IMMUNE REGULATION IN GUT AND CORD
opportunities for directing the immune system

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opportunities for directing the immune system

Immuun regulatie in darm en navelstreng;
mogelijkheden om het immuunsysteem te sturen

(met een samenvatting in het Nederlands)

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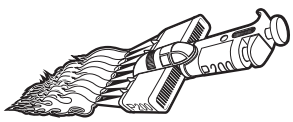
CHAPTER 1



General Introduction

The human body is an attractive source of nutrients and replication possibilities for a large number of microbial pathogens and multi-cellular parasites. Protection against invading pathogens is necessary, which is delivered by the immune system. The fact that the immune system is able to fight off a variety of pathogens with diverse mechanisms harbours a potential hazard for the body itself. Wrongly directed, or overaggressive immune responses can cause severe damage to tissues and organs. A balance between immune activation and immune regulation is therefore very important.

$CD4^+$ T helper (T_H) cells are important players in the regulation of the immune response. Different subsets of this cell type direct the immune system towards different kinds of immune responses. As different subtypes influence each other, their relative number and activity mediates immune homeostasis. Disproportionate activity of some $CD4^+$ T cell subsets might result in auto-immune diseases or atopic and allergic disorders.

Regulation is specifically necessary on mucosal surfaces like the skin and the gut, where direct interaction with microbial life takes place. The human gut is inhabited by an entire ecosystem of microbes, called the gut microbiota or microflora. The microbiota consists of an estimate of 400 different bacterial species, with a collective number of circa 10 times more bacterial cells than human cells found in the body(1). On top of that, the combined genetic capacity of the microbiota is 100 times larger than that of the human host. This enormous amount of prokaryotic life is in close contact with the immune system, only a mucosal cell layer divides the two. Yet, no inflammatory immune response is mounted against the microbiota in a healthy system. Regulatory mechanisms are present in order to maintain homeostasis in the mammalian gut in which $CD4^+$ T cells are important players. The immune system is able to shape the composition of the gut microbiota and the microbiota on its turn is able to influence the immune system, e.g. has immune modulatory capacities.

The colonization of the mucosal tissues starts immediately after, or even during birth. The neonatal immune system needs to tolerate the skin and gut colonizing commensal bacteria and to prevent an immune response. Not only bacteria, but also airborne antigens and food components are encountered for the first time by the immune system in this neonatal period.

The neonatal immune system starts to develop during pregnancy. In this period both the foetal and the maternal immune system need to tolerate each other in order to prevent preterm pregnancy loss by an inflammatory immune reaction. Although the exact mechanisms are still not fully known(2), both foetus and mother show active immune regulation towards each other. Maternal regulatory T cells (Treg) play a profound role in this tolerance during pregnancy(3). Neonatal Treg are hypothesized to be important in the period immediately after birth(4) when the neonate is exposed to colonizing

bacteria and new antigens. Differences between the adult and neonatal immune system have been known for a long time(5). Grafts are accepted easier, even later in life, when a host animal is exposed to donor cells early in life. These neonatal regulatory mechanisms can also be important in the onset of tolerance towards food components and potential allergens and be necessary for the prevention of allergic diseases later in life(6).

More research into the neonatal immune system and into gut colonizing bacteria that influence the immune system is needed to understand and exploit these mechanisms in the prevention or treatment of immune mediated disorders.

CD4$^+$ T CELL SUBSETS

CD4$^+$ T cells are the key regulators of the immune response. Depending on the type of CD4$^+$ T cell induced, B cells are activated, neutrophils recruited or macrophages directed to kill phagocytosed pathogens. Four different subtypes of CD4$^+$ T cells are currently generally accepted, T_H1, T_H2, T_H17 and (Treg). Each subset is defined by a specific key transcription factor, respectively *T-box expressed in T cells* (Tbet), *GATA binding protein* (GATA)*-3*, *retinoid related orphan receptor* (RORγT) and *forkhead box protein 3* (FOXP3). T_H9 and T_H22 cells are suggested as separate effector cell lineages and T_r1 and T_H3 cells as regulatory cells, but no transcription factor is known for these subsets as yet. Follicular T helper cells are defined by Bcl-6 expression and have a role in the formation germinal centres in the lymph nodes.

Function of CD4$^+$ T cells

All T helper subtypes are important in the defence against different pathogens and target different (innate) immune cells to be activated against these pathogens. T_H1 cells activate macrophages against intracellular pathogens, T_H2 cells provide help towards B cells in the antibody response against parasites and T_H17 cells orchestrate neutrophil activity against extra-cellular bacteria or fungi. Treg down regulate the immune response when the infection is cleared and prevent aberrant immune responses by suppressing the activity of both antigen presenting cells (APC) and other T cells(7) (Figure 1).

Although each CD4$^+$ T cell has an important and specific role in immunity against pathogens, each of them is also known to play a role in pathogenesis of immune mediated diseases. T_H1 and T_H17 cells are known for their role in disorders like arthritis(8) and colitis(9), while T_H2 cells and cytokines are important in atopic and allergic diseases like asthma and food allergies(10). Treg cells are able to prevent aberrant immune responses induced by T_H1, T_H2 or T_H17 cells and are thereby important in the prevention of diseases where these effector T cells play a pathogenic role(11) (Figure 1). Nevertheless, Treg activity is also known to prevent immunity against tumours thereby preventing tumour clearance(12, 13).

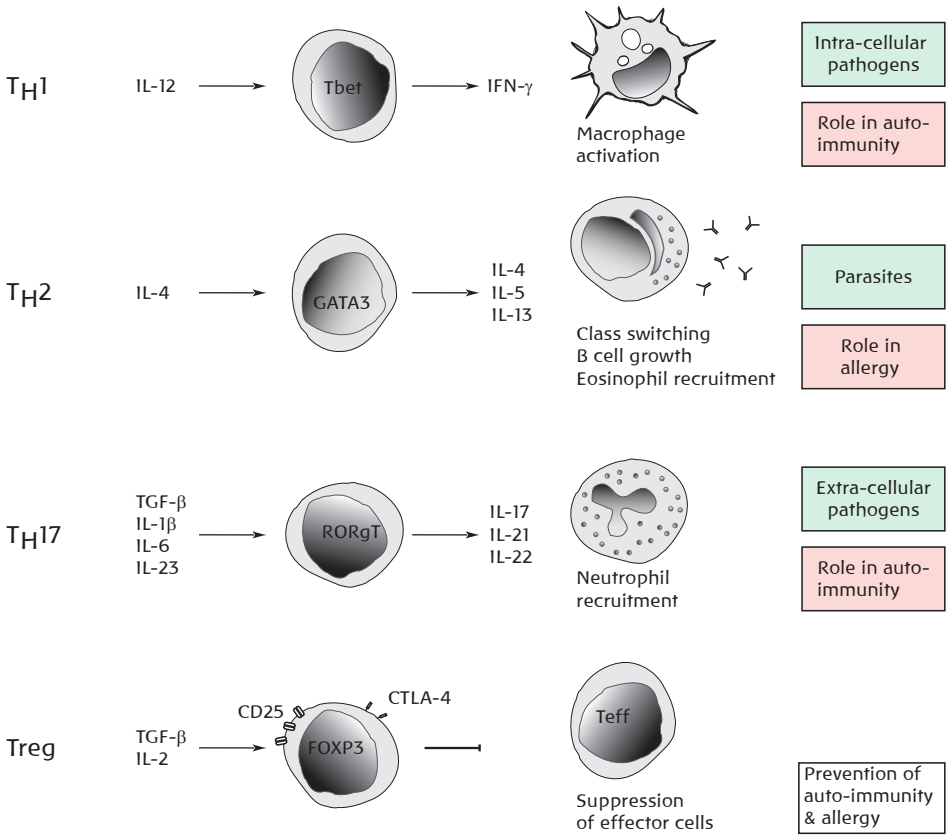


Figure 1: The 4 generally accepted T cell subsets and their function. T_H1, T_H2 and T_H17 cells are the generally accepted subsets of effector T cells and the FOXP3$^+$ Treg the commonly known regulatory cell type. For each subset, the cytokines that induce them are depicted. In each T cell nucleus the specific transcription factor that defines the lineage is shown. The cytokines produced by the effector T cells are shown, as well as the membrane bound molecules that (partially) mediate Treg function. Target cells from each subset are macrophages, B cells and eosinophils, neutrophils and effector T cells. Each subset has a role in immune reactions against specific pathogens, shown in each upper box. The effects of each cell type in pathogenesis of immune mediated disorders is shown in the lower boxes.

T cell effector molecules

In order to perform its task, each CD4$^+$ T cell has a different set of effector molecules, either soluble or cell bound. T_H1 cells are characterized by interferon (IFN)-γ production, T_H2 cells produce interleukin (IL)-4, IL-5 and IL-13, while T_H17 cells produce IL-17, IL-21 and IL-23(7). Treg are not known to produce a specific cytokine; transforming growth factor (TGF)-β is often mentioned as an effector molecule, but many other cell types can produce this cytokine, which is also true for IL-10. Treg do express several membrane bound molecules like cytotoxic T lymphocyte antigen (CTLA)-4, Glucocorticoid induced TNF receptor (GITR) and have high expression of

CD25, the high affinity receptor for IL-2. The exact mechanism by which Treg elicit their suppressive function is not known yet. Suppressive cytokines like TGF-β and IL-10 play a role. Consumption of IL-2 by regulatory T cells, thereby depriving effector cells from this survival cytokine, is also suggested as a possible mechanism. This mechanism is mediated by effector cell apoptosis in murine cells(14), but not in human cells(15). T_r1 cells are characterized by high IL-10 production(16) and T_H3 cells produce TGF-β(17) without expression of FOXP3.

Induction of $CD4^+$ T cell subsets

Upon activation of a naïve T cell by an APC, the cytokine milieu influences T cell differentiation. IL-12 production by APC directs towards T_H1 while IL-4 is essential for T_H2 differentiation. T_H17 cells and Treg both need TGF-β for their induction. T_H17 cells additionally need IL-1β and IL-6 and are propagated by IL-23. High amounts of IL-2 are also favourable for Treg induction and proliferation.

Next to cytokines, the strength of both T cell receptor and co-stimulation provide additional direction towards certain subtypes. Low TCR triggering or co-stimulation via CD28 make it more likely for a T cell to differentiate into a Treg(18, 19). Inhibitory signals that intervene with activating signals from TCR and CD28 are also likely to play a role, as has been shown for programmed death (PD)-1 signaling in mouse T cells(20). PD-1 signaling by ligation with its ligand PD-L1 prevents phosphorylation of protein kinase B (PKB/cAkt) and subsequent downstream signaling of the TCR(21-23). This results in reduced proliferation, less IL-2 production and increased apoptosis. PD-1 signaling can also induce FOXP3 transcription, thereby inducing Treg (Figure 2).

Treg & T_H17 induction

Although Treg and T_H17 cells have opposing functions, their induction in the periphery is closely related. Both RORγT and FOXP3 are upregulated in naïve T cells upon activation in the presence of TGF-β, but FOXP3 is dominant and down regulates RORγT unless inflammatory cytokines like IL-1β or IL-6 are present, with additional roles of IL-21 and IL-23(24, 25). Down stream signaling of these cytokines thus initiates a cascade directed towards T_H17 cells. It remains under debate whether T_H17 cells can be induced from any naïve T cell, or if a precursor cell exists. $CD161^+$ cells have been suggested to be such precursors(26, 27).

The intracellular signaling cascade directing towards either cell type can be modulated by the presence or absence of both extracellular and intracellular factors other than cytokines. Retinoic acid, present in the gut, induces Treg and prevents T_H17 differentiation(28, 29). Hypoxia-inducible factor (HIF)-1α expression on the other hand directs a T cell towards T_H17 cell signature by promoting RORγt expression and degradation of FOXP3-protein(30) and by promoting glycolytic metabolism in T cells(31)(Figure 2).

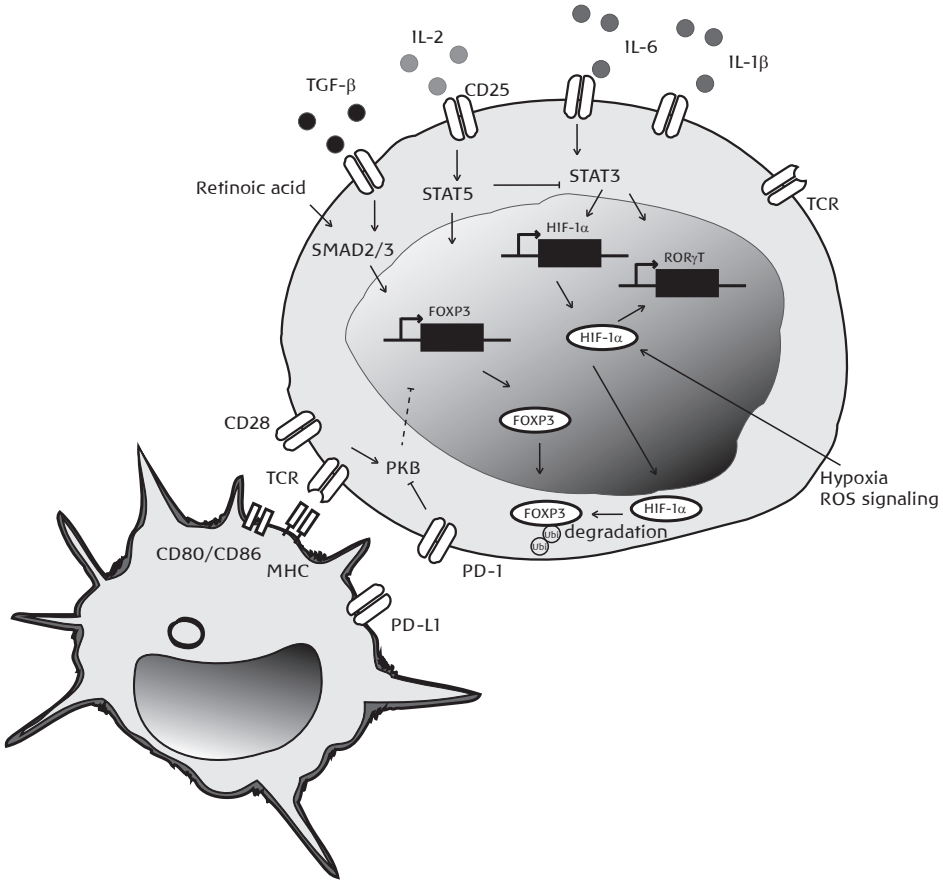


Figure 2: Treg and T_H17 induction pathways. Upon activation, a naïve T cell can differentiate into either T helper cell subset. In murine cells, PD-1 signaling by interaction with PD-L1 (expressed among others by APC) during TCR signaling and co-stimulation prevents phosphorylation of protein kinase B (PKB, cAkt). This effect results in upregulation of FOXP3 by an until now unknown mechanism. Retinoic acid and TGF-β induce FOXP3 expression by Smad2/3 activation. When TGF-β is present together with inflammatory cytokines like IL-1β and IL-6, RORγT is upregulated. IL-6 activates STAT3, which directly binds to the RORγT promoter to induce transcription. STAT3 activation is prevented by IL-2 induced STAT5 activation. The role of HIF-1α in the switch between Treg and T_H17 has only recently been described. HIF-1α can be induced or activated by hypoxic circumstances, reactive oxygen species (ROS) or IL-6 activated STAT3. Together with STAT3, HIF-1α induces T_H17 differentiation by promoting RORγT transcription and subsequently helps RORγT in IL-17 transcription. FOXP3 protein is degraded by HIF-1α via ubiquitination and proteosomal breakdown, thereby firmly establishing T_H17 commitment.

Recent data suggest that it is likely that a $CD4^+$ T helper type is able to switch from one type to the other (T cell plasticity). This phenomenon is still under research, especially for T_H17 and Treg cells(32-34), but also for T_H1 and T_H17 cells(35, 36) and in the context of allergic inflammation for T_H2 and

Treg(37), where it has been shown that reduced FOXP3 expression resulted in a T_H2 phenotype development(38). Activation of Treg in the presence of inflammatory cytokines can result in RORγT upregulation and T_H17 cell induction. This plasticity adds an important new layer to T cell biology, as induction of Treg might result in an increase in T_H17 cell numbers in a later phase.

IMMUNE REGULATION BY MICROBIOTA

Two decades ago, it was noted that the prevalence of atopic disorders like asthma and eczema was increasing in the western society(39). This phenomenon seems to correlate with the more hygienic circumstances in which children are growing up in the last decades. It was hypothesized that decreased contact with microbes and reduced prevalence of infectious diseases resulted in aberrant immune responses against non-pathogenic proteins like plant pollen and house dust mite proteins. This "hygiene hypothesis" resulted subsequently in clinical studies where children were administered bacterial species (probiotics) derived from the human gut, trying to prevent the onset of atopic disorders. The immunologic rationale for this approach was found in the T_H1/T_H2 balance which was the paradigm of that time; only these two T helper cells were described and the balance in number and activity between these two was thought to direct the immune response(32, 40). Although T_H1 cytokines prevent T_H2 differentiation and vice versa, we now know that more $CD4^+$ T cell subsets play important roles in the immune system.

The balance between T_H1 and T_H2 cells shifts from more T_H2 driven at an early age just after birth, towards more T_H1 in adulthood(41, 42). Decreased contact with bacteria that induce a T_H1-like immunity due to hygienic measures might therefore prevent the shift in this balance and leave the immune system more T_H2 polarized. T_H2 cells are known for their role in allergy and induction of T_H1 cells by probiotic, e.g. beneficial bacteria, appears to be a good approach.

The trials conducted with probiotic bacteria in order to prevent or cure atopic disorders have resulted in interesting data sets, as discussed in chapter 2 of this thesis. Clearly the administration of probiotics to children has an effect on atopic disease prevalence(43), especially when probiotics are already given to the pregnant mothers and in the first months of the child's life(44-46). Nevertheless, of all trials conducted, about half of them show no result on the prevention of the specific allergic disorder. The diseases targeted with this approach are multifaceted, both genetic background and environmental factors influence disease onset and progression. Probiotic supplementation might not be strong enough to affect this process. Other factors of influence are the complexity of the (mucosal) immune system and the choice for a bacterial strain to influence it.

Complexity of the interaction between microbiota and immune system

In the past decade, research on $CD4^+$ T helper cells has revealed that although T_H1 and T_H2 cells have profound effect on each other, the T_H1/T_H2 balance is an oversimplification of the system. Other subsets like T_H17 and Treg have been discovered and characterized and it is likely that more subtypes exist. We now know that Treg and T_H17 cells play important roles in both immune disorders and regulation of immune homeostasis in the gut. Moreover, in only the last few years, it has been shown in murine models that the role of the composition of the gut microbiota is very important for the regulation of $CD4^+$ T cells. Specific species of bacteria can induce either Treg(47, 48) or T_H17 cells(49-51), thereby inducing a tolerant milieu to prevent immune reactions directed against the microbiota, but also inducing cells that prevent outgrowth of invading pathogens. The segmented filamentous bacteria (SFB) for example are potent inducers of T_H17 cells. Mice colonized with these bacteria are protected against certain pathogens, thanks to the presence of T_H17 cells(52). Nevertheless, these T_H17 cells can also induce disease; colonization of arthritis susceptible mice with SFB is enough to induce disease onset(53). On the other hand, *Bacteroides fragilis* and Clostridium groups IV and XIVa have been shown to induce $FOXP3^+$ Treg and IL-10 production, thereby protecting against for example colitis.

Strain selection

The microbiota has profound effects on the immune balance in the gut, but also systemically. Moreover, specific species of bacteria induce characteristic immune responses. As specific strains can induce different $CD4^+$ T cells, they can influence the immune system differentially. In order to obtain an immune reaction in the desired direction, a strain able to do so should be selected. For example, when a strain is used to prevent onset of allergic diseases like asthma, it should preferentially induce Treg and/or T_H1 cells. T_H2 and T_H17 cells are pathogenic in asthmatic disorders, induction of these cells by probiotic supplementation should therefore be prevented. Intense characterization of the immunological properties of bacterial strains is therefore an important aspect in probiotic functionality.

Administration of probiotics early in life or even before birth has shown the most promising results in the prevention of allergic disorders, although even this approach does not show lasting significant effects when the children grow up. Nevertheless, the period around birth seems a likely period to influence both the microbiota and the immune system of the newborn. Firstly, no stable composition of the microbiota has developed yet. Secondly, the neonatal immune system is still naïve and more likely to respond with regulatory mechanisms, as described below.

IMMUNE REGULATION AROUND BIRTH

Immune regulation already starts before birth in both mother and foetus. In order to tolerate the presence of a semi-allogeneic foetus and prevent pregnancy loss, the mother's immune system is changed. Higher numbers of Treg are found in the peripheral blood of pregnant women(3, 54) and the T_H1/T_H2 balance is shifted towards a T_H2-type immune reaction in both mother and foetus(55). On top of that, the maternal immune system shows increased tolerance towards allogeneic proteins derived from the father. This phenomenon is reversed after birth(56). These results imply an active immune tolerance from the mother towards the foetus. IL-10(57), prostaglandin E2(58) and progesterone(59) (at least partly) mediate this effect, but exact mechanisms of maternal-foetal immune tolerance are not yet defined and still under investigation(2). $CD4^+$ T cells play important roles; an increased ratio between T_H17 and Treg cells is associated with preterm pregnancy loss(60).

The immune system of the foetus or neonate shows similar patterns of reduced immunogenicity(2). This is exemplified by more lenient transplant engraftment in neonatal mice(61) and human(62), and failure to immunize neonates through vaccination early in life, with a concomitant increase in susceptibility towards infections(55). Although no proof exists at the moment, it is likely that the biological role of this phenomenon can be found in the prevention of inflammatory immune responses against antigens encountered early after birth. The colonization of the skin and gut with microbiota and contact with new food antigens and airborne proteins will take place in the first moments of life. These components need to be tolerated by the immune system to prevent the onset of inflammation at the first encounter and importantly, later in life. Indeed, early introduction of peanut to a child's diet reduces the chance of developing peanut allergy later in life(6). This observation suggests a window of opportunity in early life for the prevention of allergic disorders. When the first activation of a T cell with an antigen results in regulatory mechanisms like Treg induction or anergy, the same antigen will not be able to elicit an (allergic) immune response later in life.

Again, the mediators of this neonatal immune tolerance are not fully known yet. Reduced production of T_H1 polarizing cytokines(41, 63, 64), reduced responsiveness of toll like receptors (TLR)(55), presence of immune suppressive soluble components(55) and differential expression of cytokine receptors(65) are all likely to play a role. Again, immune suppressive Treg have a function as well, although their activity around birth is not entirely clear(66, 67). T cells from neonatal CB are more likely to become Treg than cells upon activation than adult cells(4). The mechanisms of this induction of Treg in the neonatal system are not known yet. Both T cell intrinsic factors as well as APC directed Treg skewing can play a role.

Umbilical cord blood

The blood derived from the umbilical cord is part of the circulation of the unborn. It can be obtained relatively easily when compared to venous blood from a neonate. The immune cells isolated from cord blood (CB) are therefore frequently used as a model for the immune system *in utero* as well as after birth. However, CB reflects the immune system in the transition from a sterile environment to an environment with lots of microbes. It is therefore not per se a model for neonatal or foetal immunity, but shows the immune system ready to encounter the outside world for the first time.

Used as a model for neonatal immunity, experiments with CB would ideally have follow up experiments with neonatal PBMC, providing information on the maturation of the immune system. Nevertheless, CB derived cells give insight into the mechanisms of neonatal immune regulation, which can be confirmed in neonatal PBMC.

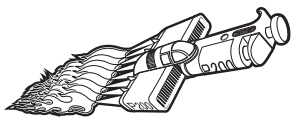
Moreover, the differences between CB regulatory, and adult peripheral blood (APB) inflammatory mechanisms, e.g. differences in the development of Treg and T_H17 cells between CB and APB provides an interesting model to study the mechanisms that underlie Treg/T_H17 induction.

SCOPE AND OUTLINE OF THIS THESIS

The first part (chapter 2, 3 and 4) of this thesis explores the potential of probiotic bacteria in modulating the immune response. Recent findings on the relation between the microbiota, the immune system and probiotics are reviewed in chapter 2. In chapter 3 the differences between several probiotic strains in their capacity to induce Treg are described. It is shown that not all strains are equally potent inducers of functional Treg in human immune cells. Chapter 4 elaborates on this finding and shows that each strain differentially modulated the $CD4^+$ T cell immunology, e.g. T_H1, T_H2, T_H17 and Treg induction. This is shown with several methods and creates the possibility to test the immune modulatory capacity of probiotic strains prior to use in clinics or experimental trials. These chapters illustrate the importance of screening the capacities of probiotic bacteria in modulating the immune system.

The second part (chapter 5 and 6) focuses on the immune response in CB. Chapter 5 shows that activation of CB cells results in relatively high numbers of $FOXP3^+$ cells as compared to cells derived from APB. This effect depends on the interaction between APC and naïve T cells, or, more specifically, on the interaction between PD-1 expressed by T cells and its ligand PD-L1 on APC. Chapter 6 uses the fact that cord blood cells preferentially become Treg instead of T_H17 cells to explore the role of a T cell intrinsic factor in this differentiation.

Chapter 7 summarizes and discusses the findings described in this thesis.



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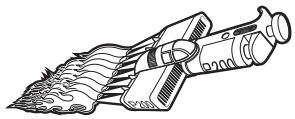
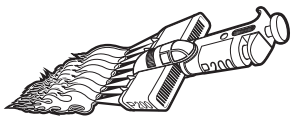


CHAPTER 2



Probiotic potential: selection criteria for immune modulation

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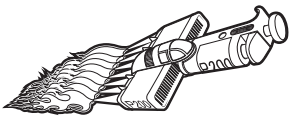
Submitted

ABSTRACT

Probiotics have been under research for their immunomodulating effects for over two decades, but clinical trials aiming at the prevention or treatment of immune mediated diseases have not always met the high expectations. With the increasing knowledge on the complexity of the mucosal immune system of the gut and the microbiotia residing there, it becomes clear that the immune modulating capacities of probiotics are multi-faceted. In the current review we focus on the effect of gut microbiota and probiotics on the host's mucosal and systemic immune system. The genetic and taxonomic background of probiotic bacterial species, the profound influence of these gut residing microbes on the immune system and selection of strains for use in clinics and experiments are discussed with a focus on improvement of probiotic functioning in future applications.



INTRODUCTION

Although professor Eli Metchnikoff received the Nobel price in 1908 for his pioneering research on phagocytosis and cellular immunology(1), nowadays he is mainly remembered as the founding father of lactic acid bacteria supplementation as probiotics. He believed that regular intake of these bacteria via dairy food increased the life expectancy of the Bulgarian people of that time. Especially the y-shaped Bifidobacteria, abundantly present in Bulgarian yoghurts, were thought to increase general health. His main rationale was that these "good bacteria" would compete with pathogenic species, thereby providing natural defence against disease and thus prolong life.

Since Metchnikoff, scientific research on the effects of probiotics as well as the underlying mechanisms of these effects really has taken off. From the idea that probiotics can exert a multitude of beneficial effects, the number of ailments for which probiotics have been used is ever growing. Most research has been performed on local, gastro-intestinal, effects. The idea of competition between commensal bacteria and pathogens has been used in the setting of traveller's diarrhoea, *Helicobacter pylori* infections, *Clostridium dificile* infections, gastroenteritis, antibiotics associated diarrhoea, etcetera(2). In the struggle for the limited amount of nutrients and space to adhere to in the guts (ecological niches), a healthy commensal gut flora (microbiotia) can prevent the colonization of the gut and subsequent outgrowth of pathogenic species. Or, in the case of antibiotics use, the harm done to the microbiota by the drugs can partly be overcome by replacing the affected commensals by administration of non-pathogenic strains.

