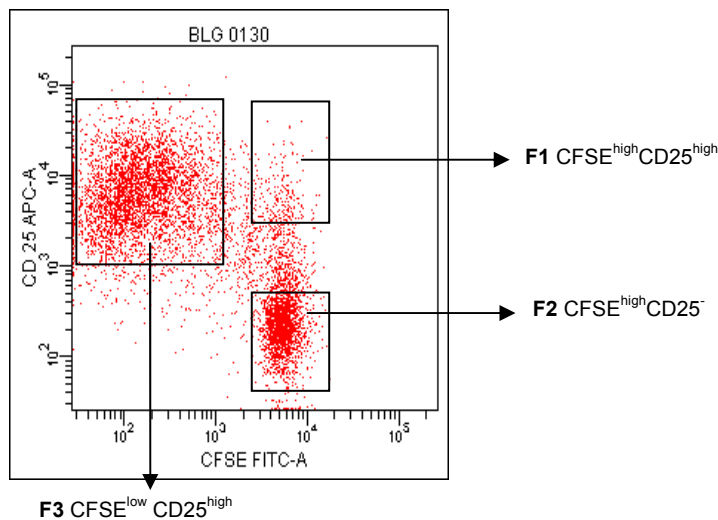
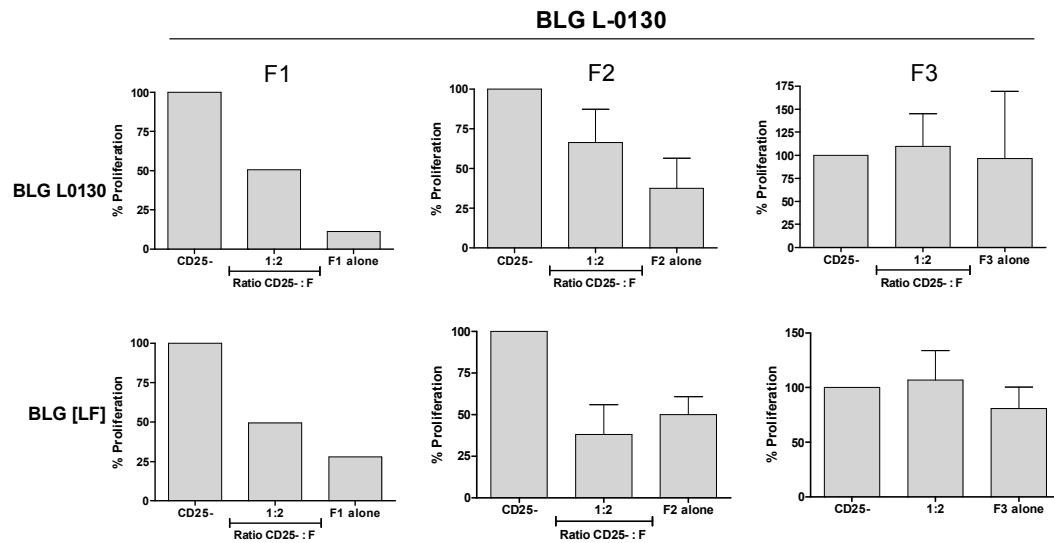


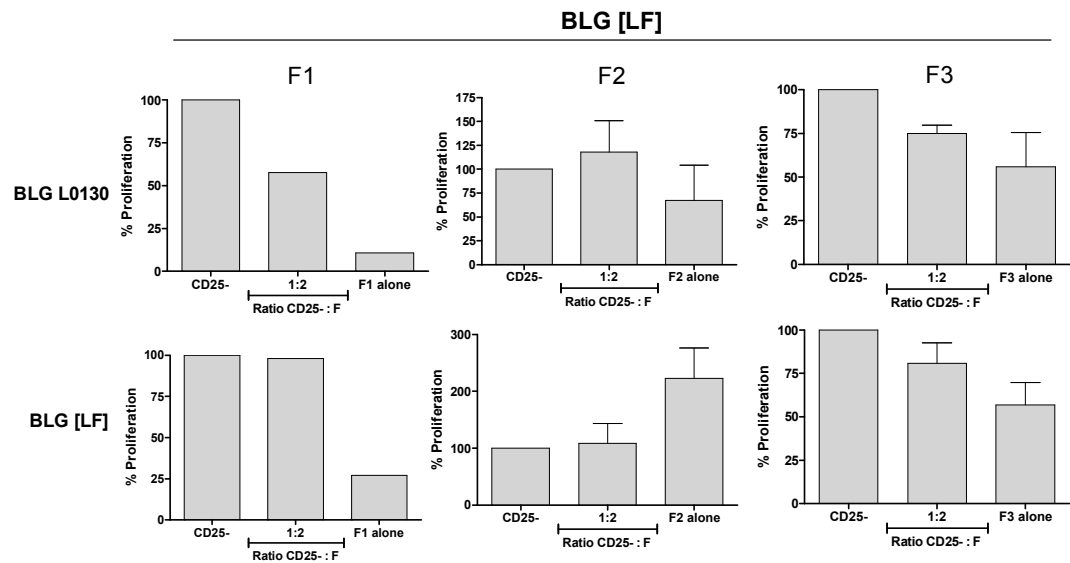
Figure 60 Fractions 1-3, sorted according to their CFSE intensity and CD25 expression

Inhibition assays were performed with 9 different fractions (3 “priming” stimuli x 3 fractions (F1-F3)) in the presence of 4 stimuli (α -CD3, BLG L-0130, BLG [LF], and OVA. Medium alone served as internal control.

