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The Soil Bacterium *Methylococcus capsulatus* Bath and its Interactions with Human Immune Cells *in vitro*

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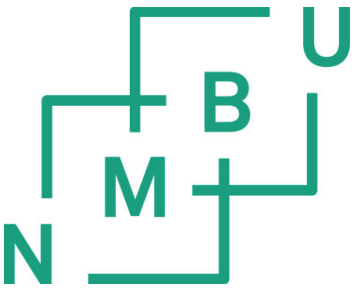

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ABBREVIATIONS

APC	Antigen-presenting cell
AMP	Antimicrobial peptide
CD	Crohn´s disease
CLR	C-type lectin receptor
DAMP	Damage-associated molecular pattern
GF	Germ-free
GAP	Goblet cell-associated antigen passages
IgA	Immunoglobulin A
IBD	Inflammatory bowel disease
IFN	Interferon
ILR	Interleukin receptor
IL	Interleukin
LP	Lamina propria
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
MAMP	Microbe-associated molecular pattern
M cells	Microfold cells
NLR	NOD-like receptor
PRR	Pattern recognition receptor
PP	Peyer's patches
Treg and Tr1	Regulatory T cells
RLR	Retinoic acid-inducible gene (RIG)-I-like receptor
SCFA	Short chain fatty acid
TCR	T-cell receptor
Th	T helper cell
TIVP	Type IV pili
TLR	Toll-like receptor
TGF	Transforming growth factor
TNF	Tumor necrosis factor
UC	Ulcerative colitis

LIST OF PAPERS

PAPER I

Indrelid, S., Mathiesen, G., Jacobsen, M., Lea, T., & Kleiveland, C. R. (2014). Computational and experimental analysis of the secretome of *Methylococcus capsulatus* (Bath). PLoS One, 9(12), e114476. doi:10.1371/journal.pone.0114476

PAPER II

Indrelid, S., Kleiveland, C., Holst, R., Jacobsen, M., & Lea, T. (2017). The Soil Bacterium *Methylococcus capsulatus* Bath Interacts with Human Dendritic Cells to Modulate Immune Function. Front Microbiol, 8, 320. doi:10.3389/fmicb.2017.00320

PAPER III

Indrelid, S., Tengs,T., Kleiveland, C. R. (2017). The non-commensal soil bacterium *Methylococcus capsulatus* Bath and the established probiotic bacteria *Lactobacillus rhamnosus* GG and *Escherichia coli* Nissle 1917, differentially modulate gene expression in in human monocyte derived DCs. *Manuscript*

SUMMARY

Whereas a role for commensal microbes in the development and normal function of the immune system is well established, a potential role for environmental bacteria in regulating immunity has been widely disregarded. However, the fact that the human immune system has evolved in the presence of, and in interaction with, microbial species from soil, water, air and fermented foods suggests that microbes from the environment may have co-evolved roles in immune system development and regulation.

In 2010 BioProtein, a feed product in which the soil bacterium, *M. capsulatus* Bath is the main component, was shown to prevent the development of soya-induced enteritis in Atlantic salmon (*Salmo salar*). In 2013 BioProtein was shown to prevent the development of inflammation in a dextran sulfate sodium induced murine colitis model. Furthermore, it was shown that the effect could be reproduced using a bacterial meal consisting of *M. capsulatus* (Bath) only, without the additional components present in BioProtein, showing that *M. capsulatus* is the active principle in BioProtein. The study suggested potent immunomodulatory properties of a non-commensal soil bacterium in mammals. However the mechanisms involved and the bacterial components responsible for these effects have not been identified.

This thesis present three studies describing characteristics of *M. capsulatus* Bath, its interactions with human immune cells and functional consequences of these interactions. Bacterial surface proteins, and proteins released to the environment, are important for how bacteria interacts with its surroundings in paper I we conducted an computational and experimental analysis of the secretome of *M. capsulatus* Bath. Results show that *M. capsulatus* (Bath) secretes proteins likely to be involved in adhesion, colonization, nutrient acquisition and homeostasis maintenance and suggests a potential for *M. capsulatus* Bath to interact with host cells.

In paper II we examined the interaction of *M. capsulatus* Bath with human leukocytes. Whereas a potential for *M. capsulatus* to engage in host interactions was suggested by results in paper I, paper II show that *M. capsulatus* Bath adheres specifically to human dendritic cells (DCs), but not to other cells present amongst peripheral blood mononuclear cells. DC play a central role in T cell immunity, and we showed that *M. capsulatus* compared to a probiotic Gram-positive bacterium and a Gram-negative bacterium of

commensal origin induced intermediate phenotypic and functional DC maturation. In a mixed lymphocyte reaction *M. capsulatus* Bath primed monocyte-derived dendritic cells (MoDCs), enhanced T cell expression of CD25, the α-chain of the high affinity IL-2 receptor, supported cell proliferation, and induced a T cell cytokine profile distinct from the control bacteria. *M. capsulatus* Bath thus modulate MoDC functions *in vitro*.

In paper III, to gain a more detail knowledge of how *M. capsulatus* Bath affects DC functions related to tolerogenic and immunogenic responses we applied genome wide transcriptomic analysis to MoDCs primed by *M. capsulatus* or two established probiotic bacteria that have also been reported to have protective effects in models of intestinal inflammation. Results confirmed that *M. capsulatus* Bath modulates MoDC gene expression and induces a state of maturation, cytokine and chemokine production intermediate between the two probiotic bacteria. Furthermore, *M. capsulatus* Bath induces MoDC expression of genes for immunosuppressive/immune regulatory cytokines, increases expression of the gene for galectin-10, a lectin involved in the suppressive function of human regulatory T cells and regulatory eosinophils, and reduces expression of the gene for CD70 a promising therapeutic target in chronic inflammatory bowel disease.

SAMMENDRAG

Det er kjent at kommensale bakterier spiller en viktig rolle både i utviklingen av immunsystemet og i regulering av normalt immunforsvar. Derimot har miljøbakteriers betydning for regulering av immunsystemet vært lite studert. Fordi det humane immunsystemet har oppstått omgitt av-, og i samspill diverse og tallrike mikrober fra jord, vann, luft, og fermenterte matvarer er det likevel rimelig å anta at disse også gjennom evolusjon kan ha fått viktige roller i utviklingen av-, og regulering av, et velfungerende immunforsvar.

BioProtein, et bakterielt fôr produkt der jordbakterien *Methylococcus capsulatus* er hovedkomponent, ble i 2010 vist å hindre utvikling av soyaindusert enteritt i Atlanterhavslaks (*Salmo salar*). I 2013 ble BioProtein vist også å beskytte mot utvikling av betennelse i en kjemisk indusert kolittmodell i mus. Samtidig ble det vist at et fôrtilskudd av *M. capsulatus* Bath alene, uten tilleggskomponentene som er tilstede i BioProtein, hadde den samme beskyttende effekten. Dette viste at *M. capsulatus* Bath er det aktive terapeutiske prinsippet i BioProtein, og at denne ikke-kommensale miljøbakterien har potente immunmodulerende egenskaper i pattedyr. Mekanismene bak, og de bakterielle faktorene som bidrar til denne beskyttende effekten er ikke kjent.

I denne avhandlingen presenteres tre studier som beskriver funksjonelle egenskaper ved *M. capsulatus* Bath*,* bakteriens interaksjoner med sentrale humane immune celler, og funksjonelle konsekvenser av disse interaksjonene.

Sekreterte proteiner er viktige for hvordan bakterier samhandler med sine omgivelser. I studien presentert i artikkel I utførte vi en analyse av sekretomet til *M. capsulatus* Bath ved hjelp av computerbaserte og eksperimentell tilnærming. Resultatene av denne studien viste at *M. capsulatus* Bath sekreterer proteiner med antatt funksjon i kolonisering, næringsopptak, homeostaseregulering og binding til overflater. Resultater fra denne analysen antydet også et potensiale for *M. capsulatus* Bath til å interagere med vert.

I artikkel II ønsket vi å undersøke hvordan *M. capsulatus* Bath påvirker humane leukocytter. Der resultater fra artikkel I antyder et potensiale for vertsinteraksjon viser resultater i artikkel II at *M. capsulatus* Bath binder seg spesifikt til dendrittiske celler, men ikke andre celletyper blant mononukleære blodceller. Dendrittiske celler spiller en viktig rolle i T celle

immunitet, og resultater fra artikkel II viser at *M. capsulatus* Bath, sammenlignet med en probiotisk Gram-positiv bakterie og en Gram-negativ bakterie av kommensalt opphav, induserer moderat fenotypisk og funksjonell DC modning. I en blandet leukocytt kultur induserte monocyttderiverte dendrittiske celler (MoDC) stimulert med *M. capsulatus* Bath økt T celle uttrykk av CD25, α-kjeden til høyaffinitets IL-2 reseptor, økt T celle proliferasjon og en T celle-cytokinprofil distinkt fra MoDC stimulert av kontrollbakteriene *M. capulatus* Bath modulerer dermed viktige DC funksjoner *in vitro*.

In arbeidet presentert i artikkel III, ønsket vi å oppnå bedre forståelse av hvordan *M. capsulatus* påvirker DC funksjoner knyttet til tolerogene og immunogene DC responser. Her gjorde vi en fullgenom transkripsjonsanalyser av MoDC som var stimulert med *M. capsulatus* Bath eller etablerte probiotiske bakterier. Resultatene fra denne studien bekrefter at *M. capsulatus* Bath modulerer MoDC genuttrykk og induserer en tilstand av MoDC modning, cytokin- og kjemokin- produksjon intermediær mellom de to probiotiske bakteriene. Resultatene viste også at *M. capsulatus* øker MoDC uttrykk av gener for immunsuppressive/immunregulatoriske cytokiner, øker uttrykket av genet for galectin-10, et lektin viktig for suppressive egenskaper ved regulatoriske T celler og eosinofiler, og reduserer uttrykket av genet for CD70 et lovende terapeutisk mål for behandling av kronisk inflammatorisk tarmsykdom.

1 INTRODUCTION

The prevalence of non-communicable, immune driven diseases has been steadily increasing since the middle of the twentieth century. Coinciding with increased urbanization and changes of lifestyle there has been a dramatic increase in diseases like asthma, atopic dermatitis, childhood type 1 diabetes, multiple sclerosis and inflammatory bowel disease (Bieber, 2008; Eder , Ege , & von Mutius 2006; Gale, 2002; M'Koma, 2013; Rosati et al., 1988). Autoimmune and chronic inflammatory diseases are typically multifactorial and arise from a complex interplay between genetic and environmental factors. However, the rapid increase of these diseases in the Western world points to environmental, rather than genetic causal factors. An increasing load of evidence suggests that reduced exposure to microbes is, at least partly, to blame (Cardwell, Carson, Yarnell, Shields, & Patterson, 2008; Ponsonby et al., 2005; Strachan, 1989, 2000). This thesis describes studies of an environmental bacterium previously shown to have anti-inflammatory properties in animal models of intestinal inflammation. The first part of the work presented in paper I focus on the bacterium and the identification of bacterial components that may be important for how this bacterium interacts with a host. The second part, presented in paper II and III focus on the immune modulatory interactions of this bacterium with human immune cells, and the functional consequences of these interactions.

1.1 The gastrointestinal immune system

Immunity is the ability of an organism to protect itself from disease by recognizing and acting against pathogens such as bacteria, parasites, viruses and tumor cells. The gastrointestinal tract (GIT) represents a potential entry point for pathogenic microorganisms. The GIT is also home to the body's largest collection of commensal bacteria, only separated from mucosal tissues by a single layer of epithelial cells. To cope with this constant challenge, the GIT also contains the largest collection of lymphoid tissues in the body and can be viewed as the body's largest immunologic organ. In addition to organized lymphoid tissues, the gut-associated immune system also contain more diffusely scattered cellular aggregates and leukocytes in the Lamina propria (LP) and the epithelium (Mowat & Agace, 2014) (Figure 1).

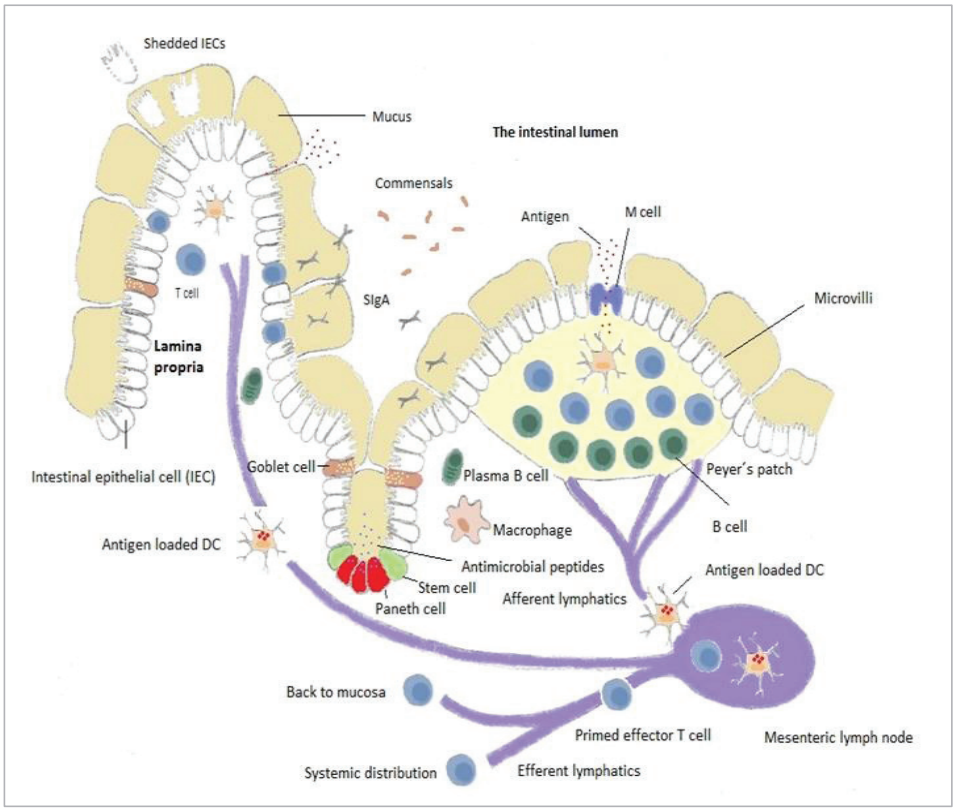

Figure 1. The gastrointestinal immune system.

Figure shows a section of the jejunum of the small intestine with long thin villi covered by surface epithelium with brush border. The central part of the villus comprises the lamina propria, containing numerous immune cells. Intraepithelial lymphocytes (IELs) can be seen lying between epithelial cells. Stem cells (Green) near the bottom of the crypts give rise to new (IECs) ensuring that old intestinal epithelial cells that are lost from the tip of the villus are continuously replaced. Mucus secreting goblet cells (brown) are found throughout the crypts, whereas Paneth cells releasing antimicrobial peptides (AMP) can be found near the bottom of the crypt (red). Antigen from the lumen may enter through microfold (M) cells in the follicle-associated epithelium (FAE), and are taken up by local dendritic cells (DCs). Antigen can be presented directly to T cells in the Peyer's patch or the antigen loaded dendritic cell may migrate via lymphatics to mesenteric lymph nodes to present antigen to naïve T cells there. Alternatively antigen may enter the lamina propria through alternative routes as described in the text and can be taken up by antigen-presenting cell (APCs) that may then migrate to MLNs for antigen presentation. Primed CD4+ T effector cells leave the MLN through efferent lymphatics (g), enter bloodstream and can home back to the mucosa as effector cells. Figure adapted from (Miller, Zhang, Kuolee, Patel, & Chen, 2007) and (Mowat & Agace, 2014).

1.1.1 Innate immunity

In mammals, the immune system can be divided in two branches, the innate and the adaptive branch, based on specificity, memory and speed of the response. Innate immunity is characterized by rapid, non-specific response and include physical barriers, humoral and cellular effector mechanisms (Romo, Perez-Martinez, & Ferrer, 2016)

1.1.2 The gastrointestinal barrier

Epithelial barriers represent the first defense mechanism that prevents pathogens from entering into the body, and maintaining an uncompromised barrier is vital to maintain homeostasis. Several mechanisms act in the intestines to prevent bacteria and other luminal components from coming in contact with the epithelium, or worse, passing through the epithelial barrier into the lamina propria. Tight junctions pins epithelial cells together to prevent unregulated transport between the cells. Goblet cells secrete gel-forming glycoproteins that forms a dynamic mucus layer covering the epithelium (Johansson, Larsson, & Hansson, 2011). In the colon the mucus layer is structured in two distinct layers, a looser and non-attached outer layer (Atuma, Strugala, Allen, & Holm, 2001), and a denser inner layer that is kept sterile to separate bacteria from the colon epithelium (Johansson et al., 2008).

In addition to the physical hindrances created by the mucus layers, molecular and mechanical mechanisms contribute to ensure sterility near the epithelium. Intestinal epithelial cells and specialized enterocytes produce antimicrobial peptides (AMPs) and enzymes, constitutively and in response to microbial stimuli or inflammation (Ostaff, Stange, & Wehkamp, 2013). Plasma cells in the LP secrete immunoglobulin A (IgA) that is transcytosed across the epithelial cell layer and released from the apical surface of epithelial cells. This IgA accumulates in the outer layer of colonic mucus (Rogier, Frantz, Bruno, & Kaetzel, 2014) and contributes to trapping bacteria in the mucus layer limiting the numbers of bacteria that reach the inner mucus layer.

Bacteria that manage to withstand biochemical barriers and colonize mucus or the epithelial cell layer, can be removed by mechanical forces. Stem cells within the intestinal crypts give rise to new epithelial cells, ensuring that the epithelial cell layer is constantly renewed. Dead cells and mucus are moved distally together with the stool by peristaltic movements contributing to keeping the epithelial surface free from bacteria. Collectively, physical and

mechanical barriers prevent microbes from entering into tissues, and ensure that most of the luminal bacteria never need to be handled by the immune system. However, if the primary barriers fail and pathogens manage to penetrate into body tissues, acute inflammatory cellular and humoral responses are initiated.

1.1.3 Innate immune cells

Beneath the mucosal lining, a large number of innate immune cells are found, ready to deal with anything that has passed through the epithelial barrier. Natural killer cells, mast cells, innate lymphoid cells, eosinophils, neutrophils, phagocytic macrophages and dendritic cells are found intraepithelial and in the lamina propria, or are quickly recruited to the site of infection.

The main cellular effectors of the innate immune system are professional phagocytes such as macrophages, dendritic cells and neutrophils that can engulf and destroy pathogens. Professional phagocytes have several central roles in both immediate pathogen clearance and activation of additional immune responses.

Although macrophages, neutrophils and dendritic cells are all efficient phagocytes, the specific role of each cell type is different. Macrophages and neutrophils are specialized for microbe elimination. Phagocytosis is a triggered process and require activation of surface receptors on the phagocyte to elicit the intracellular signaling that initiate the phagocytic response (Alberts et al., 2008). Particles that are to be phagocytized must first bind to surface receptors. Several different classes of receptors promotes phagocytosis, but the best known example is Fc receptors. As part of protective immune responses invading microorganisms can be coated by antibody molecules. Fc receptors recognized and bind the Fc region of these antibodies triggering uptake of the microbe. After engulfment, microorganisms are trapped, together with extracellular fluid, in membrane-surrounded vacuoles, the early phagosome. Once internalized, the phagosome vacuole undergo a series of fusions with membrane bound structures, a process known as phagosomal maturation. Through these fusion events the phagosome is increasingly acidified and acquires microbicidal features such as reactive oxygen and nitrogen species, antimicrobial proteins and peptides. The end result of this fusion events is the formation of a mature phagolysosome, a highly acidic and microbicidal organelle in which the microbe is eliminated (Flannagan, Cosio, & Grinstein, 2009).

The intestinal mucosa is one of the richest sources of macrophages in the body, and these tissue macrophages readily engulf both symbiotic commensals and pathogenic bacteria that crosses the epithelial barrier. However, macrophages in the LP exhibit a unique phenotype characterized by high phagocytic and bactericidal potential, but weak production of pro-inflammatory cytokines (Gordon & Taylor, 2005). In the bacteria-rich intestines, these are important features to maintain homeostasis.

In contrast to macrophages and neutrophils, dendritic cells are not directly involved in pathogen clearance. Instead, they are involved in pathogen recognition and in initiating adaptive immune responses. Their role is to sample the environment to identify pathogens, and to present this information to the adaptive immune system in the form of short microbe-derived peptides, so called antigens. Because dendritic cells must preserve information from the ingested particles DCs have developed various specializations of their phagosomal pathways, and acidification is much lower in DCs than in macrophages or neutrophils, resulting in reduced degradation and the conservation of antigenic peptides (Savina & Amigorena, 2007). How DCs translate information about a particular pathogen into instructions for the adaptive immune response is discussed in a later chapter.

1.1.4 Innate immune cells recognize pathogens through pattern recognizing receptors

To develop an appropriate response to pathogens, and to avoid autoimmunity, cells of the immune system must be able to distinguish self from non-self and to differentiate between diverse types of pathogens. Although the innate immune system lacks antigen specific receptors and is considered non-specific, this does not mean that it has no discriminatory capacity. The initial sensing of infection by the innate immune system is mediated by pattern recognition receptors (PRRs). These are germline-encoded receptors expressed by various cell types including epithelial cells and immune cells. Rather than recognizing specific microbes, a range of surface-associated and intracellular PRRs recognize microbe-associated molecular patterns (MAMPs), evolutionary conserved molecules typically present on microbes, but not host cells.