In recent decades, research on probiotics has developed a new focus due to the postulated association between hygienic life style and the increase in atopic disorders in western societies as described by Strachan in 1989. His hygiene hypothesis states that the reduced encounter with (pathogenic) micro-organisms due to hygiene influences the immune system in such a manner that allergies and asthma become more prevalent(3). The immunological background of this hypothesis was based on the observations that contact with bacteria induces T helper (T_H)1-like responses, influencing the balance between T_H1 and T_H2 cells. T_H2 cells are known for their function in immunity against parasites but also for their pathogenic role in atopy(4). The rationale for the use of probiotics in the prevention or treatment of allergy and atopy is easily adopted from these findings; probiotic, non-harmful bacteria will induce T_H1 responses in the human host, thereby skewing the balance between T_H1 and T_H2 away from T_H2 reactions and hence away from atopic immune reactions(5).

It now has become clear that T-helper cell biology is more complex than anticipated when the original T_H1/T_H2 balance was put forward. Regulatory T cells (Treg), T_H17 cells and most recent T_h9 cells have been discovered and characterized in more or less detail and play particular roles in the interactions

with the microbiota and probiotics, but also in the pathogenesis of allergic and auto-immune disorders(6, 7). Diseases that were previously thought to be T_H1 mediated are now found to originate from aberrant T_H17 and Treg cell reactions. These findings open up new ways for probiotics to interact with the immune system. Especially Treg, key players in the maintenance of immune homeostasis are attractive targets in both atopy and auto-immunity. The induction or activation of these cells by gastrointestinal bacteria can be of influence on diseases mediated by T_H1, T_H2 and/or T_H17 cells. The effect of probiotics on for example arthritis and diabetes, ailments that occur more frequently in recent years (possibly due to hygiene(8)), has therefore been under investigation as well.

Nevertheless, it has also become clear, mainly in mouse models, that commensal bacteria and probiotics can induce not only T_H1 and Treg cells, but also T_H2 and T_H17 cells. The perception that most or all strains of lactic acid bacteria will have a similar effect on the mucosal immune system probably is not correct. The growing body of knowledge on the complexity of the human gut ecosystem, the effects of the microbiota and probiotics on the different compartments of the human and murine immune system and the results of recent trials with probiotics shows that probiotic supplementation is a potentially powerful tool. Full exploitation of these beneficial effects will depend on basic insight into the mechanisms of (immune mediated) diseases as well as on the interaction of the molecular components of the probiotic bacteria with relevant counter structures in the human host. An adequate choice for a certain probiotic strain needs to be made based on the desired immune modulation and the matching properties of the strain.

THE RESIDENT MICROBIOTA AND PROBIOTIC SPECIES

Research into a black box

The majority of research on the functioning of microbial ecosystems has been performed in agricultural context, i.e. soil and aquatic ecosystems. Soil bacteria are important for plants or crops on several levels, from nutrient availability to providing defence towards plant pathogens(9-12). Soil and aquatic ecosystems can be sampled relatively easily compared to gut ecosystems. Techniques used for monitoring species composition of soil communities have therefore been implemented in research into the human intestinal tract(13).

Until recently the human microbiota could only be monitored by culturing samples from faeces, thereby ignoring the fact that most microbes can not be cultured, e.g. because of their anaerobic nature. Molecular techniques have improved our view of the gut microbiota considerably. Comparison of 16S ribosomal RNA in faeces with PCR or temperature gradient gel electrophoresis allows monitoring of both culturable and unculturable species, but also provides information on species from the entire GI tract

as viability of the microbes in the faeces is not an issue anymore(14). Even more information on niches for different species and communities can be obtained from biopsies from the different parts of the GI tract or with ingestible devices(15). These approaches make it also possible to correlate microbial composition with health status of the host more accurately(14).

The microbial diversity in some soil types is with 21 phyla present(16, 17) much higher than in the human gut, where 9 phyla have been described with predominating Bacteroidetes and Firmicutes(16, 18-20). Nevertheless, the human microbiota is still one of the most diverse microbial communities known. The bacterial cells in the human system are now estimated to outnumber the host by 10 fold and the combined genetic capacity of the microbiota (the microbiome) contains 100 times more genes than the host itself. The highest numbers of bacterial cells are found in the colon with around 10^{11-13} colony forming units (CFU) per ml. The largest diversity is also found in the colon whereas the lowest numbers and diversity are found in the stomach. **Figure 1** shows the main taxonomic divisions of gut residing bacteria.

Bacterial composition and stability

In order to understand the mechanisms by which probiotics can influence the immune system, it is necessary to understand the composition of the resident gut microbiota. The number of bacterial species inhabiting the human gut is now estimated to be around 400 of which about 200 are found in a given individual host(21). It thus can be postulated that each individual has a unique composition of microbiota, as theoretically more different compositions might be possible than individuals exist on this earth. Fortunately, this huge interindividual variation in gut microbiota is not likely because the recent sequencing of the microbiota of 39 individuals suggests that only 3 core compositions exist. Each composition, tentatively named "enterotype" is centred around a key species, with which the occurrence of other species has a positive or negative correlation. Named after these three key species, the three enterotypes are Prevotella, Ruminococcus and Bacteroides(22). Enterotypes appear to be rather robust and stable, as the three types described now can be observed within different ethnical populations and cultures. The stability of the microbiota has been shown before(23, 24). Antibiotics, probiotics or travelling can alter the composition temporarily, but within due time the original composition will prevail(25). Short and long term dietary changes result in changes in microbiome and the microbiota's key species(26), showing that changes in enterotypes within an individuals life is possible.

Enterotypes will make it more easy to correlate individual's health status with microbial composition. Relations with diabetes, obesity and asthma have been described before(27-29). Nevertheless, only 39 individual's microbiota have been sequenced so far, leaving the possibility that more enterotypes

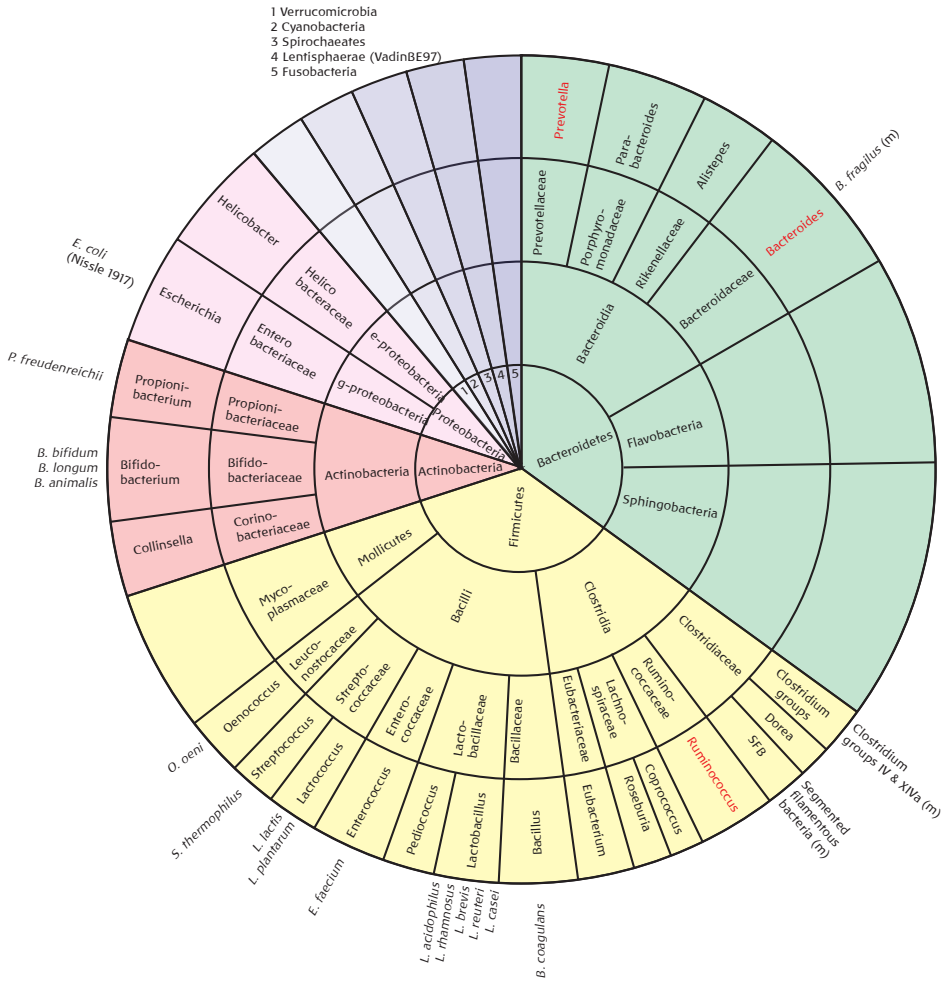


Figure 1: The composition of the human gut microbiota. Nine phyla of bacteria are known to compose the human microbiota and are depicted in the inner circle of the graph. An estimate of the relative abundance of each specific group is shown by the size of each partition. Although the exact composition is individual dependent, the three largest phyla are the Firmicutes, the Bacteroidetes and the Actinobacteria. The other 6 groups are present in smaller numbers, or even only present in some individuals, like the Cyanobacteria. In the more outer circles of the diagram the lower taxonomic ranks are shown, subsequently Classes, Families and Genera. For some groups only the presence in the human gut of members of a phylum or Class has been described, without a further classification into lower ranks. The genera of bacteria that are described as key species in Enterotypes are printed in red. Outside the outer circle, species used as probiotics are depicted. Species with a profound effect on mouse immunology are also shown, highlighted with an (m). Note that probiotic bacteria are mostly derived from the Bifidobacteria and the Lactobacilli. Of the species depicted, many strains are described and used. Other groups are known to have effect on the murine immune system (Clostridia, Bacteroidia) but are not investigated for human probiotic supplementation.

might be discovered. The usefulness of enterotypes in understanding correlations of the microbiota with health will only prevail when their number of types is limited.

Host-microbiota interplay

Pattern recognition receptors on and in the gut epithelium cells like TLRs and Nod2, play important roles in the detection of micro-organisms, the immune reaction towards these micro-organisms and the subsequent composition of the microbiota. Chronic stimulation of Nod2 in human macrophages with its ligand muramyldipeptide (MDP) induces tolerance towards both MDP and several TLR-ligands via downregulation of IL-1 receptor associated kinase (IRAK)-1 resulting in reduced cytokine production. Macrophages derived from Crohn's patients with a Leu1007insC Nod2 mutation however do not become tolerant and produce TNF-α readily towards LPS stimulation(30). Mice with Nod2 mutations show an affected microbiota(31), with relatively more Bacteroidetes and Firmicutes present(32, 33).

The interaction between host and microbiota is further regulated by the inflammasome NLRP-6. Knockout mice for this molecule show dysbiosis with an increased prevalence of Prevotellaceae in their microbiota resulting in a higher production of CCL5 in the colon and an increased susceptibility towards dextran sulphate sodium (DSS) induced colitis(34). Interestingly, transfer of the microbiota found in a NLRP-6 knockout mouse to a germ free (GF) mouse on a wild type background induced identical susceptibility towards DSS(34, 35) clearly showing that the host induced microbial composition influences disease onset. Activation of the inflammasome by the microbiota occurs both locally and systemically, resulting in an adequate response towards viral infections in for example the lungs. Mice with normal microbiota are therefore less susceptible for influenza infections than mice treated with antibiotics(36).

These findings indicate that the phenotype of the host is able to shape the composition of its microbiota, but that the microbiota is subsequently able to prevent or promote immune mediated disease susceptibility. Importantly, these effects are not only observed in inbred mouse models, but are also operative in the human system (**Figure 2**).

Probiotic species

Probiotic bacteria are defined as live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host(37) (FAO/WHO 2001). Three words in this definition are cause for lengthy discussions: "live", "adequate" and "health benefit". "Live" is defined as the state of the micro-organism at the moment of entry into the digestive tract. Bacterial products, or killed bacteria that have an effect on the immune system like OM-85 BV (Broncho-Vaxom)(38), are not probiotics according to the definition. Probiotics are thought to be able to survive

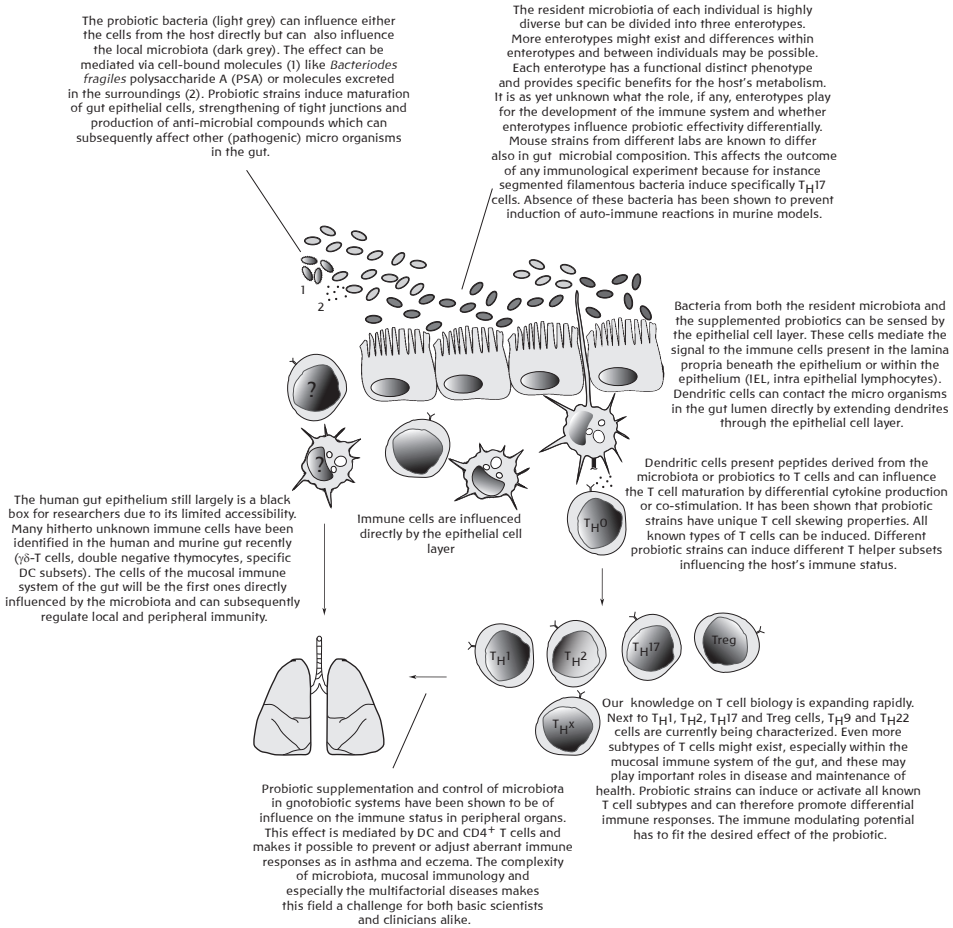


Figure 2: Interactions between immune cells, epithelial cells, resident microbiota and probiotic bacteria.

in the human gut for at least a few weeks, but do not, or not necessarily lead to permanent colonization. As such, they therefore do not fill an ecological niche that otherwise would remain unoccupied. It should also be noted that for few, if any, of the probiotics it is known whether they still are alive at the moment when they reach the relevant site within the gastro-intestinal tract.

Adequate amounts is a part of the definition which can be different for each setting. The immunological response of monocyte derived DC towards probiotics differs depending on the amount of bacteria added to *in vitro* cultures(39). For clinical *in vivo* situations daily intake is generally recommended, but differences in immune response due to different intake regimes have not been tested. Dose dependent differences in both the

magnitude and the direction of the immune response can be expected. Finally, the definition of a "health benefit" is as complex as the definition of "health".

Bacterial species selected for probiotic applications are generally derived from the human gut, directly from faeces or from dairy products where gut micro-organisms are used for fermentation. The human gut microbiota (as we know it at the moment) is depicted in Figure 1, as well as the known species and strains of probiotics. It is obvious from Figure 1 that from the many species which make up the microbiota, only a small proportion is exploited as probiotics. Moreover, even species with profound effects on the immune system are not (yet) used as probiotics. Research into new species for probiotics is complicated by the fact that many species are anaerobic and can not be cultured easily. Nevertheless, new species, and new classes of bacteria are being tested for their immune modulating capacities(40, 41).

IMMUNOLOGICAL SELECTION CRITERIA FOR PROBIOTICS

The interaction between gut residing bacteria and the immune system is clearly shown in germ free (GF) mouse systems(42). The immune system of these mice differs from conventional mice in many different ways(36, 42-50). This significant effect of the gut microbiota on the immune system suggest that an important part of the immune system specifically resides in the gut epithelium. Moreover, it is even possible that the gut harbours a second central immune organ like bone marrow, where progenitor cells differentiate and mature into (mucosa specific) effector cells. Both Treg and T_H17 cells are closely associated with the gut's microbiotia, other cell types are specifically found in the gut and not in the periphery. For example $\gamma\delta$-T cells, CD8$\alpha\alpha$ expressing T cells, but also many subsets of DC are enriched in the gut(51). In future research, the potential effects of probiotic strains directly on these cells should be tested.

Induction of specific $CD4^+$ T cells

The regulatory effect of the microbiota on the immune system is clearly shown by the reduced number of Treg present in GF mice(47, 48). Treg are key players in maintaining homeostasis of the immune system; absence of these cells in mice knock out for the Treg defining transcription factor Forkhead Box P3 (FOXP3) (Scurfy mice) and humans with a mutation in the FOXP3 allele (IPEX) show polyendocrinopathy, enteropathy, multiple organ autoimmunity and atopy(52-54). Most of the Treg are thymus derived, but a considerable part is induced by contact with the gut microbiota. The absence of microbiota in GF mice prevents the induction but also influences the efficacy of the Treg that are present as these cells are less able to prevent proliferation of effector T cells(48, 49). Colonization with a full microbial community induces Treg(55), but this effect can also be observed with a

single strain of bacteria. Colonization of GF mice with *Bacteroides fragilis*, a species that colonizes the human gut in relatively high numbers(56), induces $CD4^+$ T cell proliferation, restoring normal numbers(45). Remarkably, this effect is only observed in bacteria carrying the surface polysaccharide PSA; $PSA^{-/-}$ bacteria are ineffective in this respect, while PSA alone was able to induce maturation of the immune system in GF mice(45). PSA is specifically recognized by colonic $CD11c^+$ DC(45) that promote IL-10 production by $CD4^+$ T cells(57) and $FOXP3^+$ upregulation(58).

B. fragilis colonization protects against the induction of experimental colitis with a profound role for PSA; PSA alone could mimic the effect of full colonization(44, 45, 49, 57). While these data are very convincing, it is hard to imagine that a single polysaccharide (next to PSA, *B. fragilis* expresses 7 other polysaccharides(59)) of a single bacterial species would be sufficient, but also unique to support the balanced development of the (mucosal) immune system. Indeed, colonization with a balanced microbiota like the standardized Altered Schaedler Flora (ASF; consisting of 8 bacterial species) also induced Treg in an IL-10 dependent manner(55). Clostridia groups IV and XIVa have been shown to induce Treg by TGF-β and IDO signaling(60).

Gut residing bacteria not only induce Treg, but also have an important role for inflammatory T cells like T_H17, which are regulated in the gut(61). Remarkably, $PSA^{-/-}$ *B. fragilis* still is able to induce T_H17 cells. The best known example at the moment of bacterial activation of T_H17 cells are the Segmented Filamentous Bacteria (SFB), which has recently been sequenced(62). These bacteria potently induce T_H17 cells, which subsequently have an effect on local immunity when colitis is induced, but also systemically in an arthritis model where joint inflammation is easily induced when SFB are present in the gut(63). Similar results have been observed with experimental encephalomyelitis(64). Absence of SFB in the microbiota abrogates the possibility to induce inflammatory responses showing the profound effects of specific bacterial species. The importance of T_H17 inducing species has been shown in *Citrobacter rodentium* disease models; absence of SFB results in decreased resistance towards *Citrobacter*(65).

It thus can be concluded that gut microbes induce inflammatory immune cells protecting against pathogens, and regulatory mechanisms protecting against aberrant immune responses. A full, balanced, microbial composition (like ASF) induces a balanced immune reaction and induces all subsets of T helper cells(55, 66). The animal models described above also could be used to assess the efficacy of other probiotics species intended to be used for control of mucosal inflammation and/or protection against infection.

Effects of bacterial products on the gut mucosal immune system.

From the *B. fragilis* data summarized above it is clear that the immunomodulatory effects can be induced by the specific polysaccharide and that the complete, live micro-organism is not needed. The immunomodulating

effects of bacterial products have been exploited in clinical settings with products like Subreum and Broncho-Vaxom which consist of heat-killed *E. coli* or a mix of heat-killed bacterial species. These products were originally developed as oral vaccines to boost the immune response of patients experiencing recurrent infections(67). Indeed, heat-killed *E. coli* is effective in the treatment of urinary tract infections(68), probably by inducing T_H1 immune responses against *E. coli* strains that cause the infection. Remarkably, the same bacterial product has positive effects on the development of rheumatoid arthritis(RA)(69), which is thought to be mediated by aberrant T_H1 and T_H17 responses.

This positive effect of Subreum is probably mediated by induction of oral tolerance towards a bacterial component in the product which plays a role in RA disease development. Culture of PBMC of RA patients with Subreum showed a increased production of IL-10(69). It has been hypothesized that this component could be a heat shock protein(70), evolutionary conserved proteins with an important role in protein folding and stress reaction. Oral administration of bacterial HSP epitopes has also been tested in clinical setting(71).

Thus, the immunomodulation by bacteria in the gut can be achieved by administration of bacterial products in both mouse and man. This approach clearly has benefits as compared to probiotics, as viability is not a problem and standardization is easier. Bacterial products will not reside in the human gut as probiotic bacteria might do for a certain amount of time, which could be both an advantage and a disadvantage.

In other settings it has been difficult to deliver the (immune modulating capacity of a) probiotic strain to the gut. The presence of *Faecalibacterium prausnitzii* in the microbiota has been shown to associate with maintenance of remission of Crohn's Disease patients after surgery(72). Nevertheless, exploitation of this bacterium or its immune modulating properties has been difficult, as it can not be cultured in adequate amounts for human use. A solution for this problem could be to clone the DNA of *F. prausnitzii* into a different bacterial species that can be cultured more easily. This approach has not been successful so far.

INTERACTION OF PROBIOTICS WITH THE HUMAN MUCOSAL IMMUNE SYSTEM

Data on the influence of gut derived bacteria on the human immune system are largely derived from *in vitro* experiments testing the potential effects and mechanisms of possible probiotic strains. Co-culture of strains of lactic acid bacteria with PBMC or selected cells derived from healthy volunteers shows that different strains modulate the cytokine production of the PBMC(73) or dendritic cells(74-76) differentially. A variation on this approach also includes gut epithelial cell lines. Human *in vivo* experiments where the

interaction of probiotics with the human mucosal immune system is studied are scarce; in this approach biopsies from gut epithelial tissues are taken from volunteers taking probiotics and subsequently tested for changes in cellular activity. **Table 1** shows the influence of selected strains on the mouse or human immune system.

It needs to be pointed out that many different strains are being used as probiotics. Both Figure 1 and Table 1 show that probiotics species are derived from relatively few taxonomic groups, but many different strains from single species (like *Lactobacillus rhamnosus*) can be found in literature. In what way species and strains differ genetically from each other is usually not known and strains known under different names might actually prove to be genetically similar if not identical(77). Different techniques used to characterize the resident microbiota can also be used to fingerprint probiotic species and strains, aiding the standardization of probiotics. Nevertheless, this approach does not provide information on functional differences between strains. Genetically closely related strains might differ in the expression of proteins or structures that elicit beneficial effects to the host, while unrelated strains can express identical beneficial molecules(78). Indeed, $PSA^{-/-}$ *B. fragilis* has lost all beneficial effects as compared to the wild type strain(49, 57), yet both would appear identical in most of the molecular characterization techniques.

In vitro culture with human immune cells

All known subsets of human T helper cells can be induced by probiotic strains, at least *in vitro*(79, 80). The capacity of a given probiotic bacterium to induce one type of T helper cell may vary greatly; *L. salivarius* induces relatively high numbers of $FOXP3^+$ Treg, while *Lactococcus lactis* also induce RORγT expression and IL-17 production(81). Because many immune mediated diseases are associated with impaired Treg function, induction of Treg is a desired function of probiotic strains in human *in vitro* experiments. These cells can be induced by some strains very potently, while other strains are completely negative in this respect(79, 82). The mechanisms by which Treg, and other T helper cell subsets for that matter, are induced differ between studies and strains. The capability of *Lactobacillus reuteri* and *L. casei* to bind to DC-SIGN expressed by DC is pivotal to the induction of IL-10 producing Tregs by these DC(82). An *L. acidophilus* strain induces $FOXP3^+$ Treg in an IL-10 independent manner in PBMC(79) while the mixture VSL#3 prevents T_H1 cell induction via IL-10(83).

Dendritic cells activated by commensal (probiotic) bacteria do generally not fully mature as evidenced by B7-molecule (CD80/CD86) and HLA-DR expression(76). This in contrast to DC activated by pathogenic bacteria or LPS(76, 84). The immature phenotype of DC results in the induction of Treg rather than effector T cells(75, 84), although it has also been reported that probiotic strains induce fully mature DC that modulate T cell responses differentially via cytokine production(85). Differential cytokine production

(IL-10/IL-12 ratio) by human macrophages depends on the ability of the bacterial strain to activate SOCS3 in the macrophage(86). Thus, *in vitro* co-cultures of immune cells with probiotic bacteria show that each strain has a different immunomodulating effect.

Mucosal epithelial cell lines as a screen for probiotic action

The role of mucosal epithelial cells in the response to probiotic bacteria has been investigated with trans-well systems where mucosal cell lines like Caco-2 or HT-29 separate immune cells from lactic acid bacteria as a model to reflect the *in vivo* situation(87, 88). Mucosal cell lines respond differentially to pathogenic and commensal bacteria, specifically by producing increasing amounts of TGF-β in reaction to commensals and less proinflammatory cytokines, which subsequently induces higher IL-10 production by immune cells(87, 89, 90). These trans-well systems provide the possibility to culture several components of the gut mucosal system *in vitro* and prevent direct contact between lymphocytes and (commensal) bacteria which could obscure the data obtained in direct co-cultures of PBMC with bacteria. Nevertheless, these systems make use of transformed cell lines that might be altered in their ability to sense the microbiota correctly and prevention of leakage of microbial products throughout the mucosal cell line to the lymphocytes can not be guaranteed. Culturing bacteria with cell lines like Caco-2 and HT-29 without the addition of PBMC shows clearly a differential reaction of the cell lines towards the bacteria in NFκB activation, proliferation of the cells and cytokine production(91, 92), indicating that the epithelium is likely to influence the activation of lamina propria residing lymphocytes in a differential manner depending on the microbiotal composition or probiotics administered.