(a) F1-F3, stimulated with L-0130



(b) F1-F3, stimulated with BLG [LF]

**Figure 61 Inhibition assay with CFSE and CD25 sorted fractions**

After a 6 day priming with 2 different stimuli (BLG L-0130, and BLG [LF]), cells were FACS sorted according to their CFSE intensity and CD25 expression. Three fractions of each stimulus have been gained ($CFSE^{high}CD25^{high}$, $CFSE^{high}CD25^{-}$, $CFSE^{low/int}CD25^{high}$), an inhibition assays were performed with BLG L-0130 (a), and BLG [LF] (b).

The experiments suggest that the undividing fraction bears the highest suppressive potential. The dividing fraction however, also possessed some inhibitory capacity after the allergen exposure. Marginal differences could be found after the “priming” with the BLG L-0130 (which contained trace amounts of LPS) compared to the LPS-free BLG [LF]. The $CD4^{+}CD25^{high}CFSE^{high}$ fraction stimulated with BLG L-0130 showed generally a diminished proliferative response (~ 3 times lower cpms). The assumption that cells are exhausted after the weekly allergen-experience however can be disproved. We could demonstrate that cells are still able to respond to various allergens and even showed suppressive potential after the strong stimulus BLG L-0130.

5 DISCUSSION

Two main issues were addressed in this thesis: First, the differential impact of endotoxin contamination of commercially available antigen preparations on cord blood and peripheral blood from healthy adult donors *in vitro*. Second, cord blood specific features of the regulatory T cell subset and its impact on allergen-specific responses and tolerance.

Endotoxin contamination in antigen preparations is one of the most important confounders of *in vitro* assays. Interestingly, despite general importance, literature about this issue is relatively rare. We performed a detailed investigation to define the impact of such contaminations.

Although indirect evidence is strongly pointing towards a bias of *in vitro* assays by such contaminations the precise impact on an antigen-specific read-out pattern in a human system in general and cord blood in specific needed to be elucidated^{413, 414}. We demonstrated both, the high frequency of detectable endotoxin contamination in allergen preparations and the pro-proliferative effects and induction of cytokine production by these contaminants.

Firstly, we could demonstrate that endotoxin in commercially available antigen preparations strongly affects *in vitro* cellular measurements. The proliferative responses as well as the cytokine production were significantly elevated of mononuclear cells from human sources (both cord and adult blood). This effect could be validated for food and inhalative allergens and for the Th1-type recall antigen tetanus toxoid.

Secondly, we defined the differential impact of endotoxin on peripheral and cord blood derived mononuclear cells. Cord blood mononuclear cells are significantly more prone to proliferation in response to endotoxin and allergens than PBMCs from adult non-allergic donors. This is also true for antigens the fetus was most likely not exposed to (e.g. camel caseinate). Therefore the underlying mechanism may be more complex than originally suggested. Cord blood does not only harbour a higher percentage of a naïve T cell population but also a subset that is directly derived from the thymus and differs from the naïve T cells in adult blood. The naïve nature of cord blood may

contribute to the higher frequency of endotoxins responses. Cord blood cells from pregnancies without complications have not encountered endotoxin exposure and consequently did not develop LPS tolerance. This phenomenon has been described upon continuous, low LPS dose exposure in adults^{415, 416}. LPS tolerance may contribute to a higher heterogeneity of the peripheral blood mononuclear cell response.

However, not only LPS-dependent responses, but also allergen-specific responses were significantly enhanced. Data from Thornton et al may provide another explanation: thymic emigrants in cord blood are highly responsive to a short initial proliferation boost resulting in apoptosis thereafter²⁴⁹. Moreover it has been postulated that allergens may cross the placenta from mother to child and thereby prime cord blood derived T cells *in utero*^{395, 405}. This explanation was supported by the fact that allergen-specific IgE could be detected in the amniotic fluid. This is of particular interest as IgE was thought not to cross the placenta. However, the origin of amniotic IgE is still subject of debate²³⁰.

Insufficient regulatory mechanisms may be a reason for the heightened response in cord blood. This is supported by the fact that un-primed, naïve cord blood derived CD4⁺CD25⁺ regulatory T cells display less inhibitory potential than CD4⁺CD25⁺ Tregs from adults^{248, 417}.