Four different classes of PRR families have been identified: the transmembrane Toll-like receptors (TLRs), the C-type lectin receptors (CLRs), the intracellular retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) and the NOD-like receptors (NLRs) (Takeuchi & Akira, 2010). Together these PRRs recognize diverse microbial components

and can differentiate between different types of microbes in the extra- and intracellular environment. Cell surface-expressed TLRs (TLR1, TLR2, TLR4, TLR5 and TLR6) recognize a wide range of viral proteins, bacterial and fungal cell wall components, and can differentiate between different groups of microorganisms. TLR4, for example, recognizes lipopolysaccharides (LPS), a highly potent immune-stimulant that is a major component of the cell wall of Gram-negative bacteria. TLR2 recognizes cell wall lipoproteins, lipoteichoic acids and mycobacterial lipoglycans from the envelope of Gram-positive bacteria, while TLR5 recognizes flagellin, proteins part of the flagella of motile bacteria. Other members of the TLR family (TLR3, TLR7, TLR8, and TLR9) are expressed in endolysosomal compartments, and recognize bacteria- and virus-derived nucleic acids, whereas CLRs recognize bacterial, fungal and viral carbohydrates. The intracellular NLRs and RLRs receptors are well positioned to detect intracellular pathogens. NLRs sense various ligands from pathogens in the cytoplasm as well as damage-associated molecular patterns (DAMPs), biomolecules released from the host cell in response to cell death and stress, whereas RLRs detect viral nucleic acids in the cytosol (Matsumiya & Stafforini, 2010; Motta, Soares, Sun, & Philpott, 2015). Recognition of MAMPs (and DAMPs) triggers intracellular signaling cascades, and ultimately the expression of genes for proteins involved in pathogen elimination including proinflammatory cytokines, antimicrobial proteins and chemokines (Takeuchi & Akira, 2010).

1.1.5 NF-κB signaling pathways

The NF-κB family of transcription factors is a critical component the immune response. Five gene products combine to form various active homo- and heterodimers responsible for directing expression of hundreds of genes. Active NF-κB transcription factors promotes the expression of over 150 target genes, a majority of which participates in the host immune response and includes genes for cytokines and chemokines, receptors required for immune recognition, such as MHC molecules, proteins involved in antigen presentation and receptors required for neutrophil adhesion and transmigration across blood vessel walls (Pahl, 1999). NF-κB activity therefore plays a central role in inflammatory responses by coupling signaling through PRRs with immune function (figure 2). Depending on the type of PRR engaged, the inducing stimuli and the specific cell type activated, distinct sets of genes can be upregulated to tailor the responses to the threat encountered. NF-κB activity is regulated on multiple levels (Dev, Iyer, Razani, & Cheng, 2010). NF-κB dimers form stable

complexes with inhibitor of NF-κB proteins, keeping the transcription factor in the cytoplasm. Signaling through PRRs, cytokine or antigen receptors activates a kinase complex known as inhibitor of NF-κB kinase, responsible for phosphorylating the NF-κB: IκB complex leading to its targeted ubiquitination and proteasomal degradation. Free NF-κB can be further activated or modified by phosphorylation, acetylation or glycosylation and can now translocate to the nucleus. Within the nucleus NF-κB transcription factors recognize and bind specific DNA sequences affecting expression of hundreds of genes involved in various aspects of cell physiology including anti-microbial immunity, inflammation, apoptosis, cell survival, with, and proliferation (Dev et al., 2010).

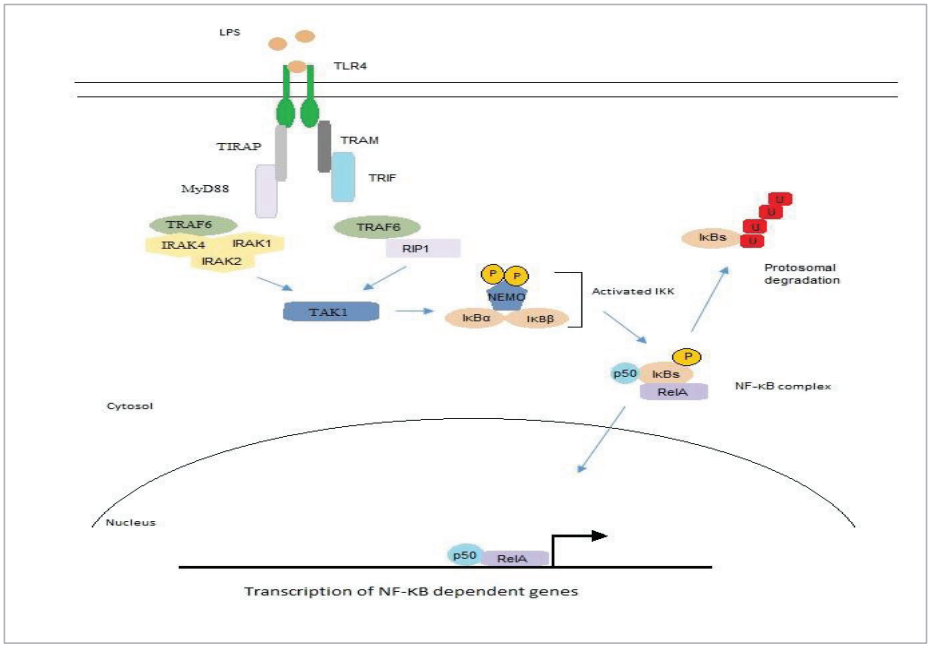

Figure 2. Activation of the canonical NF-κB signaling pathway

Signaling through various cell-surface receptors that can recognize a wide range of ligands can activate the NF-κB signaling pathway. The figure shows the activation of NF-κB by TLR4. Binding of LPS results in rearrangement of the cytoplasmic tail of TLR4 and recruitment of various adaptor and signaling proteins. TLR4 can activate NF-κB through MyD88 dependent or TRIF dependent pathways. Either pathway can activate TAK1 which subsequently activates the I-kappa B kinase complex (IKK). The NF-κB protein RelA in the cytosol is bound to inhibitory proteins (IκBs). The active IKK complex phosphorylates IκB marking the protein for ubiquitination and protosomal degradation. NF-κB is released and free to migrate into the nucleus where it can stimulate the transcription of target genes including inflammatory cytokines, chemokines and other genes central to inflammatory immune responses.

1.1.6 Cytokines and chemokines

Innate and adaptive immune responses depend on cooperation between various cell types. Intercellular communication is therefore essential to coordinate the response. Small signaling peptides called cytokines are major players in this communication. Cytokines are produced by, and affect, diverse cell types and tissues through binding to specific surface receptors that trigger intracellular signaling processes.

Cytokines are divided into different classes based on biological function, target receptors and associated signaling pathways (Romo et al., 2016). Chemokines constitute the largest family of cytokines (Griffith, Sokol, & Luster, 2014). These are a group of small chemotactic cytokines that are involved in directing migration and residence of immune cells during homeostasis and immune response. Cytokines such as the interleukins (ILs), tumor necrosis factors (TNFs), transforming growth factors (TGFs) and interferons (IFNs), modulate homeostasis and inflammation through a network of synergistic and antagonistic interactions. Therefore, although often classified as proinflammatory (IL-1, IL-6, TNFα, IL-17, IFNγ) or anti-inflammatory (IL-4, IL-10, IL27, IL-13, TGF-β), the same cytokine may have different effects depending on concentration, timing, the type of target cell affected and the local environment (Cavaillon, 2001). In fact, even prototypic pro-inflammatory cytokines may have additional functions related to tissue repair, remodeling and inflammation resolution contributing to steady-state (Scheller, Chalaris, Schmidt-Arras, & Rose-John, 2011).

1.1.7 Inflammation and innate immunity

Inflammation is a strategy to protect the organism from damage caused by microbial infections, tissue injury and other noxious conditions (Medzhitov, 2010). The inflammatory response to infection has traditionally been classified in four distinct phases: recognition of infection, recruitment of cells to the site of infection, elimination of the microbe, and resolution of inflammation and return to homeostasis (Barton, 2008). Upon microbial infection or tissue damage, innate inflammatory responses are alerted by recognition of evolutionary conserved MAMPs from pathogens, or damaged associated molecular patterns (DAMPs) from damaged cells (Broggi & Granucci, 2015).

Pattern recognition triggers inflammatory pathways resulting in the production of inflammatory mediators such as cytokines, chemokines, bioactive amines and eicosanoids.

These inflammatory mediators act on various cells and tissues, such as blood vessels, to induce vasodilation and increased vascular permeability, allowing increase of the blood flow to infected/damaged areas and extravasation of neutrophils and plasma influx (Medzhitov, 2010). Neutrophils and tissue resident macrophages seek and destroy invading pathogens. This process can be aided by serum components such as complement proteins or antibodies and then taken up via complement or Fc receptors.

The initiation, activation and resolution of innate inflammatory responses are mediated by a complex network of interactions among the numerous cellular and molecular components of immune and non-immune system (Liu & Cao, 2016). The type of inflammatory pathways induced, inflammatory mediators formed, inflammatory responses initiated and tissues affected depend on the nature of the inflammatory trigger ensuring appropriate responses (Medzhitov, 2010).

Whereas the ability to launch efficient inflammatory responses is essential to enable survival during infection, it is equally important to prevent the progression from non-resolving acute inflammation to persistent chronic inflammation (M. A. Sugimoto, Sousa, Pinho, Perretti, & Teixeira, 2016). The process of inflammation resolution initiate shortly after initiation of the inflammatory responses. Chemokines that were formed as part of the inflammation process are depleted by proteolysis, sequestration by atypical receptors, and degradation by neutrophil extracellular traps, restricting the influx of neutrophils once sufficient numbers of cells have been recruited. In addition, inflammatory mediators like prostaglandins may induce negative-feedback loops to downregulate inflammatory cytokines, and pro-resolution mediators like lipoxins, resolvins, protectins, and maresins provide anti-inflammatory signals to prevent further granulocyte ingress, activate apoptosis of inflammatory granulocytes and turn on tissue reparative programs (M. A. Sugimoto et al., 2016).

Whereas the first line of defense by innate immune cells and humoral factors may be sufficient to eliminate an infection, sometimes an adaptive immune response is also required. Adaptive immune responses take several days to develop, and the innate immune response makes crucial contribution to the activation of adaptive immunity.

1.1.8 Adaptive immunity

The adaptive immune system is characterized by specificity and immunologic memory to allow faster and more efficient attacks upon subsequent encounters with pathogens. Whereas innate immunity is rapid and depends on recognition of pathogens by germline-encoded receptors, adaptive immunity develops later, because adaptive immune cells specific for the pathogen must first be clonally expanded, differentiate into effector cells and migrate to the site of infection to assert their effector functions.

1.1.9 Lymphocytes are the cells of the adaptive immune system

The cells of the adaptive immune system, T cells and B cells, are lymphocytes that carry membrane-attached antigen specific receptors. B cell antigen receptors (BCRs) and the T cell receptors (TCRs) recognize foreign structures from bacteria, virus or cells, so called antigens.

Antigen receptors are generated through a process of random genetic recombination independent of antigenic stimulation. This ensures that although each lymphocyte carries only one receptor specificity, among all the lymphocytes of different specificities, there exists a highly diverse repertoire of receptors for any antigenic structure. Many different lymphocytes each carrying a different antigen receptors can typically recognize different parts of the same antigen. The part of the antigen that is recognized by the binding site of any particular antigen receptor is known as the epitope.

1.1.10 T cell development

T cell development takes place in the thymus. Progenitor cells (thymocytes) lacking most of the characteristic T cell markers arrive from the bone marrow. The thymocytes interact with thymic epithelial cells triggering an initial differentiation along the T-cell lineage pathway. The cells undergo rapid proliferation and begin to express cell-surface molecules specific for T cells. Developing thymocytes pass through a series of distinct phases that are marked by changes in the status of T-cell receptor genes and in the expression of the T-cell receptor, and by changes in expression of the cell-surface co-receptor proteins CD4 and CD8 (Janeway CA Jr, 2001).

As the antigen binding site of T cell receptors is produced by random recombination events, there is a need for control mechanisms to ensure functionality and avoid auto-reactivity. T cells with all kinds of receptor specificities are produced, and it is vital to make sure that only T cells with functional and not strongly self-reactive T cell receptors are allowed to circulate in the body. Developing T cells therefore undergo positive and negative selection processes before they can leave the thymus ensuring that only functional, but not self-reactive T cells reaches maturity. T cells leaving the thymus to enter the blood, lymph and secondary lymphoid organs have not yet encountered foreign antigen and are considered naïve T cells. T cell activation and development of effector functions requires physical interactions with innate immune cells carrying an antigen recognized by the T cell receptor. The process of antigen acquisition and antigen presentation is discussed next.

1.1.11 Routes of antigen acquisition

As discussed above, professional phagocytes are frequent in the lamina propria. Some of these are specialized antigen presenting cells (APCs). They acquire information of microbes that has escaped through the epithelium and then present information about the encountered threat to cells of the adaptive immune system. However, even when the epithelial barrier is intact, luminal content is continuously monitored by the mucosal immune system.

In the absence of barrier disruption antigen can be delivered through the epithelium by four different pathways: M cells, goblet cell-associated antigen passages (GAPs), intraepithelial dendritic cells and paracellular leak (Knoop, Miller, & Newberry, 2013). Mucosal lymphoid tissues, such as the Peyer's patches (PP) of the small intestines, colonic patches of the colon and isolated lymphoid follicles and cryptopatches of the small intestines and colon, are lymphoid tissues found embedded directly in the submucosa and protruding into the mucosa. The follicle associated epithelium is highly specialized to sample luminal antigens and bacteria: It contains few goblet cells and enteroendocrine cells. Instead, the follicle-associated epithelial layer contains specialized enterocytes termed microfold cells (M cells) dedicated to the transport of luminal material to the follicles. In the PPs immediately below the follicle-associated epithelium aggregates of dendritic cells are found ready to pick up and process antigen.

However, follicle-associated epithelium represents only a small proportion of the surface area of the intestine, and other delivery routes exist to transport antigen into LP. In addition to their well-known role in mucus production, goblet cells via GAPs deliver small soluble antigens from the intestinal lumen to underlying APCs in the steady-state (McDole et al., 2012). GAPs have been suggested best suited to deliver small soluble antigens, such as those derived from the diet (Knoop et al., 2013).

In addition to sampling of intact luminal antigens in lymphoid follicles, and sampling of small molecules through GAPs, scattered APCs may also be involved in direct luminal sampling. Rescigno et al. (2001) observed that CD11c-expressing cells with the gut epithelium were capable of sample luminal microbes by protruding dendrites through tight junctions between epithelial cells. There has later been some controversy around whether these cells in fact represent DCs capable of antigen presentation, or non migratory $CX3CR1^+$ macrophages representing immune effectors rather than immune inducers (Niess et al., 2005), but recent data suggest that also a $CD103^+$ cell population can sample luminal antigen. Bacterial challenge was shown to recruit $CD103^+$ DCs from the lamina propria (LP) and into the epithelium. These $CD103^+$ DCs extended dendrites trough the epithelium and actively sampled luminal *Salmonella* (Farache et al., 2013). $CD103^+$ DC was shown by others to be capable of inducing antigen specific T cell responses and consist a DC population distinct from $CX3CR1^+$ cells (Schulz et al., 2009).

Paracellular leak is a fourth route of antigen delivery across the epithelium. Tight junctions can be locally regulated to form larger pores in the epithelium to allow transport of small molecules and carbohydrates between epithelial cells. Antigen delivered through paracellular leak did not appear to be efficiently picked up by DC in the lamina propria in a study by McDole et al. (2012), but may be delivered directly to mesenteric lymph nodes via prelymphactic channels and lacteals in the villus (Knoop et al., 2013).

The mode of antigen delivery might be functionally important. In a review discussing the different routes of trans-epithelial antigen delivery Knoop et al. (2013) suggests that antigen introduced through M cells are particularly important in inducing IgA responses, antigen delivered by GAPs contributes to peripheral tolerance, and antigen delivered by paracellular leak initiates immune responses in the MLN.

Finally, luminal sampling by intracellular DC may play an important role in host protection during pathogen infection by grabbing potential pathogens and quickly initiating inflammatory responses before the barrier is breached.

1.1.12 Dendritic cells and T cell activation

The adaptive immune system does not operate on its own, but requires tight co-operation with the innate immune system to function. In order proceed from a naïve to an activated and functionally mature state; T cells depend on signals from, and physical interactions with, innate APCs. Dendritic cells, when activated by microbial stimuli, matures into a highly effective antigen-presenting cell and play a particularly important role in shaping T cell immunity.

Three different signals are required for the activation, proliferation and differentiation of naïve T cells into Th effector cells or regulatory T cells (Kapsenberg, 2003) (Figure 3). Signal 1 is delivered through the T-cell antigen receptor (TCR). The T cell receptor recognizes peptide antigens displayed on cell surfaces together with specialized glycoproteins, major histocompatibility complex (MHC) molecules. Two different classes of MHC molecules exist. The function of the two classes is similar, but they display antigens from different sources and obtained by different pathways (Neefjes, Jongsma, Paul, & Bakke, 2011). The two main T cell linages, $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells, each interacts with only one of the two classes.

MHC class I molecules are expressed on all nucleated cells and present peptide fragments from invading intracellular pathogens or other peptide fragments from the cytosol to $CD8^+$ cells. In contrast, MHC class II proteins are expressed only by professional APCs, and are responsible for presenting antigens to $CD4^+$ T cells, so called T-helper cells. Signal 1 is necessary but not sufficient for T cell activation. It ensures that only T cells with the right antigen specificity are activated, but additional signaling is necessary for full T cell activation.

Signal 2, often referred to as “co-stimulation” is mediated by interactions between co-stimulatory molecules on APC through co-signaling receptors on the T cell. In the classical two-signal model of T cell activation co-stimulation is often illustrated by the signaling through co-receptor CD28 constitutively expressed on the surface of T cells interacting with co-stimulatory molecules of the B7 class (CD80/86) on the surface of APCs (Slavik,

Hutchcroft, & Bierer, 1999; Smith-Garvin, Koretzky, & Jordan, 2009). Activation of the TCR in the presence of co-stimulatory signals supports T cell survival, proliferation and development of effector functions (Slavik et al., 1999).

The process of developing into specialized effector T cells starts in parallel with antigen-specific activation and clonal expansion. In addition to signal 1 and signal 2, the local cytokine milieu during T cell activation is highly important to determine the functional outcome of the process. DC cytokines act as a third signal to guide this process, and are important decisive polarizing factors. The balance of DC-released cytokines contribute to determine what kind of T effector cells are produced and thus the functional direction of the adaptive immune response.

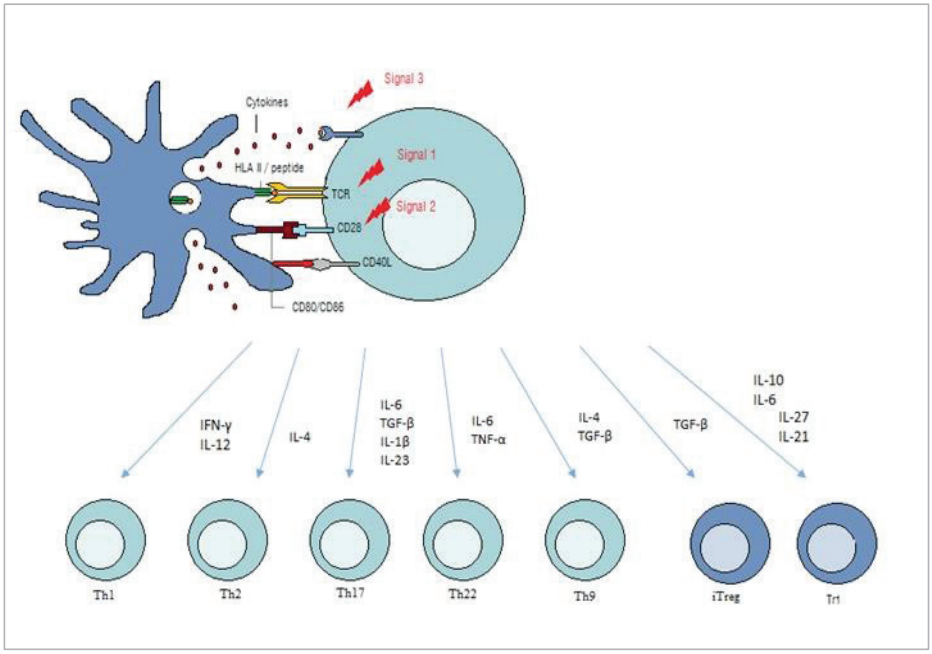

Figure 3. DC meditated activation and instruction of naïve T cells.

Three different signals are required for activation of naïve T cells. HLA class II molecules presenting antigen to T cell receptor mediates the antigen-specific signal 1. Co-stimulatory molecules CD80/CD86 on the dendritic cell interacts with the CD28 receptor on the T cell to confer the co-stimulatory signal 2. Cytokines released by the dendritic cell contribute T cell polarizing signal 3 that promote the development of individual T helper or regulatory T cell subsets. Figure is adapted from (Kaplan, Hufford, & Olson, 2015; Kapsenberg, 2003; Swain, McKinstry, & Strutt, 2012)

1.1.13 CD4+ T effector cells functions

$CD4^+$ T helper cells are central effector cells of the adaptive immune system, and play important roles in both protective immunity and tolerance. As implied by their name, the functionality of T helper cells is linked to their ability to help or enhance the function of other immune cells by inducing (or inhibiting) cell proliferation, differentiation and activation. $CD4^+$ T cells play a leading role in orchestrating homeostasis and inflammatory responses in the gut, and they do so mainly by producing various cytokines to recruit cells or to change the activation status or behavior of nearby cells.

$CD4^+$ T-cell populations can be broadly divided into T helper cells (Th1, Th2, Th9, Th17, Th22) and regulatory T cell populations, each with distinct cytokine-secretion phenotypes and unique functional characteristics. The initial classification of $CD4^+$ effector populations encompassed two subsets of murine $CD4^+$ cells, Th1 and Th2 cells, that could be distinguished based on the cytokines they produced and the surface molecules they expressed (Mosmann, Cherwinski, Bond, Giedlin, & Coffman, 1986). The distinct cytokine profile characteristic for each linage was reflected in different functional roles in the immune response. Th1 cell development is favored in response to IL-12 and IFN-γ. Th1 cells produce IFN-γ, lymphotoxin and are good IL-2 producers. Th1 cytokines activate macrophages and phagocyte-dependent protective responses (Mosmann & Sad, 1996; Romagnani, 1999) and are primarily involved in cell-mediated responses against intracellular bacteria and viruses.