Human *in vivo* studies

The central role of the NFκB pathway in the reaction of mucosal epithelial cells has also been demonstrated *in vivo* in experiments in which probiotic bacteria were administered to healthy volunteers and gene activation in gut biopsies from the duodenum was assessed with micro arrays. Live *Lactobacillus plantarum* bacteria induce a gene expression profile preventing the onset of an adaptive immune response(93). With the administration of different strains of probiotic bacteria discrete expression profiles are found in the epithelium showing that the gut mucosa is able to distinguish between strains. Interestingly, the gene expression profiles induced by specific strains are compatible with the (intended) clinical effects. Thus, *L. rhamnosus* GG which is effective in relieving the symptoms of atopic disorders induces a mucosal T_H1 response with the upregulation of IFN-γ induced genes indicating an effect on the T_H1/T_H2 balance. Similar relations are found between the effect of *L. acidophilus* Lafti L10 on the mucosal gene expression (down regulation of the IL-23 subunit p19) and the role of IL-23 in the pathophysiology of IBD(94, 95).

Table 1: Immune modulatory effects of key probiotic species and resident microbiota species.

Species (Strain)	Host Species	*in vitro/ in vivo*	CD4 T cell subsets				Cytokine production					Remarks	Reference
			T_H1	T_H2	T_H17	Treg	IL-12	IFN-γ	IL-4	IL-10	IL-17		
Bacteroides fragilus	M	*in vivo*				↑				↑			(58)
PSA$^{-/-}$ *B. fragilus*	M	*in vivo*			↑								(49, 57)
Clostridium group IV	M	*in vivo*				↑							(60)
Clostridium group XIVa	M	*in vivo*				↑							(60)
Lactobacillus plantarum (WCFS 1)	H	*in vitro*										↑ NFκB activity	(93)
Lactobacillus acidophilus (Lafti-L10)	H	*in vivo*										Mucosal activation	(94)
Lactobacillus casei (CRL-431)	H	*in vivo*										Mucosal activation	(94)
Lactobacillus rhamnosus (GG)	H	*in vivo*										Mucosal activation	(94)
Lactobacillus rhamnosus (GG)	H	*in vitro*					↓			↑		Mϕ SOCS3 modulation	(86)
Streptococcus thermophilus (THS)	H	*in vitro*					↓			↑		Mϕ SOCS3 modulation	(86)
Lactobacillus reuteri (ASM20016)	H	*in vitro*								↑			(82)
Lactobacillus casei (NIZO B255)	H	*in vitro*								↑			(82)
Lactobacillus casei	R	*in vivo*					↓	↓		↑	↓		(122)
Streptococcus thermophilus (W67)	H	*in vitro*		↑									(81)
Lactobacillus acidophilus (W55)	H	*in vitro*				↑							(79)
Lactococcus lactis (W19)	H	*in vitro*	↑					↑	↑				(81)
Lactobacillus salivarius (W57)	H	*in vitro*			↑	↑				↑	↑		(81)
Lactobaccilus plantarum (W21)	H	*in vitro*				↑							(81)

Lactobacillus gasseri (ATCC 19992)	H	*in vitro*	↑		↑			(75)
Lactobacillus johnsonii (ATCC 33200)	H	*in vitro*	↑		↑			(75)
Lactobacillus reuteri (ATCC 23272)	H	*in vitro*	↑		↑			(75)
L. rhamnosus	H	*ex vivo*				↓		(123)
L. rhamnosus Lcr35	H	*in vitro*			↑		Dose dependent	(39)
E. coli Nissle 1917	H	*in vitro*			↓		MoDC	(124)
B. infantis 35624	H	*in vitro*			↓		MoDC	(124)
Propionibacterium species	H	*in vitro*			↓	↑		(41)
Oenococcus species	H	*in vitro*			↓	↑		(125)
Lactobacillus acidophilus (NCFM)	M	*in vitro*			↓	↑		(126)
E. coli Nissle 1917	M	*in vitro*				↑	BMDC	(127)

Listed are the known effects of selected species on $CD4^+$ T cells and on cytokine production, tested either *in vitro* or *in vivo*, in mouse models or with human cells. More studies investigating the immune modulating effects of gut bacteria can be found and some listed references investigate more species than shown here.

Although the direction in which a given probiotic strain skews the immune system may be rather consistent *in vitro*(81), the occurrence of this effect is highly donor dependent both *in vitro* and *in vivo*(81, 94). Under *in vivo* conditions the net effect might be affected by the host's specific enterotype and/or genetic background. Therefore, depending on the specific disease or condition targeted by a probiotic intervention, the strain used should be selected carefully(79, 96) with an emphasis on both bacterial characteristics as well as characteristics of the host. Whether the variation in host gene expression profiles as observed for probiotic bacteria would also hold true for pathogens is unknown.

CLINICAL TRIALS WITH PROBIOTICS: PAST RESULTS AND FUTURE APPLICATIONS

Probiotics have been used for the promotion of general health but also in many different disease settings, in most cases without prior knowledge of their interactions with the human host. We here focus on the use of probiotic bacteria in the prevention or management of atopic disorders like eczema and asthma. Systemic reviews on this topic have recently been published(97-100), comparing the outcome of around 25 trials. Some of these trials have been rather successful, resulting in a decrease in incidence of eczema(101-103) or in a decrease in the severity of eczema(104-108). Nevertheless, other trials showed no effect at all(109-114), or the effects were not sustained for more than 5 years after administration. The overall conclusion is that probiotics have a potential for the prevention of eczema and asthma. However, not any strain will have a beneficial effect in any protocol or any individual patient. With the current knowledge on the effects of the microbiota and probiotics on the immune system as described in this review, several reasons for the differential outcome of these trials can be suggested.

Selection of probiotic strains

Not all probiotic strains are identical. While this is a sweeping generalization, it is important to realize that the specific immunomodulatory profile of a given probiotic strain should be matched with the desired immunoregulatory effect. Some probiotic strains are potent IL-17 inducers like SFB(46) or *Lactobacillus salivarius* W57(81), whereas others will likely induce Treg like Clostridium groups IV and XIVa(60) and *Lactobacillus acidophilus* W55(79). Indeed, also differences in clinical effects between probiotic strains have been observed (115). In most clinical trials conducted until now, the rationale for the selection of a specific strain is not given. Obviously, commercial reasons for testing a certain strain which is already on the market as a probiotic in dairy products like *Lactobacillus rhamnosus* GG or *L. casei* Shirota may play a decisive role in some cases. An advantage of using such commercially available strains is the considerable amount of supportive data, especially safety data.

Only in some trials a scientific rationale for the particular strain(s) which are selected is given; for example IL-10 production(73) or T cell differentiation into Treg in co-cultures of bacteria with immune cells(116). Nevertheless, this approach still could be improved and expanded. The relation between *in vitro* experiments and *in vivo* results needs further attention. The increasing knowledge on the composition of the human microbiota provides an opportunity to further characterize relations between certain strains and disease. Although no clear relation between enterotypes and disease has been described as yet, it is possible that individuals with atopic disorders carry or miss certain species in their microbiota that have a profound effect on disease induction or progression(14, 117). Colonization of GF mice with the microbiota of atopic patients might provide valuable information on the effects of certain species on the immune system. The use of humanized mice (i.e. mice with the microbiota of humans) will improve such experiments even further(118, 119).

Up till now virtually all probiotic bacteria in use belong to the lactic acid bacteria. Yet, a bacterial species that can tip the balance between health and disease might be a member of a totally different phylogenetic group. Our view of which species can be used as probiotics needs to be broadened; Clostridium species known in mice to induce Treg, in rats to prevent proinflammatory cytokine production(120) and in humans to be beneficial in IBD are waiting to be explored(121).

Patient specific responses

Each individual person reacts differently on a probiotic strain(81, 94). This can be due to a differences in microbial composition, but also the genetic background of an individual, more specifically the polymorphisms of (innate) immunity genes, is likely to play a role as well. Mutations in Nod2 and the inflammasome NLRP6 are now known to affect the immune systems reaction towards the microbiota and probiotic strains. Other molecules can play important or subtle roles, determining the ultimate response. Ideally, in the era of personalized medicine, personalized gut microbiota management should include assessment of the individual immune reaction towards a given probiotic strain.

Disease complexity

The immune mediated diseases that are targeted by probiotic supplementation are generally complex and multi-factorial. The pathology of diseases like asthma is not fully understood yet; the complexity increases with progression of science. Whether probiotics can influence the development of such a disease is part of that complexity. From performed trials it is clear that the moment of supplementation is very important; best results (in the infant) are obtained when the pregnant mother already uses probiotics in the last weeks of gestation and the child immediately after birth and during the first

months of life(101-103). The fact that the aberrant inflammation is localized in a distant organ (in case of asthma) warrants a strain that is capable to influence both local mucosal and systemic immunity.

In order to investigate the *in vivo* immunologic properties of probiotic strains, clinical trials in diseases like asthma should be carefully designed. The appropriate immunologic biomarkers should be tested during and after the intervention period in order to relate the clinical effect with immune effects of the strain.

Probiotics clearly have a high potential in clinical settings. Nevertheless, the above mentioned aspects need full attention of scientists and clinicians before the promises can be fulfilled. More knowledge on the bacterial strains, their interaction with the immune system, the diseases in distant organs they can influence, and patient specific reactions towards probiotics is necessary.

The fact that the human immune system and the gut microbiota are so intertwined is only now, a century after Metchnikoff, becoming clear. It is unlikely that probiotics will further improve life expectancy, as was Metchnikoffs vision. Hygienic and other measures since Metchnikoff have prolonged life considerably already. Nevertheless, the fact that the microbiota and probiotics are potent modulators of the human immune system give probiotics the potential to improve immune mediated diseases and improve the quality of life. The complexity of the gut ecosystem and its interaction with the mucosal immune system warrants further research in this fascinating field in order to obtain optimal health promoting effects of probiotics.

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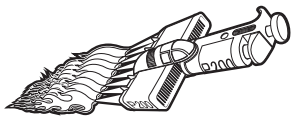

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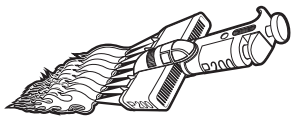
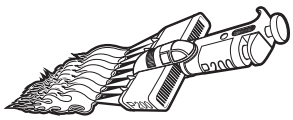


CHAPTER 3



Lactic acid bacteria differ in their ability to induce functional regulatory T cells in humans

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SUMMARY

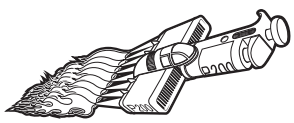
Background: Trials with probiotic lactic acid bacteria have yielded different results, which may be due to the strains used. Lactobacilli and bifidobacteria are known to be potent modulators of the immune system. The capacity of these bacteria used as probiotics to influence both T helper type 1 (T_H1)- and T_H2-mediated diseases has been shown before. However, the ability of strains to induce forkhead box P3 ($FOXP3^+$) expressing regulatory T cells has not yet been investigated.

Objective: Test the inherent differences between strains in their capacity to induce functional regulatory T cells in human peripheral blood mononuclear cells (PBMC).

Methods: Human PBMC were co-cultured *in vitro* with either *Bifidobacterium lactis* W51, *Lactobacillus acidophilus* W55 or *Lactobacillus plantarum* W62 or *an Escherichia coli* control strain. The percentage of $FOXP3^+$ cells, the origin of the induced cells and the functionality of these cells were assessed.

Results: Probiotic strains differ in their capacity to induce regulatory T cells. $FOXP3^+$ cells were induced from $CD25^-$ cells and were able to suppress effector T cells. Naturally occurring regulatory T cells were not affected by co-culture with lactobacilli. IL-10 concentrations found in the supernatant showed a trend towards the same differences between strains. Blockade of IL-10 did not influence the up-regulation of FOXP3. No differences between lactic acid bacteria were found in IL-17, IFN-γ or IL-13.

Conclusions: Some probiotic strains are potent inducers of regulatory cells, while others are not. The clear differences between strains imply that an *in vitro* characterization of probiotic strains before application is recommended.



INTRODUCTION

The prevalence of immune-mediated disorders has increased in recent years(1). An explanation for this can be found in the hygiene hypothesis, which states that a reduced contact with microbes and microbial products leads to an increased chance of developing atopic disorders like eczema and asthma(2). In an attempt to counteract the increase in atopic disorders, several groups have recently performed trials with orally administered microbial products. Lactic acid bacteria were used to modulate the immune system, aiming at the prevention or the treatment of allergic disorders. However, different results have been obtained in these trials. For example, Kalliomäki *et al.*(3, 4) found a positive effect of *Lactobacillus rhamnosus* GG (LGG) on the prevention of atopic dermatitis (AD), where Taylor *et al.* and Prescott *et al.*(5, 6) showed no effect of a *Lactobacillus acidophilus* strain. In addition, Wickens *et al.*(7) show in one clinical trial that supplementation with an *L. rhamnosus* strain does not influence the chance of developing AD, while a strain of *Bifidobacterium animalis* does.

The rationale behind the hygiene hypothesis and supplementation with lactic acid bacteria is originally found in the T helper type 1 (T_H1)/T_H2 immune balance. However, this hypothesis is insufficient in explaining the complex interplay between immune cells. The forkhead box P3 (FOXP3)$^+$ regulatory T cell (Treg) and the T_H17 cell are now recognized as important players in immune homeostasis next to T_H1 and T_H2 cells(8, 9). Thus, the effect of supplementation with lactic acid bacteria on the immune system is likely to be more complex than skewing the T_H1/T_H2 balance towards a T_H1 profile. Indeed, LGG is able to influence both T_H2- and T_H1- or T_H17-mediated disorders(4, 10, 11). In addition, increases in FOXP3 mRNA expression in peri-bronchial lymph nodes have been observed upon administration of *Bifidobacterium lactis* Bb12 and LGG, suggesting the induction of regulatory cells by these strains(12).

Treg are thought to be the master regulators of the immune response in both humans and rodents. Defects in the transcription factor FOXP3, which defines the Treg lineage, result in multiple auto-immune diseases and atopy(13-15), showing the central role of FOXP3$^+$ CD4 cells in immune homeostasis. It has therefore been suggested that a deficient function or insufficient numbers of FOXP3$^+$ Treg are an important co-founder of auto-immune and atopic disorders. Next to naturally occurring regulatory T cells (nTreg), which are generated in the thymus, FOXP3$^+$ cells can also be induced in the periphery (iTreg)(16). Induction of Treg is an important target for treating both auto-immune and atopic disorders as these cells can suppress the activity of effector cells. The ability of lactic acid bacteria to induce functional FOXP3$^+$ cells in humans has not been assessed before, but is obviously important when these microbes are used to treat or prevent immune-mediated diseases.

The human gut microbiota has been recently estimated to consist of at least 400 different species(17), and it is likely that the potency of each of these species to influence immune homeostasis is different. Indeed, cytokine profiles in *in vitro* co-cultures of bacteria with peripheral blood mononuclear cells (PBMC) show marked differences between strains(18). Concomitantly, the effects of lactobacilli supplementation on experimental autoimmune encephalomyelitis have been shown to be highly strain dependent(19). It is therefore conceivable that the contradicting results found in the human trials can be partly explained by differences in the immunomodulatory capacity of the strains used.

Thus, strain diversity and the complexity of their interplay with the immune system warrant a careful selection process before using them in clinical trials. Therefore, it is necessary to conduct appropriate *in vitro* studies to characterize and compare the immune modulating capacity of different strains of bacteria.

In this paper, we show that probiotic bacteria can induce functional $FOXP3^+$ regulatory cells *in vitro*, but differ in their ability to do so.

METHODS

Cells and bacteria

PBMC were isolated from healthy controls using Ficoll-Isopaque density centrifugation (Ficoll-Paque, Pharmacia, Uppsala, Sweden). PBMC were depleted from $CD25^+$ cells using anti-CD25 magnetic-activated cell sorting (MACS) magnetic beads (BD Biosciences, San Jose, CA, USA).

CD4 T cells were obtained by enrichment of PBMC using BD's MACS CD4 T cell enrichment kit. $CD25^-$ effector cells were subsequently isolated using anti-CD25 magnetic beads (BD Biosciences). $CD25^+CD127^-$ regulatory cells were sorted using fluorescence-activated cell sorting (FACS) Aria (BD Biosciences). Antigen-presenting cells (APC) were obtained by depleting PBMC from $CD3^+$ cells with anti-CD3 magnetic beads (BD Biosciences).

Lactic acid bacterial strains *B. lactis* W51, *L. acidophilus* W55 and *L. plantarum* W62 were obtained from Winclove Bio Industries BV, Amsterdam, the Netherlands. *Escherichia coli* AMC B12G1, used as a Gram-negative control strain, was kindly provided by M. Kapsenberg, Academic Medical Center, University of Amsterdam. The strain selection is based on the cytokine profile of PBMC cultured with these strains as described by Niers *et al.*(18). Strains were washed three times in phosphate-buffered solution (PBS) and subsequently frozen at -80°C in aliquots of 10^8 colony-forming units (CFU)/vial in RPMI 1640 (Gibco, Breda, the Netherlands) supplemented with 1% L-glutamine (Gibco). For each experiment, an aliquot of bacteria was thawed and used.

Culturing conditions

Cells were cultured in RPMI 1640 supplemented with 10% AB-serum, 1% L-glutamine, 100 mg/ml streptomycin and 100U/ml penicillin (Gibco). PBMC were cultured with bacteria in a 1 : 10 ratio (cells : CFU) for 168 or 120 h as indicated in 96-well plates. This ratio was found to be the most optimal for detecting differences between strains without inducing apoptosis of PBMC. IL-10 blocking antibody (JES3-19F1; BD Biosciences) or isotype (R35-95; BD Biosciences) was added to co-cultures at a concentration of 10 mg/ml.

For suppression and proliferation assays, 96-well plates were pre-coated overnight at 4°C, with 1.0 or 1.5 mg/ml anti-CD3 (OKT3, BD Biosciences) as indicated. $CD25^+CD127^-$ regulatory cells were sorted from freshly isolated PBMC (nTreg) or from culture with *L. acidophilus* W55 (iTreg). $CD25^-$ effector cells in suppression assays with iTreg were labelled with 30 mM carboxyfluorescein succimidyl ester (CFSE, Invitrogen/Molecular Probes, Breda, the Netherlands). APC were frozen until use, thawed, irradiated (3.5Gy) and added to the cultures (30 000/well). ^{3}H-labelled thymidine was added to suppression assays with nTreg and co-cultures of nTreg with *L. acidophilus* W55 after 96 h of culture for another 16 h. Proliferation in assays with nTreg was determined by calculating the difference in the proliferative response (mean ^{3}H-thymidine incorporation) between $CD4^+CD25^-$ effector cells alone and $CD4^+CD25^-$ effector cells co-cultured with $CD4^+CD25^+CD127^-$ regulatory cells. CFSE assays with iTreg were cultured for 5 days and subsequently surface stained using BD Biosciences fluorescent antibodies against CD3, CD4 and CD25 (PE, PerCP and APC, respectively).

Recombinant human IL-2 (eBiosciences, San Diego, CA, USA) was added to cultures of $CD4^+CD25^+CD127^-$ cells at a concentration of 1000 U/ml.

Flow cytometry, cell sorting and multiplex immunoassay

Cells were washed twice with PBS containing 2mM Na-EDTA supplemented with 2% FCS. Cells were surface stained using BD Biosciences fluorescent antibodies against CD4 (FITC or PerCP), CD25 (FITC or APC) CD127 (PE), CD30 (PE) and GITR (PE). For sorting experiments, CD4 cells were enriched with the CD4 T cell enrichment kit from BD Biosciences before staining. The viability of the PBMC after co-culture was assessed by FACS staining with Annexin V and 7AAD (both BD Biosciences).

For intracellular cytokine staining, cells were treated with monensin protein transport inhibitor (BD Biosciences) for 4 h before FACS staining. Cells were permeabilized with the permeabilization kit of eBioscience (eBioscience) and FOXP3 was stained with APC- or PE-labelled antibody from eBioscience (clone PCH101). CTLA-4 (CD152) and IL-10 were stained with a PE-labelled antibody (BD Biosciences).

Cells were subsequently analysed or sorted on FACS Calibur (BD Biosciences) or FACS Aria (BD Biosciences), respectively. A specific binding was prevented by incubating with mouse or rat serum before adding

antibodies. FACS data were analysed using CellQuest software from BD Biosciences.

Various cytokines were measured in the supernatant of co-cultures of PBMC with bacteria using a multiplex immunoassay (Luminex, Austin, TX, USA) as described previously(20). The detection limits of IL-4, IL-13, IL-17, IL-10 and IFN-γ are 1.22, 2.44, 2.44, 4.88 and 4.88 pg/ml, respectively.

Statistics

The statistical significance of the observed differences was tested using the Kruskal–Wallis test. Subsequent pairwise comparisons were performed with the Wilcoxon rank sum test with Bonferroni's correction. All analyses were performed in R 2.5.1 for Macintosh.

RESULTS

Lactic acid bacteria differ in their potential to induce forkhead box P3

To investigate whether lactic acid bacteria are able to induce FOXP3$^+$ T cells, PBMC from healthy donors were co-cultured with different lactic acid bacterial strains and an *E. coli* control strain. After 168 h of culture, the percentage of FOXP3$^+$ cells was analysed by intracellular FACS staining. PBMC of all co-cultures showed an increase in the percentage of CD25$^+$FOXP3$^+$ cells as compared with PBMC cultured in medium alone (Figure 1A–C). This percentage differed significantly among the lactic acid bacteria used, with *L. acidophilus* W55 being the most potent strain ($p<0.01$). *L. plantarum* W62 did not induce significant up-regulation of FOXP3 as compared with medium. The observed differences in FOXP3 induction could not be explained by differences in the viability of the PBMC, as tested by 7AAD and Annexin V stainings (not shown). In addition, the difference in FOXP3 expression was not the result of differences in the proliferation rate, because the strains induced equal ^{3}H-labelled thymidine incorporation by the PBMC (not shown).

Besides differences in the increase in regulatory cells or cytokines, the bacteria could also differ in their potential to induce T_H1, T_H2 or T_H17 cells. Therefore, cytokines specific for these T cell subsets were measured in the supernatant (Figure 2B–D). All lactic acid bacteria induced IFN-γ production; no differences were observed between the strains. The *E. coli* strain induced less IFN-γ production. IL-17 and IL-13 were produced in very low amounts, but above the detection limit. IL-4 could not be detected.

The induction of FOXP3 had the same profile as the production of the regulatory cytokine IL-10 in the supernatants of the co-cultures (Figure 2A). However, this trend was not significant. The production of IL-10 by APC present in the co-cultures could be an important factor in the induction of FOXP3$^+$ cells. Therefore, IL-10 was blocked with a monoclonal antibody during co-culture with *L. acidophilus* W55. However, this did not result in a significant reduction of the percentage of CD25$^+$FOXP3$^+$ cells (Figure 3).

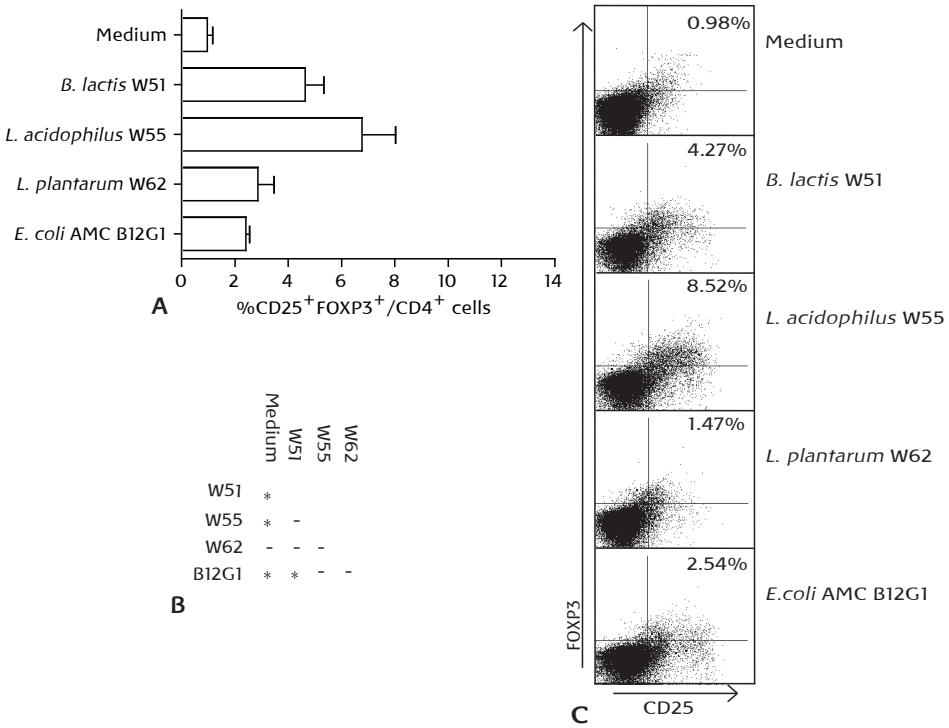


Figure 1. Induction of CD25⁺forkhead box P3 (FOXP3⁺) cells by bacteria in peripheral blood mononuclear cells (PBMC). (**A**) PBMC were co-cultured with *Bifidobacterium lactis* W51, *Lactobacillus acidophilus* W55, *L. plantarum* W62 or *Escherichia coli* in a 1 : 10 ratio or in medium alone. The percentage of CD25⁺FOXP3⁺ cells was assessed by intracellular fluorescence-activated cell sorting (FACS) staining after 168 h of culture. Data are expressed as mean ± SEM of six independent experiments. (**B**) Significant differences between treatments of (**A**), *P<0.05, -P>0.05. (**C**) Representative FACS plots for cultures described in (**A**).

Forkhead box P3 cells are induced from CD25⁻ cells

The observed increase in CD25⁺FOXP3⁺ cells between co-cultures and PBMC cultured in medium alone (Figure 1) can originate from proliferating nTreg, present in the PBMC, or from CD25⁻ T cells becoming CD25⁺FOXP3⁺ due to co-culture with the bacteria. When nTreg were depleted from the PBMC before co-culture by removing CD25⁺ cells, the percentage of FOXP3⁺ cells after co-culture was increased when compared with the total PBMC (Figure 4A–C). The differences between the strains became even more apparent (compare Figure 1 and 4).