Thirdly, we could demonstrate that the contamination has to be ascribed to LPS as it acts through TLR-4 dependent mechanism. LPS is certainly not the only form of contamination. Numerous potential other confounders could be responsible for the measured effects (reviewed in^{418, 419}) such as CpG, RNA- or DNA- fragments, flagellin, or other bacterial cell fragments that induce an innate immune response. LPS, however, seems to be the most probable candidate, since high amounts were detected in different BLG preparations. In addition, we could prove its ability to induce proliferation and cytokine production via spiking experiments, where LPS was added in the same amount as detected by limulus assay in the contaminated BLG preparations. To date, a reliable assay for detection is only available for LPS. It can also be considered as a surrogate marker for contamination in general. The involvement of other contaminants that represent ligands for TLR-2, TLR-3, TLR-5, TLR-7, and TLR-9 cannot be ruled out

completely. Nevertheless, we believe that LPS is the main contaminant as we could clearly illustrate a significant cytokine decrease after TLR-4 blockade. Ergo a TLR-4 and LPS engagement can most be certainly ascribed to contamination in the BLG preparation. Moreover we could confirm this data with size-exclusion chromatography as well as TLR-4 transfected HEK-cells. TLR-2 dependend mechanisms were not affected at all.

Purification protocols are commercially available. The most popular systems are based on polymyxin B columns. This leads to decrease the LPS amount to a certain extent. They are not efficient enough to be used in practice, as trace amounts of LPS remain. Trace amounts present (0.7ng/mL) affect the cellular level as we have demonstarted in various experiments. This fact investigated in this study is of central importance when standardized amounts of LPS are tested. Moreover, other TLR ligands may not be removed by this approach and still confound measurements.

Another important methodological consideration is the amount of antigen used for limulus measurement (1 mg/mL or 1 µg/mL) since the sensitivity of the limulus assay is limited. However the impact of these contaminations on antigen-specific proliferation testing is strongly related to the amount of LPS present and the thereof resulting kinetics.

BLG, one of the major milk allergens, is one of the most frequent food allergens in infancy. Milk allergy often resolves spontaneously in the first years of life and is therefore suitable for studying tolerance induction. Moreover, several commercially available BLG preparations have been reported to contain high amounts of endotoxin^{420, 421}. Thus, BLG, an allergen well-described to very efficiently and frequently induce proliferative responses in cord blood makes BLG a perfect model-allergen to investigate. Brix et al proved the endotoxin fraction of contaminated BLG to be the more important to induce a proliferative response of naïve T cells in BALB/c mice than to the allergen fraction itself^{393, 420}. Human cord blood mainly harbours naïve T cells and consequently represents a perfect model to assess whether endotoxins equally contribute to the proliferative capacity of un-purified BLG in the human system. However, human mononuclear cells are known to react with less proliferation to endotoxin. Therefore, the relevance of endotoxin as a confounder could be less prominent in human cells than in mice. Other food allergens, such as Ara h 1 (the major

peanut allergen) and OVA (egg allergen) were used as representative allergens that have not shown that great proliferative impact on cord blood. Moreover, the allergen rBet v 1 (major birch pollen allergen) was chosen as a representative inhalational allergen known to induce proliferation in peripheral blood and to a lesser extent in cord blood. To assess the whole picture a Th1 recall-antigen, tetanus toxoid (TT), and plate-bound anti-CD3 a polyclonal TCR dependent T cell activator, were used for proliferation assays and anti-CD3 also for inhibition assays.

Pure BLG was capable of inducing proliferation in cord blood and led to a percentage of positive proliferative responses comparable to published data^{248, 392, 422, 423}. This strengthens the hypothesis that measured proliferation in cord blood is also induced by the allergen itself, thus reflecting allergen-specificity.

PBMCs from adult donors (whether allergic or healthy) shows a wholly different pattern. Little or no proliferation in response to purified BLG is measurable in non-allergic individuals. Moreover, endotoxin related effects become visible at a later time point and are less prominent.

Recombinant allergens and antigens display an important feature of immunological research. Although endotoxin free conditions are very difficult to achieve due to the expression system in *E.coli*, preparations with low levels are available. This is the case for rBet v 1 used in the present publication. Our data suggest that contaminations [20 pg/mL] as measured in purified recombinant allergen preparations are acceptable.

Interestingly, no positive proliferative response to rBet v 1 was observed in cord blood while 3 out of 6 of the adult group responded to rBet v 1. The later observation is in line with data of Ebner et al demonstrating allergen-specific proliferation of PBMC from non allergic individuals⁴⁰³.