In contrast, Th2 development from naïve $CD4^+$ cells is favored by the presence of IL-4 and IL-2. Th2 cells fail to produce IFN-γ, lymphotoxin, and are characterized by IL-4, IL-5, and IL-13 production and only modest levels of IL-2 (J. Zhu, Yamane, & Paul, 2010). Th2 cells predominate in response to infestations by gastrointestinal nematodes and are associated with strong antibody and allergic responses. They encourage antibody production and enhance eosinophil proliferation and function (Mosmann & Sad, 1996).

More recently, Th17, a third major $CD4^+$ effector linage has been described. Th17 cells are characterized by production of the signature cytokines IL-17A, IL-17F, and IL-22 and are good IL-21 producers. Th17 cells are involved in the elimination of extracellular pathogens and fungal infections, coordinating tissue inflammation, and has been linked to autoimmunity (Noack & Miossec, 2014). The Th17 cytokines IL-17A and IL17F act on a variety of cells to induce production of proinflammatory cytokines, chemokines and

metalloproteases to recruit neutrophils and other immune cells to sites of infection (Akdis, Palomares, van de Veen, van Splunter, & Akdis, 2012). IL-22 targets epithelial cells and induces secretion of defensins and mucus, thereby strengthening the intestinal barrier function (K. Sugimoto et al., 2008).

IL-22 production is also found in Th22 cells, another Th subset distinct from Th1, Th2 and Th17 cells (Trifari, Kaplan, Tran, Crellin, & Spits, 2009). This subset is characterized by production of IL-22 and TNF-α, but with little or no IL-17, IFN-γ and IL-4. Similar to the Th17 subset, Th22 cells are involved in inflammatory immune responses against bacterial infections (Basu et al., 2012) and have been linked to autoimmunity (J. Yang, Sundrud, Skepner, & Yamagata, 2014). However, the defining Th22 cytokine IL-22 has also been linked to improved barrier function and wound healing (Eyerich et al., 2009; Mizoguchi, 2012), and there is still controversy as to whether Th22 cells should be regarded as pathogenic or protective in an intestinal setting (Azizi, Yazdani, & Mirshafiey, 2015; Symons, Budelsky, & Towne, 2012).

Th9 cells are one of the more recently described subsets of effector T cells. Th9 development is promoted by IL-4 and TGF-β. Functional Th9 cells are characterized by IL-9 production and have also been shown to produce IL-10 and IL-21 (Kaplan, 2013). IL-9 is a pleiotropic cytokine that can function as both a positive and negative regulator of immune responses. It has detrimental roles during allergy and autoimmunity but acts protective in pathogen clearance and can promote the maintenance of a tolerant environment during skin transplantations (Noelle & Nowak, 2010). IL-9 is important in immune responses against helminths, and participates in the pathogenic process of allergy and allergic asthma by promoting proliferation and survival of mast cells, $CD4^+$ T cells and other leukocytes in the respiratory tract and gut, and to modulate intestinal permeability function via the regulation of tight junction molecules (Gerlach, McKenzie, Neurath, & Weigmann, 2015; Tan & Gery, 2012).

1.1.14 Regulatory T cells are involved in cell-mediated immune suppression

Some $CD4^+$ T cells have regulatory functions rather than defensive functions. Whereas Th cells promote inflammation, regulatory T cells (Tregs) are involved in negative regulation of inflammation. They are characterized by the capacity to suppress the activation, proliferation and effector functions of a range of other immune cells, and therefore have

important functions in preventing autoimmunity by maintaining self-tolerance and by controlling expansion and activation of autoreactive $CD4^+$ T effector cells.

Different subsets of regulatory T cells have been identified. Tregs that express high levels of CD4, the α-chain of the high affinity IL-2 receptor (CD25), and the transcription factor FoxP3, include both naturally occurring Tregs (nTregs) generated in the thymus and induced Tregs (iTregs), that can be generated from naïve T cells in the periphery driven by TGF-β and IL-2 (Sakaguchi, Miyara, Costantino, & Hafler, 2010; Schmitt & Williams, 2013). More recently, another regulatory T-cell subsets have been identified and is referred to as Tr1 cells. Tr1 do not express FoxP3, but produce IL-10 and, similar to nTregs and iTregs, have potent immunosuppressive properties. As reviewed by (Zeng, Zhang, Jin, & Chen, 2015) several cytokines, IL-21, IL-6, IL-27, and in particular IL-10 are essential for Tr1-cell development.

The understanding of molecular mechanisms of T cell-mediated suppression is still limited. However, several cell-surface molecules have been suggested to play a role (Josefowicz, Lu, & Rudensky, 2012). CD25, a subunit of IL-2 receptor (IL-2R) is upregulated on effector T cells and expressed at a high levels on Tregs. Thus, high-levels of high affinity IL-2R expression on Treg cells could deprive other effector T cells of IL-2 and inhibit their proliferation. Other proposed mechanisms include suppression mediated by cell–cell contact, metabolic disruption, and the secretion of immunosuppressive cytokines such as IL-10, TGF-β and IL-35 (Sakaguchi et al., 2010; Schmitt & Williams, 2013). Because of their capacity to suppress T cell function Tregs were early on predicted to be involved in prevention of autoimmune diseases by maintaining self-tolerance, but Tregs have also been proposed to be involved in tolerance to dietary antigen, suppression of allergy and repression of pathogen-induced immunopathology as well as several other functions (Corthay, 2009).

1.1.15 DC control peripheral T cell tolerance

The fact that antigen specific T cell receptors are formed through random genetic recombination ensures a receptor repertoire capable of recognizing virtually any kind of antigen, to recognize both previously encountered, and novel pathogens. However, it also poses a serious problem, because some of these receptors will inevitably recognize innocuous antigens and worse, self-antigens. Strong control mechanisms must therefore be

in place to avoid autoimmunity and excessive inflammatory responses. The mechanisms by which the immune system prevent self-reactivity are known as immunological tolerance. Central tolerance to self-antigens is established before developing T cells leave the thymus when cells that bind with high affinity to MHC molecules presenting self-antigen are eliminated. However, cells bearing T cell receptors with low-affinity to self-antigens can escape to the periphery. Peripheral tolerance serves as a backup to prevent such autoreactive cells to create damage, as well as suppressing detrimental immune responses against other harmless materials, such as food components, or commensal organisms.

DCs not only play a central role in initiating immune defense against pathogens, but also in controlling peripheral T cell tolerance (Manicassamy & Pulendran, 2011; Steinman, Inaba, Turley, Pierre, & Mellman, 1999; Xing & Hogquist, 2012). The potential of DCs to induce T cell tolerance is related to their maturation status and, but also exposure to environmental factors such as immunosuppressive agents, microbial stimuli, or environmental cues from the tissue environment can induce DC tolerogenic properties (Li & Shi, 2015).

1.1.16 DC maturation state is connected to immunogenic vs tolerogenic DC functions

DCs can exist in two different states associated with distinctive gene expression patterns phenotype and function (Dalod, Chelbi, Malissen, & Lawrence, 2014). In an immature, resting, state immature DCs (imDCs) are highly phagocytic and efficient in antigen capture, but express low levels of co-stimulatory molecules and MHC class II molecules necessary for antigen-presentation. However, stimulation of the cell´s PRRs can trigger a program of DC maturation that prepares the cell for antigen presentation, co-stimulation and cytokine production. The cell loses its capacity for phagocytosis. At the same time MHC class II and co-stimulatory molecules are upregulated, as is the expression of chemokine receptors necessary for the cell to migrate to T cell rich zones of secondary lymphoid tissues where the cell can present antigen to naïve T cells. It has been proposed that antigen presentation by mature DCs, expressing high levels of MHC class II and co-stimulatory molecules, induces T cell activation, proliferation, and effector cell differentiation, promoting immunogenic responses. In contrast, antigen presentation in the absence of co-stimulation by imDCs expressing low levels of MHC molecules results in T cell deletion or Treg development promoting immune suppression (Manicassamy & Pulendran, 2011). However, whereas mature DCs are generally considered immunogenic, certain stimuli induce DC maturation, yet stimulates T cell tolerance.

DCs can mediate tolerance through various interrelated mechanisms including deletion of T cells, induction of T cell anergy, the shaping effector T cell subsets and Tregs, expression of immunomodulatory molecules and the production and release of immunosuppressive factors (Ezzelarab & Thomson, 2011; Li & Shi, 2015; Manicassamy & Pulendran, 2011).

Not only the absence or presence of co-stimulation defines DCs as tolerogenic vs immunogenic. Rather a complex network of transmembrane receptor/ligand pairs acts to enhance or modify T cell activation. The pattern of co-stimulation hence is a central feature distinguishing tolerogenic and immunogenic DCs (Hubo et al., 2013). Whereas co-stimulation often is considered to result from the engagement of T cell-derived CD28 with DC-expressed B7 molecules (CD80/CD86), numerous other co-stimulatory molecules and co-receptors have been identified on APCs and T cells respectively. Costimulatory signaling mediated by these molecules can be divided into activating or inhibitory pathways depending on whether they promote T cell proliferation or attenuate T cell responses (Bakdash, Sittig, van Dijk, Figdor, & de Vries, 2013). The balance between such co-stimulatory and co-inhibitory signaling determines the outcome of antigen-presentation in terms of T cell activation and quality of T cell responses (Bakdash et al., 2013).

Furthermore, DC-produced cytokines and other immunomodulatory factors play important roles in DC tolerogenic responses. Tolerogenic DCs can produce and secrete immune suppressive cytokines such as IL-10, TGF-β and IL-27 or other immunosuppressive factors such as indoleamine dioxygenase that can act by promoting Treg development, inhibit T cell proliferation or assert immune suppressive effects on other cell types (Ezzelarab & Thomson, 2011). Finally, mature DCs produce a range of cytokines that can both promote and modulate T effector cell differentiation. DCs are thus critical in maintaining peripheral tolerance by generation of anergic or regulatory T cells, and by fine-tuning responses by altering the T-helper (Th1/Th2/Th17) cell balance (Manicassamy & Pulendran, 2011).

1.2 Microbial contributions to health

The prevalence of several immune driven diseases like asthma, atopic dermatitis, childhood type 1 diabetes, multiple sclerosis and inflammatory bowel disease has been steadily increasing since the middle of the twentieth century (Bieber, 2008; Eder et al., 2006; Gale, 2002; M'Koma, 2013; Rosati et al., 1988). Although the increased prevalence of autoimmune, allergic and chronic inflammatory diseases has been reported to rise also in developing countries, the trend is particularly prominent for urbanized communities in developed countries (Bach, 2002; Patterson, Carson, & Hadden, 1996). Several lines of evidence suggest that reduced microbial exposure may be an important contributing factor. The rapid increase in prevalence suggests that environmental, rather than genetic causal factors are responsible. Furthermore, an overall North-South gradient for these disorders has been noted in North America, Europe and China, and a west–east gradient in Europe, mirroring the geographical distribution of infectious diseases including hepatitis A, gastrointestinal infections and parasitic infections (Okada, Kuhn, Feillet, & Bach, 2010).

1.2.1 Hygiene hypothesis

The hygiene hypothesis explains the increased prevalence of allergic and autoimmune diseases as a result of reduced exposure to childhood infections. In 1989, Strachan observed that the risk of developing hay fever in childhood was inversely related to family size and the and number of older siblings. Strachan proposed that childhood infections may somehow protect against atopy later in life, and that increased hygienic standards have reduced our exposure to such protective infections. Similar negative correlations between number of siblings and risk of developing MS and T1D have since been noticed (Cardwell, Carson, Yarnell, Shields, & Patterson, 2008; Ponsonby et al., 2005). Other studies have found protective effects of bacterial lipopolysaccharide, helminth parasites and lactobacilli (Bjorksten, Naaber, Sepp, & Mikelsaar, 1999; Braun-Fahrlander et al., 2002; Yazdanbakhsh, Kremsner, & van Ree, 2002), supporting a role for microbes and microbial components in regulating normal immune function.

1.2.2 The commensal microbiome

The contribution of microbes to human health is well established for intestinal commensal microorganisms. Bacteria colonize all human surfaces, but the highest numbers, and most diverse communities, of commensal microbes are found in the human gut. 10^{13} to 10^{14}

microorganisms inhabit the human intestines, with the highest numbers found in the colon (Gill et al., 2006). The mutual regulatory interactions that exists between intestinal commensal bacteria and mammalian hosts illustrates well how microorganism can beneficially influence human health.

Colonization by commensal microbes starts at birth. The fetal gastrointestinal tract is sterile, but during birth the infant leaves the sterile environment of the uterus, and is exposed to a variety of microbes from the environment. For children delivered by vaginal birth the first microbes encountered are those of the mother´s birth canal and gastrointestinal tract. In contrast, in caesarian births the initial exposure is to microbes of the mother´s skin and the hospital delivery room. Accordingly, children delivered by caesarian section have a different composition of commensal bacteria than children delivered vaginally (Dominguez-Bello et al., 2010). This initial colonization and the resulting immune- and metabolic programming is expected to have implications for health later in life. In a Danish study of two million children, babies delivered by cesarean section had significantly increased risk of asthma, systemic connective tissue disorders, juvenile arthritis, inflammatory bowel disease, immune deficiencies, and leukemia later in life (Sevelsted, Stokholm, Bonnelykke, & Bisgaard, 2015). Caesarian section is different from vaginal birth with respect to several factors (e.g. use of anesthetic agents and antibiotics, physiologic effects on the newborn, and hospital environment after birth). Nevertheless, differences in the infant microbiome was suggested responsible for the increased risk of developing immune-related diseases later in life for children delivered by caesarian section (Sevelsted et al., 2015).

Colonization continues during early childhood in a stepwise manner, to form a mature microbiome. A stable, diverse and well-balanced human microbiome is expected to be important to human health for several reasons. Firstly, a stable microbiome confer resilience to invasion by new bacterial species by direct microbe–microbe interactions, by competing for nutrients and niches or by creating conditions within the intestine that can inhibit growth and virulence gene expression (Lawley & Walker, 2013). Secondly, a diverse microbiome adds metabolic flexibility to its host: Estimates suggest that the commensal genome outnumber human somatic and germ cells by at least an order of magnitude, and may encode a 100-fold more unique genes than the human genome, adding substantial genetic and functional flexibility to a relatively small human genome (Qin et al., 2010; Turnbaugh et al., 2007). Humans, for example, lack the biosynthetic capacity for many vitamins, but certain members of the microbiome can synthesize and supply vitamin K and several of the

B-group vitamins to the human host (LeBlanc et al., 2013). Furthermore, the intestinal microbiota can also ferment complex carbohydrates indigestible by the host. 10-20 % of the dietary carbohydrate ingested by humans are resistant to small intestinal digestion, such as pectin or starch (Ramakrishna, 2013). Non-digestible dietary carbohydrates can be fermented by colonic bacteria to produce short chain fatty acids (SCFA) such as butyrate, propionate and acetate. SCFA, serve as energy sources for intestinal epithelial cells liver- and muscle cells, promote epithelial barrier function and have important immune regulatory functions. The contribution of commensal microbes to human health is however not restricted to pathogen resistance and nutritional benefits to their hosts. Commensal microbes also have important regulatory functions in mammalian immune function.

1.2.3 Commensals provide signals necessary for immune system development and function

Studies of germ-free (GF) animals, animals delivered and raised under sterile conditions have highlighted the importance of commensal microbes in immune development and function. Microbial stimuli are necessary for the development of the mucosal immune system and the epithelium (Belkaid & Hand, 2014; Round & Mazmanian, 2010; Smith, McCoy, & Macpherson, 2007). GF animals display developmental defects such as fewer/smaller Peyer's patches and mesenteric lymph nodes, and reduced numbers of $CD4^+$T cells and IgA-producing plasma cells. The microbiota also contribute regulatory signals necessary for a normal immune function. Intestinal bacteria influence the expression and localization of PRR in the gut, and GF animals are deficient in defensins and other antimicrobial substances and are more susceptible to infections than conventional mice (Round & Mazmanian, 2010). This shows that microbial signals are important to maintain the first line of defense and epithelial barrier function. Furthermore, commensal microbes are also important in homeostatic immune regulation.

A complex network of regulatory pathways are involved in maintaining homeostasis in the gut. Commensals and their metabolites may stimulate multiple host pathways affecting various innate and adaptive immune functions and tissue repair.

Th17 and Treg cells are common in the intestinal mucosa, where they may have contrasting roles. Whereas Th17 cells function to protect the host from pathogenic microorganisms, Tregs function to restrain excessive effector T-cell responses (Omenetti & Pizarro, 2015). The balance between Treg and Th17 effector cells is thought to determine the intestinal

status where a shift towards Th17 or Treg corresponds to an inflammatory and immune tolerant status, respectively. Gut commensals, their metabolites and secreted proteins have been reported to regulate the differentiation and expansion of several T effector subsets in animal models (Furusawa et al., 2013; Ivanov et al., 2009). Evolutionary diverse groups of commensal bacteria like the Gram-negative *Bacteroides fragilis*, and the Gram-positive *Bifidobacterium infantis* have been reported to drive the generation and function of Tregs (O'Mahony et al., 2008; Round & Mazmanian, 2010). Other groups may induce Th17 cells and thereby antimicrobial responses. For example, segmented filamentous bacteria, murine commensals, induces $CD4^+$ cells that release IL-17 and IL-22 resulting in enhanced resistance to the intestinal pathogen *Citrobacter rodentium* in mice (Ivanov et al., 2009).

1.2.4 "Old-friends" provide regulatory input for the immune system

Not only stably colonizing commensals, but also microbes transiently associated with the gastrointestinal tract have been claimed to provide important 'educational' input for the immune system. Despite the popularity of the hygiene hypothesis, several studies have failed to show a causal relationship between childhood infections and autoimmune diseases as suggested by the hypothesis (Benn, Melbye, Wohlfahrt, Bjorksten, & Aaby, 2004; Matricardi et al., 2000). As a refinement of the hygiene hypothesis Rook et al. (2004) postulated the "old-friends" hypothesis. Rook, Raison, and Lowry (2014) suggest that a general reduced exposure to environmental bacteria and old chronic infections, not modern childhood infections, is responsible for the recent "epidemic" of autoimmune-, allergic- and chronic inflammatory diseases (Rook & Brunet, 2005). The "old-friends" hypothesis emphasizes the shared evolutionary story of men and microbes. Throughout human evolution man has been exposed to environmental microbes, pseudo-commensals from vegetation, soil and water as well as chronic bacterial, single- and multicellular parasitic infections (Rook, 2010; Rook et al., 2014). The authors argue that modern childhood infections are 'crowd infections' that evolved late in human history and that can only survive in large communities. Such crowd infections typically either kill the host or leave him immunized, and therefore have little immune regulatory potential. In contrast, commensal bacteria, old chronic infections that persisted in small isolated hunter-gatherer groups as relatively harmless subclinical carrier states, as well as pseudo-commensal environmental bacteria from soils, water and fermented foods, are much more likely to contribute to normal immune regulation. Because such microbes could not be avoided, they

have had to be tolerated, and may over time have taken on important immune regulatory functions. Over time, evolution may have turned the inevitable into a necessity (Rook, 2010). Thus, we may have developed dependence on diverse microbial stimulation, not only for educating the immune system early in life, and for maintaining background activation of the innate immune system. Input from "old friends" may also contribute to setting up regulatory control mechanisms to stop the immune system from causing inappropriate inflammatory responses (Rook et al., 2014). Whereas urbanization maintains exposure to the crowd infections, people living in urbanized communities are deprived of important immune regulatory input due to reduced exposure to microbial "old friends" from the natural environment. This lack of microbial exposure may be part of the explanation why autoimmune and chronic inflammatory diseases are on the rise.

1.2.5 Therapeutic potential of bacteria

Following increased appreciation for microbial contribution to human health, many have suggested a potential for microbes and microbial compounds for therapeutic purposes.

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). These are single strains, or defined mixtures of bacteria that often originate from the commensal microbiome, or from fermented foods that humans have a long tradition of eating. Several beneficial health effects are claimed for probiotic bacteria transitionally associated with the gastrointestinal tract. Meta-analyses confirm the efficacy of probiotics in acute infectious diarrhea and prevention of antibiotic-associated diarrhea, and there is evidence that probiotics can treat and prevent enteric infections and post-antibiotic syndromes (Sazawal et al., 2006).

Microbes and microbial compounds have also been suggested for prophylactic and therapeutic use to modulate or tune immune responses in immune-driven diseases. Loss of commensal-host mutualistic relationship is associated with the development of IBD, and probiotics have been suggested to restore homeostasis in IBD patients. Several studies have described protective properties of probiotic bacteria, commensals, and their metabolites against experimental colitis in animal models of chronic inflammatory bowel disease (IBD) (Mileti, Matteoli, Iliev, & Rescigno, 2009; Pils et al., 2011; Qiu, Zhang, Yang, Hong, & Yu, 2013; Souza et al., 2016; Toumi et al., 2014). Although a number of *in vitro* and animal studies support immune-modulatory effects of such bacteria, clinical studies are less conclusive (Goldin & Gorbach, 2008; Isaacs & Herfarth, 2008). Currently there is little

evidence to support effects of probiotics in maintaining remission and preventing recurrence in Crohn´s disease (CD) (Rahimi et al., 2008), but promising results have been obtained for maintaining remission in ulcerative colitis (UC) as reviewed by Scaldaferri et al. (2013). However, the efficacy of probiotic treatment is likely to depend on the choice of probiotic organism, the variability in concentrations of organisms administered, and the etiology of the diseases being treated (Isaacs & Herfarth, 2008), emphasizing the need for extended knowledge of properties and modes of action for individual species/strains intended for therapeutic use.

1.2.6 Mechanisms behind anti-inflammatory properties of immune modulatory bacteria

How some bacteria may prevent inflammatory responses in mammalian hosts is not fully known. However, three general, not mutually exclusive modes of action, have been suggested for probiotic bacteria (Lebeer, Vanderleyden, & De Keersmaecker, 2008). 1) they may inhibit pathogens, directly by pathogen exclusion or indirectly by stimulating host defenses; 2) they may stimulate host functions to enhance epithelial barrier function or 3) they may modulate host immune responses through interaction with immune cells such as lymphocytes and dendritic cells resulting in both local and systemic effects (Lebeer et al., 2008). Although several mechanisms have been suggested for bacteria to prevent inflammatory responses, there is growing evidence that DCs play a key role in commensal and probiotic bacterial stimulation of innate and adaptive immunity (Drakes, Blanchard, & Czinn, 2004; Foligne, Zoumpopoulou, et al., 2007; Hart et al., 2004). As discussed in a previous chapter, DC maturation state is important for DC functionality and tolerogenic vs. immunogenic properties. Probiotic bacteria have been shown to differentially modulate DC maturation and cytokine production (Christensen, Frokiaer, & Pestka, 2002), suggesting that bacteria may modulate adaptive immunity through interactions with DCs strengthening their tolerogenic or immunogenic properties.