The strains used in this study induce FOXP3⁺ cells from CD25⁻ cells. Nevertheless, it is still possible that nTreg proliferate in response to direct contact with lactobacilli. To test this, nTreg cells were sorted and co-cultured with *L. acidophilus* W55 in the presence of irradiated APC. As cells have to be

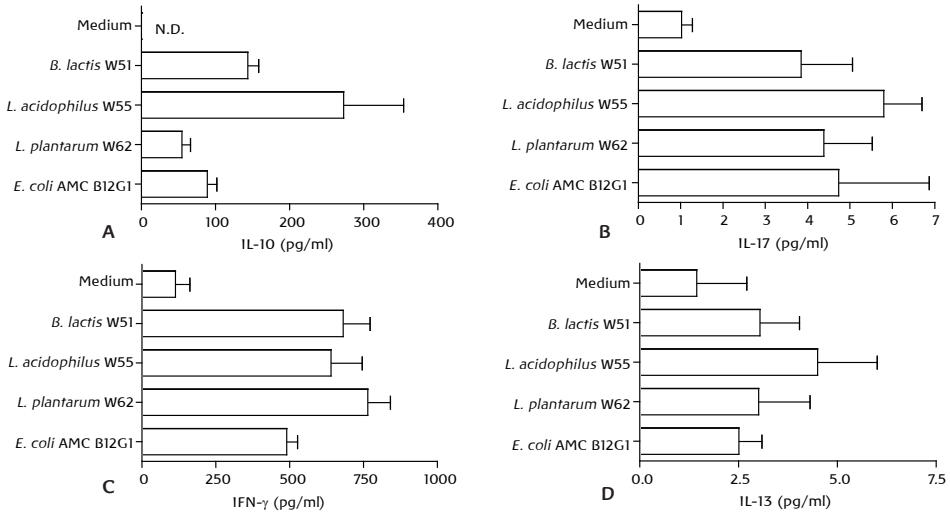


Figure 2. *In vitro* production of (A) IL-10, (B) IL-17, (C) IFN-γ and (D) IL-13. Supernatants of 72 or 96 h co-cultures of peripheral blood mononuclear cells (PBMC) with *Bifidobacterium lactis* W51, *Lactobacillus acidophilus* W55, *L. plantarum* W62 or *Escherichia coli* AMC B12G1 or PBMC in medium alone were collected. The concentration of cytokines was subsequently determined using a multiplex immunoassay. Data are expressed as mean ± SEM of three independent experiments. ND, not detected.

permeabilized to FACS-stain the intracellularly located transcription factor FOXP3, no functional assays with FOXP3-stained cells can be performed. As a consequence, Treg were sorted as $CD4^+CD25^+CD127^-$ cells, which is a tested combination of surface markers for Treg(21, 22). *L. acidophilus* W55 was chosen for these experiments as this strain induced the highest percentages of $FOXP3^+$ cells.

nTreg did not proliferate when cultured without a T cell receptor stimulus, with anti-CD3 alone or with IL-2 alone. The cells only proliferated when

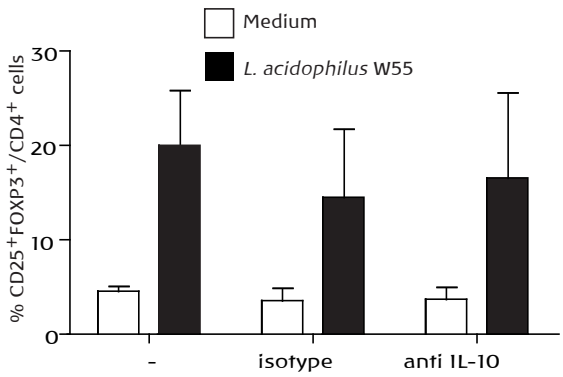


Figure 3. Induction of forkhead box P3 (FOXP3) by *Lactobacillus acidophilus* W55 in the presence of IL-10 blocking antibody. Peripheral blood mononuclear cells were cultured as described in Figure 1A. IL-10 blocking antibody or isotype was added before culture. Percentage of $CD25^+FOXP3^+$ cells was assessed by intracellular fluorescence-activated cell sorting staining after 168 h of culture.

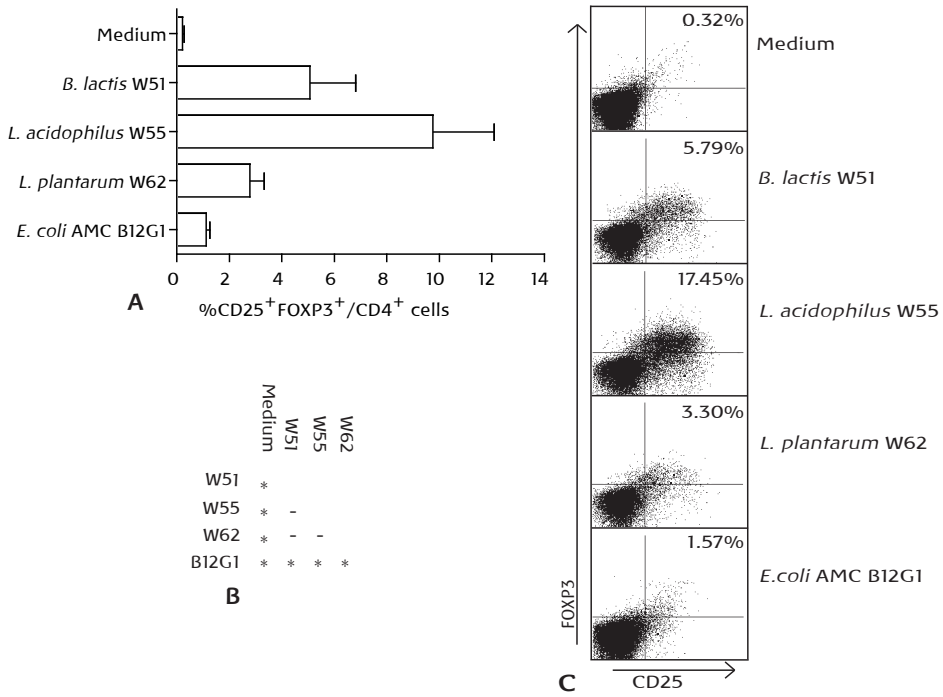


Figure 4. Induction of CD25⁺forkhead box P3 (FOXP3⁺) cells by bacteria in CD25-depleted peripheral blood mononuclear cells (PBMC). (**A**) PBMC were co-cultured with *Bifidobacterium lactis* W51, *Lactobacillus acidophilus* W55, *L. plantarum* W62 or *Escherichia coli* in a 1 : 10 ratio or in medium alone. The percentage of CD25⁺FOXP3⁺ cells was assessed as described in Figure 1. Data are expressed as mean ± SEM of six independent experiments. (**B**) Significant differences between treatments of (**A**), *P<0.05, -P>0.05. (**C**) Representative fluorescence-activated cell sorting plots for cultures described in (**A**). The same donor as in Figure 1C is depicted.

cultured in the presence of both anti-CD3 and IL-2. Addition *of L. acidophilus* to any of these treatments did not significantly alter the proliferation of the cells (Figure 5A).

Altogether, these data indicate that the increase in FOXP3⁺ T cells seen in co-cultures of lactobacilli with PBMC can be explained by newly induced FOXP3⁺ iTreg derived from CD25⁻FOXP3⁻ cells.

Lactobacilli do not influence the suppressive capacity of naturally occuring regulatory T cell

In a co-culture of nTreg with bacteria, as in a culture of PBMC with *L. acidophilus* W55, the suppressive function of nTreg may be altered by the bacteria. To address this issue, CD25⁺CD127⁻ nTreg were co-cultured with *L. acidophilus* W55 or cultured in medium alone. Subsequently, a suppression assay was conducted with these cells. As shown in Figure 5B, co-culture of

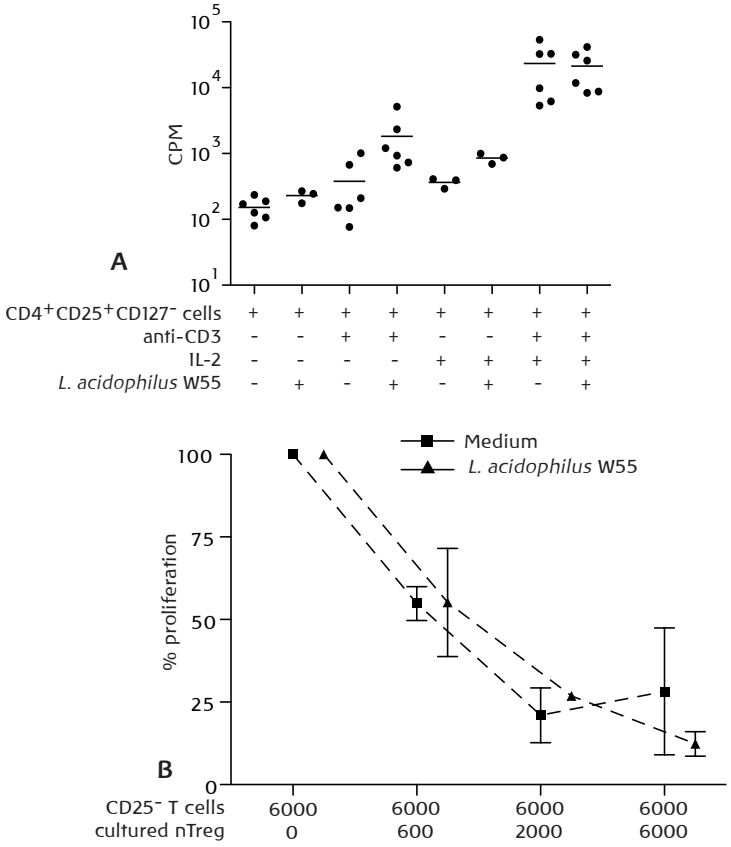


Figure 5. Effect of bacteria on nTreg. (**A**) $CD25^+CD127^-$ cells were selected by fluorescence-activated cell sorting (FACS) sorting from freshly isolated peripheral blood mononuclear cells (PBMC). These cells (50 000/well) were subsequently activated by plate-bound anti-CD3 (1.5 mg/ml), recombinant human IL-2 and *Lactobacillus acidophilus* W55 (1 : 10) in the presence of 30 000 irradiated antigen-presenting cells (APC)/well. Proliferation was assessed by ^3H incorporation after 96 h. Counts per minute (c.p.m.) is used as an indication of the amount of incorporated ^3H. 200 c.p.m. is equal to background. Data from six independent experiments, and the geometric mean values are indicated. (**B**) $CD25^+CD127^-$ cells were selected by FACS sorting from freshly isolated PBMC and subsequently co-cultured with *L. acidophilus* W55 for 96 h in a 1 : 10 ratio or in medium alone. After co-culture, cells were FACS sorted in different ratios with $CD25^-$ effector T cells in the presence of 30 000 irradiated APC. Effector T cells were activated with 1.5 mg/ml plate-bound anti-CD3. Proliferation was assessed by ^3H incorporation after 96 h, n=3.

nTreg with bacteria did not alter the suppressive capacity of nTreg. There was no difference in the expression of FOXP3 in these cells either (not shown).

Stability and suppressive capacity of induced-regulatory T cell

To date, no definitive surface markers for human Treg have been found. The currently most accepted way to determine the number of Treg is to measure the percentage of $FOXP3^+$ cells. However, the transcription factor FOXP3

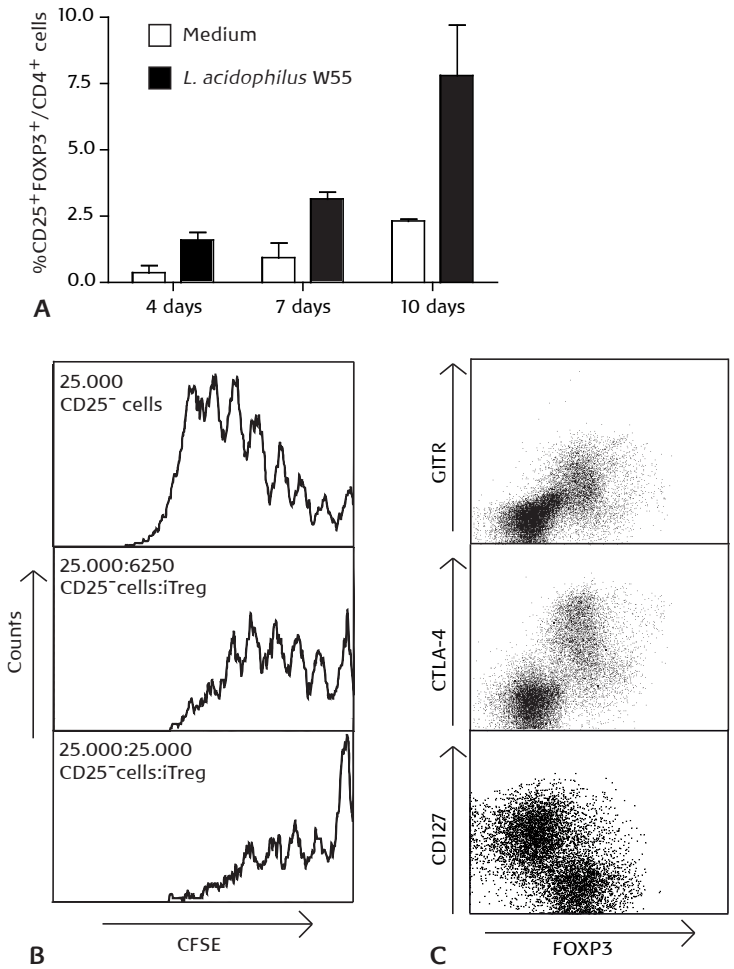


Figure 6. Kinetics of forkhead box P3 (FOXP3) expression and suppressive capacity of iTreg. (**A**) peripheral blood mononuclear cells (PBMC) were co-cultured with *Lactobacillus acidophilus* W55 in a 1 : 10 ratio or in medium alone. The percentage of $CD25^+FOXP3^+$ cells was assessed by intracellular fluorescence-activated cell sorting staining at several timepoints, n = 4. (**B**) *In vitro* suppression assay with *L. acidophilus* W55-induced Treg. $CD4^+CD25^+CD127^-$ regulatory cells were sorted from a 168-h co-culture of CD25-depleted PBMC with *L. acidophilus* W55. $CD4^+CD25^-$ cells were negatively selected with magnetic-activated cell sorting, carboxyfluorescein succimidyl ester (CFSE) labelled and activated with plate-bound anti-CD3 (1.5 mg/ml). Irradiated antigen presenting cells (30 000/well) were added. Proliferation of cells was assessed by flow cytometry after a 120-h culture. Plots are gated on $CD3^+CD4^+CFSE^+$ effector cells. Data are representative for two donors. (**C**) Co-expression of CTLA-4, GITR and CD127 with FOXP3. Plots are gated on $CD4^+$ T cells.

is also upregulated transiently in activated T cells, not representing Treg. Therefore, we first analysed whether the FOXP3$^+$ population found after co-culture was stable, by culturing these cells for 10 days in the presence of *L. acidophilus* W55. As shown in Figure 6A, the population of FOXP3$^+$ cells was stable and still increasing even after 10 days.

Second, we tested whether FOXP3$^+$ iTreg were able to suppress effector T cells. As shown in Figure 6B, CD25$^+$CD127$^-$ (iTreg), sorted from a co-culture of CD25-depleted PBMC and *L. acidophilus* W55, were able to suppress effector cells activated by anti-CD3 in a dose-dependent manner.

Finally, we investigated whether the iTreg expressed markers known to be induced on Treg. *L. acidophilus* W55-induced FOXP3$^+$ cells had a high expression of GITR and CTLA-4 and a low expression of CD127 (Figure 6C).

DISCUSSION

In this paper, we show that strains of lactic acid bacteria differ in their capacity to induce FOXP3$^+$ cells *in vitro*. *L. acidophilus* W55-induced large percentages of FOXP3$^+$ cells, but co-culture with *L. plantarum* W62 had no additional effect above medium alone in inducing FOXP3. The FOXP3$^+$ cells induced in co-cultures originate from CD25$^-$ cells, as no proliferation of nTreg in response to lactobacilli is observed. Depletion of CD25$^+$ cells from PBMC before co-culture with lactobacilli does not reduce but rather results in even higher percentages of FOXP3$^+$ cells. Removal of CD25$^+$ cells from PBMC yields a cell population that is devoid of regulatory cells. This population is able to respond more vigorously to the lactic acid bacteria than PBMC that are still under the control of a population of naturally occurring Treg. This results in more activated cells and subsequently more induced FOXP3$^+$ cells.

The strains that were most capable of inducing FOXP3 also induced the highest levels of the suppressive cytokine IL-10 in the supernatant of the co-cultures. This IL-10 can be produced by the FOXP3$^+$ T cells, but no IL-10$^+$FOXP3$^+$ cells were found by FACS analysis. IL-10 can also be produced by monocytes, which can subsequently be the cause of FOXP3 up-regulation in T cells. However, adding IL-10 blocking antibodies did not influence the induction of regulatory T cells. This suggests that the induction of FOXP3 accompanied by IL-10 production could be a coincidence of the strains used.

Besides to differences in the induction of Treg and cytokines, we also assessed the induction of effector molecules from T_H1, T_H2 and T_H17 cells. The amount of IL-13 and IL-17 found was very low, suggesting that T_H2 and T_H17 differentiation is not induced by stimulation with bacteria alone. IFN-γ production was observed in all co-cultures. These data indicate that the main difference between the strains tested is the induction of regulatory cells and IL-10.

The mechanism of FOXP3$^+$ Treg induction in the co-cultures remains unclear. TGF-β is the key cytokine in the induction of Treg and could be

important for lactic acid bacteria-induced Treg. Co-cultures of PBMC and bacteria with a TGF-β blocking antibody could provide an insight into this. DC-SIGN signalling has also been shown to be important in the induction of IL-10-producing T cells by lactic acid bacteria and could also be a factor in the induction of Treg(23). However, the number of DC-SIGN expressing dendritic cells in PBMC is very small and therefore unlikely to play a key role in these experiments.

Treg are characterized by the presence of the transcription factor FOXP3. Nevertheless, recently activated T cells also up-regulate FOXP3 transiently, but do not have a persistent regulatory phenotype(24). We tested whether the induction of FOXP3$^+$ cells by lactic acid bacteria reflected induction of Treg in two ways. We analysed whether the FOXP3 up-regulation was stable during a long running co-culture, and we tested whether the FOXP3$^+$ cells induced in CD25-depleted PBMC actually have a suppressive phenotype. FOXP3 is stably expressed for at least 10 days in co-culture with *L. acidophilus* W55, which is far longer than the transient up-regulation of FOXP3 observed in activated effector cells(24, 25). This indicates that the FOXP3 up-regulation is stable. In addition, the induced FOXP3$^+$ cells showed suppression in *in vitro* functional assays.

Besides *de novo* Treg induction, we investigated whether lactobacilli are able to influence the function of naturally occurring Treg. Therefore, we tested the proliferation and suppressive capacity of nTreg after co-culture with bacteria. Treg are known to be anergic when stimulated with anti-CD3 alone, and only proliferate when stimulated in the presence of high concentrations of IL-2. We show that the presence of L. acidophilus does not have an influence on these characteristics of Treg. Even when we co-cultured W55 with Treg in the presence of IL-2 and irradiated APC, no proliferation was observed. We also demonstrated that a co-culture of Treg with W55 does not influence the suppressive function of Treg. Thus, lactic acid bacteria are able to induce functionally suppressive, Treg *de novo*, but do not influence the phenotype or the function of existing nTreg *in vitro*.

Other groups have previously presented data on the immunological differences between strains of lactobacilli. For example, Smits *et al.*(23) show that dendritic cells primed with *L. reuteri* or *L. casei* are able to induce IL-10-producing T cells, but dendritic cells primed with *L. plantarum* are not. Niers *et al.*(18) have demonstrated that strains of lactobacilli differ significantly in their ability to induce the production of certain cytokines in PBMC. Here we presented data showing that strains of lactobacilli differ significantly in their capacity to induce FOXP3$^+$ regulatory cells *in vitro* that is not dependent on the production of IL-10.

It is important to keep in mind that strains can differ in their characteristics even within a species. As mentioned, *L. acidophilus* strain LAVRI-A1 had no clinical effect on eczema in the study performed by Taylor *et al.*(5). It is possible that the LAVRI-A1 strain, although from the same species as the

W55 strain, is not capable of inducing FOXP3 in these high percentages. We expect the W55 strain to be a good candidate for prevention of eczema when used in a protocol as used by Taylor *et al*. It is interesting to pinpoint the exact differences between these two related strains that might explain the clinical and *in vitro* differences. This could for example be differences in the expression of cell wall proteins or sugars as has been shown for polysaccharide A by Mazmanian *et al*.(26).

It is tempting to hypothesize that different outcomes among clinical trials performed with different bacteria are due to differences in FOXP3$^+$ Treg-inducing capacity between the strains. Of course, it is still necessary to show that these differences between strains are also observed *in vivo*, when the strains are administrated orally to mice or human subjects but it is already clear that not just any lactic acid bacteria are able to influence the immune system in a desired direction. Next to induction of regulatory FOXP3$^+$ cells, T_H1, T_H2 and T_H17 cells could also be differentially induced by strains. Therefore, before using strains clinically, they should be screened for their immune modulating abilities.

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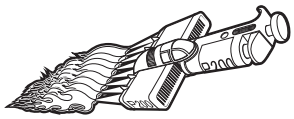

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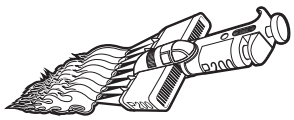
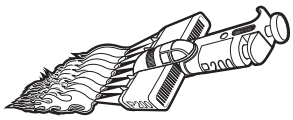


CHAPTER 4



Gut derived lactic acid bacteria induce strain specific CD4+ T cell responses in human PBMC

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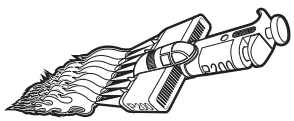
ABSTRACT

Background & aims: Probiotic bacteria are used as food supplement in many different disease settings. The immune modulating capacity of different strains is not always properly tested which might result in a suboptimal choice of strains for clinical use.

Methods: The CD4 T cell responses to 19 different gut derived lactic acid bacteria were tested with different methods to show their diversity in immune modulation and to make a well-founded choice on which strains to use in future clinical trials. After co-culture of PBMC with bacteria, the induction of $CD4^+$ T cell subsets (regulatory T cells, T helper type (T_H)1, T_H2 and T_H17) was analysed by rtPCR of transcription factor mRNA, intracellular FACS staining of transcription factors and cytokine production.

Results: Bacterial strains all have diverse, unique immune modulatory properties. Strains can induce Treg, T_H1, T_H2 and T_H17 cells which can be shown at different levels of T cell activation, and is consistent for most strains tested. For T_H1, T_H17 and Treg, a positive correlation between the different methods was found. For T_H2 cells the correlation was less consistent.

Conclusions: Probiotic bacteria have very different immune modulating capacities. Analysis of transcription factor mRNA is a suitable method for *in vitro* characterization of strains prior to clinical application.



INTRODUCTION

Human gut derived bacterial strains are frequently used as probiotic supplements for a variety of medical affections, including diarrhoea, constipation, and infections.(1) One of the most challenging objectives of probiotic supplementation however, is to influence the immune system.(2) Dendritic cells residing in the lamina propria of the gut are influenced by food antigens and the gut microbiota by direct contact of dendrites sampling the gut lumen and by cytokine production of the epithelial cell layer. These dendritic cells will subsequently move to draining lymph nodes and activate T cells there.(3) The first experiments with supplementation of lactic acid bacteria were based on the hygiene hypothesis and had the objective to prevent or cure atopic diseases. In the original dogma of the T_H1-T_H2 balance, it was thought that probiotic bacteria will mainly induce T helper (T_H) type 1 cells, thereby skewing the balance away from the T_H2 cells which have an important role in allergic inflammation.(4, 5)

However, in recent years, it became evident that T helper cell functions are considerably more complex and heterogeneous than originally thought. The original characterization of the T_H1 and T_H2 pathways has now been expanded to include additional T_H cell subsets each with their own cytokine repertoire and transcription factors. These include T_H9,(6) T_H17(7) and FOXP3$^+$ regulatory T (Treg) cells.(8-11) These subsets also have a role in the initiation and layers of complexity to the pathogenesis of such diseases. This enforces revisiting and reinterpreting existing ideas on the concept of probiotic supplementation as a therapeutic option for auto-immune and allergic diseases. Combating a T_H2/T_H17 mediated disease such as allergic asthma through immune regulatory actions of gut derived bacteria should not only involve the induction of T_H1 cells but also the induction of FOXP3$^+$ Treg. In contrast, probiotics targeting rheumatoid arthritis or multiple sclerosis should inhibit T_H1 and T_H17 responses and promote the induction of T_H2 and Treg cells.

Recent papers suggest that gut derived bacterial strains all have a different influence on the immune system.(12, 13) To be able to select strains suitable for clinical supplementation, the immune influencing and other characteristics of each strain should be documented accurately.(14) The choice of strains should subsequently fit the experimental setup. We recently showed that the capacity to induce FOXP3$^+$ regulatory T cells is highly variable between strains. Some strains were able to induce significant numbers of FOXP3$^+$ T cells with clear regulatory function whereas other strains were not.(15) It is likely that the capacity of gut derived bacterial strains to either down regulate or induce the differentiation of other T_H subtypes is just as variable.

To select potential strains for future clinical trials we investigated the immune influencing capacity of 19 bacterial strains of commonly used species using a high throughput method. The strains were co-cultured with

peripheral blood mononuclear cells (PBMC) of healthy adult donors and the induction of the different $CD4^+$ T cell subtypes was evaluated on mRNA and protein level of signature transcription factors and cytokines. We show that the capacity of each strain to influence the immune system is highly different and that some strains have immune modulatory capacities that are favourable or not.

MATERIALS AND METHODS

Cells and bacteria

PBMC were isolated from healthy adult volunteers using Ficoll-Isopaque density gradient centrifugation (Ficoll-Paque, Pharmacia,Upsalla, Sweden). For some experiments, PBMC were depleted of $CD25^+$ cells using anti-CD25 magnetic beads (BD Biosciences, San José, CA, USA). PBMC or $CD25^-$ PBMC were subsequently co-cultured with bacteria in a 1:10 ratio (PBMC:CFU) in RPMI 1640 (Gibco, Breda, The Netherlands) supplemented with 1% L-glutamine (Gibco), 0.5% Penicillin-Streptomycin (Gibco) to prevent bacterial overgrowth and 10% human AB serum. The 1:10 ratio (based on viable bacteria) was shown before to induce T cell responses without apoptosis.(15) Bacterial viability was tested by FACS analysis using the BacLight-kit (Invitrogen, Breda, The Netherlands) Since the presence of $CD4^+CD25^+$ Treg within the total PBMC population inhibits proliferation of the T cells and protein synthesis, we increased the sensitivity of some tests by using $CD25^+$ cells depleted PBMC.

Bacteria (*Streptococcus thermophilus* W67, *Bacillus coagulans* W64, *Bifidobacterium bifidum* W28, *Bifidobacterium breve* W25, *B. breve* W29, *B. breve* W6, *Bifidobacterium lactis* W51, *B. lactis* W52, *Bifidobacterium longum* W108, *Lactobacillus acidophilus* W22, *L. acidophilus* W55, *L. acidophilus* W74C, *Lactobacillus casei* W20, *L. casei* W79, *Lactobacillus plantarum* W21, *L. plantarum* W62, *Lactobacillus rhamnosus* W71, *Lactobacillus salivarius* W57, *Lactococcus lactis* W19) were obtained from Winclove BioIndustries BV, Amsterdam, The Netherlands. Bacteria were frozen at -80°C in RPMI 1640 (Gibco) supplemented with 1% glutamine (Gibco). For each experiment, a vial was thawed, diluted to the correct concentration and co-cultured with PBMC. Viability and bacterial cell number of the strains used was assessed by FACS using the BacLight-kit from Molecular Probers (Eugene, OR, USA).

For rtPCR, $CD4^+$ T cells were isolated from PBMC after 6 days of culture using a CD4 T cell enrichment kit (BD Bioscience). CD4 T cell purity was >95%. Cells were subsequently lysed in Tripure (Roche Diagnostics, Basel, Switzerland) and frozen at -80°C.

rtPCR

mRNA was isolated from lysed $CD4^+$ T cells and cDNA prepared using the iScript cDNA synthese kit from BioRad (Hercules, CA, USA). cDNA was frozen

at -20 °C till use for rtPCR. Primers for β2M (Fw: CCAGCAGAGAATGGAAAGTC, Rv: GATGCTGCTTACATGTCTCG), FOXP3 (Fw: TCAAGCACTGCCAGGCG, Rv: CAGGAGCCCTTGTCGGAT), RORγT (Fw: AAGACTCATCGCCAAAGCAT, Rv: TCCACATGCTGGCTACACA), GATA3 (Fw: CTGCAATGCCTGTGGGCTC, Rv: GACTGCAGGGACTCTCGCT) and T-bet(Fw: CCCCAAGGAATTGACAGTTG, Rv: GGGAAACTAAAGCTCACAAAC) were obtained from TIB Molbiol (Berlin, Germany). PCR reaction was performed with the LightCycler system from Roche, using SYBR Green I kit or SYBR Green FastStart kit from Roche. Ct values were compared to values obtained from mRNA samples isolated from tetanus toxoid stimulated PBMC.