Regarding cord blood related responses to inhalational allergens it is quite interesting that none of the investigated samples responded to rBet v 1. A possible explanation may be provided by intra-uterine priming with food allergens present at high concentrations in maternal peripheral blood and known to cross the placenta³⁹⁵. The amount of inhaled birch pollen allergen is extremely low and makes intrauterine priming less likely. Thus BLG-specific responses are more frequent by far than proliferation to inhalational

allergens. Recent studies have clearly pointed out that allergen-specific proliferation to inhalant allergen does also occur in cord blood but at lower frequencies^{248, 423}.

Our data supports the difficulty of differentiating between allergen-specific and unspecific responses. The results highly depend on the cellular system (cord versus adult blood cells), the read out assay (proliferation, cytokine production) and the allergen used. While cord blood mononuclear cells readily respond at day three to both contamination and antigen at very low levels, this is not the case for PBMCs from adult donors, being allergic or not. Consequently, proliferation data should be based on an extensive setting with caution on positive responses to low antigen concentrations. In our experiments, CBMCs were significantly more prone to proliferate in response to endotoxin and purified BLG than PBMCs from adult donors.

In conclusion we were able to demonstrate that endotoxins present as contamination in commercially available allergen preparations act as a confounder on cellular responses via a TLR-4 dependent mechanism. However, the impact of these contaminations on antigen-specific proliferation is strongly dependent on the amount of endotoxin, the thereof resulting kinetics, the type of allergen used and the regulatory properties related to the maturity of the donor system.

The increased proliferation of cells in cord blood upon allergen stimulation is poorly understood. On one hand we have many confounders in CB as a different response to endotoxins⁴¹¹. On the other hand, it could be linked to recent thymic emigrants (RTE) in cord blood as postulated by Thornton et al²⁴⁹. RTE, frequent in CB, are known to initiate a quick and high proliferative response resulting in apoptosis accompanied by the generation of regulatory T cells and, importantly, do not possess memory function. In fact this is considered as one of the main mechanisms to exclude responses to innocuous antigens. However there is evidence for active responses that are linked to tolerance induction via generation of antigen specific regulatory cells. It is not known whether the heightened proliferative responses to allergens in cord blood are linked to effector cells that have been primed *in utero* or due to lack of control by regulatory T cells.

Allergen-specific proliferative responses in CB vs adult blood cells were in general significantly higher, quantitatively and qualitatively. We provide evidence that freshly isolated CB derived CD4⁺CD25^{high} Tregs share many phenotypic characteristics with their PB derived counterpart. They are both anergic with the main difference that CB derived CD4⁺CD25⁺ Tregs are incapable of suppressing CD4⁺CD25⁻ T cells in the presence of irradiated APCs when compared to PB-derived Tregs. This lack of their regulatory function is consistent with findings of other groups^{120, 143, 248-250, 417, 424}. We hypothesize that allergen exposure directly after birth is pivotal to gain functional regulatory T cells.

Re-stimulation experiments suggest that functionally active CD4⁺CD25⁺ T regulatory cells emerge after antigen exposure in cord blood. It is tempting to speculate that this mechanism plays a key role in tolerance development after birth. Some recent studies⁴²⁵⁻⁴²⁷ can fortify this hypothesis as they suggest an antigen-specific tolerance that is already induced *in utero*. Observations and experiments described that the graft survival of kidney transplanted children of a sibling donor is higher when the donor has maternal HLA antigens not inherited by the recipient than when the donor has paternal HLA antigens not inherited by the recipient. This is extremely interesting in reference to our data, as it indicates that the regulatory T cells are generated *in utero* which is the case for maternal cells that cross the placenta and induce

CD4⁺CD25^{high}FoxP3⁺ Tregs. Those cells suppress anti-maternal immunity and last until early adulthood ⁴²⁷. Contrary to popular belief strict food avoidance also has to be scrutinized with the data. It has been widely held that strict avoidance of food allergens may reduce the risk or prevent the IgE-mediated sensitization and immunological reactions ⁴²⁸. However, no association between the level of environmental exposure and the extent of allergen-induced proliferation could be found ⁴²⁹. Allergens might be important to prime Tregs towards potent suppressor cells after birth and therefor induce tolerance mechanisms and prevent allergy later on.

We could confirm this theory by several experiments: Firstly we could prime CD4⁺CD25^{high} T cells in the presence of different allergens (BLG, Ara h1) to become functional.

Secondly, an expansion of the Treg-compartment upon antigen exposure is detected. Thirdly, both the nTreg compartment (upon activation, without proliferation) and the iTreg compartment (upon expansion in response to the antigen) confer to suppression. The fact that medium “treated” cord blood derived CD4⁺CD25⁺ cells gained suppressive potential can be explained by the fact that nTregs are less prone to undergo apoptosis and unspecific activation may take place as a result of the culture condition ¹⁸⁴. The expanding fraction, which possessed suppressive properties after allergen stimulation per contra, does not fortify an allergen-unspecific effect.