1.2.7 M. capsulatus, an environmental bacterium with anti-inflammatory properties

Although bacteria from soils and water are suggested to play a role in normal immune regulation (Rook, 2010), surprisingly few studies have focused on immune regulatory properties of environmental bacteria. However, in 2011 (Romarheim, Overland, Mydland, Skrede, & Landsverk) showed that dietary inclusion of BioProtein, a feed product based on bacterial protein, in which a soil bacterium is the main ingredient, could prevent soybean-

induced enteritis in Atlantic salmon (*Salmo salar*). The mechanism(s) involved in soybean-induced enteritis in fish is not fully known. However, it has been suggested that soy saponins trigger the onset of enteritis by increasing epithelial permeability, exposing the local mucosal immune system to dietary and bacterial antigens from the gut lumen (Bakke-McKellep, Penn, et al., 2007; Knudsen, Uran, Arnous, Koppe, & Frokiaer, 2007; Krogdahl et al., 2015) and initiating a T cell- and cytokine mediated inflammation (Bakke-McKellep, Froystad, et al., 2007; Lilleeng et al., 2009). Development of soybean-induced enteritis thus resembles the etiology of inflammatory bowel disease (IBD) in mammals.

In 2013 Kleiveland et al. investigated whether inclusion of BioProtein in the diet could also abrogate disease development in a mammalian models of IBD. They used a murine dextran sulfate sodium (DSS)-induced colitis model, a well established model of ulcerative colitis. Whereas mice that received a standard diet developed intestinal injury and inflammation, mice fed the experimental diet combined with BioProtein showed markedly reduced symptoms of DSS colitis as assessed by inflammation markers, clinical signs and histological evaluation. The BioProtein is based on the environmental bacterium *Methylococcus capsulatus* Bath grown in a fermentor. Other bacteria: *Alcaligenes acidovorans, Bacillus firmus* and *Aneurinibacillus danicus* are also added to the fermentor to remove waste products formed in the fermentation process. *M. capsulatus* constitute 88% of the final fermentation product, *A. acidovorans 11%* and *B. firmus* and *A. danicus* the remaining 1% (Norwegian Scientific Committee for Food Safety, 2006). Kleiveland et al. (2013) showed that results achieved with BioProtein could be reproduced in an experiment where mice were fed a standard diet including 20% *M. capsulatus* (Bath) only, without the additional bacteria present in BioProtein, proposing that *M. capsulatus* is the active principle in BioProtein and that this non-commensal bacterium has potent anti-inflammatory properties in mammals. The studies by Romarheim et al. (2011) and Kleiveland et al. (2013) show that the non-commensal soil bacterium *M. capsulatus* has immune modulatory, anti-inflammatory properties in teleost- and mammalian models of intestinal inflammation. However, the mechanism(s) behind the effects has not yet been identified.

1.2.8 M. capsulatus, history and taxonomy

M. capsulatus Bath belongs to the methanotrophic bacteria, a group of Gram-negative bacteria unique in their ability to use the C_1 compound methane as a sole source of carbon and energy (Hanson & Hanson, 1996). In 1966 Foster and Davis (1966) isolated a

methanotroph bacterium from sewage sludge from the Austin municipal sewage plant. The new species was described as Gram-negative, non-motile, coccoid aerobic bacteria capable of growth in a mineral salts medium at temperatures up to 50°C with a temperature optimum at around 37°C. The cells were encapsulated and frequently seen as diplococci. The name *M. capsulatus* was suggested for the new species. The strain isolated by Foster and Davis is commercially available today under the strain designation *M. capsulatus* Texas.

In 1970 (Whittenbury, Phillips, & Wilkinson) isolated more than 100 methane-utilizing bacteria from mud, water and soil samples from numerous locations in Europe, Africa, North- and South America. These methanotrophs were classified based on morphology, fine structure and resting stage formed (J. P. Bowman, Sly, & Stackebrandt, 1995; Hanson & Hanson, 1996). Among the many methanotroph species isolated by Whittenbury et al. (1970) a species was found that was considered to be identical with the species previously described by Foster and Davis (1966). The new strain was named *M. capsulatus* Bath. It is this strain that has later become the type strain for the species.

All methanotrophs can be broadly categorized in two groups, type I and type II methanotrophs, based on phylogeny, internal ultrastructure, biochemical aspects and whether the serine pathway or ribulose monophosphate pathway is used for carbon assimilation (Hanson & Hanson, 1996). *M. capsulatus* is a Gammaproteobacterium (class), belonging to the order Methylococcales, the family Methylococcaceae and the genus Methylococcus (J. Bowman, 2006). Members of the *Methylococcacea* family, includes the type I methanotrophs, bacterial taxa that all share some common features. They possess a particular methane monooxygenase, an integral membrane metalloenzyme that oxidizes methane to methanol; they all utilize the ribulose monophosphate pathway to assimilate C1 carbon units, and they all form characteristic intracellular membranes arranged in lamellar stacks (John P. Bowman, 2014) (Figure 4). Methanotrophs have received much interest for their involvement in global carbon cycles. *Methylococcus* is the type genus of type I methanotrophs, and *M. capsulatus* a type species for *Methylococcus.* The strain *M. capsulatus* Bath has therefore been much studied as a representative for methanotroph prokaryotes (John P. Bowman, 2014). The complete genome sequence of *M. capsulatus* Bath was released in 2004 (Ward et al., 2004), and suggested a unexpected genetic potential for metabolic flexibility, ability to grow on sugars, oxidize chemolithotrophic hydrogen and sulfur and live under reduced oxygen tension (Ward et al., 2004).

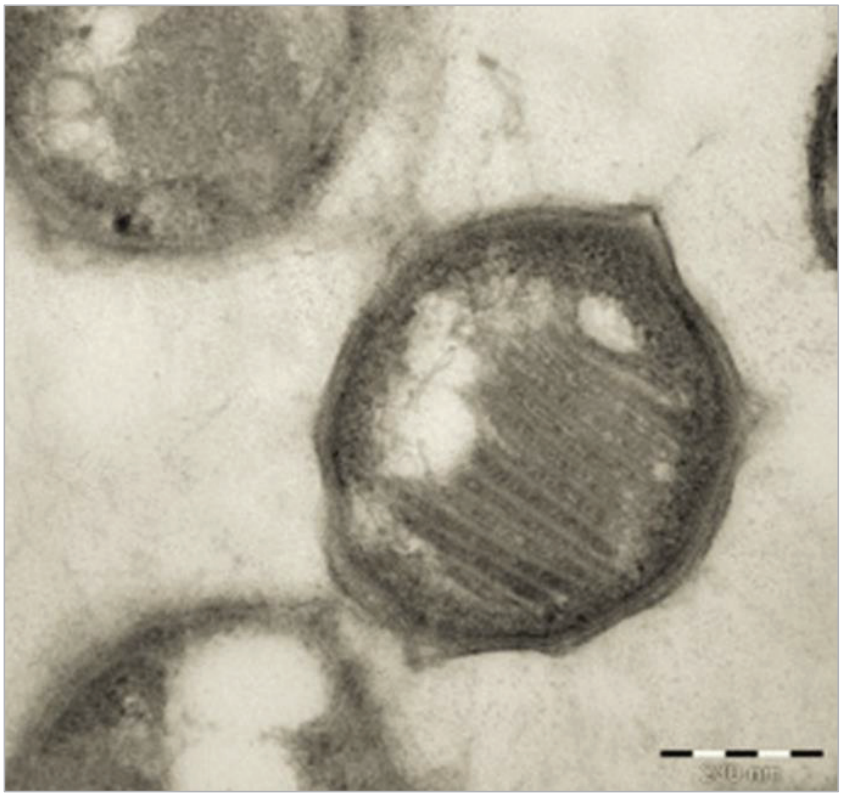

Figure 4 Methylococcus capsulatus Bath

M. capsulatus Bath with characteristic intracellular membranes arranged in lamellar stacks visualized by transmission electron microscopy. Image from Christoffersen et al. (2015) used with permission from author.

1.2.9 Biochemical properties of M. capuslatus Bath

Methanotrophs are ubiquitous in nature. They play an important role in maintaining a balance of atmospheric methane by consuming methane from non-biogenic sources such as natural gas fields, hydrocarbon seeps and cold mines, and from biogenic sources such as methanogenic archaea in wetlands and enteric fermentation in animals (J. Bowman, 2006). *M. capsulatus* strains are widely distributed in nature, and are found in many terrestrial soils and sediments. *M. capsulatus* Bath has a relatively high DNA GC content (59-66%) and fix atmospheric nitrogen and CO_2 in the presence of methane (Baxter et al., 2002; John P. Bowman, 2014). All strains form cysts and accumulate granules of polyhydroxyalkanoate (PHA), high molecular weight, linear thermoplastic polymers that has been suggested as

candidates to replace conventional petroleum-derived polymers (Strong et al., 2016). *M. capsulatus* strains contain several types of lipids: bacteriohopanepolyols, squalene, and sterols, the latter considered very rare in bacteria (John P. Bowman, 2014). Furthermore, an unusual lipoquinone, a 18 carbon methylenated version of ubiquinone-8, is found in *M. capsulatus* (Collins & Green, 1985). The main phospholipid fatty acid (PLFA) found in *M. capsulatus* is palmitic acid C16:0 and palmitoleic acid C16:1 ω7c whereas minor PLFAs includes C14:0, the C16:1 isomers C16:1 w6c, C16:1 ω5c, C16:1 ω5t and C18:ω7c (John P. Bowman, Skerratt, Nichols, & Sly, 1991; Jahnke & Nichols, 1986). Dietary lipids have been shown to have modulatory effects on immune system functions and may affect immune parameters such as lymphocyte proliferation, cytokine synthesis, natural killer (NK) cell activity, phagocytosis and cell survival (de Pablo & Alvarez de Cienfuegos, 2000). The lipid profile of *M. capsulatus* Bath may therefore be relevant to its anti-inflammatory properties, but has not been the focus of this study.

The cell envelope of Gram-negative bacteria consists of an inner membrane (the plasma membrane) and an outer membrane, separated by a periplasmic space containing a thin layer of supportive peptidoglycan. Proteins are found in all cellular compartments, in the cytosol and periplasm, embedded in or bound to the inner and outer membrane. There are two major types of outer membrane proteins, β-barrel proteins integrated in the membrane that function in active and passive transport of ions and nutrients, whereas lipoproteins associate with the outer or inner leaflet of OM through lipid anchors (Koebnik, Locher, & Van Gelder, 2000; Zuckert, 2014). Finally, proteins may be peripherally associated with the outer membrane or they may be secreted and released to the environment. The OM proteome of *M. capsulatus* (Bath) has previously been analyzed using proteomic and computational approaches (Berven et al., 2006; Fjellbirkeland, Kleivdal, Joergensen, Thestrup, & Jensen, 1997). Proteins peripherally associated with the surface was further characterized by proteomic methods (Karlsen, Lillehaug, & Jensen, 2008), demonstrating that the surface proteome of *M. capsulatus* (Bath) is highly dynamic. Little attention has been given to *M. capsulatus* proteins released to the extracellular milieu.

The reported protective effects of *M. capsulatus* Bath in and animal model of colitis suggest that *M. capsulatus* components have the potential to interact with cells of the epithelium and lamina propria involved in maintaining mucosal homeostasis, but the mechanisms involved and bacterial components responsible has not yet been identified (Kleiveland et al., 2013). This thesis presents studies attempting to understand immunomodulatory interactions

between host cells and a non-commensal environmental bacterium shown to have anti-inflammatory effects in a murine model of colitis. As bacterial surface attached proteins and proteins secreted and released to the environment are central to how bacteria interacts with their environment we first examined the secretome of *M. capsulatus* Bath using computational and experimental methods. Secondly, we describe specific interactions between *M. capsulatus* and immune cells central in regulating innate and adaptive immunity as well as functional effects of these interactions. Finally, we have searched for mechanisms involved in immune modulatory effects of *M. capsulatus* Bath and two other immunomodulatory bacteria by analyzing how these bacteria affect global gene expression in a model for human dendritic cells.

2 AIMS OF STUDY

PAPER 1

Methylococcus capsulatus (Bath) has been shown to abrogate inflammation in a murine model of inflammatory bowel disease, emphasizing the importance of understanding behavior and properties of this bacterium. Surface proteins and proteins released to the environment are important for how bacteria interacts with molecules, surfaces, and other cells. Whereas the outer membrane proteome of *M. capsulatus* Bath is relatively well characterized, less is known about proteins peripherally associated with the OM or released to the bacterium's surroundings. In paper I the secretome of *M. capsulatus* Bath, its secretion systems, and secreted proteins (, surface attached or released,) were characterized by computational, and proteomic methods. A novel *M. capsulatus* secretion system was identified and substrates of all the *M. capsulatus* secretion systems as well as potential moonlighting proteins were characterized. The study shows that *M. capsulatus* secretes proteins likely to be involved in adhesion, colonization, nutrient acquisition and homeostasis maintenance and suggests a potential for *M. capsulatus* Bath to interact with host cells.

PAPER II

Reported anti-inflammatory effects of *M. capsulatus* (Bath) in a murine model of inflammatory bowel disease, suggests that this bacterium interacts with cells involved in maintaining mucosal homeostasis *in vivo*. In paper II immune modulatory effects of *M. capsulatus* Bath on human immune cells were characterized. When co-incubating *M. capsulatus* Bath with human peripheral blood mononuclear cells we found that the bacterium adhered specifically to a small subset of cells. This subset was identified as dendritic cells. As DC play a central role in T cell immunity and homeostasis regulation we assessed the effects of *M. capsulatus* Bath, a probiotic Gram-positive bacterium and a Gram-negative bacterium of commensal origin to modulate monocyte-derived dendritic cell function. *M. capsulatus* induced intermediate phenotypic and functional DC maturation. In a mixed lymphocyte reaction *M. capsulatus* Bath-primed monocyte-derived dendritic cells enhanced T cell expression of CD25, the α-chain of the high affinity IL-2 receptor, supported cell proliferation, and induced a T cell cytokine profile distinct from both *Escherichia coli* K12 and *Lactobacillus rhamnosus* GG.

PAPER III

DC not only play a critical role in inducing protective immune responses, but are also essential mediators of immunologic tolerance. The specific interaction between *M. capsulatus* Bath and MoDC described in paper II suggests a potential for *M. capsulatus* Bath to modulate inflammatory/ homeostatic status by modulating DC function. In paper III we searched for mechanisms involved in bacteria mediated immunomodulation. *M. capsulatus* Bath and two well characterized immune modulatory probiotic species were co-incubated with MoDCs and the effects on global gene expression were determined by RNA sequencing. DCs induce tolerogenic T cell responses by mechanism including antigen presentation by immature DC, expression of T cell suppressive/modulatory surface molecules and by production of soluble immune suppressants or immune modulatory cytokines, and the three immune modulatory bacteria tested was shown to affect genes related to all these properties of MoDC.

Results confirmed MoDC modulatory properties of the soil bacterium *M. capsulatus* Bath. *M. capsulatus* Bath induces an expression pattern distinct from both *Escherichia coli* Nissle, *Lactobacillus rhamnosus* GG and the maturation control, and induced a state of DC maturation, cytokine and chemokine production, intermediate between *L. rhamnosus* GG-primed and *E. coli* Nissle -primed MoDCs. Furthermore, *M. capsulatus* Bath induced elevated expression of regulatory T cell promoting cytokines IL10 and LIF, galactin-10, an s-type lectin important for the suppressive effects of regulatory T cells and eosinophils. Furthermore, *M. capsulatus* reduced the expression of CD70 a co-stimulatory molecule implicated in auto-immunity and suggested as a promising target for antibody-directed immunotherapy in chronic inflammatory diseases.

The study also reveals that two established probiotics reported to confer protection in animal models of colitis have very different, and partly opposing effects on the expression of genes important for DC mediated activation of innate and adaptive immune responses. *L. rhamnosus* GG prevented MoDC maturation, reduced expression of genes necessary for migration and antigen presentation, co-stimulation and chemokine and cytokine production, but increased expression of TGF-β, a cytokine with immune suppressive function. *L. rhamnosus* GG-primed MoDCs may therefore be expected to have reduced potential for driving T cell immunity and innate immune functions. In contrast, gene expression analysis suggest *E. coli* Nissle is a strong inducer of MoDC activation. *E. coli* Nissle promotes

expression of genes necessary for migration and T cell activation, but also induces increased expression levels of genes for immune suppressive/regulatory cytokines IL10 and LIF and induces MoDC expected to have high capacity for promoting antimicrobial/viral defense. Results thus not only shows a potential for environmental bacteria in MoDC modulation, but also identify important functional differences between two established probiotic species.

3 MAIN RESULTS AND DISCUSSION

3.1 Paper I: The *M. capsulatus* Bath secretome suggest a potential for host cell interaction

Cell wall molecules and proteins released to the extracellular milieu are particularly important for how bacteria interact with their surroundings. Microbial surface-attached and released, secreted proteins participate in both commensal, probiotic and pathogenic microbe-host interactions and adaptations (Lebeer, Vanderleyden, & De Keersmaecker, 2010). They mediate adhesion to surfaces and host cells to facilitate colonization of an environment, and participate in communication, host adhesion, invasion, internalization, and defense. Furthermore, surface-exposed proteins are targets of the host immune responses and are therefore important for how the host responds to the bacterium. Bacterial cell wall molecules are ligands for various host receptors, and act as typical microbe-associated molecular patterns (MAMPs). Secreted proteins are also expected to be important mediators of immunomodulatory effects of probiotic-, and commensal bacteria (Green & Mecsas, 2016; Kleerebezem et al., 2010; Lebeer et al., 2010).

Because many of the effector molecules that participate in health-promoting interactions of bacteria with the host intestinal immune system are expected to be secreted (Kleerebezem et al., 2010), it was important to define the *M. capsulatus* Bath secretome. Characterization of the secretome can provide information about how a bacterium interacts with host cells. In order to gain new insights on the molecular crosstalk between *M. capsulatus* Bath and mammalian hosts, we used an *in silico* strategy combined with experimental analysis to characterize the *M. capsulatus* secretome.

Experimental secretome analysis vs in silico prediction

The term ‘Secretome’ refer to an organism´s complete set of secreted proteins, and was originally proposed as a term to include both the components of the pathways for protein secretion and the secreted proteins (Tjalsma, Bolhuis, Jongbloed, Bron, & van Dijl, 2000). Whereas the term is sometimes used to refer exclusively to those proteins that are present in the extracellular milieu of the bacterium, we have adopted the definition by Desvaux, Hébraud, Talon, and Henderson (2009). Here ‘secretome’ refers to secreted proteins that are surface localized, are parts of surface appendages or are released into the extracellular

milieu, as well as their related transport systems. Proteins localized in the extracellular milieu, whether they are actively secreted or not, are defined as “extracellular”.

Both experimental and computational methods can be applied to characterize microbial secretomes, and both approaches have their advantages and limitations. By an experimental approach extracellular-, or extracted membrane proteins are harvested and separated by 2D gel electrophoresis followed by mass spectrometric methods to identify peptides and proteins in the sample (X. Y. Yang, Lu, Sun, & He, 2012). This gives an overview of the secreted proteins actually expressed at the time of harvest. The bacterial secretome, however, is highly dynamic and changes in response to environmental stimuli and factors such as medium composition and temperature. For example, the composition of proteins at the cellular surface of *M. capsulatus* Bath varies with the availability of copper and changes significantly with only minor changes in copper concentrations in the growth medium (Karlsen, Larsen, & Jensen, 2011). A limitation of experimental methods is that only proteins expressed under the particular conditions used can be identified. Furthermore, low expression-limited sensitivity can result in that some potentially interesting proteins may be lost by experimental approaches.

Provided the genome sequence of the organism is available, an *in silico* approach represents an alternative to experimental secretome analysis. In bacteria proteins are directed to their subcellular location by signals in their amino acid composition or sequence. Protein transport over the inner membrane is commonly directed by a targeting signal in the amino acid sequence, so called signal peptides, α-helical proteins are usually integrated in the inner membrane, whereas β-barrel proteins are directed for the outer membrane of Gram-negative bacteria (Solis & Cordwell, 2011). *In silico* methods take advantage of the information encoded in the amino acid sequence of a protein, and a plethora of predictive algorithms have been developed to determine whether a protein is localized to the surface, or a membrane based on sequence motifs or physiochemical characteristics.

T1SS, T2aSS, T2cSS, T5bSS and a T7SS are present in M. capsulatus Bath

In bacteria that only contain one membrane, so called monoderms or Gram-positive bacteria, subcellular location prediction is relatively straightforward. However, whereas the presence of a signal peptide indicates that a protein is transported across the inner membrane, no universal sequence motifs directs protein transport across the outer membrane. Therefore, *in silico* subcellular location prediction in Gram-negative bacteria is more complicated. We used a genome-wide protein localization prediction strategies for Gram negative bacteria adapted from Romine (2011), and described in figure 5. The strategy resulted in subcellular location prediction for all 2956 *M. capsulatus* proteins.