Flow cytometry

After 6 days of culture, CD25 depleted PBMC were FACS stained with CD3-PE Cy7 (Biolegend, San Diego, CA, USA), CD4-APC (eBioscience, San Diego, CA, USA), FOXP3-PerCP Cy 5.5 (clone PCH101, eBioscience), RORγT-PE (eBioscience), T-bet-FITC (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and GATA3-PE (BD Bioscience). In other experiments, after 6 days of culture, cells were stimulated with phorbol 12-myristate 13-acetate (PMA, 5 ng/ml) and Ionomycin (1 mg/ml) in the presence of monensin (Golgistop, BD Biosciences) for 4 h. Subsequently cells were intracellular FACS stained for IL-10-APC (Biolegend), IL-17a-Alexa Fluor 488 (eBioscience), IFN-γ-PE (BD Bioscience) or IL-4-PE (BD Bioscience).

Cells were analysed using a FACS Canto with FACS Diva software (BD Biosciences).

Statistics

Statistical significance was tested using Prism 4.0 for Macintosh with a Kruskal-Wallis test. $p < 0.05$ was considered significant. Dunn's multiple comparisons test was used to identify significant differences between strains. Correlations between experiments were tested with Spearman's rank correlation.

RESULTS

In order to investigate the immune modulatory properties of strains with a high throughput method, PBMC from healthy volunteers were co-cultured with the strains in a 1:10 ratio. After 6 days of culture, $CD4^+$ T cells were isolated and lysed. Subsequently, the expression of transcription factors specific for $CD4^+$ T cell subsets was investigated by real time PCR. FOXP3, T-bet, GATA3, and RORγT are thought to be specific for Treg, T_H1, T_H2 and T_H17 cells respectively.(16) The expression of the transcription factor mRNA of co-cultured PBMC was subsequently compared to the expression in the absence of bacteria in the culture medium. As shown in Figure 1A-D, many differences between strains were observed. Some strains seem to activate

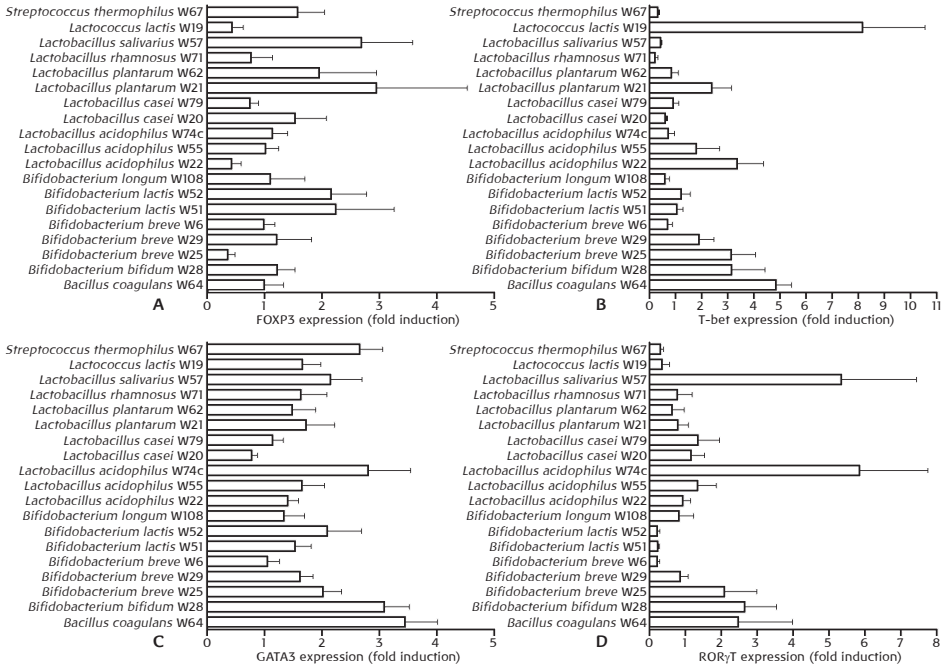


Figure 1. Transcription factor mRNA in CD4$^+$ T cells after culture. Fold induction of the transcription of mRNA of FOXP3 for Treg (**A**), T-bet for T_H1 (**B**), GATA3 for T_H2 (**C**), and RORγT for T_H17 (**D**) of CD4 T cells in PBMC co-cultured with probiotic strains compared to cells cultured in medium alone. CD4 cells were isolated from PBMC after 6 days of culture, lysed, and the transcription of specific mRNA compared to β2M mRNA transcription by rtPCR. Differences between strains in transcription of mRNA were significant for all genes tested ($P < 0.01$, Kruskal-Wallis), n = 5.

different subtypes of T helper cells (e.g. *L. salivarius*, FOXP3 and RORγT, Figure 1A, C), while others specifically induce one subtype (e.g. *L. lactis* W19, T-bet, Figure 1B). Although the differences between the strains were significant for all individual transcription factors (Kruskal-Wallis, $p < 0.01$), the magnitude of the differences varied. The differences in GATA3 expression were less pronounced than the differences in for example T-bet and RORγT mRNA expression. No post-hoc tests were performed as the Bonferroni correction with these many strains and comparisons would reduce the probability of finding significant differences.

mRNA expression of the transcription factors defining the CD4 T cells subsets is a good indication for the fate of the T cells. However, mRNA does not necessarily lead to stable protein expression. In order to show that different T cell subsets are actually induced in the co-cultures, we subsequently measured transcription factor protein expression by intracellular FACS staining with a smaller number of strains. For these experiments we selected strains that showed an interesting profile in the expression of mRNA: *L.*

plantarum W21 and *B. lactis* W52 for FOXP3 mRNA induction, *S. thermophilus* W67 for GATA3, *L. lactis* W19 T-bet, *L. salivarius* W57 both FOXP3 and RORγT. CD25 depleted PBMC were co-cultured with these strains for 6 days. At day 6 FACS staining was performed.

Figure 2A-D shows the percentages of $CD3^+CD4^+$ T cells positive for the protein of the specific transcription factor after culture with the strains mentioned. Most of the strains that induced mRNA of a specific transcription factor also induced expression of the protein. The strains *L. plantarum* W21 and *L. salivarius* that induced FOXP3 mRNA also induced high percentages of $FOXP3^+$ CD4 cells. *L. lactis* W19 and *L. salivarius* W57 also showed a similar pattern in mRNA and protein expression for T-bet and RORγT expression respectively. On top of that, significant differences between strains were observed in the induction of specific T cell subsets. *L. salivarius* W57 was shown to be the most potent inducer of $FOXP3^+$ cells, whereas *L. lactis* W19 induced the most $T\text{-}bet^+$ cells. These two strains induced the most $ROR\gamma T^+$ cells as well.

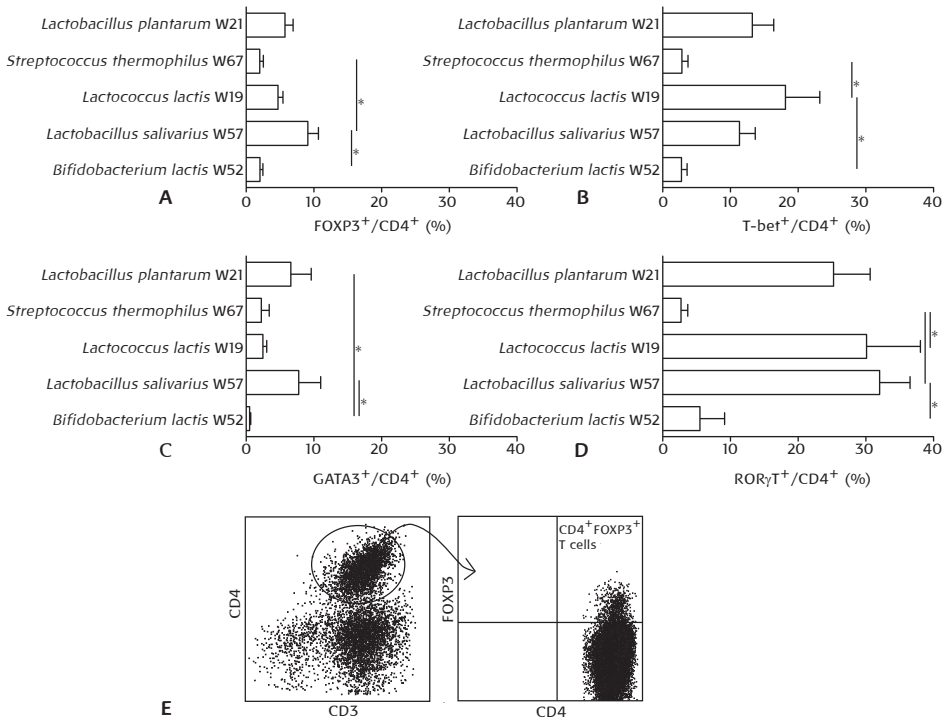


Figure 2. Transcription factor protein in $CD4^+$ T cells after culture. Percentage of $FOXP3^+$ (**A**), $T\text{-}bet^+$ (**B**), $GATA3^+$ (**C**), and $ROR\gamma T^+$ (**D**) $CD3^+CD4^+$ T cells. After 6 days of culture of CD25 depleted PBMC, cells were FACS stained for transcription factors. *, significant difference according to Dunn's multiple comparisons test. Gating strategy is shown in **E**. n = 6.

Most CD4$^+$ T cells produce cytokines in order to perform their biological function. For each T helper cell subset, the profile of cytokines produced is different (IL-10 for cells with a regulatory phenotype, IFN-γ, IL-4 and IL-17 for T_H1, T_H2, T_H17 cells respectively). To show that the T cells induced in our previous experiments really produce their specific effector molecules, we again co-cultured CD25 depleted PBMC with the selected strains and performed FACS staining after 4 h incubation with PMA/Ionomycin and monensin. Figure 3A-D shows that most cytokine profiles are comparable with the profiles of transcription factor mRNA and protein. Specifically, the strains that induced protein expression of the transcription factors also induced specific cytokine production. This is nicely shown for T-bet expression and IFN-γ production, and for RORγT and IL-17 production. The strains that significantly induced the highest percentage of T-bet$^+$ and RORγT$^+$ cells, also did so for the corresponding cytokines. GATA3 expression is less in accordance with IL-4 production. Finally, *L. plantarum* W21 and *L. salivarius* W57 induce both FOXP3$^+$ cells and IL-10 producing CD4 T cells although there was no significant difference found for the IL-10 producing cells.

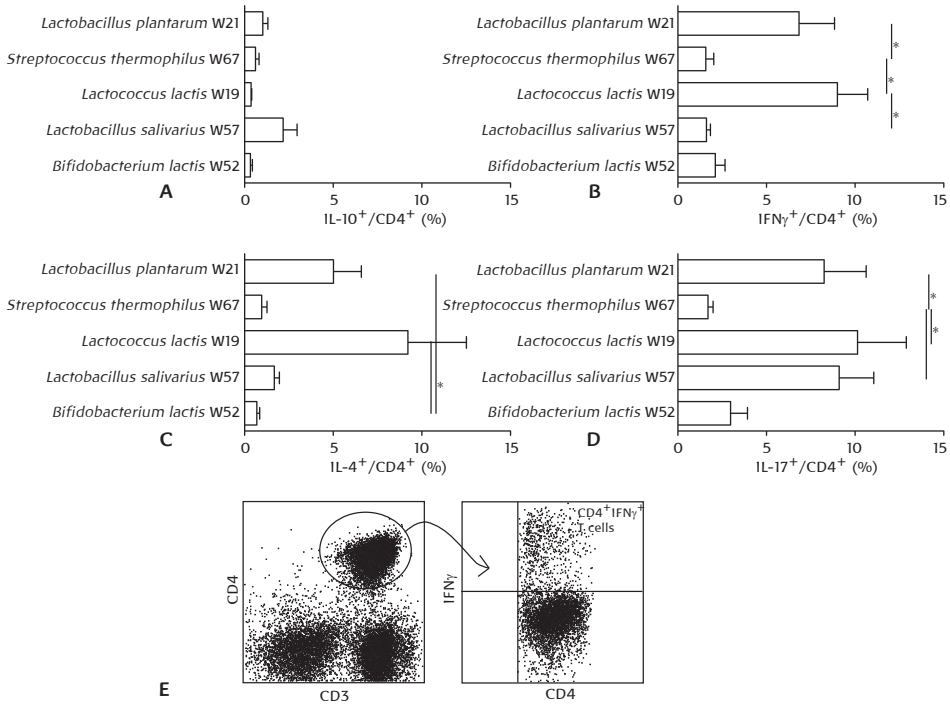


Figure 3. Cytokine production by CD4$^+$ T cells after culture. Percentage of IL-10$^+$ (**A**), IFNγ$^+$ (**B**), IL-4$^+$ (**C**) and IL-17$^+$ (**D**) CD3$^+$CD4$^+$ T cells. After 6 days of culture of CD25 depleted PBMC, cells were restimulated with PMA/Ionomycin for 4 h in the presence of monensin. Cells were subsequently FACS stained for intracellular cytokines. *, significant difference according to Dunn's multiple comparisons test. Gating strategy is shown in **E**. n = 6.

In order to show that our methods are consistent with each other, we performed a Spearman's rank correlation test with the strains that were tested with all methods. We analysed the correlation between the percentage of cytokine producing cells and the upregulation of mRNA for the specific transcription factor or the percentage of transcription factor positive cells measured by FACS (Figure 4). Although for most correlations significance was not reached, the methods used to test the induction of Treg, T_H1 and T_H17 cells all have a positive relation (Spearman $\rho > 0$). The measurements on the induction of T_H2 cells however also showed a negative correlation (Spearman $\rho < 0$) between GATA3 mRNA and IL-4 producing cell percentage, indicating that our methods were not consistent for this cell type.

As shown in Figure 4A, we observed a considerable inter-donor variation with most techniques used. Therefore, we constructed a heat map to find out whether most donors had a comparable pattern of reaction towards the different strains (Figure 4B). Indeed, some donors react more or different than others. However, most donors show consistent reaction towards the strains, forming clusters of differentiation towards a certain T helper subtype. This was observed for all strains and techniques used.

DISCUSSION

Strains of human gut derived lactic acid bacteria have potent capacities to influence the immune system when used as a supplement. Each strain seems to be unique in the subtypes of CD4 T cells that it promotes.We and others already showed this for Treg induction(15) and for the production of cytokines.(17) We now extend these observations by showing that lactic acid bacteria significantly differ in their capacity to promote T_H1 cells, but also T_H2, Treg and T_H17 cells. This finding adds important information to the concept of probiotic use as a food supplement for the prevention or treatment of immune mediated diseases such as allergies and auto-immune diseases. Our data suggest that not any strain of probiotic bacteria is able to mount a favourable immune response for any disease and that the strain used can make an important difference for the outcome of clinical experiments. For example, it is likely that the inconsistencies in the outcome of studies such as on the prevention of atopic disorders is due to subtle differences in the strains used.(18-21) Studies conducted with the same strain have more consistent outcome.(22)

As it is important to monitor the immune influencing capacities of strains, a reliable high throughput method is a helpful tool to screen for wanted and unwanted effects of probiotics. Co-culture of strains with PBMC and subsequent transcription factor mRNA analyses seems suitable for this purpose. The activation or induction of T cell subtypes was shown consistently on several levels; the transcription factor mRNA content, transcription factor protein as well as cytokine production. These methods correlated well for

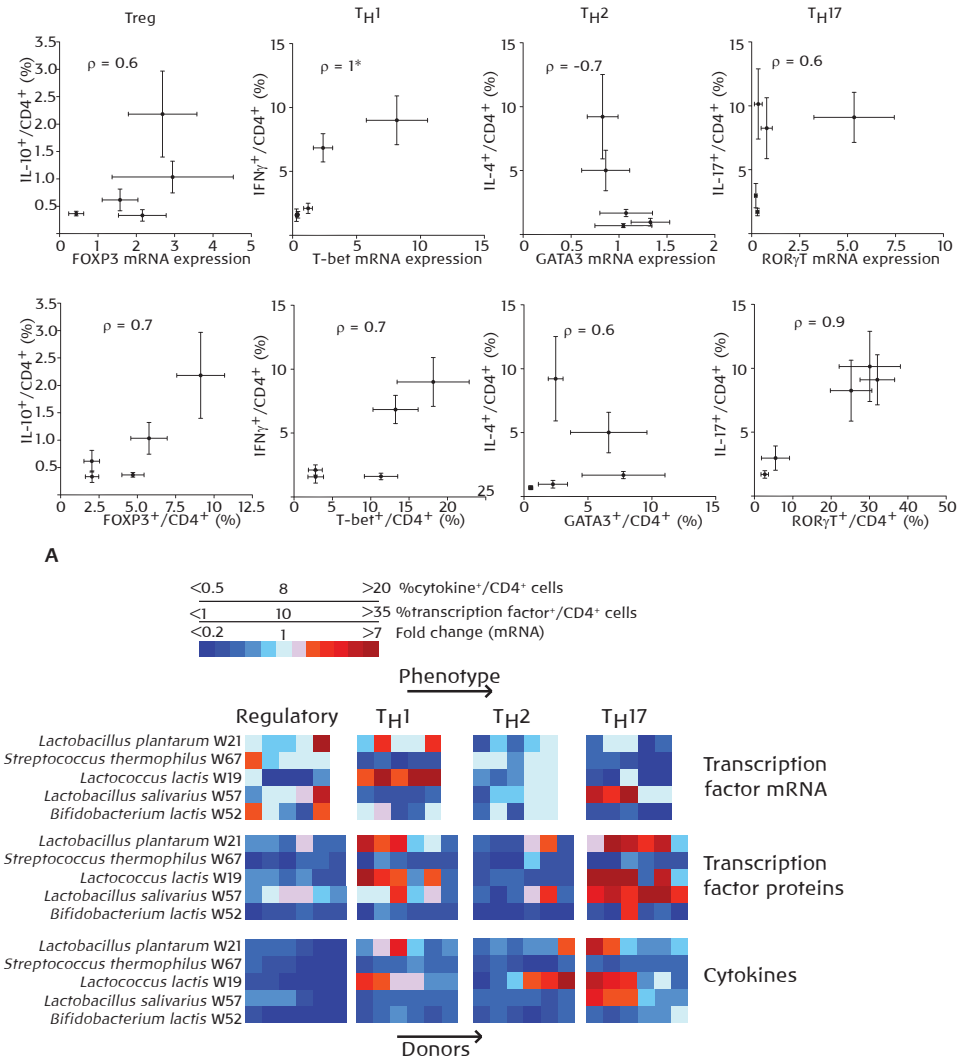


Figure 4. Analysis of correlations and donor variability. Correlation between expression of transcription factor mRNA and percentage cytokine producing cells (**A**, upper part) and percentage of transcription factor positive cells and percentage cytokine producing cells (**A**, lower part). Spearman ρ is given as a measure of correlation. *, significant correlation according to Spearman's signed rank test. n = 5 for mRNA data and n = 6 for FACS data of transcription factor expression and cytokine production. Heat map (**B**) indicating the responses of all donors towards the strains used measured by transcription factor mRNA PCR (FOXP3, T-bet, GATA3, RORγT respectively), transcription factor protein FACS (FOXP3, T-bet, GATA3, RORγT respectively) or cytokine FACS (IL-10, IFNγ, IL-4, IL-17 respectively) as depicted in the figures 1-3. The legend indicates the values corresponding to the colours for each technique

most T cell subsets, making all methods used in this research possible candidates for high throughput measurements of large numbers of strains, depending on a laboratory's possibilities.

Most *in vitro* analyses of immune modulating properties of bacterial strains are performed by measurements on cytokines in the supernatants of PBMC or other cell cultures in the presence of bacteria.(17, 23) Up- or downregulation of surface markers on dendritic cells is also a common approach.(13) Although these methods definitely have benefits (broad scan of immune responses), the response of the adaptive immune system needs to be addressed as well. T cells are likely candidates for local immune activity outside the gut making these cells the targets in settings as for example eczema and asthma. Analysis of T cell responses as described here can perfectly be combined with analysis of cytokines in the supernatant of the same culture, increasing the value and reliability of the screening.

Because of high variability between donors, and the use of different donors for each experiment, the correlations were not significant for all tests. Nevertheless, the correlation was shown to be positive for Treg, T_H1 and T_H17 cell induction, indicating that the strains that induce mRNA of a specific T cell type will also induce a high percentage of specific cytokine producing cells. Our methods showed not to be suitable for the measurement of T_H2 cell induction by probiotics. This can be due to the fact that GATA3 is known to be necessary for more cellular processes than T_H2 differentiation,(24) or timing of the measurements. Of course, each mRNA, and protein has its own kinetics, making it hard to measure all processes with a few tests on a few time points. Possibly the timing for T_H2 mRNA and proteins was suboptimal.

Although strains have a clear tendency to induce either subtype of T helper cell, this effect is not shown with identical strength for all donors tested, resulting in considerable variation in our results. This finding, as depicted in Figure 4B, adds another layer to the complex field of immune modulation by probiotics. Possibly the variation in the human population in reactivity towards a certain bacterial strain warrants a personalized approach. Before supplementing a patient with a certain strain, the quality and quantity of the patients T cell response towards this strain can be determined with either of the described methods. On the other hand, supplementation with a probiotic mixture (e.g. a multi-species probiotics) of strains with a certain immune modulating capacity might be beneficial for a broader group of individuals. (25) *In vitro* analysis of T cell responses towards such a mixture would be of great interest.

Whether the induction of different T cell subsets is also consistent *in vivo* and whether these cells are able to influence immune mediated diseases still remains to be tested. It has been shown by several authors that *in vitro* experiments do not necessarily correlate with clinical outcome.(26, 27) Nevertheless, application of probiotic strains without proper knowledge on their immunological capacities can also have unwanted effects.(28) *In vitro*

research using human PBMC cultures can give a good indication, although this does not perfectly mimic the *in vivo* situation. Both *in vitro* and *in vivo* parameters should be selected carefully in order to find and correlate effects of probiotics. Testing the strains used here in several experimental models including colitis and atopic dermatitis models, and comparing the results to our *in vitro* data would be of great interest. Treg inducing strains will likely have a beneficial effect on these diseases, whereas T_H1, T_H17 or T_H2 inducing strains might aggravate disease.

There is still much to learn regarding the determinants of the diverse immune responses elicited by different bacterial strains. The identification of closely related strains that differ in the type of immune response they elicit is of special interest. In our study *L. acidophilus* W74c and *L. acidophilus* W22 showed remarkable differences in their capacity to induce RORγT. Further screening of the genomes of these strains using micro-array might give more insight in the specific gene(s) responsible for the immune modulation. That the type of immune response induced by gut derived bacteria can depend on one single molecule has elegantly been shown by a study of Mazmanian *et al.*(29) In this study it was demonstrated that *Bacteroides fragilis* depends on polysaccharide A (PSA) to protect against inflammation in an experimental colitis model and that PSA is sufficient for the protective effect. Also, the capacity of strains to ligate certain receptors expressed by the innate immune system can differentiate between activation of the immune system. Attachment to DC-SIGN expressed by DC has been shown to prime DC to induce $FOXP3^+$ regulatory T cells.(30) The differences we observe in *in vitro* immune modulation between strains might also be partially explained by ligation of DC-SIGN or other receptors.

We conclude that to make well-founded choices on the type of probiotic strains to be used for clinical application, *in vitro* monitoring of the immunological effects of the strains using a high throughput method is highly recommendable.(15) By building on our current knowledge of strain specific immune modulatory effects it may become possible to design clinically effective, bacteria based strategies to maintain and promote health.

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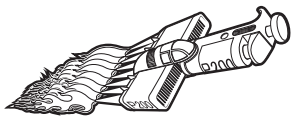

CHAPTER 5

Critical role for programmed death 1 signaling and protein kinase B in augmented regulatory T-cell induction in cord blood

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In newborns the induction of productive immune responses is generally blunted in comparison with adults, resulting in tolerogenic immune reactivity(1) This immune status results in an increased potency to engraft neonatal animals, ineffective vaccination responses in newborns, and reduced occurrence of graft-versus-host disease when cord blood (CB)-derived allografts are used.(2, 3) We hypothesized that forkhead box protein 3 (FOXP3)-positive regulatory T (Treg) cells are pivotal in this phenomenon because these cells are key players in immune homeostasis. (4) When comparing the number of $FOXP3^+$ cells in human CB and adult peripheral blood (APB), we found less $FOXP3^+$ cells in CB than APB (Figure 1, A, *ex vivo*, uncultured cells). However, when naive ($CD25^-CD45RO^-$) T cells were activated by plate-bound anti-CD3, significantly more $FOXP3^+$ T cells were induced from CB precursors. Remarkably, we only observed this difference between CB and APB when viable antigen-presenting cells (APCs; T cell-depleted CB or APB mononuclear cell fraction) were included in the culture; replacement with irradiated APCs abrogated the effect, whereas costimulation by soluble anti-CD28 showed a less pronounced difference (Figure 1, A). Induced CB $FOXP3^+$ T cells (sorted as $CD4^+CD25^+CD127^{low}$ cells) were able to suppress dose dependently the proliferation of both CD4 and CD8 effector T cells when cultured together in different ratios (not shown), confirming the Treg nature of these cells. Thus on the first activation, CB T cells have a tendency to become functional $FOXP3^+$ Treg cells.

To substantiate the role of CB APCs in the induction of high percentages of Treg cells, we activated naive T cells in an alloreaction with APCs. Again we observed a higher number of Treg cells when CB T cells were cultured. CB APCs were able to induce higher numbers of Treg cells than APB APCs from both CB and APB precursor T cells (Figure 1, B). Nevertheless, CB T cells always contained higher percentages of Treg cells, indicating a T cell intrinsic mechanism as well.

We investigated the percentages of different APC populations because the APCs used in our previous experiments consisted of a mixture of different cell types. In both APB and CB, we found comparable numbers of conventional dendritic cells ($HLA\text{-}DR^+CD11c^+$) and plasmacytoid dendritic cells ($HLA\text{-}DR^+CD123^+$), with slightly more $CD14^+$ monocytes in APB (not shown). When we sorted different subsets as APCs and cultured them with anti-CD3-activated T cells, we observed that all APC subsets induced more Treg cells from CB than from APB precursor cells (Figure 1, C).