Some other studies ^{122, 251} demonstrated a strong suppressive potential of CB-derived Tregs. At second look however, achieved potent suppression only after pre-activation with polyclonal stimuli. This entirely supports our data. Why the CB-derived Treg fraction does not possess inhibitory function in first case remains to be answered. However, we found out that this observation cannot be explained by a significant lower frequency of FoxP3⁺CD4⁺CD25^{high} cells or mean percentages of CD4⁺CD25^{high}CD127^{low} only increase slightly throughout life (6.1% in CB, 7.22% adults (age 20-25) and 7.5 (age 60+)) ⁴³⁰. Moreover, expression of the Treg markers RUNX 1, RUNX 3, and GARP, or IL-10 and TGF-β, did not substantially differ between CB-Tregs and PB-Tregs in our experiments.

CB and PB derived mononuclear cells mainly differ in their percentage of naïve T cells that express a $CD45RA^+CD45RO^-CD62L^{bright}$ phenotype whereas the adult $CD4^+$ T cells exhibit a memory phenotype ($CD45RA^-CD45RO^+$)^{105, 431}. It is possible that freshly isolated CB $CD4^+CD25^+$ T cells are less suppressive due to their phenotypic immaturity. However, Valmori et al²⁴⁷ described a naïve natural Treg population in adult individuals. These are more prominent in young adults and decrease with age together with the total naïve $CD4^+$ population. In cord blood 7% of total $CD4^+$ T cells display this phenotype whereas in adults only 0.2-3.3% of the $CD4^+$ T cells exhibit this naïve Treg phenotype²⁴⁵. The TREC content of naïve Tregs was similar to that of naïve $CD25^-$ T cells and higher than that of the antigen-experienced $CD25^-$ fraction. These results are also consistent with the concept that naïve Tregs are in an early differentiation stage and have high resemblance to cord blood derived Tregs. CB derived naïve Tregs only differ to naïve adult Tregs due to their suppression profile. Peripheral blood derived naïve Tregs from adult donors showed inhibitory potential compared to those of cord blood²⁴⁷. The cord blood derived Tregs seem to be even more naïve than the naïve counterpart in adult blood. Fritzsche et al (2006)²⁴⁶ and Santner-Nanan et al (2008)⁴³⁰ could show differences in the functional properties of naïve and memory Tregs. The majority of adult blood derived Tregs possess high $CD45RO$ and $CD95$ levels, prone to $CD95L$ -mediated apoptosis. The $CD95L$ -resistant Tregs in adult blood show augmented naïve markers comparable to the Tregs in cord blood. When stimulated with monoclonal antibodies (e.g. $CD3CD28$ antibodies) CB cells up-regulate $CD95$ as well as memory markers and show sensitization towards $CD95L$ hypothesizing that $CD95$ and the memory markers are important to create potent regulatory T cells.

It is possible that a specific subset of naïve CB $CD4^+CD25^-$ can be “educated” and expanded to become suppressive $CD25^+FoxP3^+$ Tregs as also suggested by Zelenay et al⁴³². Our study is the first study that could show inhibitory potential of the regulatory T cell subset upon priming with allergens. These, newly generated Tregs were highly suppressive in an antigen-nonspecific manner. This corresponds with the observation in other studies as $CD25^+$ Tregs need antigen specific stimulation via the TCR to become suppressive but once activated, they unspecifically suppress the antigen.¹⁸⁴ After the initial antigen dependent activation Tregs seem to be stimulation-independent in their

mechanism and are capable of suppression even in the absence of the appropriate antigen¹⁸⁴. Conventional T cells with different antigen-specificities can be suppressed in vicinity to the Tregs. This mechanism is known as “bystander suppression”.

Naïve CB cells resemble recent thymic emigrants cells (RTEs)²³⁹ and express a number of characteristics that distinguish them from naïve T cells that are present later in life. RTEs express "thymocyte-like" characteristics with regard to rapid rate of apoptosis which goes along with the observation that CB cells in contact with allergens are prone to apoptosis as well^{216, 389-392, 403}. In our experiments however, apoptotic markers were not of primary importance. Apoptosis is a big phenomenon regarding the absolute cell count upon stimulation. However as we always put our main focus on forward side-scatter characteristics and the CD4 expression (^{high/normal}) of T cells and have always verified the data with functional assays apoptotic cells are highly unlikely to act as confounder in our experimental setting. The significantly increased proliferation of CBMCs compared to PBMCs can be related to an immature state of the Treg compartment. Nevertheless, after FACS sorting, a small CB-derived regulatory fraction (CD127^{low}) possessed inhibitory capacity without pre-activation. This subset admittedly was not able to exert net-inhibitory effects. The CD4⁺CD25^{+(high and int)}CD127^{low} compartment showed a suppressive capacity whereas an increased proliferation was observed at the CD4⁺CD25⁺CD127^{high} compartment.