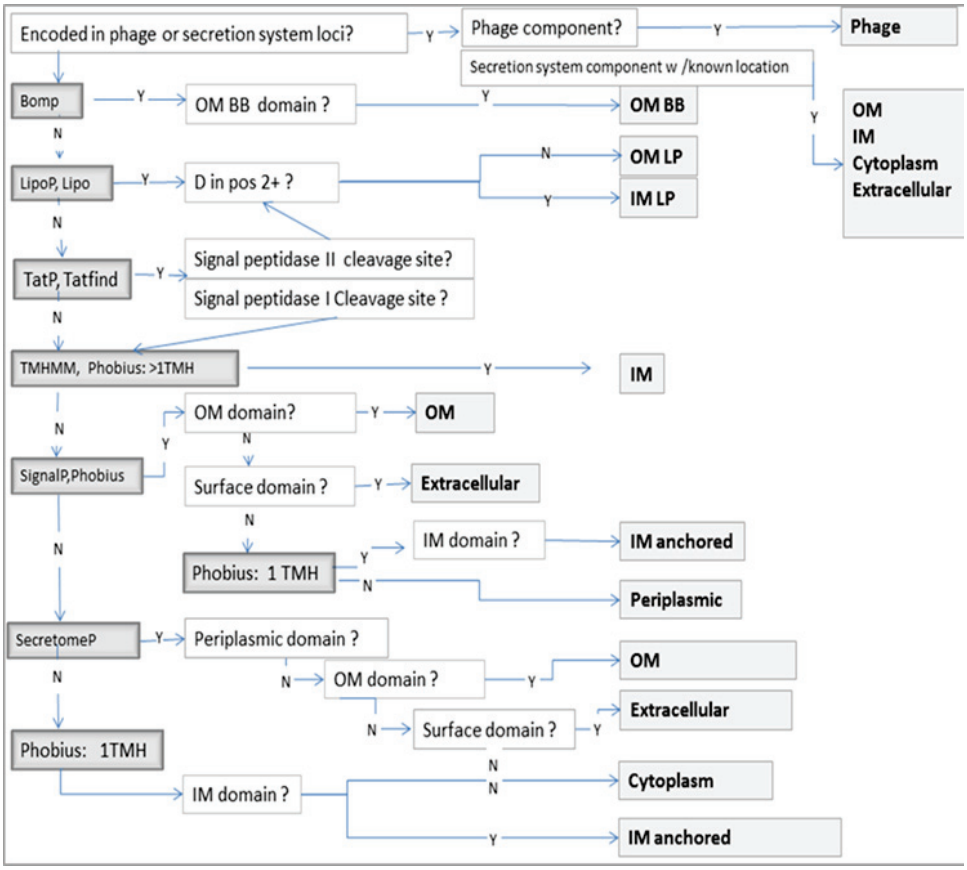

Figure 5. Decision tree for sub cellular location analysis.

Figure show the decision tree used to predict subcellular location for all *M. capsulatus* Bath proteins.

Nine different types of secretion systems have been identified in diderm bacteria and have been numerically classified as type I to type IX (T1SS-T9SS) (Chagnot, Zorgani, Astruc, & Desvaux, 2013). A conventional type II secretion system (T2aSS), a type IV pili system (T2cSS) and a chaperon-usher secretion system (T7SS) has already been recognized in *M. capsulatus* Bath (Cianciotto, 2005; Nuccio & Baumler, 2007; Pelicic, 2008). We showed that an additional type Vb (two-partner) secretion system and putative type I secretion systems are present in *M. capsulatus* (Bath).

M. capsulatus Bath secreted proteins and their functions

Twenty-one proteins predicted to be secreted in *M. capsulatus* had putative functions in adhesion, colonization, nutrient acquisition and homeostasis maintenance. Gram-negative bacteria secrete a wide variety of enzymes into the extracellular environment, these extracellular enzymes, so called exoenzymes, have many functions important for defense and colonization. Many of the predicted secreted *M. capsulatus* proteins are likely to have enzymatic function. Two predicted substrates of *M. capsulatus* T1SSs contained domains suggesting hydrolase and amylo-alpha-1,6-glucosidase activity. Enzymes are also typical substrates of bacterial T2SS. Among proteins predicted to be secreted by a *M. capsulatus* Bath T2aSS, were a serine protease, an endonuclease, a metalloprotease and an acid phosphatase. The serine protease and a methanol dehydrogenase were also identified in the culture supernatant proving their expression under the culture condition used in our setup. Two proteins contained a polycystic kidney disease domain (PKD) domain, a domain found in the human PKD1 gene encoding the cell surface glycoprotein polycystin-1. PKD domains are often found in biopolymer hydrolases, and may be involved in degradation of biopolymers, such as collagen (Y. K. Wang et al., 2010). Typical exoenzymes are toxins and virulence factors, or function in nutrient acquisition by breaking down large macromolecules to smaller components that can be taken up and utilized by the bacterium (Wandersman, 1989).

Secreted proteins provide information about a bacterium´s lifestyle. *M. capsulaus* Bath is a methanotroph bacterium that oxidize methane to methanol by methane monooxygenases (MMOs), and methanol to formaldehyde by methanol dehydrogenases (MDHs). A methanol dehydrogenase was present in the culture supernatant in early-mid-and late logarithmic growth stage. The availability of copper during growth of *M. capsulatus* Bath plays an important role in the physiology of *M. capsulatus* Bath, and in the switch between the two

MMOs expressed by this bacterium (Nielsen, Gerdes, & Murrell, 1997). Among predicted T2aSS substrates expressed in the culture supernatant were a protein with a copper-binding domain. Furthermore, three members of the cytochrome c5530 family were predicted to be secreted, and the presence of one of these was confirmed in the culture supernatant of *M. capsulatus* during logarithmic growth. Karlsen et al. (2008) has previously shown that multiple c-type cytochromes are peripherally associated with the *M. capsulatus* Bath surface. Such surface-exposed cytochromes are uncommon in bacteria (Heidelberg et al., 2004), but c-type cytochromes on the surface of other bacteria have functions related to the reduction of inorganic metals, and multihaem c-type proteins at the surface of *M. capsulatus* are likely to participate in similar redox reactions possibly related to copper homeostasis (Karlsen et al., 2008).

Furthermore, several proteins of unknown function were predicted to be secreted and/or identified in culture supernatants of bacteria in logarithmic growth. No functionally annotated homologs were found for MCA0155, a protein consistently found in culture supernatant of all parallels in mid- and late logarithmic growth. Given the importance of secreted proteins in bacterial communication and in host-interactions, further efforts should be made to define the function of uncharacterized secreted proteins to understand how *M. capsulatus* Bath interacts with its environment.

The M. capsulatus secretome suggests a potential for host-interactions

Although *M. capsulatus* is described as an environmental bacterium that is not expected to engage in a host-associated lifestyle, proteins suggesting the potential for host interaction were found among the predicted secreted proteins and/or were isolated from the culture supernatant. A lipoprotein annotated as VacJ was among the proteins predicted to be secreted by the *M. capsulatus* Bath T2aSS. Bacterial lipoproteins are hydrophilic proteins that are anchored to a cell membrane. They have been described to possess a variety of virulence-associated functions, and contribute to processes such as colonization, invasion, evasion of host defense, and immunomodulation (Kovacs-Simon, Titball, & Michell, 2011). In Gram-negative bacteria, surface-exposed lipoproteins were previously considered to be rare. However, recent studies indicate that they are actually widespread (Wilson & Bernstein, 2016). *VacJ* was identified in *Shigella flexneri* as a virulence factor that promote intercellular spreading of this pathogen (Suzuki et al., 1994). The function of this protein in the non-pathogenic *M. capsulatus* Bath is not known.

Interestingly, three proteins, a glutamine synthetase and two 60 kDa chaperonin (Cpn60) family proteins, without predicted signal peptides, were found in the culture supernatants. All three belong to families of proteins previously described to act as moonlighting proteins (V. Kainulainen et al., 2012). Moonlighting proteins are characterized by multiple biologically unrelated functions that often localize to separate cellular compartments (V. Kainulainen et al., 2012). They lack typical signal peptides, yet are often found on the bacterial surface or are released to the environment (V. Kainulainen et al., 2012; Pancholi & Chhatwal, 2003). Moonlighting proteins have been described to contribute both to bacterial virulence and to commensal and probiotic functionality (Veera Kainulainen & Korhonen, 2014; G. Wang et al., 2014). Moonlighting functions include binding to host epithelial and phagocytic cells, cytoskeleton, mucins and circulating proteins of the immune and hemostatic systems (Veera Kainulainen & Korhonen, 2014).

Secreted glutamine synthetase have been described as adhesive proteins in *Lactobacillus crispatus* (V. Kainulainen et al., 2012), whereas *Mycobacterium tuberculosis* release glutamine synthase into the phagosome in infected human monocytes, contributing to this pathogens capacity to inhibit phagosome-lysosome fusion and phagosome acidification (Harth, Clemens, & Horwitz, 1994). Surface-associated and released members of the Cpn60 family represent the most diverse range of moonlighting activities for any protein family known. Cpn60 family proteins have been described to function in host adhesion, invasion, and stimulation or inhibitation of host cell cytokine production (Henderson, Fares, & Lund, 2013). The mechanisms by which moonlighting proteins translocate to the cell exterior is not known. It has been suggested that they are either released from dead or traumatized bacterial cells, or that they are secreted onto the cell surface by an as yet-undescribed mechanism (Veera Kainulainen & Korhonen, 2014). However, the lack of ribosomal proteins, and the fact that all signal-less proteins identified in culture supernatants belonged to protein families with functions in both cytoplasm and extracellular locations, suggest that these proteins were indeeed secreted by an unknown mechanism.

In addition to the potential virulence factor VacJ and moonlighting proteins with potential relevance in host-interactions, several of the proteins predicted to be secreted are expected to be adhesins or parts of adhesion complexes known to be involved in host-interactions in other species. The ability to attach to surfaces allow bacteria to colonize different niches and contribute to define their species-specific lifestyles. Along with the transport of proteins

necessay for nutrient acquisition and defence, the transport of adhesins and assembly of ahesion protein complexes are important tasks for Gram-negative secretion systems.

We identified a novel *M. capsulatus* T5bSS (two-partner secretion system) consisting of a β-barrel transporter protein and a typical T5bSS passenger polypeptide. T5SSs, so called autotransporters, are mainly involved in secretion of virulence factors and adhesion proteins (Leo, Grin, & Linke, 2012). The predicted substrate of the *M. capsulatus* Bath T5bSS secretion system is a large hemagglutinin-like protein with homology to filamentous hemagglutinin (FHA) in other species. In *Bordetella pertussis* filamentous hemagglutinin is a surface-associated adhesin with at least three different binding specificities, including carbohydrate-, heparin sulfate- and integrin binding (Locht, Bertin, Menozzi, & Renauld, 1993). FHA mediates adhesion of *B. pertussis* to macrophages by binding to integrin αMβ2 recognized by an Arg-Gly-Asp (RGD) sequence in FHA (Relman et al., 1990). A RGD domain was found in the hemagglutinin-like protein of *M. capsulatus* (Bath) (unpublished results) suggesting an integrin-binding potential for this adhesin. Interestingly FHA from *B. pertussis* induced IL-10 producing dendritic cells (DCs) with regulatory function, and these DCs directed naive T cells into a regulatory subtype (McGuirk, McCann, & Mills, 2002) proposing that a *M. capsulatus* Bath hemagglutinin-like protein may also be functionally relevant for regulating DC mediated immunity/tolerance.

A T2cSS responsible for the secretion of pilins, and assembly of type IV pili (TIVP) has previously been identified in *M. capsulatus* Bath (Pelicic, 2008). *In silico* analysis confirmed the presence of a complete TIVP system in *M. capsulatus* Bath. Four proteins expected to be part of the TIVP complex were predicted to be extracellular, based on the presence of a signal sequence and surface domain or homology to extracellular proteins. One of these, a fimbrial protein, was identified in culture supernatants at all sampled time points during logarithmic growth confirming its expression, and is therefore likely to be the major *M. capsulatus* pilin, the major structural unit of the *M. capsulatus* Bath TIVP. Additionally, MCA0087, a protein predicted to be secreted and found in supernatants in the stationary growth phase (unpublished data.), and was shown by BLAST protein search to be homologous to the TIVP protein PilY1 across a range of Proteobacteria. TIVP are involved in prompting bacterial adhesion to both host cells and abiotic surfaces (Pelicic, 2008). In *Pseudomonas aeruginosa* PilY1 is a tip adhesin assumed to play an important role in the adhesion of *P. aeruginosa* to host epithelial cells. An integrin-binding arginine-glycine-aspartic acid (RGD) motif is present in the *Pseudomonas aeruginosa* PilY1. This motif is

responsible for TIVP binding to integrin and is expected to mediate host epithelial cell binding (Johnson et al., 2011). Interestingly, the *M. capsulatus* Bath PilY1 homolog does not contain an RGD motif, but two other integrin-binding sites are identified in the predicted *M. capsulatus* tip adhesin (unpublished results): A leucine-aspartic acid-tyrosine (LDV) motif associated with binding to α4β1 and α4β7 (Ruoslahti, 1996) and the leucine-aspartic acid-threonine (LDT) motif responsible for binding α4β7 (Viney et al., 1996). α4 integrins are expected to play a critical role in the development of IBD. Crohn's disease (CD) is associated with the accumulation of lymphocytes in inflamed gut tissues, and experimental and clinical evidence suggests that leukocyte-associated α4 integrins may play an important role in the recruitment of these cells to intestinal tissues, contributing to the induction and perpetuation of chronic intestinal inflammation (Kurmaeva et al., 2014). The role of α4 integrins in directing site-specific homing has made them attractive therapeutic targets for treatment of IBD, and monoclonal antibodies that targets and block α4β1 and α4β7 molecule have been shown to be effective of increasing the rates of clinical remission and the response rates in Crohn´s disease (Kurmaeva et al., 2014). The presence of α4β1 and α4β7 integrin binding motifs in a secreted tip adhesin from a bacterium with anti-inflammatory effects in a murine model of IBD is therefore particularly interesting.

In addition to a complete TIVP complex, another secretion system involved in pilus biogenesis, the chaperone-usher (CU) pathway, has been identified in *M. capsulatus* (Nuccio & Baumler, 2007). The chaperone-usher (CU) pathway is used for assembly and secretion of a large family of pili and is widespread among Gram-negative pathogens (Geibel & Waksman, 2014). We predicted two *M. capsulatus* Bath proteins to be secreted by the chaperon-usher system. Nuccio and Baumler (2007) reviewed the phylogeny of operons belonging to the chaperone-usher assembly class and subdivided the class into six major phylogenetic clades. The *M. capsulatus* chaperone-usher operons was classified as belonging to the σ-clade. Surprisingly little is known about the morphology or function of the surface structures encoded in these operons, however σ-fimbriae have been implicated in both biofilm formation and spore coat formation and assembly of both fimbrial and afimbrial surface structures (Nuccio & Baumler, 2007). Whether the *M. capsulatus* Bath proteins secreted by this secretion system do form fimbrial adhesins, or have other functions is thus not known.

In conclusion, results from paper I point to several uncharacterized proteins and moonlighting proteins identified in the *M. capsulatus* culture medium as candidates for future efforts to understand how *M. capsulatus* interacts with its environment. Furthermore, the presence of virulence factors and adhesion proteins with integrin-binding sites suggest that *M. capsulatus* Bath, a soil bacterium not previously described to engage in host interactions, may have potential for interacting with host cells.

3.2 Paper II: *M. capsulatus* interacts with human DCs to modulate DC function

M. capsulatus Bath interacts specifically with human DC

Whereas dietary inclusion of *M. capsulatus* Bath in the DSS-colitis model was shown to have beneficial anti-inflammatory effects, direct effects on cells of the immune system was not scrutinized in that study (Kleiveland et al., 2013). In paper II we set out to investigate a possible immunomodulatory capacity of *M. capsulatus* on blood leukocytes. Interestingly, whereas results presented in paper I suggested that *M. capsulatus* had potential for host interactions, paper II shows that *M. capsulatus* Bath adheres specifically to human immune cells *in vitro.* When *M. capsulatus* Bath were co-incubated with human peripheral blood mononuclear cells, bacteria clustered around a small subset of the cells. The appearance of these cells, and the low frequency of target cells, were consistent with the size and expected frequency of DCs among PBMCs. To determine whether these cells were in fact DC we co-incubated *M. capsulatus* Bath with $CD14^+$ monocytes and MoDCs respectively. *M. capsulatus* Bath quickly clustered around a majority of cells when added to differentiated MoDCs, but not $CD14^+$ cells prior to differentiation, showing that *M. capsulatus* specifically interacts with MoDCs. Adhesion to the cells were strong as shown by scanning electron microscopy (figure 5). *M. capsulatus* Bath were found in large clusters on MoDCs even after several washes with PBS. Kinetic studies showed that intact bacteria were found associated with MoDCs even after 72 hours of co-incubation suggesting that *M. capsulatus* are able to avoid phagocytosis for an extended time period.

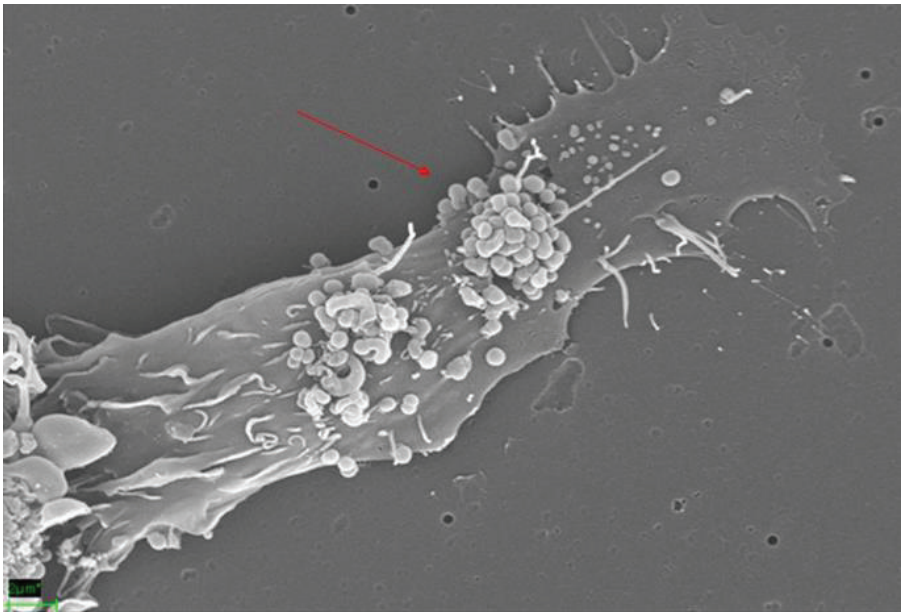

Figure 1. *M. capsulatus* Bath binds to DCs.

Image show *M. capsulatus* Bath (arrow) adhering to a monocyte derived dendritic cell as visualized by scanning electron microscopy.

M. capsulatus affects MoDC maturation and cytokine release

Given the anti-inflammatory effects of *M. capsulatus* Bath in the DSS colitis model and the central role of dendritic cells (DC) in the activation and modulation of adaptive immune responses, we asked whether *M. capsulatus* could affect inflammatory responses by modulating dendritic cell function. In paper II we therefore focused on immunomodulatory effects of *M. capsulatus* Bath on MoDCs. MoDCs were co-incubated for 24 h with *M. capsulatus* or control bacteria. Functional effects were characterized and compared to effects of the Gram-positive probiotic *Lactobacillus rhamnosus* GG, and a Gram-negative, non-pathogenic bacterium of commensal origin, *E. coli* K12.

The regulatory functions of DCs are closely related to the maturation state of the cells (Manicassamy & Pulendran, 2011). In paper II the ability of *M. capsulatus* to induce DC maturation, in low MoDC: bacteria ratio (1:10) was assessed. LPS, the major constituent of Gram-negative cell walls is a strong maturation signal for DCs. Gram-negative *M. capsulatus* Bath and the commensal *E.coli K12* both induced MoDC maturation as shown

by upregulation of phenotypic maturation markers. However, there was a clear difference in the ability of the two species in activating MoDCs. Compared to *E.coli* K12, *M. capsulatus* Bath stimulation resulted in weaker upregulation of maturation markers together with fewer, and reduced levels, of cytokines released by MoDCs. The Gram-positive *L. rhamnosus* GG was a weak inducer of DC maturation compared to the two Gram-negative bacteria and resulted in a phenotype similar to unstimulated cells, and low cytokine production.

We next evaluated the functional consequences of interactions of *M. capsulatus* or control bacteria *L. rhamnosus* GG or *E. coli* K12 with MoDC *in vitro*. The differential ability of the three bacteria to induce MoDC maturation was reflected in substantial differences in MoDC functions important for the activation of innate and adaptive immune responses. In accordance with observed phenotypic MoDC activation, both the Gram-negative bacteria were stronger inducers of MoDC cytokine release than the Gram-positive *L. rhamnosus* GG. However, *M. capsulatus* not only produced a less mature MoDC phenotype, but also reduced cytokine release compared to *E. coli* K12. *E. coli* K12 was the strongest inducer of MoDC cytokine release and induced higher levels of the pro-inflammatory cytokines (IL-1β, IL-12p70, IFN-λ, TNF-α, IL-6, IL-23) and the anti-inflammatory cytokine IL-10 compared to the other two bacteria. The Gram-negative bacteria, however induced similar levels of IL-2, a growth factor necessary for T cell clonal expansion.

M. capsulatus Bath, and control bacteria, differentially affect the ability of MoDCs to activate expand, and polarize T cells.

To evaluate whether priming MoDC with *M. capsulatus* Bath or control bacteria affected the ability of primed MoDC to activate naïve T cells, bacteria primed MoDCs were co-incubated with allogenic T cells for 5 days. Cells were collected and phenotyped for surface expression of CD4 and the α-chain of the high affinity IL-2 receptor (CD25), a marker of T cell activation. MoDCs primed by *M. capsulatus* expressed higher levels of CD25 than unstimulated control cells and cells stimulated by any of the control bacteria. To test the ability of bacteria-primed DCs to induce clonal T cell expansion bacteria, primed MoDCs were incubated with naïve T cells in the presence of IL-2. Interestingly, despite exhibiting a less mature phenotype than *E. coli*-primed MoDCs, *M. capsulatus*-primed MoDCs were more efficient in inducing T cell proliferation, possibly due to increased levels of high affinity IL-2 receptor on T cells activated by *M. capsulatus*-primed DCs.