The augmented Treg cell induction was not correlated with a reduced proliferation of CB T cells, which we showed to be identical in CB and APB by using carboxyfluorescein succinimidyl ester dilution assays (data not shown). Moreover, we found no difference in the kinetics of FOXP3 upregulation when we measured the percentage of $FOXP3^+$ cells daily; both APB and CB showed a peak of $FOXP3^+$ cells around 4 days of culture, after which the percentage of $FOXP3^+$ cells decreased to stable expression at day 6 (Figure 1,

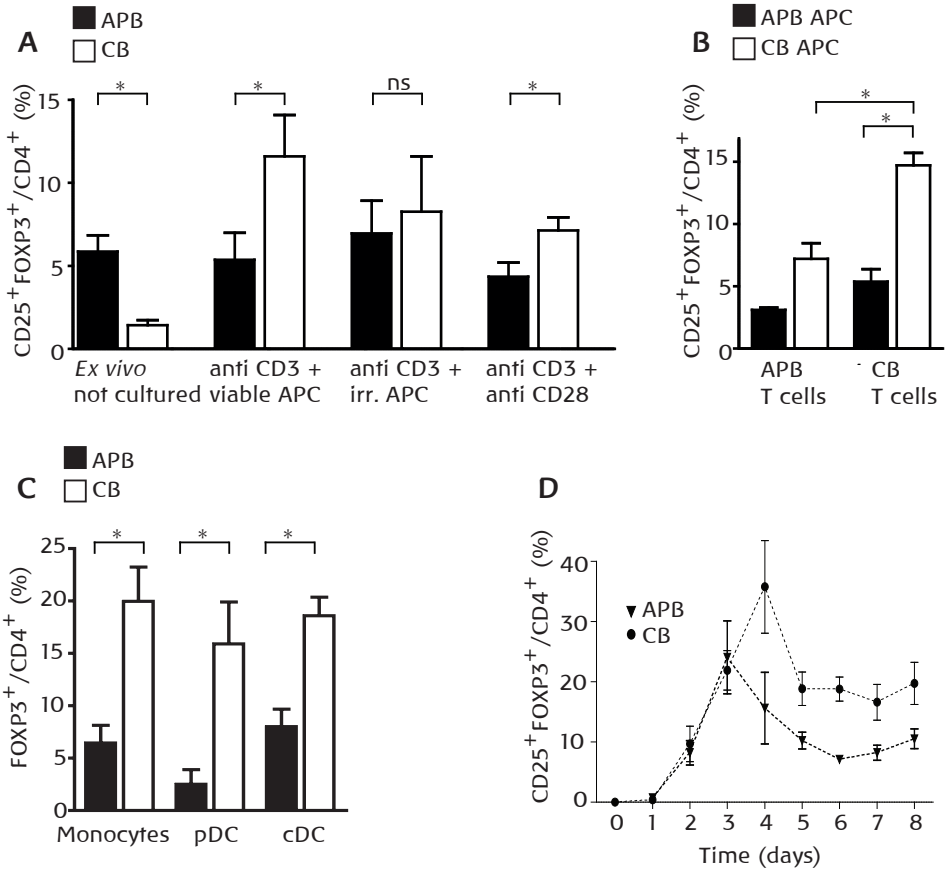


Figure 1. CB APCs stimulate Treg induction. A, FOXP3+ Treg cell percentages *ex vivo* or after 6-day naive ($CD4^+CD25^-CD45RO^-$) T cell stimulation, as indicated in the figure (left to right, n = 3, 6, 4, and 10). ns, not significant. **B**, Treg cell percentage after 6-day alloreaction between naive T cells and APCs (n = 4). **C**, Treg cell percentage of anti-CD3–activated naive T cells after 6-day culture in the presence of sorted indicated APCs (left to right, n = 5, 3, and 3). cDC, Conventional dendritic cells; pDC, plasmacytoid dendritic cells. **D**, Time course of Treg cell expansion of anti-CD3–activated naive T cells cultured with viable APCs (n = 4). *$P < .05$.

D). Both APB and CB cells had a similar stable FOXP3-expressing population compared with the initial peak around day 4; 65% of the FOXP3+ T cells at day 4 remain stable FOXP3-expressing cells during the 8-day culture.

The induction of Treg cells in the periphery on activation can be mediated by several factors. Cytokines like TGF-β and IL-2 are known to induce FOXP3,(5) whereas inflammatory cytokines prevent this. To test whether possible differences in secreted mediators, such as cytokines and chemokines, can explain the difference in Treg cell induction, we exchanged culture supernatants between CB and APB every day during the 6-day culture

period of naive T cells with APCs and anti-CD3. This approach did not result in altered differences between CB and APB Treg cell induction, making a role for secreted mediators unlikely (not shown).

Another factor influencing FOXP3 induction is the strength of the T cell receptor (TCR) signal and costimulation.(6, 7) In our settings the TCR signal was standardized by plate-bound anti-CD3, but costimulation is provided by the APCs. High costimulation through CD28 signaling prevents the upregulation of FOXP3. We therefore investigated the role of the CD28 ligands CD80 and CD86 on APCs.We observed no difference in the expression of these molecules. When blocking antibodies toward CD80, CD86, or both were added to the cultures, this did not result in a difference in FOXP3 expression (Figure 2, A). This finding was confirmed by adding increasing concentrations of cytotoxic T lymphocyte-associated antigen 4-immunoglobulin (CTLA4-Ig) to the cultures, blocking both CD80 and CD86, which had no obvious effect (data not shown).

Inhibition of downstream signaling pathways of TCR and CD28 on T cell activation is able to promote FOXP3 induction as well. The phosphoinositide 3-kinase/protein kinase B (PKB) pathway is central in T cell differentiation. (8) Prevention of activation of this pathway by limited costimulation or signaling through inhibitory molecules, such as programmed death 1 (PD-1), promotes FOXP3 upregulation.(7,9) We hypothesized that differential downstream signaling on activation might be pivotal to the high levels of FOXP3 found on day 6 of activation and onward (Figure 1, D). We therefore investigated the role of PD-1 and PKB in the induction of FOXP3 in CB-naive T cells. On activation, CB $CD4^+$ T cells have an increased expression of the PD-1 molecule compared with APB (Figure 2, B). When we blocked the interaction between PD-1 and its ligand PD-L1 by including a blocking mAb to PD-L1 in the culture, a significant reduction in the percentage of $FOXP3^+$ T cells was observed (Figure 2, C), isotype mAb had no effect (not shown). A PD-1-blocking mAb showed comparable results. As a consequence, the phosphorylation status of PKB on activation by different concentrations of anti-CD3 was lower for CB than APB cells (Figure 2, D). Additionally, CB T cells required approximately 10-fold more TCR triggering than APB T cells before an increase in PKB phosphorylation was observed.

Shortly after birth, the immune system of the newborn encounters all kinds of neoantigens. The low percentages of Treg cells found *ex vivo* in CB are not likely to play a major role in maintaining tolerance to these neoantigens. However, we here show that PD-1 signaling in CB T cells facilitates their differentiation into induced functional $FOXP3^+$ Treg cells through a mechanism involving reduced PKB signaling. This phenomenon might well represent a mechanism that is developed to ensure active tolerance in the neonatal immune system.

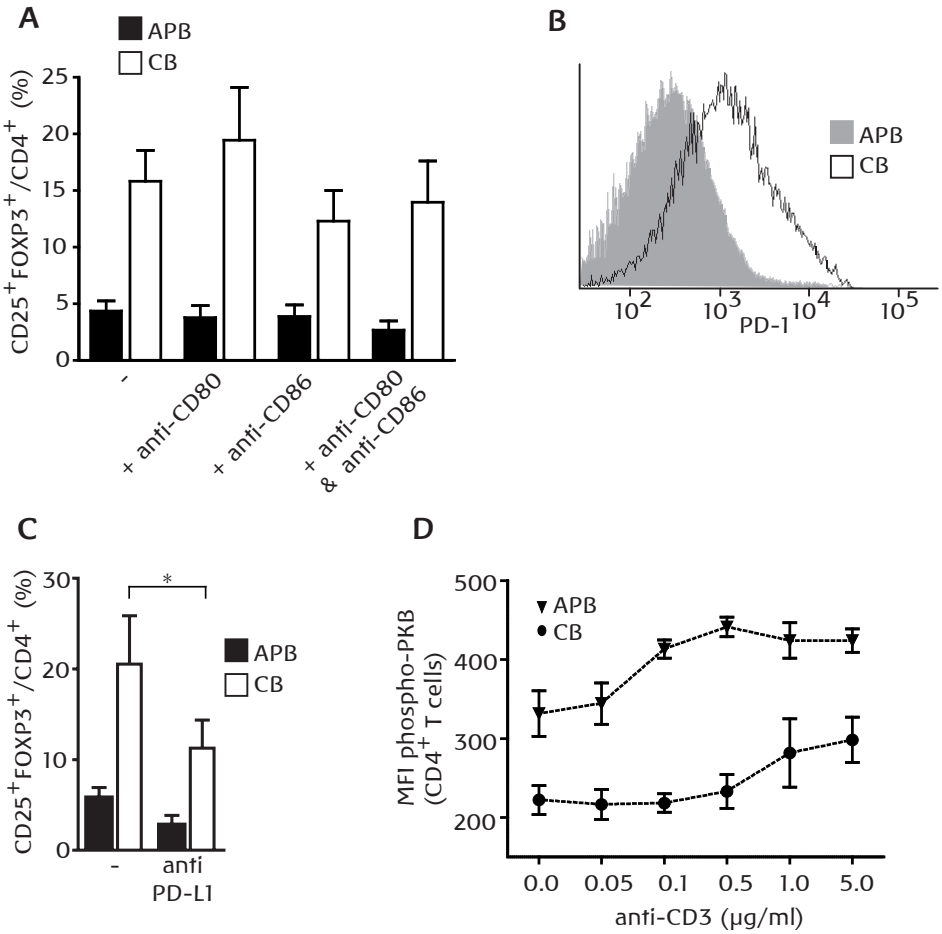
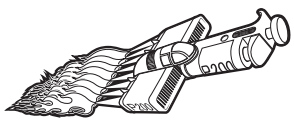


Figure 2. PD-1/PD-L1 interaction stimulates Treg cell induction. A, Treg cell percentage after 6-day stimulation of naive T cells in the presence of anti-CD3/viable APCs with blocking mAb as indicated (n = 6). **B**, PD-1 expression on 6-day anti-CD3–activated T cells in the presence of viable monocytes. **C**, Treg cell percentages of anti-CD3–activated naive T cells with viable monocytes in the presence of blocking mAb (CB, n = 11; APB, n = 3). **D**, Relative phosphorylated PKB levels in $CD4^+$ T cells cultured with a graded amount of plate-bound anti-CD3 (n = 3). *$P < .05$.



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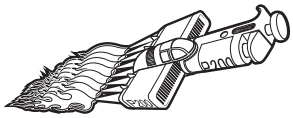
CHAPTER 6



T cell fate decision between human peripherally induced FOXP3^{+} regulatory T/T_H17 cell involves a signal downstream of RORC gene expression

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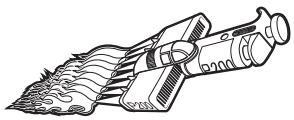
ABSTRACT

A well-balanced $CD4^+$ T cell repertoire is important for immunological health, as is supported by the exacerbating role of T_H17 cells and protective role of regulatory T cells (Treg) in inflammatory autoimmune diseases such as rheumatoid arthritis. The mechanisms that govern the cell fate decisions in human naïve T cells have remained incompletely understood. The maturing immune system provides a means to study cell fate decision between T_H17 and Treg: naïve $CD4^+$ T cells in human cord blood have increased propensity to differentiate into Treg, while being restrained in T_H17 lineage commitment, compared to adult counterparts. We here investigated the mechanisms involved in human $CD4^+$ Treg/T_H17 T cell commitment, by use of CD4 T cell cultures from cord blood (CB) and adult peripheral blood (APB). We show that the cytokines IL-23, IL-1β, and IL-6 control the human Treg/T_H17 balance, as they promote T_H17 cells while simultaneously inhibiting Treg differentiation. However, we show a differential reaction of CB and APB towards these cytokines, suggesting the existence of T cell intrinsic factors. One such factor operates downstream of RORC and upstream of IL-17 cytokine release, as we observed RORC transcript without IL-17 production in CB. Taken together, we propose that in humans, the Treg/T_H17 balance involves not only exogenous factors (i.e., cytokines), but is further controlled by a $CD4^+$ T cell intrinsic factor, for directing cell differentiation into either the T_H17 or Treg lineage.



INTRODUCTION

Regulatory T cells (Treg) are important mediators of immune homeostasis, whereas T_H17 cells facilitate immune activation and are associated with pathology in auto-immune disorders including rheumatoid arthritis(1-3). The switch between T_H17 and Treg cell development is therefore an important issue in clarifying disease mechanisms and also the treatment of auto-immunity. Peripheral induction of Treg is closely related to the induction of T_H17 cells, as both lineages are dependent on TGF-β. The lineage defining transcription factors RAR-related orphan receptor(ROR)γT and Forkhead box (FOX)P3 are both upregulated in naïve T cells upon activation in the presence of TGF-β, but FOXP3 is predominates and down regulates RORγT unless inflammatory cytokines are present. IL-6 is indispensable in mice for T_H17 induction(4, 5), in humans IL-1β, IL-6 and IL-23 are necessary for the induction and propagation of this cell type(6, 7).

It is still under debate whether T_H17 cells derive from any naïve cell when activated in the correct cytokine milieu, or whether a precursor cell exists. In humans, $CD161^+$ $CD4^+$ T cells are such precursors to T_H17 lineage cells(8, 9). While TGF-β, IL-1β, IL-6 and IL-23 facilitate T_H17 cell development, additional exogenous factors play a role as well; Retinoic acid prevents T_H17 development via Smad3 signaling(10) and induces Treg cells(11, 12). The cell's intrinsic metabolic pathways also influences T_H17 and Treg development; activation of murine cells which results in glycolytic metabolism directs its fate towards T_H17 and prevents Treg induction(13). Mice knockout for the transcription factor that regulates the metabolic switch from oxidative phosphorylation to aerobic glycolysis in hypoxic conditions, Hypoxia Inducible Factor (HIF)-1α, show an increased Treg induction(13).

HIF-1α now appears to have an additional steering role in Treg/T_H17 lineage control(14). HIF-1α regulates the transcription of RORC (encoding RORγT-protein) in cooperation with STAT3, thereby promoting T_H17 cell development. Additionally, HIF-1α binds to FOXP3-protein and ubiquinates it, thereby directing FOXP3 protein towards proteosomal degradation. The presence of HIF-1α is therefore considered to be a T cell intrinsic switch for either Treg or T_H17 cell induction, at least in mice.

The role of hypoxia in this phenomenon is not entirely clear. Certain tissues that are more hypoxic might steer ongoing antigen-driven immune responses towards T_H17 cell differentiation. Indeed, T_H17 induction and activation seems to occur largely in the small intestines(15). Production of reactive oxygen species (ROS) can also induce HIF-1α, and mediate signal transduction ultimately resulting in the induction of effector T cells(14). Whether ROS induces or prevents T_H17 cells is not entirely clear, as cells derived from a NOX2 knockout mouse produce less ROS, especially in phagocytes, but show an increase in T_H17 induction with a concomitant decrease in Treg(16).

We hypothesized that a T cell intrinsic factor regulates the induction of T_H17 and Treg cells in humans, besides the cytokine signals that are already known to bias Treg/T_H17 cell fate decision. In order to test this, we compared naïve T cells derived from human umbilical cord blood (CB) that do not produce IL-17 upon activation with naive T cells from adult peripheral blood (APB). We show that addition of T_H17 inducing cytokines does not result in IL-17 production in CB, but rather prevents the upregulation of FOXP3. On the other hand, blockade of T_H17 inducing cytokine signaling results in reduced IL-17 production in APB without a concomitant increase in Treg numbers. These findings suggest the involvement of a human T cell-intrinsic factor, which acts downstream of RORC as the mRNA of this transcription factor is upregulated in cells from both origins.

METHODS

Cells

Cord blood samples were obtained from normal deliveries of full-term neonates (approved by the local Medical Ethical Committee of the University Medical Centre Utrecht). Cord blood mononuclear cells (CBMC) and peripheral blood mononuclear cells (PBMC) from healthy adult volunteers were isolated using Ficoll-Isopaque density gradient centrifugation (Ficoll-Paque, Pharmacia, Upsalla, Sweden). Naïve $CD4^+$ T cells were selected as $CD4^+CD25^-CD45RO^-$ lymphocytes by sorting with Magnetic Activated Cell Sorting (MACS) magnetic beads. $CD4^+$ T cell enrichment kit (BD Bioscience, San Jose, CA, USA), CD25-beads (BD Bioscience) and CD45RO-beads (BD Bioscience) were used. APC were enriched by CD3-bead-assisted T cell depletion from CBMC or PBMC (BD Bioscience). Population purity was > 95%.

Culture

Naïve T cells were cultured in RPMI 1640 (Gibco, Breda, the Netherlands), supplemented with 1% L-glutamine (Gibco), 100 mg/ml streptomycin, 100 U/ml penicillin (Gibco)) and 10% human AB-serum. $5.0x10^4$ T cells per well of a 96 well plate were cultured with $1.0x10^5$ APC. Cells were activated by plate bound anti-CD3 (1.0 µg/ml or mentioned in the figure legends; clone OKT3, eBioscience, San Diego, CA, USA) in the presence of 30 units IL-2/ml (Novartis, Arnhem, The Netherlands) and cultured for 5 or 6 days. Recombinant human cytokines were added to the cultures: 10 ng/ml IL-1β (Miltenyi Biotec Bergisch Gladbach, Germany), 50 ng/ml IL-6 (BD Bioscience) and/or 10 ng/ml IL-23 (BD Bioscience). Cytokine signaling was blocked for IL-10 with 5µg/ml anti-human IL-10 (JES3-9D7; eBioscience), 1µg/ml IL-1receptor antagonist (IL-1RA, Anakinra, Kineret®) and/or 1µg/ml IL-6 receptor blocker (Tocilizumab, RoActemra®). In some experiments, CD4 T cells were labelled with 1µM CFSE prior to culture.

Cytokines

Cell culture supernatants were collected each day of the cultures, or at the end of the culture period. Cytokine concentrations in the supernatants were determined by Luminex (Austin, TX, USA) Multiplex assay as described elsewhere(17).

Flow cytometry

Prior to intracellular cytokine stainings, cells were restimulated with PMA(20 ng/ml) and Ionomycin (1µg/ml) in the presence of Brefeldin A (Golgistop, BD Bioscience). Cells were subsequently stained with CD3 APC or CD3 PE-Cy7, CD4 PerCP Cy5.5, and CD25 FITC. After permeabilization with the appropriate buffers (eBioscience), FOXP3 eFluor and IL-17 PE were used. Cells were analyzed using a FACS Canto II (BD Bioscience) with FACS Diva software (BD Bioscience). Subsequent data analysis was performed using Flow Jo.

qPCR

Cells were lysed for mRNA isolation with Tripure (Roche Diagnostics, Basel, Switzerland) at several time points after start of culture. Primers for RORC were used and expression compared to Actin and GAPDH expression.
Actin, Fw: CATGTACGTTGCTATCCAGGC, Rv: CTCCTTAATGTCACGCACGAT
GAPDH, Fw: GTCGGAGTCAACGGATT, Rv: AAGCTTCCCGTTCTCAG
RORC, Fw: AAGACTCATCGCCAAAGCAT, Rv: TCCACATGCTGGCTACACA

Statistics

Statistical relevance of the data was analysed using SPSS 15.0 for Windows. Specific tests are mentioned in the figure legends. $p \leq 0.05$ was considered significant.

RESULTS

Treg/T_H17 induction in APB and CB

CB naive $CD4^+$ T cells are known to differentiate into $FOXP3^+$ regulatory T cells (Treg) more easily than APB cells upon *in vitro* activation, especially in the presence of viable antigen presenting cells (APC)(18). As Treg and T_H17 cells share a reciprocal development pathway(5), we tested whether T_H17 differentiation is comparable in both APB and CB.

Upon activation by plate bound anti-CD3, APB naive $CD4^+$ T cells in the presence of viable APC differentiate in both IL-17 producing cells and FOXP3 expressing cells (Figure 1A). CB cells however only differentiate into Treg, negligible numbers IL-17 producing cells are found after 5 days of culture. Measurement of cytokines in the culture supernatant showed IL-17 production in APB cultures, but not in CB cultures. The regulatory cytokine IL-10 is more produced by CB cells (Figure 1B).

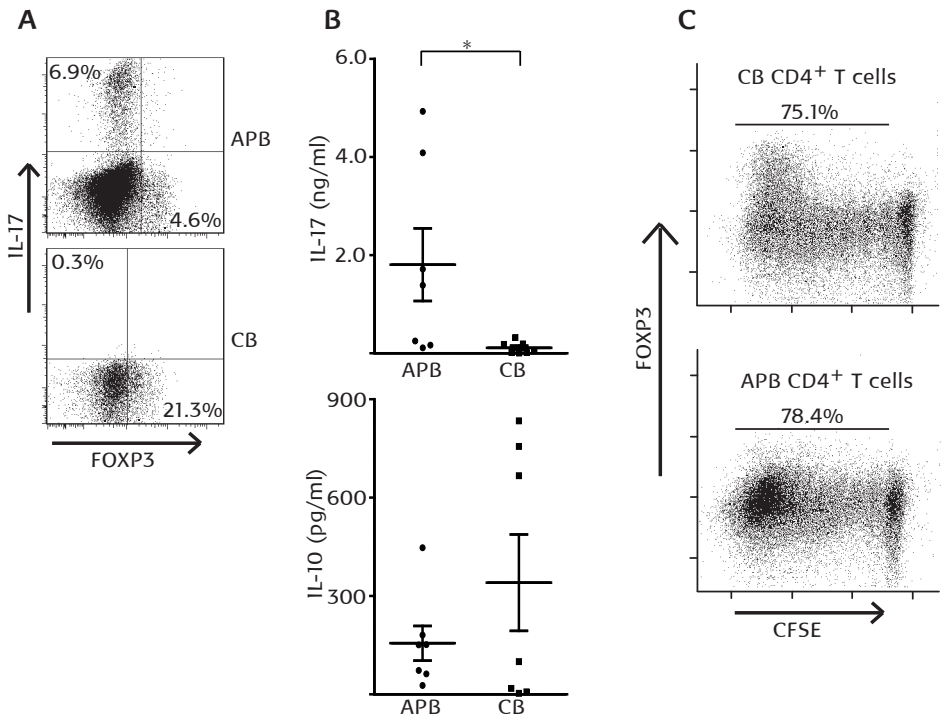


Figure 1: Differences in Treg and T_H17 cell induction between CB and APB cell cultures. 50.000 $CD4^+CD25^-CD45RO^-$ naïve T cells were cultured with 100.000 viable APC and activated by 1µg/ml plate bound anti-CD3. After 5 days of culture, cells were restimulated with PMA and Ionomycin and Brefeldin A was added for 4 hours. IL-17$^+$ and FOXP3$^+$ cell numbers were assessed by flow cytometry (**A**, representative for n=6). IL-17 and IL-10 were measured in culture supernatant of identically activated cells (without PMA-Ionomycin restimulation or Brefeldin A) (**B,** n=7 for IL-10, n=5 for IL-17). $CD4^+CD25^-CD45RO^-$ naïve T cells were CFSE labelled prior to culture and again activated by plate bound anti-CD3 and viable APC. FOXP3 expression and CFSE dilution were measured after 6 days of culture by flow cytometry (**C**). Significance of the differences was tested with a Mann Whitney U test.

To assess whether the differences in Treg and T_H17 differentiation could be explained by differences in activation status, CD4 cells were CFSE labelled prior to culture. No difference in proliferation could be observed (Figure 1C).

Production of T_H17 inducing cytokines

Treg cells are induced upon activation by TCR signaling in the presence of TGF-β while T_H17 cells are induced by additional IL-1β and/or IL-6 and are propagated by IL-23(4, 19, 20). We tested the presence of these T_H17 driving cytokines in the culture supernatants and found significantly less IL-1β and less IL-6 in CB cultures compared to APB cultures (Figure 2A & B). No difference in IL-23 production was found (not shown). IL-1β and IL-6

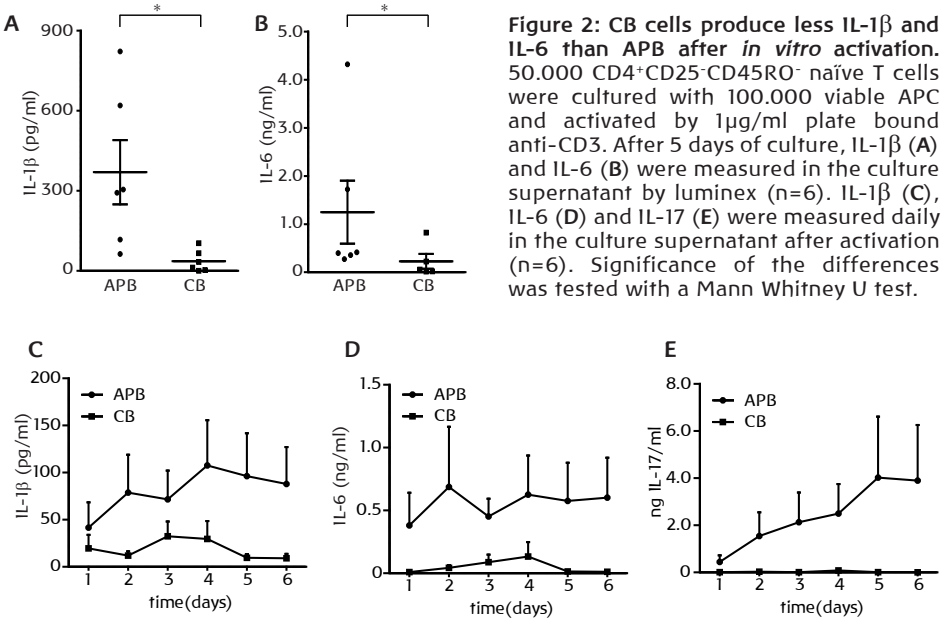


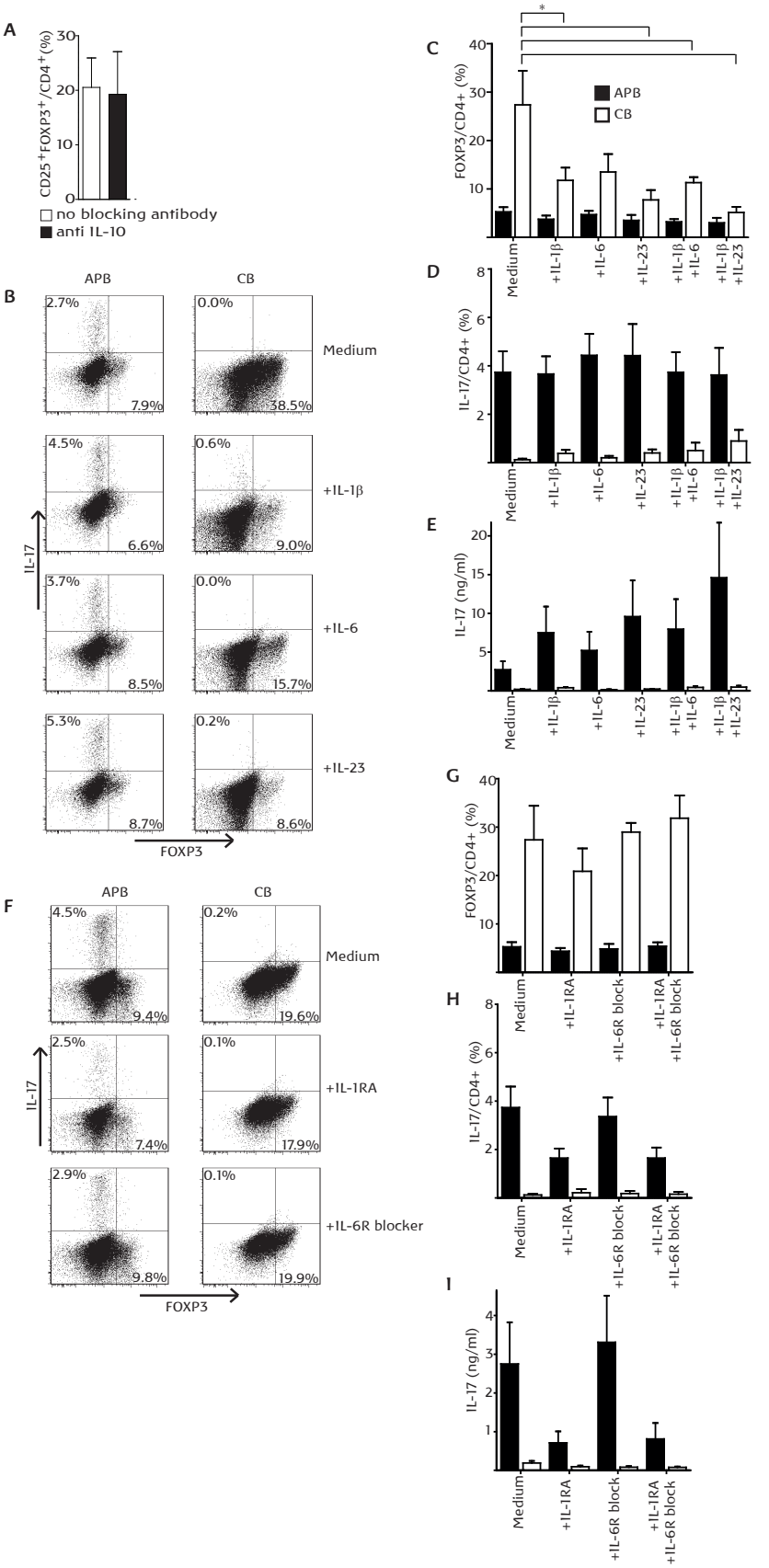
Figure 2: CB cells produce less IL-1β and IL-6 than APB after *in vitro* activation. 50.000 $CD4^+CD25^-CD45RO^-$ naïve T cells were cultured with 100.000 viable APC and activated by 1µg/ml plate bound anti-CD3. After 5 days of culture, IL-1β (**A**) and IL-6 (**B**) were measured in the culture supernatant by luminex (n=6). IL-1β (**C**), IL-6 (**D**) and IL-17 (**E**) were measured daily in the culture supernatant after activation (n=6). Significance of the differences was tested with a Mann Whitney U test.

production could be observed in the culture supernatants from the first day of activation onward, consistently higher in APB than CB cultures (Figure 2C & D). IL-17 is increasingly found in APB culture supernatant from the first days of culture onward, but not observed in CB cultures at all (Figure 2E).