Grindebacke et al (2009)⁴¹² and Santner-Nanan et al (2008)⁴³⁰ could demonstrate a significantly reduced FoxP3 expression in cord blood compared to peripheral blood. This difference can be ascribed to the gating strategy as gating on the top 2% of CD4 cells that express high CD25 results in a lower FoxP3 expression. On the other hand when gating on the defined CD25FoxP3 expression, no statistically significant difference can be found.

In conclusion this study contributes substantially to a better understanding of *in vitro* responses in cord blood. In particular the unique features of cord blood derived T cells are defined in detail. This study suggests an active acquisition of allergen-specific tolerance via induction of allergen specific regulatory T-cells after birth.

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7 APPENDIX

7.1 *Abstract english*

Background: Upon antigen exposure cord blood derived T cells respond to ubiquitous environmental antigens by proliferation. To date it remains unclear whether these “heightened” responses relate to different regulatory properties of the regulatory T cell (Treg) compartment or whether this can be explained due to endotoxin contamination detected in allergen preparations. Distinct effects of endotoxin on cells in cord and adult blood are poorly defined.

Objectives: To examine the specific nature of allergen-specific responses of cord blood derived T-cell *in vitro* in detail with special focus on regulatory T cells and determine the impact endotoxin contamination in allergen preparations in this context.

Methods: The endotoxin content was assessed via limulus assay. Proliferative and cytokine response of mononuclear cells were evaluated via ($[^3\text{H}]$ thymidine incorporation and ELISA. Size exclusion chromatography of endotoxin contaminated BLG was performed to define the impact of the protein on the proliferative responses measured. The involvement of toll like receptor 2 and 4 was investigated by blocking antibodies and toll like receptor-transfected HEK cells. The Treg compartment in cord blood and peripheral blood was characterized via flow cytometry using MACS and FACS sorting. The suppressive potential per se and upon *in vitro* priming was evaluated via functional inhibition assays ($[^3\text{H}]$ thymidine incorporation assay and CFSE staining). Suppressive properties of expanding cell populations were investigated using FACS sorting.

Results: Allergen- and endotoxin-induced proliferative responses in cord blood were significantly enhanced compared to those in adult blood. Compared to un-primed $\text{CD4}^+\text{CD25}^{\text{high}}$ cells from adult blood, the respective cord blood derived population did not show inhibitory capacities upon TCR activation. This could not be explained by a significantly lower frequency of $\text{FoxP3}^+\text{CD4}^+\text{CD25}^{\text{high}}$ cells or different expression of the Treg markers GARP, CD127, or the Treg associated transcription factors RUNX 1,

RUNX 3 and FOXP3. FACS sorting experiments of unstimulated cells revealed subsets in cord blood ($CD4^+CD25^{high}CD127^{low}$ and $CD4^+CD25^{int}CD127^{low}$) that possessed inhibitory potential. In vitro exposure to allergens leads to both functional activation of both an expanding and a resting Treg population.

Conclusion: Endotoxins in allergen preparations act as confounder in the assessment of allergen-specific responses. The impact of these contaminations is strongly related to the blood source (cord vs peripheral blood), the type of allergen, the amount of endotoxin and the thereof resulting kinetics and regulatory properties. Cord blood harbours a very small subset of Tregs that is not able to exert net inhibitory effects within the mononuclear cell fraction. However in case of antigen-exposure the putative Treg subset ($CD4^+CD25^{high}$) becomes highly suppressive and expands.

Key words: BLG: β -lactoglobulin; cord blood; endotoxin contamination; food allergens; regulatory T cells; T-cells; toll like receptors

7.2 Abstract german

Hintergrund: Nabelschnurblut T Zellen proliferieren trotz ihres naïven Phänotyps auf unterschiedlichste Allergene. Diese erhöhte Immunantwort kann nicht vollends erklärt werden. Sie könnte einerseits auf eine funktionelle Unreife der regulatorischen T Zellen (Tregs) im Nabelschnurblut als auch auf Endotoxinkontaminationen in Allergenpräparaten zurückgeführt werden. Funktionelle Unterschiede im T regulatorischen Kompartiment von Nabelschnurblut und Erwachsenenblut und deren mögliche Relevanz für die Entstehung allergen-spezifischer Toleranz werden jedoch noch ungenügend verstanden.

Ziel: Ziel ist es die Eigenheiten allergen-spezifischer Immunantworten von Nabelschnurblutzellen *in vitro* zu definieren. Spezieller Fokus wird dabei auf regulatorische T Zellen und auf den Einfluss von Endotoxinkontaminationen gelegt.