MoDC-derived cytokines are important for promoting differentiation of T cell into distinct T effector cell populations. $CD4^+$ T cells play a major role in mediating immune response through the secretion of specific cytokines. Several subsets of CD4 helper cells (Th1, Th2, Th17, Th22, Th9) and regulatory cells (iTreg and Tr1) have been identified, and each subset is defined by expression of different transcription factors and are associated with distinct cytokine profiles and functions in immunity (J. Zhu et al., 2010). As *M. capsulatus* and the two control bacteria induced distinct DC cytokine profiles we examined whether this resulted in different T cell polarizing ability of the three species. Culture media from T cells activated by bacteria primed DC was collected and analyzed for cytokines associated with different T effector cell subsets. MoDCs primed by any of the three bacteria increased T cell expression of the Th1 signature cytokine IFN-γ and reduced Th2 cytokines IL-13 and IL-5 compared to control cells. *L. rhamnosus* GG resulted in lower levels of all cytokines compared to the Gram-negative bacteria. However, the three bacteria resulted in considerably different DC cytokine profiles. *M. capsulatus* Bath and *E. coli* K12 induced expression of the proinflammatory cytokines (IL-6, IL-1β, and IL-1α), cytokines associated with generation, maintenance and function of the Th17 subset (IL-23, IL-17A, IL21, IL22), Th22 cytokines (IL-22, TNF-α) and Th9 cytokines (IL-9 and IL-21). However, compared to *E. coli* K12 *M. capsulatus* Bath resulted in significantly less of the pro-inflammatory cytokines (IL1-α, IL-1β and IL-6) and the anti-inflammatory cytokine IL-10. There was no significant difference in *M. capsulatus* Bath and *E.coli* in levels of the Th1 signature cytokine IFN-γ or the Th9 cytokines IL-9 or IL-21, but T cells activated by *M. capsulatus*-primed MoDCs released significantly less of the Th17 associated cytokines IL-23, IL-17A and the Th17/Th22 cytokine IL-22 *in vitro.* The cytokine profile indicated that different T effector populations dominated in response to MoDCs primed by *M. capsulatus* Bath and *E. coli* K12. *E.coli* is a stronger inducer of Th17 effector cells, whereas *M. capsulatus* Bath appear to induce Th1/Th9 effector cells more than Th17 cells *in vitro*.

Commensal and probiotic bacteria may confer their benefits through several principally different, but not mutually exclusive modes of action (Lebeer et al., 2008). They may inhibit pathogens directly by microbe-microbe interaction, competitive exclusion, by producing antimicrobial substances or indirectly by stimulating host antimicrobial defenses. They may stimulate host epithelial barrier function or they may modulate host immune responses through interaction with immune cells such as lymphocytes and dendritic cells (DCs). A potential for *M. capsulatus* Bath to affect mechanisms essential for maintenance of the

colonic barrier function has already been described (Kleiveland et al., 2013), and there is evidence that *M. capsulatus* Bath do modify the composition of the microbiome (manuscript in preparation). The protective effects of *M. capsulatus* Bath may therefore act through mechanisms unrelated to DC. Nevertheless, paper II describes several effects of *M. capsulatus* Bath on MoDC gene expression that can be expected to have functional consequences for the ability of these cells to activate and shape innate and adaptive immune responses

3.3 Paper III: Transcriptome analysis reveal DC modulatory bacterial properties

MoDCs respond to bacteria with general and microbe specific responses

DC not only initiates adaptive immunity, but also have a critical role in establishing tolerance, limiting undesired responses against self- or innocuous antigens, food-components and commensals (Manicassamy & Pulendran, 2011; Steinman & Nussenzweig, 2002). The close interaction between *M. capsulatus* Bath and MoDCs that was observed and described in paper II suggested a potential of *M. capsulatus* Bath to modulate inflammatory/ homeostatic status by modulating DC function. Paper II showed that *M. capsulatus* Bath affects MoDC maturation markers and cytokine release and had functional effects on the ability of MoDCs to activate and instruct naïve T cells, their maturation markers and cytokine profile. Nevertheless, phenotypic markers and cytokines only provide a crude picture of how interactions between bacteria and DCs elicit functional responses against the detected microorganisms (Lebeer et al., 2010).

In paper III, we co-incubated *M. capsulatus* Bath with MoDCs and determined effects on global gene expression by RNA sequencing. The transcriptomic profile of *M. capsulatus* Bath-primed MoDCs was compared to that of MoDCs primed by to established probiotic species, the Gram-positive *L. rhamnosus* GG and the Gram-negative *Escherichia coli* Nissle 1917. Both have previously been reported to be protective in models of IBD (Amit-Romach, Uni, & Reifen, 2008; Foligne, Nutten, et al., 2007; Grabig et al., 2006) and to have DC modulatory effects (Adam et al., 2010; Braat et al., 2004; Vlasova et al., 2016).

MoDCs responded to all three bacteria with a major change in gene expression profile. A high proportion of the differentially expressed genes (genes that showed significantly different expression compared to control cells) were related to immune system processes. Dendritic cells maintain peripheral tolerance and immune homeostasis through several different mechanisms that include: 1) Antigen presentation by immature DCs with low co-stimulatory ability, 2) by expression of immunomodulatory surface molecules and 3) by the production and release of immunosuppressive factors (Li & Shi, 2015). The investigated bacteria regulate genes related to all these mechanisms.

M. capsulatus Bath and E.coli Nissle induce DC maturation whereas L. rhamnosus prevents full DC maturation.

The ability of DCs to promote immunogenic and tolerogenic responses is closely related to their maturation stage. Activated, mature DCs express high levels of MHC class II, CD80 and CD86, have strong capacity for antigen presentation and co-stimulation and are efficient inducers of naïve T cell proliferation and differentiation. In contrast, under homeostatic conditions, immature DCs express high phagocytic capacity, low levels of MHC class II and co-stimulatory molecules (CD40, CD80 and CD86), and low migratory capacity. Antigen presentation by immature DCs is thought to promote T-cell anergy, deletion, or regulatory cell fate leading to tolerance (Barratt-Boyes & Thomson, 2005; Ezzelarab & Thomson, 2011).

M. capsulatus Bath, *E. coli* Nissle and *L. rhamnosus* GG demonstrated different potential to induce DC maturation. The Gram-negative *M. capsulatus* Bath and *E. coli* Nissle both induced MoDC maturation with *E. coli* Nissle being the stronger stimuli of the two. In sharp contrast to the two Gram-negative bacteria *L. rhamnosus* GG was found to be a weak inducer of phenotypic MoDC maturation. It moderately increased surface expression of CD83 compared to unstimulated cells, suggesting partial activation of MoDCs, but resulted in expression levels similar to negative control for CD80, CD40 and HLA class II, suggesting reduced ability for antigen-presentation and co-stimulation.

Gene expression analysis confirmed reduced ability of *L. rhamnosus* GG to induce MoDC maturation. DEGs for a high number of co-stimulatory molecules were reduced in response to *L. rhamnosus* GG. Furthermore, *L. rhamnosus* GG reduced the expression of chemokine receptors required for migration of mature DCs to lymph nodes, genes for proinflammatory cytokines and chemokines but elevated the expression of genes for surface-associated pattern recognizing receptors of the C-type lectin and TLR families, consistent with immature MoDC function.

As immature DCs have been postulated to promote tolerogenic responses, and probiotic bacteria have been shown to modulate MoDC maturation, prevention of MoDC maturation is a feasible mechanisms for probiotics to induce tolerance. Results from paper III suggest that inhibitory effects on MoDC maturation is not a likely mode of action for *M. capsulatus* Bath or *E. coli* Nissle, but may be one mechanism behind *L. rhamnosus* probiotic functionality. *L. rhamnosus* GG primed MoDCs did not induce T cell anergy in a co-culture

with allogenic T cells as presented in paper II, however, in our setup MoDCs were cultured in the presence of IL-2. It has been shown that anergy in T cells caused by antigen presentation in the absence of a positive co-stimulatory environment can be reversed by stimulating T cells in the presence of IL-2 (Dure & Macian, 2009). It can therefore not be excluded that *L. rhamnosus* GG primed MoDCs induce T cell hypo-responsiveness in the absence of exogenous IL-2.

Co-stimulatory and co-inhibitory surface molecules were differentially regulated in response to bacterial stimulation

Although co-stimulatory signaling is usually represented by the binding of CD28 to CD80/86 molecules on DCs, co-stimulation is more likely to involve a number of different DC ligands interacting with various co-stimulatory or co-inhibitory receptors on T cells (Bakdash et al., 2013; Chen & Flies, 2013; Pletinckx, Dohler, Pavlovic, & Lutz, 2011). A high number of genes for co-stimulatory molecules were regulated in response to bacterial stimulation, and different sets of co-stimulatory molecules were up- or down-regulated in response to the three species. Co-stimulatory and co-inhibitory pathways are promising targets for immunotherapies in transplantation settings, cancer, and autoimmune diseases. *M. capsulatus* Bath, *E. coli* Nissle and *L. rhamnosus* GG each characteristically regulated the expression of genes for co-stimulatory/co-inhibitory molecules. Interestingly, *L. rhamnosus* GG and *M. capsulatus* Bath both reduced the expression of the *CD70* gene. Binding of CD70 to its receptor, CD27, is important in priming, differentiation and memory formation in T-cells (Boursalian T.E., 2009). The co-stimulatory CD70/CD27 pathway has been implicated in autoimmunity, and blocking the CD70/CD27 pathway has been shown to ameliorate inflammation in a murine model of IBD (Manocha et al., 2009). The ability of *M. capsulatus* Bath and *L. rhamnosus* GG to downregulate CD70 in DCs may be functionally relevant to their protective functions in animal models of IBD.

Furthermore, DEGs for ligands of both co-inhibitory and co-stimulatory receptors were downregulated in response to *L. rhamnosus* GG including 4-1BB, CD40, PDL1 and PDL2. Furthermore, *E. coli* Nissle and *L. rhamnosus* GG, but not *M. capsulatus* Bath, downregulated ICOSLG (B7-H2). Co-stimulation via the ICOS-L/ICOS pathway supports proliferation and production of cytokines in activated T cells (Hutloff et al., 1999), and is likely to play roles in both T cell activation and tolerance complicating the exploitation of this pathway for therapeutic purposes (Bakdash et al., 2013).

Given current advances in targeting co-stimulatory/ co-stimulatory pathways to modulate immunity in diseases including cancer, autoimmunity, inflammation and transplant rejection, the toning of co-stimulatory molecules may be a feature of immune modulatory bacteria that could be exploited for therapeutic purposes.

Differential cytokine expression in response to three immunomodulatory bacteria

Whereas immature DCs are generally assumed to promote tolerance, mature DCs do not always induce immunogenic responses. The balance between different Th effector subsets (Th1, Th2, Th17, Th22) and tolerogenic regulatory cells is essential for immune homeostasis, and dysregulation of this balance has been implicated in a variety of inflammatory conditions including inflammatory bowel disease. (Raphael, Nalawade, Eagar, & Forsthuber, 2015; Ueno, Ghosh, Hung, Li, & Jijon, 2015). DCs produced cytokines are important in regulating the balance between different T effector cell subsets.

L. rhamnosus GG was shown in paper II to be a poor inducer of MoDC cytokines compared to the two Gram-negative bacteria. Results presented in paper III supports this conclusion as *L. rhamnosus* GG-primed MoDCs reduced the expression levels of genes for a high number of pro-inflammatory cytokines compared to maturation control. Both Gram-negative bacteria, *M. capsulatus* Bath and *E. coli* Nissle, elevated the expression of genes for colony stimulating factors and the pro-inflammatory cytokine TNF-α. However, only *E.coli* Nissle upregulated the expression of DEGs for IL-1α, IL1β, IL-23, IL-6 and oncostatin M, and induced higher levels of expression for TNF-α than *M. capsulatus* Bath, suggesting a higher pro-inflammatory potential for *E.coli* Nissle than *M. capsulatus* Bath. TNF-α promotes the generation of Th22 cells and is critical in the pathogenesis of inflammatory bowel disease (IBD) (Duhen, Geiger, Jarossay, Lanzavecchia, & Sallusto, 2009; Pache, Rogler, & Felley, 2009). The observation that *M. capsulatus* and *E.coli* Nissle, two bacteria with anti-inflammatory potential in models of IBD, both upregulates expression of TNF-α in MoDCs suggests that protective effects of these bacteria are not a result of counterbalancing proinflammatory cytokines as was suggested for a probiotic species of Lactobacillus (Christensen et al., 2002).

Whereas it was initially suggested that that once polarized, T cells are committed to a particular functional state, recent evidence suggest that $CD4^+$ T cell subsets are much more flexible. IL-17 secreting Th17 cells, is a Th subset that have been strongly implicated in immune pathologies including IBD murine models of IBD, human Crohn's disease and ulcerative colitis (Ueno et al., 2015). Substantial plasticity within this subset is suggested by the existence of IL-17 secreting cells, which can also secrete interferon-γ, the signature cytokine for Th1 cells, or that can co-express the transcription factor FoxP3, a signature transcription factor of Tregs (Ueno et al., 2015). The commensal microbiome has been shown to regulate the balance between Treg and Th17 cells by stimulating host cytokine production (Omenetti & Pizarro, 2015) suggesting a potential also of probiotic bacteria for shifting the Th17/ Treg balance in IBD by modulating host cytokines.

M. capsulatus Bath and *E. coli* Nissle increased MoDC expression of cytokines that regulate the development of Treg and Th17 lineages. Two members of the same cytokine family, leukemia inhibitory factor LIF and IL-6 have been shown to counter-regulate the development of the Treg and Th17 cells. LIF promotes the Treg lineage-specific transcription factor, Foxp3, and represses the Th17 lineage-specific transcription factor RORγt while, conversely, IL-6 inhibits expression of Foxp3 and promotes RORγt (Metcalfe, 2011). Whereas both *M. capsulatus* and *E. coli* Nissle significantly upregulated LIF expression in MoDCs, only IL6 was upregulated by *E. coli* Nissle suggesting that *M. capsulatus* may be a better choice to tune Treg/ Th17 responses in the direction of Tregs.

The immune regulatory cytokines IL-10 and TGF-β are critical for maintaining tolerance in the intestines (Manicassamy & Pulendran, 2011). Both have pleiotropic functions, but play particularly important roles in induction of regulatory T cells. IL-10 and TGF-β are well recognized drivers of regulatory T cell development, but these cytokines are associated with the induction of different subsets of regulatory T cells. IL-10 is the most important cytokine driving $CD4^+FoxP3^-$ type 1 regulatory T cells (Tr1s) (Zeng et al., 2015) whereas TGF-β is a main driver of classical $CD4^+FoxP3^+$ Tregs, but also Th9 and Th17 development depending on the general cytokine environment (Zheng, 2013).

Interestingly, we found that both Gram-negative bacteria induced expression of IL-10, but not TGF-β whereas the Gram-positive *L. rhamnosus* GG enhanced expression of TGF-β, but not IL-10. *L. rhamnosus* GG also reduced expression of the gene for the β subunit of IL27, another cytokine involved in the induction of Tr1 (Zeng et al., 2015). Thus, MoDCs

stimulated by Gram-positive and Gram-negative bacteria may preferentially induce different types of regulatory T cell subsets. Gram-positive bacteria appear to promote Tregs and Gram-negative Tr1 cells. Whereas both types of regulatory T cells contributes to maintain peripheral immune tolerance, different types of regulatory T cells may have specific roles in distinct disease models (Zeng et al., 2015), again proposing that the Gram-negative *M. capsulatus* Bath and *E. coli* Nissle and the Gram-positive *L. rhamnosus* GG may stimulate peripheral tolerance by different mechanisms.

Bacteria modulates expression of galectins involved in tolerance promotion

Galectins, are a family of lectins with pleiotropic roles in microbe-host interactions, innate and adaptive immune responses T cell function and tolerance (Baum, Garner, Schaefer, & Lee, 2014; Manicassamy & Pulendran, 2011; Rabinovich & Toscano, 2009; Zeng, Zhang, Jin, & Chen, 2015). Accumulating evidence suggests a role for galectins in tuning the immune response and galactins have been suggested to have potential as novel therapies for autoimmune diseases, chronic inflammation and cancer (Rabinovich, Liu, Hirashima, & Anderson, 2007). *M. capsulatus*, *E.coli* Nissle and *L. rhamnosus* GG each exclusively induced or downregulated expression of genes for galectins, Of notice, *M. capsulatus* Bath and *L. rhamnosus* GG both upregulated expression of genes for galectins described to promote tolerance. The gene for galectin-10, also known as the Charcot-Leyden crystal protein (CLC), was upregulated in *M. capsulatus* Bath-stimulated MoDCs. Originally considered an eosinophil/basophil-specific protein, galectin-10 has more recently been shown to be an immune regulatory molecule expressed also by other immune cell types. Kubach et al. (2007) compared protein expression in $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ cells and showed that galectin-10 showed the most striking difference in expression between the two T cell subsets, and was critical for the suppressive function of Tregs. Moreover, galectin-10 was described as responsible for to the T cell suppressive activity of a newly described regulatory subset of eosinophils (Lingblom, Andersson, Andersson, & Wenneras, 2017). To our knowledge galectin-10 expression and function in DCs have not been described, but based on functions of galectin-10 in other cell types, MoDC-expressed galectin-10 may be involved in regulating DC-mediated T cell activation.

L. rhamnosus GG increased the expression of *LGALS9*, the genes for galectin-9. Galectin-9 is a ligand for the co-inhibitory receptor TIM3 expressed by T cells and have been described to induce Th1 apoptosis, promote Tregs and suppress Th17 differentiation (de Kivit et al.,

2012; C. Zhu et al., 2005). Interestingly, a previous study suggested that modulation of galectin-9 expression may contribute to probiotic functionality. De Kivit et al. (2012) showed that the administration of a symbiotic combination of *Bifidobacterium breve* and prebiotic oligosaccharides increase galectin-9 expression by intestinal epithelial cells and reduce acute allergic skin reaction and mast cell degranulation in a murine model for cow's milk allergy. Furthermore, serum galectin-9 levels were increased in human infants suffering from IgE-mediated eczema treated with *B. breve*, and galectin-9 levels were correlated with reduced allergic symptoms (de Kivit et al., 2012). The authors suggested that galectin-9 is involved in the suppression of allergic disease and showed that galectin-9 induced the development of Th1 and Treg in PBMCs. The highly differential expression of galectins observed in response to different bacteria presented in paper III, together with reported roles of galectins in tolerance, makes them good candidates for modulation of DC tolerance/immunogenic properties by probiotics.

Whereas transcriptome analysis allows the investigation of differences in gene expression patterns of MoDCs after bacterial treatments, the real quantity of interest is not the intermediate mRNA, but the final protein products of the cell, and it is important to note that mRNA concentrations are only proxies for the concentrations and activities of the corresponding proteins. Several regulatory processes, post-transcriptional, translational and protein degradation occur after mRNA is made, and transcript abundance may only partially predict protein abundance (Vogel & Marcotte, 2012). Therefore, whereas transcriptome analysis is a useful explorative approach for evaluating different cellular responses to different stimuli and forming hypotheses about functional effects, results should be interpreted by caution, and interesting results should be followed up by proteomic approaches.

In conclusion, paper III defines DC modulatory properties of three bacteria with demonstrated potential for therapeutic use. The results shows that even a non-commensal environmental bacterium profoundly modulates DC gene expression. *M. capsulatus* Bath induces a state of DC maturation, cytokine- and chemokine production different from *L. rhamnosus* GG -primed and *E. coli* Nissle -primed MoDCs and modulates expression of genes for surface molecules and released immunosuppressive/ immunomodulatory cytokines. The results thus support a potential even for environmental bacteria in immune modulation, and points to soils and water as novel sources of microbes and microbial components that can be exploited for therapeutic purposes. Furthermore, paper III suggests

very different effects of the three tested bacteria on MoDC tolerogenic/ immunogenic functions. The two established probiotic species, *L. rhamnosus* GG and *E. coli* Nissle, were found to have very different and partly opposite effects on genes important for immunogenic and tolerogenic MoDC functions. *L. rhamnosus* GG seemed to maintain an immature MoDC phenotype and reduced the expression of genes involved in migration and antigen presentation, co-stimulation and cytokine production, but increased the expression of TGF-β, a cytokine enhancing Treg generation. In contrast, *E. coli* Nissle appears to be a strong inducer of MoDC activation, and generate MoDCs expected to have high capacity for promoting antimicrobial/antiviral responses. *E. coli* Nissle promotes expression of genes necessary for migration and T cell activation, but also induces increased expression levels of genes for immune suppressive regulatory cytokines like IL-10 and LIF. Paper III may form the basis for hypothesis formation about how immunomodulatory bacteria work, and provide a knowledge basis to make educated decisions about their appropriate applications.

4 CONCLUSIONS AND FUTURE PERSPECTIVES

This thesis describes properties of the non-commensal soil bacterium *Methylococcus capsulatus* Bath and its immune modulatory interactions of with human immune cells. *M. capsulatus* Bath was previously shown to have protective effects in a salmonid and murine model of intestinal inflammation, but the responsible mechanisms have not been identified. *M. capsulatus* Bath has not previously been described to engage in a host-associated life style. The aim of our studies was therefore to examine how this bacterium interacts with host cells involved in maintaining mucosal homeostasis.

Bacterial cell wall molecules and proteins released to the extracellular milieu are particularly important for how bacteria interact with their surroundings. In paper I we conducted a secretome analysis and showed that *M. capsulatus* secretes proteins likely to be involved in adhesion, colonization, nutrient acquisition and homeostasis maintenance. Although *M. capsulatus* has not previously been described to engage in pathogenic or commensal host-interactions results presented in paper I, suggests a potential for host interactions. A type Vb (two-partner) secretion system was identified in *M. capsulatus* Bath consisting of a hemagglutinin-like protein and its associated transporter. Furthermore, a complete type IV pili system is encoded in the genome of *M. capsulatus Bath* and was confirmed to be expressed by presence of its pilin and putative tip-adhesin in the culture supernatant during growth. Integrin binding sites were identified both in the hemagglutinin-like protein and in the *M. capsulatus* tip-adhesin.