Role for cytokines in T_H17 and Treg development

To test whether the differences in cytokine production could underlie the differences in FOXP3 and T_H17 induction, we selectively blocked or added certain cytokines. First, we blocked IL-10 signaling by adding a blocking antibody to the cultures and tested Treg induction in CB cultures. No difference in $FOXP3^+$ cell percentages are observed, showing that the higher IL-10 production in CB cultures is not likely to influence FOXP3 induction (Figure 3A).

Next, we tested whether inclusion of T_H17 driving cytokines affects Treg and T_H17 differentiation to discernible extent, when comparing naïve CD4 T cells from APB and CB sources. The addition of IL-1β, IL-6 and IL-23 clearly reduced the number of CB Treg found after 5 days of culture from naïve CD4 T cell precursors (Figure 3B & C). The number of Treg derived from APB naïve CD4 T cells was only slightly affected. Addition of IL-6 and especially IL-23 increased the number of adult T_H17 cells (Figure 3B & D) as well as the amount of IL-17 found in the adult culture supernatants (Figure 3E). None of these cytokines individually, or combinations of these cytokines induced T_H17 cells in CB cultures. Blockade of IL-1β by addition of IL-1R antagonist (Anakinra) inhibited the induction of APB T_H17 cells (Figure 3F & H) and the


A
CD25+FOXP3+/CD4+(%)
no blocking antibody
anti IL-10
B
APB
CB
Medium
+IL-1β
+IL-6
+IL-23
IL-17
FOXP3
C
FOXP3/CD4+ (%)
APB
CB
D
IL-17/CD4+ (%)
E
IL-17 (ng/ml)
F
+IL-1RA
+IL-6R blocker
G
H
I

amount of IL-17 found in the APB culture supernatant (Figure 3I). Blockade of IL-6 by adding IL-6R blocker (Tocilizumab) to the cultures did not have an effect on T_H17 cell induction, nor did it have an additional effect to IL-1β blockade (Figure 3F, H & I). Finally, blockade of either IL-1β or IL-6 or both did not affect the CB or APB Treg cell number after 5 days of culture.

Taken together, the presence of especially IL-1β in APB cell cultures as shown in figure 2, induces T_H17 cells, as blockade of this cytokine reduces T_H17 cell number. Blockade of IL-17 inducing cytokines did not result in significant increase of Treg cells in APB. In CB cell cultures however, addition of inflammatory cytokines resulted in significant decrease in Treg, but did not affect the absence of IL-17 producing cells. We hypothesized that the presence or absence of a T cell intrinsic factor regulates propensity in naïve CD4 T cells to balance the Treg/T_H17 polarization via inhibition of T_H17 cell differentiation, rather than stimulating Treg development.

RORC mRNA content

We tested the concentration of RORC mRNA (encoding RORγT protein) in our T cell cultures. This transcription factor is directly induces IL-17 transcription, and its absence or presence might control the subsequent IL-17 production.

Figure 4 shows the content of RORC mRNA as compared to both actin and GAPDH mRNA content during a 5 day culture at several time points. Both APB and CB derived cells show an increase in RORC transcript after 24 hours of culture and a gradual decline afterwards. CB cells do express mRNA of the T_H17 defining transcription factor without subsequent IL-17 production, suggesting the existence of an additional check-point for IL-17 secretion by $CD4^+$ effector T cells.

◀ **Figure 3: IL-1β, IL-6 and IL-23 influence Treg/T_H17 balance differentially between CB and APB cell cultures.** 50.000 $CD4^+CD25^-CD45RO^-$ naïve T cells were cultured with 100.000 viable APC and activated by 1μg/ml plate bound anti-CD3 in the presence of a blocking antibody towards IL-10 (JES3-9D7; eBioscience, 5μg/ml) FOXP3 expression was measured by flow cytometry after 6 days of culture (**A**). 50.000 $CD4^+CD25^-CD45RO^-$ naïve T cells were cultured with 100.000 viable APC and activated by 1μg/ml plate bound anti-CD3 in the presence of recombinant human cytokines as indicated. After 5 days of culture, cells were restimulated with PMA and Ionomycin and Brefeldin A was added for 4 hours and $IL\text{-}17^+$ and $FOXP3^+$ cell numbers was assessed by flow cytometry (**B**, **C**, **D**)(APB n=4-6, CB n=3-5). IL-17 was measured in culture supernatant after 5 days of culture without PMA/Ionomycin stimulation (**E**, n=6). 50.000 $CD4^+CD25^-CD45RO^-$ naïve T cells were cultured with 100.000 viable APC and activated by 1μg/ml plate bound anti-CD3 in the presence of monoclonal antibodies as indicated. After 5 days of culture, cells were restimulated with PMA and Ionomycin and Brefeldin A was added for 4 hours and $IL\text{-}17^+$ and $FOXP3^+$ cell numbers was assessed by flow cytometry (**F**, **G**, **H**)(APB n=4-6, CB n=3-5). IL-17 was measured in culture supernatant after 5 days of culture without PMA/Ionomycin stimulation (**I**, n=6). Significance of the differences was tested with ANOVA with subsequent pairwise comparisons using Dunnett's test.

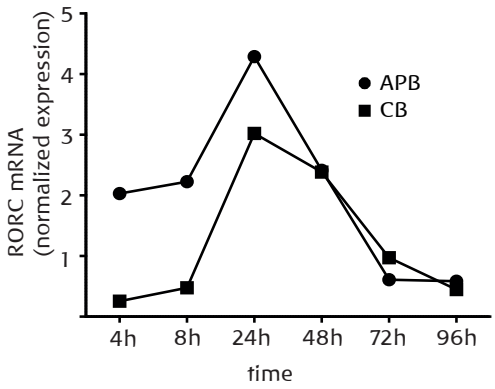


Figure 4: Expression of RORC mRNA. 50.000 $CD4^+CD25^-CD45RO^-$ naïve T cells were cultured with 100.000 viable APC and activated by 1µg/ml plate bound anti-CD3. Cells were lysed at several time points and mRNA was isolated and tested for RORC transcript, n=1.

DISCUSSION

Regulatory T cells and T_H17 cells have opposing roles in auto-immune disorders, but have a closely related differentiation pathway when induced from a common naïve CD4 T cell precursor. The differentiation of both cell types in the periphery has therefore been under intense research in the past few years(21). Both cell types need TGF-β, but the upregulation of RORC transcription needs additional inflammatory cytokines. Interestingly, the inflammatory cytokines that lead to RORγT upregulation and T_H17 cell induction differ between mouse and man. STAT3 activation via IL-6 is shown to be indispensable for murine T_H17 cells, whereas IL-1β has a major role for human T_H17 cells(22, 23). Indeed, we here show that IL-1β presence is instrumental for the induction of IL-17 producing T cells as blockade with IL-1RA reduced IL-17 production. We also confirmed the role of IL-23 in the T_H17-axis. Nevertheless, we show that in cells that are refractory at IL-17 production, i.e. cord blood derived naïve T cells cultured with autologous APC, these cytokines are not able to skew the cell differentiation towards T_H17. Although the number of induced $FOXP3^+$ Treg is reduced when inflammatory cytokines are added to the cultures, no IL-17 production was induced.

This discrepancy prompted our hypothesis that another, T cell intrinsic factor would play a role in the cell fate decision between T_H17 and Treg induction. The role of HIF-1α in the induction of T_H17 and prevention of Treg development has been described recently for murine cells(13, 14). For human cells however, it has been described that HIF-1α expression induces Treg(24). To clarify the role of RORγT and HIF-1α in lineage commitment of differentiating CD4 T cells, we first tested mRNA expression of RORC, as HIF-1α co-operates with other factors in the transcription of this T_H17 cell defining transcription factor. RORC mRNA was low at the start of culture, but increased after 24 hours of activation for both CB and APB. Other papers have described the presence of RORC mRNA and absence of IL-17 mRNA as well in total CBMC cultures(25).

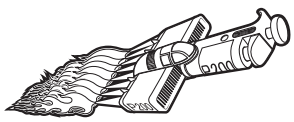
These findings suggest that in human T cells, a factor downstream of RORC mRNA upregulation prevents IL-17 production. The potent induction of FOXP3 in CB cells as described here and before(18) suggest a role for this transcription factor. Indeed, FOXP3 is able to directly bind RORγT, thereby preventing its activity, e.a. transcriptional regulation of IL-17(26). Nevertheless, addition of IL-1β and IL-23 to the CB cell cultures resulted in decreased FOXP3 expression without IL-17 production which makes FOXP3 as a regulator of IL-17 production unlikely.

Another candidate in the prevention of IL-17 production is STAT-5. STAT-5 is activated by IL-2 signaling and can subsequently bind to the area of the IL-17 promoter where STAT-3 (activated by IL-6) would activate IL-17 transcription. IL-2 and STAT-5 can thereby prevent IL-17 transcription, even in the presence of RORγT(27). Nevertheless, we observed no difference in IL-2 production between APB and CB (not shown). Moreover, the role of IL-6 signaling and thereby STAT3 seems to be small for human T_H17 differentiation; addition or blockade of IL-6 signaling had only minor implications for IL-17 production. Until now, the factor preventing IL-17 production in human T cells still needs to be elucidated.

The role of HIF-1α in murine Treg/T_H17 lineage decision has recently been described in two individual papers(13, 14). This factor induces RORγT transcription and subsequently induces IL-17 transcription together with the RORγT protein. In our human system, where CB cells are more prone to become Treg and APB can also become T_H17, RORC transcript is found in cells from both origins. A role for human HIF-1α would therefore be likely specifically at the check-point resulting in IL-17 transcription.

Activated CB cells contain high percentages of $FOXP3^+$ cells. Our data do not show whether the RORC transcript is expressed in the $FOXP3^+$ or the $FOXP3^-$ cells. The recent questioning of the stability of T cell lineages makes it possible that the CB cells expressing FOXP3 represent cells that co-express RORγT and can still switch towards an IL-17 producing T_H17 cell lineage. FOXP3 upregulation might be the default pathway for neonatal or CB CD4 T cells, in order to prevent inflammation during pregnancy or birth. Flexibility of the T cells might result in commitment towards a different lineage upon second activation.

The mechanisms by which human Treg and T_H17 cells are induced, and their flexibility or stability are very important in understanding the onset and treatment of auto-immunity. The factors that prevent IL-17 production in CB can be an important switch in the choice between Treg and T_H17 and could be exploited for reversing auto-immune pathogenic T cells into Treg.



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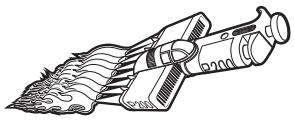
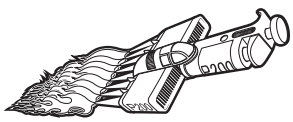


CHAPTER 7



General Discussion

Gut commensal bacteria have profound effects on the immune status of the host. The first part of this thesis describes the clear differences that exist between different bacterial strains in their immune modulatory capacities; some strains are potent inducers of regulatory T cells (Treg), whereas others skew the immune system towards inflammatory T_H1 or T_H17 cells(1-5). Moreover, the effect of bacteria on the immune status is highly donor-dependent(6, 7). The combination of these two features (variability between bacterial strains and among hosts) has important consequences for the use of these bacterial strains as probiotics in clinical settings in order to modulate the immune status.

The neonatal period appears to be a good moment for immune modulation, especially when focussed on Treg. The second part of this thesis describes the potency of T cells derived from cord blood (CB) to become Treg upon activation. This effect is based on the interaction between antigen presenting cells and T cells, where ligation of programmed death (PD)-1 by PD-ligand (L)1 is crucial. Moreover, this thesis shows that CB cells cannot be skewed towards T_H17 cells *in vitro* like adult peripheral blood cells (APB). This effect is based on a T cell intrinsic factor, which is still to be identified. The switch between T_H17 and Treg is crucial in human auto-immune disorders; a human T cell intrinsic factor controlling T cell fate can be investigated in CB and APB.

Both parts of this thesis are discussed below.

IMMUNE REGULATION BY THE GUT MICROBIOTA

As described in chapter 2, the gut microbiota has profound effects on the immune system. Changes in the microbial composition due to dietary habits or probiotic supplementation are therefore likely to influence the immune system and diseases mediated by the immune system. These effects have been shown in several clinical trials in the prevention of allergic disorders, but the great expectations of this approach have not been met(8-11).

Probiotic functionality

The question is what might be the reason why the results of supplementation with probiotic bacteria has so far been quite disappointing. Various aspects of supplementation could account for this and a few of those are stressed in this discussion.

First of all, an important aspect is the complexity of the diseases that are targeted by probiotics. Allergic diseases like asthma and eczema are multifaceted(12). Both genetic and environmental factors play important roles in the development of these ailments(13). On top of that, many different cell types from the immune system play a role in the pathogenesis of for example asthma; eosinophils, neutrophils, NK cells(14), T_H2 cells, T_H17 cells, Treg(15), T_H9 cells(16), NKT cells(17) and dendritic cells(12) and even more cell types have all been reported to be active in this disease. Administration of a single species, or maybe multiple species of bacteria to the gut is not likely to be strong enough to influence the progression of this complex disease. Nevertheless, combination of administration of immune modulating bacteria with conventional therapy could benefit the patient and prolong disease free periods. This approach is under research(18), but deserves more attention.

Secondly, timing of probiotic administration is clearly of importance for its efficacy. Once the composition of an individuals microbiota has developed, it is rather stable and difficult to change(19, 20). Manipulating the composition will therefore be easiest before a stable microbiota has developed, early after birth. When probiotic bacteria are taken by the pregnant mother, these bacteria will be among the first to colonize the newborn's gut as has been shown for different animal species(21) and humans(22). Trials using this approach have shown the most promising results on the prevention of atopy(23-25), although even here significant clinical effects between probiotics and placebo are so far only observed in the first years of the newborn's life. Valuable information can be obtained from clinical trials where probiotics are administered in early life when these trials are combined with monitoring the change in composition of the gut microbiota of the included infants. The presence and absence of strains that correlate with disease is of special interest, as well as the question whether it is feasible that early life probiotics can influence or change the composition of the microbiota.

Finally, the selection of bacterial strains to use as probiotics can be improved. In chapter 3 and 4 of this thesis, we show dramatic differences between several strains in their capacity to influence the immune system. We not only observed differences in Treg induction, but found that each strain differs in which direction it affects the immune system. The fact that probiotic strains can induce T_H17 cells even harbours a dangerous risk in the use of probiotics; T_H17 cells are pathogenic in many auto-immune and allergic disorders. Administration of segmented filamentous bacteria (SFB) to mice susceptible to arthritis develop joint inflammation readily without further disease induction(26). So, intense strain characterization prior to clinical application will not only improve clinical outcome, but might also prevent aberrant, unwanted effects. Moreover, chapter 4 of this thesis shows that important differences between individuals exist in their immune reaction towards probiotic strains. Similar conclusions have been made for *in vivo* experiments(7). In a ideal clinical setting, each individual would receive a different strain, based on *in vitro* results from the lab showing that the particular strain is most likely to direct the individual's immune system in the desired direction. Such "personalized medicine" might be a possibility in the future for probiotics and will improve the effects of probiotics for each individual.

SUGGESTIONS FOR STRAIN SELECTION

Scientific research on the effects of the microbiota on the immune system of mice and men is an interesting challenge for translational medicine. Lots of appealing data are available in murine immunology, but the translation towards a human setting is problematic. The effects of bacteria on the human immune system are hard to investigate. Contact between bacteria and the immune system takes primarily place in the gut, an organ which is not easily accessible for studies. Only three different approaches are being used at the moment. 1) Direct co-culture of PBMC with bacteria(27-29), 2) Co-culture of gut epithelial cell lines with bacteria (30-32) or 3) Administration of bacteria to volunteers and subsequent monitoring of gene transcription in biopsies of epithelial cells(7, 33). Other approaches where biomarkers are measured in the blood or clinical parameters measured after probiotic supplementation in clinical trials provide indirect data on the effects of the selected strain.

In order to improve efficacy of probiotics, the capacities of a strain to influence the immune system should be measured, ideally with all three approaches. Each approach has its own benefits and downsides. *In vitro* culture with PBMC induces direct contact between immune cells and bacteria, which is obviously not the case *in vivo*. The use of transformed epithelial cell lines overcomes this problem, and adds another layer of immune modulating cells. The exact effect of transformed cells and the relation to *in vivo* epithelium however, is not clear. Epithelial cell lines separating PBMC from

bacteria, do not guarantee prevention of contact between immune cells and bacterial components like LPS as leakage still may occur. The use of biopsies from healthy volunteers provides an elegant way to obtain *in vivo* data, but depends on an invasive technique used on volunteers.

In vitro screening with PBMC is therefore a good option for a primary screening of bacterial strains. Co-culture of PBMC with different strains, and measurement of the relative amount of T cell subsets after culture, will provide general information on the immune modulating capacity of strains. In chapter 4 we show that the differentiation of T cells by bacteria can be measured consistently with several techniques, providing options for a researcher for what protocol to use. Strains with the desired effect on the immune system can subsequently be selected for use in clinical settings.

The immune modulating capacities of strains we observed *in vitro* still need validation *in vivo*. Other studies show that in vitro IL-10 production by human PBMC correlated well with protection against colitis *in vivo* in a mouse(29). Ideally, *in vitro* experiments with human PBMC should be combined with *in vivo* screening of mucosal cell activation by biopsies of the human gut.

Alternative strains

From recent literature and data presented in this thesis (Chapter 2), it is clear that some strains that are not classically used as probiotics, can still have pronounced effects on the murine or human immune system. Immunologic effects of strains derived from a large variety of species, genera and phyla should therefore be tested. *Clostridium* species for example should be taken into account(34), just like *Propionibacteria*(35) and *Oenococcus*(36) species have been tested.

The rationale for testing a species for its immunologic effects in a human system should be scientific data derived from mouse experiments or association with human diseases. Until now, most strains tested in *in vitro* experiments and *in vivo* clinical trials seem to be selected for other, more commercial reasons. For industries, it is more lucrative to commercialize a strain which is already on the market than to extensively test new species. Nevertheless, from our data it is clear that one single strain is not likely to benefit the user in different clinical settings. Different species of probiotics should be investigated for different ailments. *Lactobacillus rhamnosus* GG (LGG), *L. casei* Shirota and *L. acidophilus* W55 do have beneficial effects, but more powerful strains might be available. Scientist should therefore conduct their own investigations without a dependency on commercial institutions for providing probiotic strains.

Complications of *in vitro* testing

For both *in vitro* approaches described above, the choice between dead or viable bacteria need to be made. It is generally thought that probiotics

should be alive when used, in order to have their beneficial effects(37). This is obviously true when the probiotic effect is dependent on microbial metabolites or when the probiotics are used to compete with (pathogenic) species in the gut. Nevertheless, in case of *in vitro* cultures the viability of bacteria has downsides. Co-culture of PBMC with bacteria in a ratio which is based on viable bacterial cells (e.g. a 1:10 ratio PBMC:viable bacteria), neglects the number of dead bacteria (which can not be counted as a colony forming unit in an assay) that remain in the medium in which the bacteria are cultured. Unpublished data show that less stable strains (e.g. *Lactobacillus salivarius* W57), have a high number of dead cells in the medium as measured by flow cytometry. Standardization on viable bacteria can result in tenfold more dead bacterial cells in the co-culture with PBMC. This bacterial debris contains components like LPS and peptidoglycan that will have pronounced effects on the immune cells. Indeed, co-culture with these strains results in increased death of immune cells. Disturbingly, the same cultures show high concentrations of IL-10 in the supernatant which can be due to the increased apoptosis(38). IL-10 concentration in the supernatant of co-cultures is often a selection criterion for a certain strain(27, 39), but might represent an experimental artefact.

In our experiments we use viable bacteria based on number of colony forming units as well, which might have influenced the results. It is therefore recommended to use several experimental designs (e.g. include a control with heat killed bacteria) to investigate the immunological properties of different strains. Gene transcription in biopsies from healthy donors after probiotic intake can be used to confirm the *in vitro* data.

Suggestions for further research into probiotics

Both the *in vitro* experiments described in this thesis and *in vivo* experiments with probiotics have provided valuable information next to mouse data on the influence of the microbiota on the immune system. Nevertheless, research into probiotics should take a step back from the bedside, to the bench again. The immunological properties of gut bacteria should be further investigated in a laboratory setting with human cells. Different species, including less conventional species, should be intensively characterized before use in a clinical setting. When used in clinical trials, the correct immunological biomarkers should be identified and monitored in order to obtain a complete view of the possibilities of a strain. For example biopsies from the gut can be taken to see direct effects, but also a complete set of blood biomarkers (e.g. cytokine profiles, T helper cell subsets, etc.) can be tested. Distinction between responders and non-responders at the end of a trial, combined with data from adequate biomarker tests will provide useful information on the immunological mechanisms by which probiotics function. This approach can further improve our view of the possibilities of specific strains and probiotics as a whole.

NEONATAL CORD BLOOD AND ADULT PERIPHERAL CELLS

Treg/T_H17

Neonatal cells derived from CB have not had contact with a gut microbiota yet, just like cells from germ free (GF) mice. Interestingly, $CD4^+$ T cells from GF mice show reduced ability to induce experimental autoimmune encephalomyelitis (EAE) when compared to cells derived from conventional mice. This effect is mediated by increased regulatory T cell induction in GF mice with a concomitant decreased induction of T_H17 cells upon activation with EAE-inducing MOG peptide(40). In this case, the microbiota primes the immune system towards inflammatory cells, which subsequently have a role in EAE. The induction of T_H17 cells by the microbiota has a biological role in the protection against pathogens as has been shown in models with *Citrobacter rodentium* infections(41).

We observed similar effects in naïve T cells from CB, as described in Chapter 5 and 6 of this thesis. These cells have not encountered any (commensal) bacteria yet, and respond primarily by induction of Treg. T_H17 cells on the contrary are not easily induced in CB cell cultures. Addition of T_H17 inducing cytokines like IL-1β, IL-6 and IL-23 do reduce the number of CB $FOXP3^+$ cells, but has no clear effect on IL-17 production.

The fact that T_H17 cells seem to be regulated in the gut(42), probably by contact with the microbiota(4), suggests that these cells can only be induced after contact with microbes has been established. The similarities between T_H17 and Treg inducing milieus and pathways might result in a remarkably increased number of Treg induced upon activation in CB cells that had no contact with gut microbes yet.

The role of the microbiota in this discrepancy between CB and APB cells in Treg/T_H17 cell induction as described in the second part of this thesis is for now purely hypothetical. Nevertheless, another interesting hypothesis combining the gut immune system and the switch between Treg and T_H17 cell induction is found in the hypoxia induced factor (HIF)-1α. The expression of HIF-1α, which is stabilized by hypoxic circumstances and reactive oxygen species provides a switch towards T_H17 development(43, 44). FOXP3 protein is ubiquitinated by HIF-1α and degraded by the proteosome, while RORγT and subsequently IL-17 transcription is induced by this factor. An hypoxic milieu is therefore likely to induce T_H17 cells rather than Treg cells. Indeed, the gut is relatively hypoxic when compared to other tissues, and harbours large numbers of T_H17 cells(42, 45). Hypoxia is certainly not enough to induce T_H17 cells in the gut, as absence of certain bacteria like SFB prevents T_H17 cell induction(26, 40). The combination of hypoxia with microbes however might be necessary to induce stable HIF-1α expression and the possibility to become a T_H17 cell. The impossibility to induce T_H17 cells in CB might result

from the lack of contact with specific gut microbes and subsequent reduced HIF-1α expression.

Role for Antigen Presenting Cells in Treg induction

We observed major differences between CB and APB derived cells in their capacity to differentiate into Treg or T_H17 cells. T_H17 cells could not be induced in CB cultures, not even when high amounts of T_H17 cell inducing cytokines were added to the medium. Interestingly, other papers described potent T_H17 cell differentiation of CB T cells when activated by anti-CD3 and anti-CD28 and differentiating cytokines(46-48). We typically needed APC in our cultures to observe the differences between CB and APB. When APC were replaced by anti-CD28, the differences in Treg induction between CB and APB cells was less pronounced. CB APC likely prevent the differentiation into T_H17 cells.

The role of PD-1 for iTregs in neonatal cord blood has been established in chapter 5. PD-1/PD-L1 interaction between CB APC and T cell establishes FOXP3 induction and could play a role in the prevention of T_H17 differentiation. Absence of APC in the cultures results in absence of PD-1/PD-L1 signaling and subsequently reduced Treg number. This could concomitantly open up the possibility of T_H17 differentiation. This role for PD-1 could be of importance in the feto-maternal tolerance. Indeed, in mice, it was shown that PD-1/PD-L1 plays an important role in the protection of the foetus. Blockade of PD-L1 results in resorption of the foetuses and reduced litter size, due to reduced number of $FOXP3^+$ cells and increased numbers of IL-17 producing cells(49).