Methoden: Der Endotoxingehalt unterschiedlicher Allergenpräparationen wurde mittels Limulus assay bestimmt. Der Einfluss von Endotoxinkontaminationen auf die Proliferations- und Zytokinantwort mononukleärer Zellen wurde mit [³H]Thymidin-assay und ELISA bestimmt. Mittels „Size Exclusion Chromatography“ wurde der relative Anteil gereinigter Proteine im Vergleich zu Endotoxinkontaminationen im Bezug auf die proliferative Kapazität getestet. Die Abhängigkeit der Toll-like Rezeptoren (TLR2 und 4) der Kontaminationsprodukte wurde mittels Blockierungsversuchen und TLR-transfizierter HEK-Zellen ausgetestet. T regulatorische Zellen wurden durch RT-PCR und FACS charakterisiert. Sub-Populationen wurden mit Hilfe von MACS und FACS aufgereinigt und deren suppressives Potential *in vitro* durch Inhibitionsassays ([³H]-Thymidineinbau und CFSE Färbung) bestimmt.

Ergebnisse: Allergen- als auch Endotoxin induzierte Immunantworten im Nabelschnurblut sind im Vergleich zum Erwachsenenblut erhöht. Im Gegensatz zu den CD4⁺CD25^{high} T Zellen im Erwachsenenblut zeigten die Nabelschnurblut T regulatorischen Zellen eine stark verminderte suppressive Kapazität. Dieser Effekt konnte weder mit einer geringeren Frequenz der FoxP3⁺CD4⁺CD25^{high} noch durch differentielle Expression der Treg Marker GARP, CD127, oder den Treg-assoziierten

Transkriptionsfaktoren RUNX 1, RUNX 3 und FoxP3 erklärt werden. Lediglich eine Untergruppe der regulatorischen T Zellen ($CD4^+CD25^{high}CD127^{low}$ und $CD4^+CD25^{int}CD127^{low}$) besaß inhibitorisches Potenzial. *In vitro* Exposition von Allergenen führt jedoch zu einer funktionellen Aktivierung regulatorischer T Zellen im Nabelschnurblut. Es kommt dabei einerseits zu einer Aktivierung und andererseits zu einer Proliferation der $CD4^+CD25^{high}$ T Zellen, die in beiden Fällen allergen-spezifische suppressive Kapazität besitzen.

Schlussfolgerung: Endotoxine in Allergenpräparaten wirken als systematische Störvariabel in *in vitro* Versuchen. Der Einfluss dieser Kontamination hängt stark von der Blutquelle (Nabelschnur- vs. Erwachsenenblut), dem Allergen, der Endotoxinmenge und der daraus resultierende Kinetik ab. Nabelschnurblut besitzt abgesehen von einer Subgruppe regulatorischer T Zellen mit inhibitorischer Kapazität eine regulatorische Population die funktionell unreif ist. Im Falle einer Allergen-Exposition wird diese $CD4^+CD25^{high}$ Treg Population funktionell, somit suppressiv und beginnt zu expandieren.

Schlagwörter: β -Lactoglobulin, Nabelschnurblut, Endotoxin, Kontamination, Nahrungsmittelallergene, Regulatorische T Zellen, Toll-like Rezeptoren

7.3 Curriculum vitae

Elisabeth Mayer, MSc.

Personal Information

Date and place of birth	26 th of June, 1981 Villach, Austria
E-mail	elisabeth.mayer@meduniwien.ac.at

Education

University (doctorate)

Subject and duration	LIFE SCIENCES – 2005 -
PhD thesis	<i>"ALLERGEN-INDUCED REGULATORY PROPERTIES OF THE CD4⁺CD25⁺ SUBSET IN CORD BLOOD AND POSSIBLE IMPLICATIONS ON THE POST-NATAL TOLERANCE INDUCTION"</i>
Institution	UNIVERSITY OF VIENNA AND MEDICAL UNIVERSITY OF VIENNA, DEPARTMENT OF PEDIATRICS AND ADOLESCENT MEDICINE
Supervisors	PROF. PHD. I. ELMADFA AND PROF. MD. Z. SZÉPFALUSI

University (master of science)

Subject and duration	LIFE SCIENCES – 1999 - 2005
Diploma thesis	<i>"INFLUENCE OF PROBIOTIC YOGURT ON THE ACTIVATION COEFFICIENT OF TRANSKETOLASE, GLUTATHIONREDUCTASE, AND TRANSAMINASE IN HEALTHY WOMEN"</i>
Institution	UNIVERSITY OF VIENNA, INSTITUTE OF NUTRITIONAL SCIENCES
Supervisor	PROF. PHD. I. ELMADFA
Final exam (Degree)	13.04.2005; MSc. (MAG. RER. NAT.)