Several studies are planned, or in progress to follow up results from paper I. 1) Whereas secreted pilins from a type IV pili system was readily identified in culture supernatant after centrifugation of bacteria, the experimental method used in paper I does not detect proteins that remain attached to the bacterial surface after secretion. Therefore, it is not known whether a *M. capsulatus* Bath hemagglutinin-like protein, that was predicted to be secreted, is actually expressed and surface exposed. Its expression should be confirmed to evaluate whether this protein may be functional important in *M. capsulatus* host-interactions. 2) In addition to results presented in paper II, subcellular location analysis also revealed the presence of several proteins that was not predicted to be secreted, but that showed sequence similarity to eukaryotic and prokaryotic proteins with known immunomodulatory functions and that may contribute to anti-inflammatory effects *in vivo* (Unpublished results). Candidate proteins identified through this work has already been chosen, their genes have

been cloned into expression vectors and protein purified. Relevant *M. capsulatus* proteins identified through this study are next to be tested for anti-inflammatory potential in *in vitro* and *in vivo* model systems. 3) Pili and hemagglutinin like proteins are important host factors and probiotic factors. Whether any of these proteins are responsible for attachment to MoDC or DC modulatory effects can be explored by creating *M. capsulatus* knockouts for these adhesins/adhesion complexes. Systems for creating *M. capsulatus* knockouts have already been established by others.

In paper II we set out to investigate the immunomodulatory capacities of *M. capsulatus* on blood leukocytes. Results from paper I show that potential host-interaction proteins are present in *M. capsulatus* Bath. In paper II we confirmed that it does interact with human immune cells. When *M. capsulatus* Bath was incubated with human peripheral blood mononuclear cells (PBMCs), bacteria clustered around, and adhered specifically to a small subset of the cells among PBMCs. This subset was identified as dendritic cells (DC). DCs play a key role in regulating T cell immunity, and we next examined how the close interaction of *M. capsulatus* Bath and DC affect DC functions important for T cell activation and instruction. Compared to the Gram-positive *L. rhamnosus* GG and the Gram-negative *E. coli* K12 *M. capsulatus* Bath induced intermediate MoDC maturation and a distinct MoDC cytokine profile. In a mixed lymphocyte reaction *M. capsulatus* Bath primed monocyte-derived dendritic cells, and enhanced T cell expression of CD25, the α-chain of the high affinity IL-2 receptor, supported cell proliferation, and induced a T cell cytokine profile distinct from both *E. coli* K12 and *L. rhamnosus* GG. Results show that *M. capsulatus* Bath modulates MoDC functions involved in the activation and instruction of naïve T cells.

Whereas phenotypic markers and cytokine expression are often used to assess different immune modulatory potential of different bacterial species/ strains, few have addressed how individual species of non-pathogenic bacteria induce different gene expression profiles and functionally different DCs. In paper III the immunomodulatory potential of *M. capsulatus* Bath was confirmed on the level of gene-expression. *M. capsulatus* Bath was incubated with MoDCs, and the cells were then subjected to transcriptomic analysis. For reference the effect of *M. capsulatus* Bath, was compared to established probiotic bacteria with beneficial effects in animal models of intestinal inflammation. *M. capsulatus* Bath and the two probiotic bacteria affected the expression of genes involved in DC maturation, co-

stimulation, co-inhibition and T effector cell development in a manner dependent on microbe characteristics. *M. capsulatus* Bath and *L. rhamnosus* GG both upregulated the expression of galectins described to have a role in tolerance and induction of regulatory T cells (galectin-10 and galectin-9 respectively). Furthermore *M. capsulatus* Bath and *L. rhamnosus* both reduced the expression of CD70, a co-stimulatory molecule implicated in auto-immunity and a suggested target for antibody-mediated immunotherapy in chronic inflammatory diseases. Furthermore, all tested bacteria induced expression of key cytokines involved in the generation of regulatory T cells. Interestingly, whereas the Gram-negative bacteria induced expression of IL-10, but not TGF-β, the Gram-positive *L. rhamnosus* GG induced expression of TGF-β, but not IL-10, suggesting that MoDCs stimulated by Gram-positive and Gram-negative bacteria may preferentially induce different subsets of regulatory T cells.

Finally, the two probiotics, both reported to have protective effects in animal models of IBD, were shown to have very different and partially opposing effects on the expression of functionally important genes. *L. rhamnosus* GG prevented MoDC maturation, reduced expression of genes necessary for migration and antigen presentation, co-stimulation and chemokine and cytokine production. *L. rhamnosus* GG primed MoDCs are therefore be expected to have reduced potential for driving T cell- and innate immune functions. In contrast, *E. coli* Nissle is suggested as a strong inducer of MoDC activation, by promoting expression of interferon responsive genes as well as genes necessary for recruitment of inflammatory cells and T cell differentiation. MoDC-primed by *E. coli* Nissle can therefore be expected to have high capacity for activating adaptive immunity as well as promoting antimicrobial/viral defense.

In Paper III we have identified important functional differences between three immune modulatory bacteria and confirmed a potential for environmental bacteria in modulating the expression of MoDC genes relevant for DC driven peripheral tolerance. Results suggests different modes of action for different immunomodulatory species and emphasize that bacteria intended for therapeutic purposes should be carefully selected based on functional properties of each species to match the etiology and state of disease. Results presented in paper III may form a basis for forming hypotheses about the mode of action for *M. capsulatus* Bath, *L. rahmnosus* GG and *E.coli* Nissle, and may form a basis to make educated decisions about their appropriate applications.

Whereas results presented in paper III suggest some possible mechanisms for the immunomodulatory functions of *M. capsulatus* and the two probiotic bacteria, the model used in this study has clear limitations. MoDCs can neither represent the complex interactions between many different cell types and signal molecules that acts in an intestinal environment, nor does it fully represent functional characteristic of DCs in peripheral blood or in relevant tissues. Therefore, caution should be exercised in extrapolating results from this simplified *in vitro* model to the *in vivo* situation, and further studies should be continued in more complex model systems.

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PAPER I

PLOS ONE

RESEARCH ARTICLE

Computational and Experimental Analysis of the Secretome of *Methylococcus capsulatus* (Bath)

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Abstract

The Gram-negative methanotroph *Methylococcus capsulatus* (Bath) was recently demonstrated to abrogate inflammation in a murine model of inflammatory bowel disease, suggesting interactions with cells involved in maintaining mucosal homeostasis and emphasizing the importance of understanding the many properties of *M. capsulatus*. Secreted proteins determine how bacteria may interact with their environment, and a comprehensive knowledge of such proteins is therefore vital to understand bacterial physiology and behavior. The aim of this study was to systematically analyze protein secretion in *M. capsulatus* (Bath) by identifying the secretion systems present and the respective secreted substrates. Computational analysis revealed that in addition to previously recognized type II secretion systems and a type VII secretion system, a type Vb (two-partner) secretion system and putative type I secretion systems are present in *M. capsulatus* (Bath). *In silico* analysis suggests that the diverse secretion systems in *M.capsulatus* transport proteins likely to be involved in adhesion, colonization, nutrient acquisition and homeostasis maintenance. Results of the computational analysis was verified and extended by an experimental approach showing that in addition an uncharacterized protein and putative moonlighting proteins are released to the medium during exponential growth of *M. capsulatus* (Bath).

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Introduction

Methylococcus capsulatus is a Gram-negative, methane-oxidizing bacterium [1] that has been isolated from marine-, fresh water- and terrestrial habitats. In 2004,

the genome sequence of *M. capsulatus* (Bath) strain NCIMB 11132, was published, and results indicated a potential for high metabolic flexibility [2]. Methylotrophs have received considerable industrial interest and a number of patents has been issued for the commercial exploitation of their proteins [3]. *M. capsulatus* (Bath) is the main ingredient in BioProtein (BP), a bacterial single cell protein (SCP) product produced by BioProteins AS (Norway) that serves as a protein source in feedstuff for animals, including salmonids. In 2011, Romarheim et al. showed that dietary inclusion of BP prevents development of soybean meal-induced enteritis in Atlantic salmon (*Salmo salar*) [4]. Recently Kleiveland et al. extended this observation to mammals, when they found a similar effect of BP on dextran sodium sulfate (DSS)-induced colitis in mice [5]. They further demonstrated a comparable effect in mice fed with only *M. capsulatus (Bath)* without the supplementary bacteria present in BP, suggesting that *M. capsulatus* represents the anti-inflammatory principle in BP.

Understanding protein secretion is a key to understanding how bacteria interact with their environment. Secreted proteins are involved in processes such as sensing, signaling, nutrient acquisition and attachment. Following secretion, effector proteins may remain attached to the bacterial surface, may be released to the environment, or may even be injected directly into a host cell. In Gram-positive bacteria, all proteins that are actively translocated across the cell membrane are per definition secreted. In Gram-negative bacteria, in contrast, proteins destined for the surface or the extracellular milieu of the bacterium must first traverse two lipid bilayers, the inner (IM) and outer membrane (OM).

Nine different secretion systems are so far identified in Gram-negative bacteria and have been numerically classified from type I to type IX secretion system (T1SS-T9SS) [6]. These systems range from rather simple systems of few components, to highly specialized multiprotein secretion machineries. Secretion may be achieved in a single step using a contiguous channel spanning both membranes, as is generally regarded to be the case with the T1SS, T3SS, T4SS and T6SS [7]. Alternatively, secretion may be a two-step process in which proteins are first translocated across the IM to the periplasm through general export pathways shared with monoderm bacteria and subsequently translocated across the OM via secretion systems exclusive to Gram-negative species (T2SS, T5SS, T7SS, T8SS, and T9SS).

Proteins contain information aiding the bacterium in assigning them to their correct location. Majority of the proteins secreted by the two-step mechanisms are translocated over the IM via the Sec-pathway in an unfolded state, while the less employed Tat-pathway translocates folded proteins. Whichever pathway being utilized, proteins are generally directed for the IM by an N-terminal signal peptide. In addition to signal peptides, physiochemical characteristics like hydrophobicity, amino acid (aa) charge or polarity are examples of cues to final location. The same type of characteristics can be exploited to predict subcellular location by *in silico* analysis, and a number of prediction programs have been constructed to this end.

Predicting secreted proteins in diderms is more complicated than in monoderm bacteria for two reasons. Firstly, although the presence of a signal peptide is indicative of translocation across the IM, it is not a predictor of final location [8]. Following Sec- or Tat- dependent export, proteins may be anchored to the IM by an uncleaved signal peptide, be released to the periplasm, anchored to the inner face of the OM, integrated in the OM or translocated across the OM by any of the specialized secretion system. Secondly, the numerous mechanisms used by diderms for the translocation of proteins across the OM adds to the complexity, as no universal conserved signal sequence defines secretion across the OM like the N-terminal signal peptide defines IM translocation. *In silico* prediction of protein secretion in Gram-negative bacteria should therefore be tailored to the secretion systems present.

The OM proteome of *M. capsulatus* (Bath) has previously been analyzed using proteomic and computer/bioinformatic approaches [9, 10]. Proteins peripherally associated with the surface was further characterized by Karlsen et al. [11], demonstrating that the surface proteome of *M. capsulatus* (Bath) is highly dynamic. However, less attention has been given to proteins released to the extracellular milieu.

The purpose of this study was to identify the secretome (the secretion/translocation systems and the protein substrates of these transport systems) to extend the knowledge of how *M. capsulatus* (Bath) interacts with its environment. We employed a prediction strategy developed by Romine [12], guided by homology and conserved domains, to predict the secretome of *M. capsulatus* (Bath). In addition to two T2SSs and a T7SS previously identified [13–15] we found that putative T1SS, and T5SS are present in *M. capsulatus* (Bath). An *in silico* prediction strategy was used to identify the substrates of each of the *M. capsulatus* (Bath) secretion systems. Analysis of proteins present in the growth medium confirmed that *M. capsulatus* (Bath) secretes adhesion proteins, extracellular enzymes and proteins previously suggested to have functions in copper homeostasis [11]. Furthermore, putative moonlighting proteins and a unique *M. capsulatus* protein not previously recognized as secreted were identified in the growth medium of *M. capsulatus* (Bath).

Materials and Methods

Media and growth conditions

M. capsulatus (Bath) NCIMB11132 (GenBank accession number AE017282) was cultivated in nitrate mineral salts medium [1] with a head-space of 75% air, 23.75% CH_4 and 1.25% CO_2. An overnight pre-culture was pelleted by centrifugation (550 × g at 4℃, 10 min.). The pellet was dissolved in fresh medium to OD_{440} of 0.2 (+/− 0.14) in three biological replicates. Cultures were grown in 350 ml shake flasks in 150 ml medium at 45℃ with orbital shaking at 200 rpm.

Preparation of proteins from culture supernatant

Cultures were sampled during early exponential, mid exponential and late exponential growth phase (Figure 1). The bacteria were pelleted by centrifugation (3500 × g at 4℃, 10 min.), the supernatant was sterile filtrated (0.2μm) and PMSF added to a final concentration 0.1 mM. Proteins in the cell free fractions were concentrated by trichloroacetic acid precipitation: Sodium deoxycholate was added to supernatant fractions to a concentration of 0.2 mg ml^{-1}, and samples incubated on ice for 30 min. Trichloroacetic acid was added to a final concentration of 16% and samples incubated on ice for 1 hour. Proteins from 10 ml of supernatant were harvested by centrifugation (25000 × g at 4℃, 15 min.), protein pellets washed twice with ice cold acetone and re-centrifuged. Acetone was removed and the pellets air dried.

In-gel digestion and protein extraction

Proteins precipitated from 10 ml of culture supernatant were resolved in a sample buffer containing 0.125M Tris HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.002% bromophenolblue (pH 6,8) and separated on a 10% separating gel with a 4% stacking gel [16] (Figure 2). Gels were stained by Coomassie Brilliant Blue R250 and destained. Gel-lanes was either treated as individual pieces or sliced into two pieces, proteins in-gel trypsinated and peptides extracted following the protocol of Shevchenko et al. [17]. Following trypsination and peptide extraction from the gel-pieces, samples were concentrated and desalted using ZipTipC18 (Millipore).

LC-MS and Database searching

Peptides were analyzed by an ESI-Orbitrap (LTQ OrbitrapXL, Thermo Scientific, Bremen, Germany) mass spectrometer coupled to an Ultimate 3000 nano-LC system (Dionex, Sunnyvale CA) as described in [33]. LC-MS/MS data were analyzed using the Mascot software [18] to search a local database of 2925 *M. capsulatus* protein sequences assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 10.0 PPM. Allowed variable modifications specified by Mascot were: S-carbamoyl-methylcysteine cyclization of the N-terminus, deamidation of asparagine and glutamine, oxidation of methionine, acetylation of the N-terminus and acrylamide adducts of cysteine. Protein identifications were validated by Scaffold version 3.3.3, Proteome Software Inc., Portland, Oregon, USA) [19] using the Protein Prophet algorithm [20]. For valid protein identification at least two peptides was required with a probability of $\geq$95% and a total protein probability of $\geq$99% and the protein had to be represented in at least two of the three biological replicates.

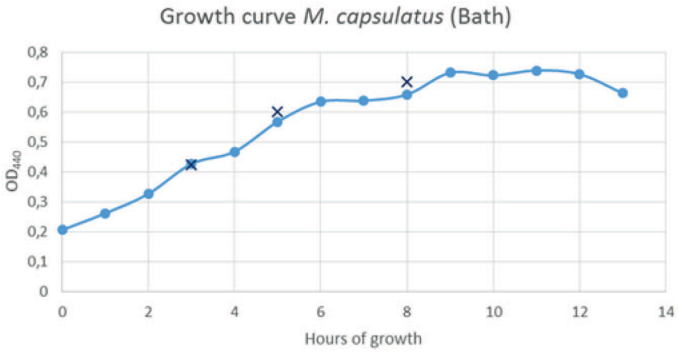

Figure 1. Growth curve showing average OD440 nm for three cultures of *M. capsulatus* (Bath) during growth. Cultures were sampled at OD440 0.424, 0.601 and 0.701 ± 0.15, indicated by X.

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Secretome analysis by computational tools

To identify novel secretion systems, the IMG Functional Profile tool [21] was used to search the *M. capsulatus* (Bath) NCIMB 11132 genome using appropriate PFAM and TIGRFAM domains and clusters of orthologous groups. To identify T1SS components the *Escherichia coli* O6:H1 (strain CFT073/ATCC 700928/ UPEC) ABC transporter (HlyB) Q8FDZ8 was used as a query in a protein BLAST [27]. The operon structures of the top twenty HlyB hits (E values ≤ 1e-15) were

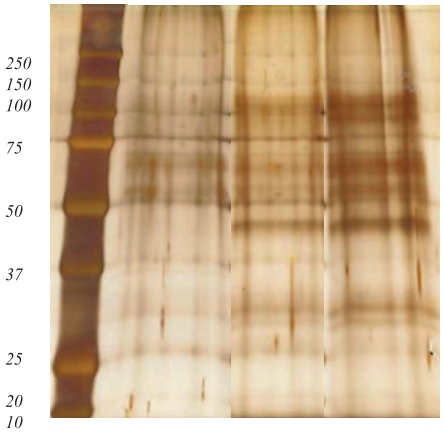

Figure 2. A representative silver stained SDS-PAGE gel showing proteins from cell free culture supernatant of *M. capsulatus* (Bath) culture supernatants from early-, mid, and late exponential growth. Precision Plus Protein Dual Color Standard; molecular weights are indicated in kDa. Proteins precipitated from 10 ml of culture supernatant were applied to the gel.

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examined using the Prokaryotic Operon DataBase [22]. To predict subcellular location of *M. capsulatus* (Bath) proteins, aa FASTA sequence of 2956 *M. capsulatus* (Bath) proteins were acquired from the NCBI protein database and analyzed using available online prediction programs (table S1) combined in a strategy/pipeline as described in [12]. Lipoproteins were predicted using LipoP 1.0 [23] and LIPO [9]. Protein sequences were analyzed for β-barrel structure using BOMP [24]. Transmembrane helices were predicted by TMHMM server v 2.0 [25] and Phobius [26]. Signal peptide prediction was performed by SignalP4.1 [27] using the high sensitivity option, TatP [28] and TATFIND [29]. PilFind v1.0 [30] was used to predict type IV pilin-like signal peptides. All proteins predicted to contain a signal peptide, that were not predicted to be integral to the IM, and that did not display β-barrel structure or OM domains, were analyzed by Psortb v. 3.0.2, [31] and were searched for homology to secreted proteins by blastp [32] or conserved surface domains by CD-BLAST [33] and Pfam 27.0 [34]. SecretomeP 2.0 [35] was used to predict non-classical protein secretion. Additional evidence, such as functional annotations, genomic context and the localization prediction servers Cello [36] and Psortb v3.0 were consulted to support predictions and resolve inconsistencies.

Results and Discussion

The secretion systems of *Methylococcus capsulatus* (Bath)

To account for the substrates of the numerous Gram-negative secretion systems it was important to recognize which secretion systems are present. Several secretion systems are already functionally annotated in *M. capsulatus (Bath)*. T2SS are widespread in Gram-negative species, and a complete classical T2aSS system is present in *M. capsulatus* [13]. Furthermore, a closely related secretion system responsible for type IV pili assembly was previously noted to be present [14]. Following the classification by Chagnot et al. type IV pili systems are categorized as T2cSS [6]. Similarly, a chaperone-usher secretion pathway T7SS responsible for secretion and assembly of fimbrial and non-fimbrial surface structures has previously been recognized in *M. capsulatus* [15].

The IMG Functional Profile tool [21] was used to search the *M. capsulatus* (Bath) genome for novel secretion systems as described in materials and methods. The analysis indicated the presence of a two-partner secretion system (T5bSS), a special case of a T5SS (autotransport) in which the transported protein resides on a different polypeptide chain than the transporter. Interestingly, MCA2226, an OM protein noted by Berven et al. [9] to display sequence similarity to hemolysin activation/secretion protein precursor demonstrates all the characteristics of a two-partner secretion system transporter. MCA2226 is a β-barrel protein that contains a ShlB type POTRA domain and is found in an operon with a typical T5bSS substrate.

Moreover, two putative T1SS were identified in *M. capsulatus* (Bath). T1SS are relatively simple secretion systems that function in a Sec/Tat pathway independent

manner. They consist of three components; an OM factor, an IM anchored periplasmic membrane fusion protein and an energy-providing ATP-Binding Cassette (ABC) transporter. In *M. capsulatus* (Bath) MCA1277, an ortholog of the archetypal *Escherichia coli* OM factor, TolC, is present and annotated and is recognized by the domain TIGR01844 (T1SS OM protein, TolC family). However, TolC have other roles besides protein secretion, and its presence in *M. capsulatus* (Bath) is therefore not necessarily indicative of type I secretion [37]. Therefore, to identify additional T1SS components we used the *Escherichia coli* O6:H1 ABC transporter (HlyB) as a query in a protein BLAST [32].

Typically, genes encoding the ABC transporter, membrane fusion protein, and substrates of T1SS are clustered together in the same operon [38]. We therefore examined if any of the top twenty HlyB hits (E values $\leq$ 1e-15) were found in operon structures characteristic of T1SS. Two of the HlyB hits, MCA1809 and MCA0555, were found in operons next to proteins that contained HlyD domains. Potential T1SS substrates were also found in each operon. Hydrolase function is common to many T1SS substrates [39], and MCA1811 contains an alpha/beta hydrolase domain. The SecretomeP prediction server that predicts non-classical (signal peptide independent) secretion showed a high score (0.94) for this protein giving support to MCA1811 as a secretory protein. MCA0553 display sequence similarity to putative glycosyl hydrolases and contains a discoidin/F5/8 type C domain found in both prokaryotic and eukaryotic proteins involved in various physiological functions such as adhesion, migration and developmental processes [40]. SecretomeP did not predict this protein to be secreted, but a putative secreted protein from *Ignavibacterium album* JCM 16511 was among the closest hits (E value of 0.0) of a protein BLAST [32] using this protein as query. To conclude, our results suggests that together with TolC, MCA1809-MCA1811 and MCA0553-MCA0555 may constitute T1SS components and secreted T1SS substrates in *M. capsulatus* (Bath), but further evidence is needed to confirm the presence of T1SS in *M. capsulatus* (Bath).

T3SS, T4SS and T6SS all form multiprotein nanomachines that inject effectors directly from the bacterial cytoplasm into host cells to promote infection or defense [41–43]. Using the IMG functional profile tool we did not find PFAM domains, TIGRFAM domains or clusters of orthologous groups associated specifically with T3SS, T4SS or T6SS, suggesting they are absent in *M. capsulatus* (Bath). Injectisome type secretion systems are typically found in bacteria that live in close interaction with eukaryotes. *M. capsulatus* have, to the best of our knowledge, not been reported to engage in symbiotic relationships, commensal or pathogenic lifestyles. The absence of T3SS, T4SS and T6SS secretion systems in *M. capsulatus* is therefore not surprising.