Programmed death-1

The role of PD-1 for T cell biology has been investigated mainly for $CD8^+$ T cells during chronic viral infections. $PD\text{-}1^+CD8^+$ T cells are often described as "exhausted" T cells. These cells have become anergic and produce decreased amounts of T_H1 cytokines(50) which can be resolved by blocking the PD-1/PD-L1 interaction.

The function of PD-1 on $CD4^+$ T cells however seems to be somewhat different, as it has mainly been described to have a role in FOXP3 induction and Treg development(51, 52). In mouse immunology the direct induction of Treg by PD-1 signaling has been shown. Thus far no human data on PD-1 induced Treg induction were available. In chapter 5 we show that blockade of the interaction between PD-1 and its ligand PD-L1 reduces the number of Treg, specifically in CB derived cells. The exact mechanism by which the PD-1/PD-L1 blockade reduces the number of $FOXP3^+$ cells in CB is still unknown. We observed reduced phosphorylation of protein kinase B (PKB), which has also been shown in murine PD-1 signaling induced Treg development. We hypothesize that reduced pPKB results in reduced FOXO3a phosphorylation directing it to the nucleus where it induces transcription of FOXP3(53-55).

The reduced CB FOXP3$^+$ T cell number we observed by PD-1/PD-L1 blockade might be an indirect effect. In all our experiments we never observed PD-1$^+$FOXP3$^+$ Treg (not shown), indicating that this molecule might have a more important function on non-Treg. On top of that, activation of human T cells with recombinant PD-L1 coated beads only show reduced proliferation and cytokine production, but no induction of Treg (not shown).

Other Treg inducing mechanisms

Most likely, PD-1 signaling is not the only pathway involved in the increased number of Treg and absence of T_H17 cells in our CB cultures. For example, co-stimulation via CD28 reduces the number of induced Treg(56). This effect is mediated by Lck, lymphocyte-specific protein tyrosine kinase. Activation of cells in the presence of a Lck inhibitor increases the number of induced Treg. Interestingly, CB T cells have reduced expression of Lck compared to APB derived cells(57), which can contribute to increased Treg induction. Nevertheless, blockade of co-stimulation with specific antibodies did not affect the iTreg number in either CB or APB cell cultures in our hands (Chapter 5), suggesting that CD28 signals are not important. The cumulative dose of TCR stimulation has also been shown to influence Treg induction, with a higher probability of Treg induction with lower TCR stimulation(58). In our cultures TCR triggering was standardized by plate bound anti-CD3 and cannot explain Treg induction differences.

Another possibly involved interaction between APC and T cells is the interaction between CD40 and CD40-ligand. *In vitro* activation of T cells via plate bound anti-CD3 leads to activation of APC via cytokines and CD40-CD40L interaction. The important differences we observed in APC derived cytokines, specifically IL-1β and IL-6, in the supernatants of our cultures (which affect the induction of FOXP3) might therefore be a result of differences in CD40-CD40L. Indeed, CB and APB monocytes reacted differently on activation with a CD40L(59). The CD40-CD40L interaction is probably less important *in vivo* than in our *in vitro* cultures, as *in vivo* a T cell would be activated by an already activated APC and less the other way around. We tested expression of CD40 and CD40L on CB and APB cells, but could not find differences on the expression level. Activation of CB APC by stimulation with TLR-ligands resulted in production of normal levels of inflammatory cytokines, showing that the APC can be activated and produce cytokines.

Implications and future prospects

The fact that neonatal cells are prone to become Treg cells upon their first activation, with an important role for APC as described in this thesis, adds to our view of the neonatal immune system. The known bias towards T_H2 cells as compared to T_H1 cells in neonates is now elaborated by a bias towards Treg cell induction rather than T_H17. The cells derived from CB are the same as cells circulating in the early newborn that encounter antigens for the first

time. APC will present antigen from gut and skin colonizing bacteria as well as from airborne proteins. With the data presented in this thesis, it is likely that the T cells activated by these antigens will become Treg and not T_H17 cells. These Treg will prevent inflammatory reactions towards these antigens. For our experiments, follow-ups with PBMC derived from neonates and children of differing age would be very informative. Somewhere between birth and adulthood the immune system will change from the phenotype we observed in CB samples to the phenotype we observed in APB samples. This change is likely to take place somewhere in the first months of life.

An obvious factor that can influence this change is the shift from breast feeding to solid food intake, which is also accompanied by a change in the composition of the microbiota(60). Not only colonization itself could influence the possibility to induce T_H17 cells in neonates, but also these changes in the composition of the microbiota. Ideally, data on the change in composition of a newborn's microbiota could be accompanied by experiments testing the Treg and T_H17 cell number and the possibility to differentiate these cells from naive T cells.

The neonatal period in which Treg are easily induced could be a window of opportunity for the prevention of allergic diseases, as has been suggested by others(61). When antigens are encountered during this period, Treg are induced that will subsequently prevent immune activation when this antigen is encountered again later in life. Indeed, early consumption of peanuts has been shown to prevent peanut allergy(62), and spending early life in an antigen rich environment is associated with reduced prevalence of asthma and eczema(63). Nevertheless, many other factors play a role in the induction of both allergic disorders and in the prevention of these aberrant immune responses by for example oral tolerance. Increasing knowledge on the development of the immune system will be valuable for understanding the development of immune mediated diseases and the role of each subset of $CD4^+$ T cell, as well as the role of the microbiota.

Whether the neonatal induced Treg will remain Treg is not known, as the stability of specific $CD4^+$ T cells is under debate recently. In both mouse and human immunology the plasticity of different subsets has been described. Specifically for T_H17 and Treg cells has this phenomenon been described(64-66), as these cells are closely related in their inducing pathways. Nevertheless, it has also been described that T_H17 cells can produce IFN-γ(67, 68) or that intermediate forms of these cells exist(69). Finally, the co-expression of transcription factors of different lineages has been described, for example for GATA3 and FOXP3(70), where GATA3 seems to be important for Treg function(71). Nevertheless, it has also been suggested that T_H1 and T_H17 cells are stable lineages due to epigenetic changes; T_H1 polarizing cytokines can induced IFN-γ production in T_H17 cells, but will not result in loss of RORγT and Tbet upregulation(72).

The stability of the T_H17 and Treg cells that are induced in our cultures is not tested. It is possible though, that the high numbers of Treg cells are induced in CB represent cells that can still switch to T_H17. FOXP3 upregulation might be the response upon the first activation; a second activation in the presence of mediators of inflammation could result in RORγT upregulation and FOXP3 downregulation. The specific milieu in which a T cell is activated is probably very important for the subsequent direction in which it develops. Indeed, chapter 5 of this thesis shows the importance of APC in the induction of Treg, as CB APC induce higher numbers of Treg than APB APC.

Immune regulation in other settings

CB derived cells have frequently been used for transplantation in malignant diseases because of the reduced chance of developing graft versus host disease(73, 74). The increased number of induced Treg due to PD-1/PD-L1 interaction we observed will partially underlie this phenomenon, whereas reduced numbers of T_H17 cells will also benefit the transplantation. Controlled PD-1 signaling using monoclonal antibodies might be an interesting possibility for treatment of GvHD. On the other hand, this approach might prevent proper immunogenicity and prevent proper graft versus leukaemia effects(75). Blockade of the cytokines that lead to T_H17 differentiation with Tocilizumab (IL-6) or Anakinra (IL-1β) can be another option in the treatment of GvHD, which is already under investigation(76, 77).

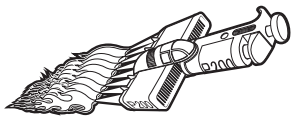
The role of PD-1 can be investigated further in auto-immune diseases as well. Type one diabetes has been under intense investigation already(78), but rheumatic disorders have been underexposed. Recent preliminary results show an increase of PD-1 and PD-L1 expression on immune cells from the inflamed tissue as compared to cells from the circulation in patients suffering from juvenile idiopathic arthritis (JIA). It is unknown whether this expression has a functional background that could be exploited in the future, or merely represents the activation status of the cells. Nevertheless, the relation of this molecule with the induction of Treg and T_H17 cells, which have profound roles in the pathology of JIA(79), make it an interesting target. The same holds true for HIF-1α, which can be the switch between inflammation and regulation. Data obtained from studies comparing CB and APB can be valuable for understanding the mechanisms in disease settings where the switch between Treg and T_H17 seems to be wrongly regulated.

CONCLUSIONS

The immune modulating capacities of probiotic bacteria as described in this thesis shows the possibilities for its use in immune mediated disorders. Further research with human material, both *in vitro* and *in vivo*, should increase our knowledge on the possibilities of individual strains. For strain selection, researchers should broaden their horizon and test unconventional

strains, as these might have great potential. In upcoming trials, more immunological parameters should be tested in order to combine the immune modulating properties with clinical outcome and to understand the mechanisms. Monitoring (changes in) the composition of the microbiotia of patients and healthy controls will give further insight into the role that gut microbes have in pathology.

The role of gut microbiotia is also interesting in the light of Treg and T_H17 cell induction. Our data on the role of APC and PD-1 and a possible T cell intrinsic switch should be followed up with experiments using PBMC from young children. Ideally, the composition of the microbiota of the children that donate PBMC for these experiments would be monitored to see whether correlations could be found. In this thesis we show that the tolerogenicity of the foetal and early neonatal immune system is partly mediated by augmented induction of Treg and reduced T_H17 cell induction. PD-1 signaling is important in the induction of Treg, whereas reduced inflammatory cytokine production underlies the low numbers of IL-17 producing T cells. These observations should be further investigated for their role in the prevention of allergic disorders, but can be translated towards other fields as well. The switch between Treg and T_H17 cells is interesting in many auto-immune disorders. The differences between CB and APB can be used as a model to study regulatory mechanisms with human material that can ultimately be used in different (clinical) settings.



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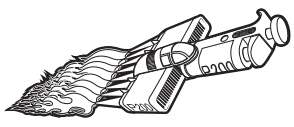
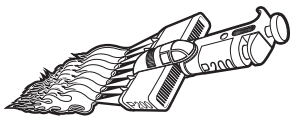


ADDENDUM



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NEDERLANDSE SAMENVATTING

Het afweersysteem

Het menselijk lichaam heeft verschillende manieren om ziekteverwekkers tegen te houden. In eerste instantie zorgen fysieke barrières als de huid en het darmepitheel ervoor dat micro-organismen moeilijk kunnen binnen dringen. Mochten ze toch binnen komen, dan zijn er twee lagen van het afweersysteem die de ziekteverwekkers kunnen aanpakken. De eerste is een aspecifieke afweer die heel snel actief wordt. De tweede herkent een specifiek onderdeel van de ziekteverwekker, meestal een eiwit, waardoor het heel doelgericht te werk kan gaan. Het heeft meer tijd nodig dan de eerste afweerlaag. Bij beide lagen van afweer ontstaat een ontstekingsreactie waarbij de ziekteverwekker verwijderd wordt.

T cellen zijn belangrijke cellen uit de specifieke afweerlaag. T cellen worden gezien als de sturende cellen in het afweersysteem. Er zijn op dit moment vier verschillende T cellen bekend, T_H1, T_H2, T_H17 en de regulatiore T cel (Treg) die alle een andere functie hebben in de bestrijding van pathogenen. T_H1 cellen zijn belangrijk in de afweer tegen intracellulaire bacteriën, T_H2 cellen bij infecties met parasieten, T_H17 zijn actief tegen extracellulaire bacteriën. Treg tenslotte reguleren het immuunsysteem. Zij zorgen ervoor dat na afloop van een infectie de afweerreactie weer stopt.

De doelgerichtheid van de T cellen in de specifieke afweer kan een probleem vormen wanneer er een eiwit wordt herkent dat geen onderdeel van een ziekteverwekker is. Dit gebeurt bijvoorbeeld bij een auto-immuun aandoening zoals reuma. Het afweersysteem ziet hier een eiwit in de gewrichten van patiënten aan voor een onderdeel van een ziekteverwekker. Vervolgens ontstaat er een ontstekingsreactie waarbij veel schade aan de gewrichten optreedt. Ook bij allergieën ziet het afweersysteem een ongevaarlijk eiwit, zoals eiwitten in graspollen, aan voor een ziekteverwekker. Het gevolg is hier een allergische ontsteking, namelijk hooikoorts. T_H1 en T_H17 cellen spelen een rol in het ontstaan van auto-immuun aandoeningen, terwijl T_H2 cellen een rol hebben in het ontstaan van allergieën en astma.

Een gezond afweersysteem zal niet met een ontstekingsreactie reageren op ongevaarlijke eiwitten of op eiwitten uit het eigen lichaam. Treg spelen een belangrijke rol in het voorkomen van ontstekingsreacties in deze gevallen. Treg zijn in staat om de activiteit van verschillende andere soorten T cellen die de ontsteking veroorzaken af te remmen. Hierdoor kunnen aandoeningen als reuma en hooikoorts niet ontstaan. Therapieën en onderzoek gericht op het voorkomen of genezen van allergieën en auto-immuniteit focussen veelal op de functie van Treg en op de aantallen Treg ten opzichte van andere afweercellen.

De darmflora beïnvloedt het afweersysteem

In de darm bevinden zich talloze bacteriën die samen de darmflora vormen. Er zijn ongeveer tien keer meer bacteriën in onze darmen dan er cellen zijn in ons eigen lichaam. Deze bacteriën zijn erg belangrijk voor de vertering van ons voedsel, en vormen een extra barrière tegen ziekteverwekkers die darminfecties kunnen veroorzaken. Vanwege deze belangrijke functie van darmbacteriën voor de gezondheid worden darmbacteriën door veel mensen ingenomen in de vorm van probiotica.

De darm is voor het afweersysteem een belangrijk orgaan. De bacteriën in de darm worden door het afweersysteem waargenomen dat vervolgens op meerdere manieren kan reageren. De verschillende T cellen spelen hier een belangrijke rol. Het afweersysteem houdt hiermee de darmflora in toom, maar de darmflora beïnvloedt ook het afweersysteem. Zo blijkt dat de aanwezigheid van bepaalde bacteriesoorten in de darmflora van muizen direct invloed heeft op het ontstaan van reumatische aandoeningen, terwijl aanwezigheid van andere bacteriesoorten dit kan voorkomen.

Het beïnvloeden van het afweersysteem door het innemen van probiotica is al een aantal jaren een grote uitdaging voor de medische wetenschap. Al geruime tijd wordt geprobeerd om het afweersysteem zo te sturen met probiotica, dat aandoeningen als allergieën voorkomen kunnen worden. Het blijkt echter lastig om daadwerkelijk een allergische aandoening als astma of eczeem te voorkomen met probiotica. Met de experimenten die in dit proefschrift beschreven worden zoeken we naar de oorzaak hiervan. De nadruk ligt hierbij op de verschillende bacteriestammen die gebruikt kunnen worden als probiotica.

Bacteriestammen verschillen in hun effect op het afweersysteem

Uit het hier beschreven laboratoriumonderzoek blijkt dat niet alle bacteriestammen hetzelfde effect hebben op het afweersysteem. Ten eerste kunnen sommige stammen duidelijk Treg induceren, terwijl andere dat helemaal niet doen. Ten tweede blijkt dat naast Treg, ook T_H1, T_H2 en T_H17 geïnduceerd kunnen worden door bepaalde bacteriestammen. Treg kunnen een positief effect op astma hebben doordat ze de ontsteking kunnen remmen. T_H2 en T_H17 cellen spelen echter een ziekteverwekkende rol bij astma. Induceren van meer T_H2 of T_H17 cellen zou dus juist nadelig kunnen zijn. Ten slotte blijkt dat niet ieder mens gelijk reageert op dezelfde bacteriestam. Dit suggereert dat de stam die goed is voor de één, misschien geen effect geeft voor de ander. We concluderen dat het uitvoerig in kaart brengen van het effect van een bacteriestam op het afweersysteem noodzakelijk is voordat deze stam gebruikt wordt in een klinische omgeving. Zo kan de beste bacteriestam voor het specifieke doel geselecteerd worden en kan het effect van probiotica op allergische en auto-immuun aandoeningen geoptimaliseerd worden. I

In onze experimenten hebben we verschillende methoden getest om het effect van bacteriestammen te onderzoeken. Wanneer in de toekomst in experimenten geprobeerd wordt om aandoeningen als astma te beïnvloeden met probiotica kan met onze methoden de juiste bacteriestam geselecteerd worden.

Navelstrengbloed

Het voorkomen van een ontstekingsreactie is erg belangrijk tijdens de zwangerschap. Een foetus is voor een zwangere vrouw lichaamsvreemd. Desondanks ontstaat er geen afweerreactie tussen moeder en foetus. Er bestaat een vorm van tolerantie in het afweersysteem van moeder en kind voor elkaar. In dit proefschrift gebruiken we navelstrengbloed als model van het afweersysteem van een pasgeborene. Hiermee onderzoeken we hoe dit nog naïeve afweersysteem verschilt van een volwassen systeem.

Het blijkt dat afweercellen uit navelstrengbloed veel gemakkelijker Treg worden dan T cellen uit volwassen bloed. Dit geeft aan dat de eerste reactie van het afweersysteem van een pasgeborene bestaat uit het voorkomen van een ontstekingsreactie. Het blijkt uit onze experimenten dat dit effect afhankelijk is van de interactie van de T cellen met andere (niet T) cellen van het afweersysteem. Het ontstaan van T_H1, T_H2, T_H17 of Treg wordt beïnvloed door signalen van deze andere cellen. Uit onze experimenten blijkt dat het ontstaan van grote aantallen Treg in navelstrengbloed wordt geïnduceerd door het molecuul *programmed death-1* (PD-1). Wanneer we de interactie tussen PD-1 en PD-ligand 1, dat door andere cellen tot expressie wordt gebracht blokkeren, ontstaan er minder Treg.

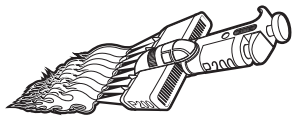
Het feit dat afweercellen uit navelstrengbloed een ontstekingsremmend fenotype hebben, suggereert dat het gunstig is om vroeg in het leven al blootgesteld te worden aan allergenen als pinda's. Dit doet immers Treg ontstaan die gunstig zijn in het voorkomen van allergie, ook later in het leven. Of dit zo werkt moet in de toekomst nog uitgezocht worden door niet alleen cellen uit navelstrengbloed te testen, maar ook cellen van pasgeboren zeer jonge kinderen. Daarnaast zal in klinische trials het effect van vroege blootstelling aan allergenen op het ontstaan Treg onderzocht moeten worden.

Treg en T_H17 in navelstrengbloed en volwassen bloed

Het blijkt dus dat T cellen uit navelstrengbloed makkelijk Treg worden. Daarnaast hebben we ontdekt dat deze cellen moeilijk te differentiëren zijn naar T_H17 cellen. Dit celtype is sterk ontsteking inducerend. Het toevoegen van signaalstoffen die bij cellen uit volwassen bloed het ontstaan van T_H17 cellen bevorderen heeft geen effect op T cellen uit navelstrengbloed. Interessant is dat het moleculaire mechanisme dat zorgt voor het ontstaan van Treg en T_H17 cellen erg dicht bij elkaar ligt, terwijl het effect van de cellen (ontsteking of juist niet!) tegenovergesteld is. In auto-immuun aandoeningen speelt de T_H17 cel een ziekteverwekkende rol, terwijl de Treg cel juist een

ziekte voorkomende rol heeft. Om het ontstaan van de ontsteking bij auto-immuniteit te begrijpen is het dus belangrijk om te onderzoeken hoe exact de keuze tussen het ontstaan van Treg of T_H17 wordt gemaakt. Het verschil tussen volwassen bloed (wel T_H17 te inductie; relatief lage aantallen Treg) en navelstrengbloed (geen T_H17 inductie, hoge aantallen Treg) vormen een mooi model om dit te onderzoeken.

Uit onze experimenten blijkt dat de T cellen uit navelstrengbloed wel verschillende kenmerken van T_H17 cellen vertonen, maar uiteindelijk niet de ontsteking inducerende signaalstoffen produceren die een T_H17 cel kenmerkt. Hoe dit gereguleerd wordt zal nog verder onderzocht moeten worden en zal verder inzicht geven in de regulatie van het afweersysteem.



DANKWOORD

Promoveren is gewoon werken. Dat vond ik toen ik er mee begon. En dat vind ik eigenlijk nog steeds wel. We moeten er vooral niet meer van maken dan het is. Dus waarom dan Jan en Alleman uitgebreid bedanken? Toch?

Maar een promotieonderzoek is wel bijzonder werk. Een groot deel ervan doe je alleen en voor je zelf. Persoonlijke motivatie voor je dagelijkse geploeter (vaak met weinig resultaat) is dus erg belangrijk. Je kan je motivatie niet alleen uit het labwerk zelf halen. Prettige omgang met collega's die in het zelfde schuitje zitten is minstens zo belangrijk. Je hebt collega's *echt* nodig om frustraties en successen mee te delen, om van te leren, om koffie mee te drinken en als publiek voor een pipettoren of een droogijs-experiment. Collega's worden daardoor meer dan alleen collega's waarop je echt kunt bouwen en dat maakt het promoveren in een lab bijzonder. Ondanks of juist dankzij de mix van eigengereide en eigenaardige persoonlijkheden, een warme capsule. Bedankt daarvoor!

Allereerst Baas B., Berent! Vaak op de achtergrond, maar altijd een bron van inspiratie en motivatie. Dank voor het vertrouwen de afgelopen jaren en in de aankomende jaren. Dank aan jou en Nico dat ik nog een tijdje in het lab mag blijven spelen!

Maarten Hoekstra, zonder jou was deze promotie er niet geweest. Voormalige bazen; Miek Jong en Adriana Contin; dankzij jullie ondersteuning heb ik terug de academische wereld in kunnen gaan.

Het "Allergiegroepje". Het is al een paar jaar terug dat we zo bekend stonden. Onze besprekingen op maandagochtend vond ik erg plezierig, nog even uitstel van de werkweek. Dankzij de vier moeders van het allergie-labje (Ismé, Mariska, Lieke en Sanne) werd ik wegwijs in het WKZ. Ismé, dank voor de discussies en het corrigeren van m'n teksten. Sanne, dank voor de samenwerking op een spannend project. Mariska, ik mis de herrie van falconbuisjes die je over het rooster in de flowkast raust nog dagelijks. Lieke, *de Reub!* Dank voor de vele kletsverhalen tijdens koffiepauzes...

Ger Rijkers, de onzichtbare begeleider. Je bent nooit officieel begeleider geworden, maar hebt voor mij zeker wel die rol vervuld. Jouw commentaar op mijn eerste artikelen was onmisbaar, en samen met jou een review schrijven was een avontuur. Zo veel kennis en inspiratie! Dank!

Marianne, dank voor het uitwisselen van gedachten en het overnemen van begeleiding op momenten dat ik echt iemand nodig had. Femke, geen begeleider, maar wel een bron van kennis, advies en gezelligheid.

M'n studenten, van jullie heb ik de afgelopen jaren nog wel het meeste geleerd! Merel, lekker tegendraads; Linda, morbide grappen; Genoveva, dansend door het lab. Dank jullie voor de mooie tijd. Merel extra dank voor de enorme experimenten die je na je stage nog gedaan hebt. Succes bij je eigen promotie! Genoveva: I'm proud you'll be joining us in the lab!

M'n paranimfen: Mark: De *Mark Klein Research Group* bestaat echt! Of je het wilt weten of niet, jij bent de hoeksteen van het lab. Zonder jou zou er geen CMCI zijn. Ik ben trots dat je mij wilt begeleiden naar het schavot! Joost, JASR waren de mooiste proeven van de afgelopen jaren. Gezelligheid gecombineerd met stoelensjoelen, filosofische discussies, en af en toe wat pipetteren. Top! Jouw oprechte interesse in andere mensen blijft me positief verrassen. Jij wordt de beste huisarts ooit.

Mijn andere speelkameraatjes in het lab: Ellen, WESR is nog op niks uitgelopen, maar veel andere dingen hebben we samen toch maar mooi afgerond. Retraites (2 stuks!), biologen-zetten-artsen-een-hak-samenkomsten, studentenbegeleiding optimaliseren, stukjes maken voor andere promovendi… etc. Altijd met veel plezier. Ik hoop dat je nog lang niet promoveert! Annick, cynische gek. De klaaguurtjes bij een koffiepauze waren onmisbaar; sinds jij op reis bent gaat het bergafwaarts met me… Al kan dat ook zijn door een gebrek aan je goede woordgrappen (die helaas te vaak onopgemerkt blijven…). Yvonne, groot voorbeeld hoe promoveren eigenlijk moet (op het maagzuur na dan). Dank voor de altijd relativerende opmerkingen. Theo, het wordt nu wel echt moeilijk nog een cartoon te vinden die de lunch aanprijst… Jenny, het lab heeft geen pipeteerrobot nodig, we hebben al de sexy bitch! Rianne, dank voor de laatste experimenten die je hebt gedaan terwijl ik achter mijn pc zat. Dat was onmisbaar.

De overige Prakken-groep: Marloes, Maja, Wilco, Bas, Ruud, Alvin, Selma, Annemarie, Annemieke, Henk, Eva, Joost S., Jorg, Eveline, Evelien, Lianne, Sarah, Erica, Angela. Dank voor gezelligheid en hulp op z'n tijd.

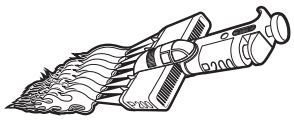
De Boes-groep. Ja, wat zal ik zeggen… niks dan positiefs? Natuurlijk: Robert, leuk dat we inmiddels kamergenoten zijn, hopelijk leer ik nu eindelijk eens wat het is. AJ, wie had ooit gedacht dat onze samenwerking zo soepel kon lopen, zelfs prettig is? Zeker nu dat haar er af is. Ewout Compaan en Flinsyflins, dank voor vogeltjes, fout vertaalde liedjes, koffie en gezelligheid. Het lab is goed gepimpt met de stereo-installatie. Lieneke, de charmantste van het stel, houd ze aub een beetje onder de duim!

De Nieuwenhuisgroep, Sylvia, Sabine, Caroline, Simone, Michal. Kerstin, dank voor de Duitse inspiratie. De bezoekjes aan de kerstmarkten waren legendarisch.

De Beekmangroep. Jeffrey, dank voor je adviezen in de afgelopen jaren. Succes met je eigen groep, Marit, Lodewijk, Pauline, Florijn.

De Coffers. Jullie gras is heus niet groener! Anders waren jullie niet constant bij ons aan het jatten. Maar toch ook wel goede buren die beter zijn dan een verre vriend. Bij een verre vriend is het lastig terug-jatten... Estel & Marije, dank voor het wekelijks leeg zwengelen van de navelstrengen!

Kamergenoten (in order of appearance), Eva, Femke, Ellen, Yvonne, Marco, Loes, Mirjam, Marcel Schijf, Niek, Koos, Robert. Een stabiele basis is het halve werk...



Now it's time to leave the capsule if you dare
David Bowie, Space Oddity

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Born December 5, 1978, Purmerend, The Netherlands

Education

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Biology, Utrecht University — finished 2002
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Research and Development Officer

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Prof. Dr. Prakken, Dr. Hoekstra, Dr. De Kleer

Postdoc under supervision of: — from 2012 onward
Prof. Dr. Prakken, Dr. Wulffraat

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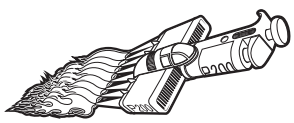

Herr Meister und Frau Meisterin,
Laßt mich in Frieden weiterziehn
Und wandern.

Franz Schubert/Wilhelm Müller, Das Wandern