<u>Prior to college</u>	HIGH SCHOOL, BG/BRG PERAU, VILLACH, CARINTHIA – 1991 - 1999 PRIMARY SCHOOL, LANDSKRON, CARINTHIA – 1987 - 1991
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Additional Qualification

Certifications

(2010, JUNE)	APPRAISAL INTERVIEW
(2009, DECEMBER)	LEADERSHIP, MOTIVATION, DELEGATION
(2009, NOVEMBER)	TRANSPORTATION DIRECTIVES FOR HAZARDOUS MATERIALS
(2009, OCTOBER)	CONFLICT MANAGEMENT
(2007, JUNE)	COMPUTER-BASED RESEARCH FOR NATURAL SCIENTISTS
(2006, JUNE)	WRITING AND SPEAKING SCIENTIFIC ENGLISH

Languages

GERMAN (NATIVE), ENGLISH (EXCELLENT), SPANISH (FLUENT), FRENCH, ITALIAN (BROKEN), LATIN (WRITTEN)

Software expertise

MS OFFICE, GRAPHPAD PRISM, SPSS, ENDNOTE, ADOBE PHOTOSHOP, ADOBE ILLUSTRATOR, CELLQUEST, CFX MANAGER, SAP R/3 INVENTORY MANAGEMENT

Technical expertise

CELL CULTURE (PRIMARY CELLS, AIR LIQUID INTERFACE (ALI), CELL LINES), MAGNETIC CELL SORTING, FLOURESCENCE ACTIVATED CELL SORTING (SURFACE-, INTRACELLULAR-, AND CFSE STAINING, CYTOMETRIC BEAD ARRAY, SETTING), INHIBITION ASSAY, ELISA, QRT-PCR, HANDLING OF RADIOACTIVE SUBSTANCES (^3H , ^{32}P), SDS PAGE/WESTERN BLOT, RNASE PROTECTION ASSAY (RPA)

Teaching experience

SUPERVISION OF DIPLOMA- AND TRAINING OF PHD STUDENTS DURING THEIR PRACTICAL WORK AT THE DEPARTMENT OF PEDIATRIC AND JUVENILE MEDICINE;
BIOCHEMISTRY PRACTICAL COURSE "BIOCHEMISCHES PRAKTIKUM", TUTOR

Publications

MAYER E*, EIWEGGER T*, BRIX S, SCHABUSSOVA I, DEHLINK E, BOHLE B, BARKHOLT V, SZÉPFALUSI Z (**PHD THESIS**); ALLERGEN-SPECIFIC RESPONSES IN CORD AND ADULT BLOOD ARE DIFFERENTIALLY MODULATED IN THE PRESENCE OF ENDOTOXINS. *CLIN EXP ALLERGY*. 2008 OCT;38(10):1627-34. *equal contribution

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EIWEGGER T, GRUBER S, GEIGER C, **MAYER E**, DEHLINK E, BANNERT C, KASPER D, JAKSCH P, KLEPETKO W, SZÉPFALUSI Z; IMPACT OF SYSTEMIC IMMUNE-SUPPRESSION AFTER SOLID ORGAN TRANSPLANTATION ON ALLERGEN-SPECIFIC RESPONSES. *Submitted*

GRUBER S, TIRINGER K, DEHLINK E, EIWEGGER T, **MAYER E**, KONSTANTIN H, KIKIC Z, GRAF A, SZÉPFALUSI Z; ALLERGIC SENSITIZATION IN KIDNEY-TRANSPLANTED PATIENTS PREVAILS UNDER TACROLIMUS TREATMENT. *Submitted*

EIWEGGER T, FUCIK P, GONA M, WITT C, **MAYER E**, SZÉPFALUSI Z; Th17 RESPONSES IN CF PATIENTS WITHOUT EXACERBATION. *In progress*

BANNERT C, **MAYER E**, GRUBER S, NACHBAUER E, SZÉPFALUSI Z; SUBLINGUAL IMMUNE THERAPY FOR MONOSENSITIZED CHILDREN AGED BETWEEN 2 AND 4 YEARS. *In progress*

Presentation and conferences

PRESENTATIONS

APRIL	2010	18 TH ANNUAL MEETING OF THE BIOMEDICAL ANALYSTS, VIENNA, AUSTRIA
NOVEMBER	2009	LANDSTEINER MEETING, SALZBURG, AUSTRIA
MARCH	2009	TRIANGLE MEETING, VIENNA, AUSTRIA
JUNE	2006	2 ND MUW PHD SYMPOSIUM, VIENNA, AUSTRIA
JUNE	2006	EAACI, VIENNA, AUSTRIA
JUNE	2006	TRIANGLE MEETING, BERLIN, GERMANY

POSTER AND SHORT TALK

JUNE	2010	MUW PHD SYMPOSIUM, VIENNA, AUSTRIA
APRIL	2010	WORLD IMMUNE REGULATION MEETING, DAVOS, SWITZERLAND
SEPTEMBER	2008	ÖGAI, VIENNA, AUSTRIA
JUNE	2007	3 RD MUW PHD SYMPOSIUM, VIENNA, AUSTRIA
JUNE	2007	(9 TH - 13 TH JUNE 2007 EAACI, GOTHENBURG, SWEDEN (POSTER PRIZE)
APRIL	2007	WORLD IMMUNE REGULATION MEETING, DAVOS, SWITZERLAND