Similarly, no evidence were found of the T8SS responsible for secretion of curli in enteric bacteria or the T9SS (porphyrin accumulation on the cell surface secretion system) previously suggested to be restricted to the Bacteroidetes phylum [44].

Genome-wide sub cellular location analysis

In an attempt to identify secreted *M. capsulatus* (Bath) proteins we applied the genome-wide protein localization prediction strategy described by Romine [12]. The strategy resulted in subcellular location prediction for 2956 proteins (table S2). To identify proteins translocated across the IM by Sec- or Tat-translocation pathways all proteins were screened for N-terminal signal peptides using LipoP, SignalP, TatP and PilFind. TMHMM was used to predict integral membrane proteins. All proteins not predicted to be integral to the inner membrane, but that contained a signal peptide, were examined for β-barrel structure or OM domains. If no such domain was found, the protein was searched for surface domains or homology to extracellular proteins. We predicted a periplasmic location for 286 proteins due to the presence of an N-terminal signal peptide and absence of surface domains or detectable extracellular homologs. In total 18 signal peptide-containing proteins displayed surface domains or homology to extracellular proteins and were predicted to be secreted. All proteins predicted to be secreted and the corresponding systems assumed to be responsible for their secretion are indicated in table 1 and are discussed in the following.

In general, several of the proteins predicted to be secreted could be found by genome context as their genes are clustered in the genome together with genes encoding components of their secretion systems. Protein transported via T2cSS (type IV piliation system), T5bSS (two-partner secretion system) and T7SS (chaperone-usher secretion pathway) could easily be identified by this approach. Additionally, pilins, the secreted substrates of T2cSS, contain characteristic signal peptides (SPIII), and can be recognized thereby using the PilFind prediction tool [30].

Four *M. capsulatus* (Bath) proteins were predicted as secreted substrates of the T2cSS/Type IV piliation system. Four pilin like proteins contain SPIII signal peptides. The T2cSS/Type IV pili system assemble polymeric fimbrial surface structures, and the major constituent of the structure is referred to as the major pilin [45]. MCA1510 contains a characteristic short leader peptide and is likely the major pilin secreted by the T2cSS. Less abundant pilin-like proteins may also be present in pili and are referred to as minor pilins. Type IV fimbrial biogenesis protein PilE (MCA0086), type IV fimbrial biogenesis protein PilV (MCA0090) and the hypothetical protein MCA0091 FimT homolog are likely minor pilins of the *M. capsulatus* (Bath) T2cSS/Type IV pili system. Reports on the subcellular location of minor pilins, have long been indecisive. Early radiolabeling experiments localized minor pilins to the IM [46], but Giltner et al. [47] demonstrated that the minor pilins PilV and PilE of *Pseudomonas aeruginosa* are incorporated throughout the pilus filament and are thus found on the outside of the bacterial cell. We therefore predict PilV and PilE to be secreted. Additionally the PilY1 homolog (MCA0087), putatively functioning as a tip adhesin of the *M. capsulatus* (Bath) type IV pilus, is expected to be secreted.

Substrates of T5bSS (two-partner secretion systems) are typically large and contain extended signal peptides and a conserved domain that that targets the

Table 1. Proteins predicted to be secreted by *M. capsulatus* (Bath).

Secretion system	Gene	Geneproduct	Pfam[a]	Predicted signal sequence
T1SS	MCA1811	Hypothetical protein MCA1811	Alpha/beta hydrolase family	No
	MCA0553	Discoidin domain-containing protein	F5/8 type C domain	No
			Amylo-alpha-1,6-glucosidase	
T2aSS	MCA0875	Serine protease	Peptidase inhibitor I9	SPI
			Subtilase family	
	MCA1028	Endonuclease	DNA/RNA non-specific endonuclease	SPI
	MCA1217	Metalloprotease	Fungalysin/Thermolysin Propeptide Motif	SPI
			Peptidase propeptide and YPEB domain	
			Fungalysin metallopeptidase (M36)	
	MCA2224	PKD domain-containing protein	Receptor for Egg Jelly (REJ) domain	SPI
	MCA2160	Cytochrome c5530 family protein	No[b]/Polycystic Kidney Disease (PKD) domain	SPI
	MCA2328	Hypothetical protein MCA2328	Domain of unknown function (DUF4082)	SPI
	MCA2512	Acid phosphatase	Phosphoesterase family	SPI
	MCA2589	Surface-associated protein	Protein metal binding site	SPI
	MCA2974	PKD domain-containing protein	REJ (Receptor for Egg Jelly) domain	SPI
	MCA0423	Cytochrome c5530	PKD (Polycystic Kidney Disease) domain	SPI
	MCA0338	Cytochrome c5530 family protein	No[b]/Polycystic Kidney Disease (PKD) domain	SPI
	MCA2076	vacJ lipoprotein	VacJ like lipoprotein	SPII
T2cSS	MCA1510	Fimbrial protein	Type IV pilin N-term methylation site GFxxxE	SPIII
			Pilin	
	MCA0086	Type 4 fimbrial biogenesis protein PilE	Prokaryotic N-terminal methylation motif	SPIII
	MCA0087	Hypothetical protein MCA0087	Neisseria PilC beta-propeller domain	SPI
	MCA0090	Type IV fimbrial biogenesis protein PilV	Prokaryotic N-terminal methylation motif	SPIII
T5bSS	MCA2227	Hemagglutinin-like protein	Haemagglutination activity domain	SPI
			Filamentous haemagglutinin family outer membrane protein	
T7SS	MCA0303	Lipoprotein[c]	Spore Coat Protein U domain	SPI
	MCA0306	Spore coat protein, late developmental	Spore Coat Protein U domain	SPI

Proteins predicted to be secreted by *M. capsulatus* (Bath) and the secretion systems responsible are shown. [a]Significant hits obtained after searches against the Pfam database [34]. [b]No conserved domain was identified in MCA2160 and MCA0338 searching Pfam 27.0, but Polycystic Kidney Disease (PKD) domains were identified by CD-search [33]. [c]Although annotated as a lipoprotein, no lipoprotein signal peptide was identified in MCA0303 by the lipoprotein signal peptide prediction tools used in this study.

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passenger for translocation across the OM [48]. Interestingly, in *M. capsulatus* (Bath) a large (3349 aa) hemagglutinin-like protein MCA2227 with an extended (43 aa) N-terminal signal peptide, is encoded in an operon with the putative

T5bSS transporter MCA2226. In *Bordetella pertussis* filamentous hemagglutinin is a surface-associated adhesin with at least three different binding specificities, including carbohydrate-, heparin sulfate- and integrin binding, and confers adhesion to a variety of cell types [49]. The hemagglutinin-like protein of *M. capsulatus* (Bath) is similarly likely to be involved in cell adhesion.

The two T7SS substrates, putative lipoprotein (MCA0303) and putative sporecoat protein (MCA0306), display sequence similarity to both sporecoat proteins and secreted pilus-adhesins and may form either fimbrial or non-fimbrial surface structures after secretion.

In total we predicted 11 proteins to be substrates of the *M. capsulatus* (Bath) T2aSS (table 1) based on the presence of surface domains or homology to secreted proteins. Enzymes are typical substrates of conventional T2aSS [50], and several of the proteins predicted to be secreted via the *M. capsulatus* (Bath) T2aSS display sequence similarity to recognized secreted enzymes. MCA0875, a serine protease displays sequence similarity to extracellular, thermostable alkaline serine protease of *Thermus* sp. [51] (E value 6e-127 and 58% identity). MCA1028, an endonuclease, displays sequence similarity to an extracellular endonuclease of *Halomonas titanicae* (E value 4e-63 and 42% identity). MCA2512, acid phosphatase displays sequence similarity (E value 5e-167 and 53% identity) to a surface-bound glycoprotein with acid phosphatase activity in *Burkholderia pseudomallei.* [52] and the metalloprotease MCA1217 displays sequence similarity (E value 1e-110 and 37% identity) to a secreted surface metalloprotease of *Psychroflexus torquis* and bacterial and fungal extracellular elastinolytic metalloproteinases of the fungalysin family. Psortb v3.0.2 supported an extracellular localization for these proteins. Moreover, two proteins predicted to be secreted based on the presence of surface domains, display sequence similarity to enzymes: MCA2224 displays sequence similarity to glycoside hydrolases limited to the central region of the protein sequence. MCA2974 displays sequence similarity to fungalysin family proteins limited to the C-terminal half of the protein.

Although the majority of T2aSS substrates are expected to be released to the extracellular milieu after secretion, some are recognized to remain bound to the cell surface, anchored by a lipid moiety, an uncleaved Tat-signal like sequence, or through association with lipopolysaccharides [53]. In *M. capsulatus* (Bath) several of the predicted T2aSS are likely to remain surface bound. The lipoprotein VacJ is surface-exposed in *Shigella flexneri* and has been defined as a virulence factor required for intercellular spread of this pathogen after invasion. VacJ is present and annotated in *M. capsulatus* (Bath) and may be surface associated also in this species. Furthermore, five assumed T2aSS substrates contained the polycystic kidney disease (PKD) domain or the related (REJ) domain found in extracellular regions of metazoan cell-surface proteins, archaeal surface layer proteins [54] and bacterial surface proteins [55]. Indeed, two of the PKD domain proteins was previously identified as peripherally attached surface proteins [11] and was suggested to be implicated in copper homeostasis regulation. Moreover, the copper binding, surface-associated protein MopE (MCA2589) is likely a T2aSS substrate.

Proteins identified in the growth supernatant

Previous research on the secretome of *M. capsulatus* (Bath) has focused on surface-attached proteins and OM proteins while less attention has been given to proteins released to the extracellular milieu. An exception is the well-studied protein MopE, shown by Fjellbirkeland et al. to occur both as a major *M. capsulatus* OM protein, and as a truncated 336 aa protein secreted into the growth supernatant [56]. Schmid et al. previously characterized the extracellular proteome of the archaeon *Pyrococcus furiosus* by LC-MS/MS-based analysis of culture supernatants [57]. In order to search for proteins released into the growth supernatant, and to confirm the location of predicted secreted *M. capsulatus* (Bath) proteins, we used a similar experimental approach. *M. capsulatus* (Bath) cultures were sampled during exponential growth, proteins were isolated from the culture supernatant and analyzed by LC/MS as described in the materials and methods section.

In an initial attempt, isolating proteins from the stationary phase resulted in identification of a large number of intracellular proteins such as ribosomal proteins, most likely due to cell death and lysis at this growth stage. Consequently, to avoid the problem of protein leakage from dead cells, we collected culture supernatants from *M. capsulatus* (Bath) during exponential growth phase. To gain an overview of whether protein expression and protein release to media changes during growth, culture supernatants were collected from cultures in early-, mid- and late exponential growth phase.

Only 10 proteins were identified in the growth medium from these growth phases (table 2). Seven of the proteins were identified at all sampled time points. Of the 10 proteins found in the growth medium, seven were predicted to contain an N-terminal signal peptides suggesting that they are translocated across the IM.

Of the 20 proteins predicted to be secreted by *in silico* analysis, four (20%) were identified in the growth medium of *M. capsulatus* (Bath). In this study we used the same media and temperature conditions as was used by Kleiveland et al. when studying the effects of dietary inclusion of *M. capsulatus* in a murine model of IBD [5]. Under these conditions we did not detect substrates of the putative T1SS, T5SS or T7SS in the *M. capsulatus* (Bath) culture supernatant. Noticeably, proteins that remain surface bound after secretion are missed by our approach. Moreover, both protein expression [11] and membrane retention/release of *M. capsulatus* (Bath) proteins [57] have previously been demonstrated to depend on media and growth conditions. Therefore, the fact that we did not detect substrates of these secretion systems in the culture supernatant does not rule out the possibility that such proteins could be expressed and/or released under different conditions.

As shown in table 2 the presence of the putative T2aSS substrates Surface-associated protein, MopE (MCA2589) and the serine protease MCA0875 was confirmed. The predicted extracellular c-type cytochrome MCA0338 expected to be peripherally attached to the surface of *M. capsulatus* (Bath) [11] was found to be released to the medium by our protocol. Similarly, fimbrial protein MCA1510

Table 2. Proteins identified in the culture supernatant of *M. capsulatus* (Bath).

Gene	Gene product	MW (kDa)	Early	Mid	Late	Unique exclusive peptides[a]	Total coverage (%) [b]	SignalP[c]	LipoP/ LIPO[d]	TatFind/ TatP[e]	PilFind[f]	TMHMM[g]	Phobius[i]	Predicted subcellular location
MCA0338	Cytochrome c5530 family protein	101	Y	Y	Y	59	34	Y	N	N	N	1 TMH[h]	SP	Extracellular
MCA0155	Uncharacterized protein	39	Y	Y	Y	21	35	Y	N	N	N	1 TMH[h]	SP	Periplasm
MCA0875	Serine protease, subtilase family	68	Y	Y	Y	20	15	Y	N	N	N	N	SP	Extracellular
MCA0779	Methanol dehydrogenase protein, large subunit	66	Y	Y	Y	23	25	Y	N	N	N	N	SP	Periplasm
MCA2589	Surface-asso-ciated protein	58	Y	Y	Y	15	21	Y	N	N	N	N	SP	Extracellular
MCA1510	Fimbrial protein	14	Y	Y	Y	6	15	N	N	N	Y	1 TMH[h]	1 TMH	Extracellular
MCA1704	60 kDa chaperonin 2	57	Y	Y	Y	14	29	N	N	N	N	N	N	Cytoplasm
MCA1677	Glutamine synthetase	52	N	Y	Y	8	18	N	N	N	N	N	N	Cytoplasm
MCA0707	60 kDa chaperonin 1	57	N	Y	Y	10	34	N	N	N	N	N	N	Cytoplasm
MCA1082	Uncharacterized protein	11	Y	N	N	3	61	Y	N	N	N	1 TMH[h]	SP	Periplasm

Proteins identified from *M. capsulatus* (Bath) culture supernatant from cultures in early, mid and late exponential growth phase. [a]Column shows the cumulative number of exclusive unique peptides from three biological replicates. Proteins were considered significant if $\geq$2 peptides were identified with a probability of $\geq$95% and a total protein probability of $\geq$99% and the protein was represented in at least two of the three biological replicates. [b]Total coverage was calculated as the percentage of all aas in a protein, after removing the signal sequence, that is identified from sample spectra. [c-f]The predicted presence (Y) or absence (N) of: [c]signal peptide, [d]lipoprotein signal peptide, [e]twin-arginine signal peptide or [f]prepilin-like signal peptide as predicted by SignalP 4.1, LipoP, LIPO, TatFind, TatP and PilFind is indicated. [g]Column shows the number of transmembrane helixes predicted by the TMHMM prediction program. An asterisk indicates that >10 of the aas found in helixes within the first 60 aas, indicating that TMH may be a signal sequence. [h]Number of TMH and/or presence of signal peptide predicted by the Phobius prediction program. [i]Subcellular location predicted *in silico*.

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predicted to be secreted via a T2cSS was identified at all sampled time points. In contrast, we did not detect other pilin-like proteins, giving support to MCA1510 being the major pilin of *M. capsulatus* (Bath).

There are some disadvantages to *in silico* approaches for prediction of subcellular locations. First, predictions rely on correct annotations and on the existence of domains associated with known protein locations or with homology to proteins of such location. Secondly, such approaches are not suitable for location prediction for proteins that have functions in more than on sub cellular compartment. Of the 10 proteins identified in the culture supernatant of *M. capsulatus* (Bath) six had been assigned a periplasmic or cytoplasmic location by *in silico* analysis.

Three signal peptide-containing proteins identified in the growth supernatant did not contain any surface domains and was predicted to be periplasmic proteins by the *in silico* analysis (MCA0155, MCA0779, MCA1082). Of these MCA1082 was found by CD-blast [57] to contain a periplasmic metal-binding domain. This protein was only detected in culture supernatants during early exponential growth. One possible explanation is that it is not expressed in later growth phases. However, LC-MS/MS is a sensitive method, and the fact that this protein is detected by only three unique peptides from early exponential growth, when few other proteins were present, suggests that it is a periplasmic contaminant that is not masked by the presence of more abundant, true extracellular proteins in these two samples.

Methanol dehydrogenase is generally assumed to be a highly abundant periplasmic protein, but has been found in OM-enriched and surface protein fractions in several studies [9, 11]. The uncharacterized protein MCA0155 was found in one parallel during early exponential growth and in all parallels during mid- and late exponential growth. No homologs were identified for this protein. Since it was consistently identified in the supernatant of all cultures we suggest that MCA0155 is actually an extracellular protein missed by the *in silico* prediction strategy. Further efforts should be made to characterize this protein, as understanding the function of unique extracellular proteins may help in understanding species-specific interactions of *M. capsulatus* (Bath) with its environment. Moreover, three proteins (MCA1677, MCA1704 and MCA0707) without predicted signal peptides were identified from the growth supernatant. All three belong to families of proteins previously described as moonlighting proteins. MCA1677 is a glutamine synthetase. The glutamine synthetase of *Mycobacterium tuberculosis* was among the first bacterial proteins reported to function as a moonlighting protein as it was found to be released into the phagosome in infected human monocytes [58]. In *Lactobacillus crispatus* a signal-less glutamine synthetase is an adhesive moonlighting protein that is surface-attached at acidic pH and released into the buffer at higher pH and that displays binding affinity for collagen, laminin, fibronectin and plasminogen [59]. The two other predicted cytoplasmic proteins identified in the growth supernatant of *M. capsulatus* (Bath) (MCA1704 and MCA0707) are closely related molecular chaperones of the 60 kDa chaperonin (Cpn60) family GroEL. Bacterial Cpn60 has been found to be released

or surface associated in a large number of studies and represents the most diverse range of moonlighting activities for any protein family know so far, including functions in adhesion, invasion, biofilm formation and stimulation of host cytokine production as reviewed by Henderson et al. [60]. As all the signal-less proteins found in the culture supernatants belong to protein families previously reported to have functions in both cytoplasm and extracellular locations, and since we did not identify ribosomal proteins or other abundant cytoplasmic proteins in the culture supernatant from exponential growth phase, we suggest that MCA1677, MCA1704, MCA0707 may represent moonlighting proteins secreted by non-classical, signal-less secretion pathways.

Possible surface associated T2aSS substrates missed by our strategy

As noted, the *in silico* strategy used here relies heavily on correct annotation of transcription start and the existence of well-characterized homologs or conserved domains. Furthermore, our experimental approach will not detect proteins that remain surface attached after secretion. We therefor searched available literature for putative secretion system substrates missed by our approaches.

Karlsen et al. has previously identified 22 proteins in fractions enriched in proteins peripherally associated with the surface of *M. capsulatus* (Bath) [11]. Of these, the authors considered seven to be periplasmic. Of the remaining 15, we found two proteins (MCA0949 and MCA2792) to belong to protein families associated with the periplasm and IM respectively. We further predicted one protein (MCA1738) to be a surface exposed OM β-barrel beyond the scope of this article, and four was predicted to be secreted via a T2aSS (MCA2589, MCA2974, MCA0423, MCA0338). The remaining proteins illustrate well the challenges of *in silico* prediction methods. In our results, these proteins were predicted either to be periplasmic due to lack of extracellular domains/homologs or to be cytoplasmic proteins because no signal peptides were detected by the signal prediction programs used in this study. Indeed, Karlsen et al. noted that three of the proteins predicted to be cytoplasmic proteins by our strategy (MCA0446, MCA0445, MCA0347) are incorrectly annotated and that N-terminal signal peptides are present in the deduced, corrected aa sequences of these proteins [11]. Thus, eight proteins that we have predicted to be located in the periplasm (MCA2590 and MCA2150) or cytoplasm (MCA0765, MCA2259, MCA0347, MCA0421, MCA0445 and MCA0446) may well be T2aSS secreted proteins that go undetected by our methodology.

Concluding remarks

Here we have combined *in silico* analysis and proteomics to define the secretome of *M. capsulatus* (Bath) to extend the knowledge of how *M. capsulatus* (Bath) may interact with eukaryotic cells, in particular. *In silico* analysis shows that multiple secretion systems are present in *M. capsulatus* (Bath). In addition to previously

described T2SS and T7SS, a T5bSS (two-partner) and putative T1SS were identified. The predicted substrates of the diverse *M. capsulatus* (Bath) systems are likely to functions in diverse processes: adhesion, colonization, nutrient acquisition and copper response. Extracellular location of predicted secreted proteins was confirmed by analyzing the growth supernatant of *M. capsulatus* (Bath) for secreted proteins. Furthermore, it was shown that proteins with potential moonlighting function, as well as a protein with no known homologs is released to the medium during exponential growth. Further functional characterization of these proteins should provide new insights in how *M. capsulatus* (Bath) interacts with its environment.

Supporting Information

Table S1. Computational tools used to analyze the secretome of *M. capsulatus* (Bath).
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Table S2. Subcellular location predictions for 2956 *M. capsulatus* (Bath) proteins.
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Author Contributions

Conceived and designed the experiments: SI GM TL MJ CRK. Performed the experiments: SI. Analyzed the data: SI GM. Contributed reagents/materials/analysis tools: MJ TL CRK. Wrote the paper: SI GM MJ TL CRK.

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PAPER II

frontiers in Microbiology

ORIGINAL RESEARCH
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The Soil Bacterium *Methylococcus capsulatus* Bath Interacts with Human Dendritic Cells to Modulate Immune Function

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The prevalence of inflammatory bowel disease (IBD) has increased in Western countries during the course of the twentieth century, and is evolving to be a global disease. Recently we showed that a bacterial meal of a non-commensal, non-pathogenic methanotrophic soil bacterium, *Methylococcus capsulatus* Bath prevents experimentally induced colitis in a murine model of IBD. The mechanism behind the effect has this far not been identified. Here, for the first time we show that *M. capsulatus*, a soil bacterium adheres specifically to human dendritic cells, influencing DC maturation, cytokine production, and subsequent T cell activation, proliferation and differentiation. We characterize the immune modulatory properties of *M. capsulatus* and compare its immunological properties to those of another Gram-negative gammaproteobacterium, the commensal *Escherichia coli* K12, and the immune modulatory Gram-positive probiotic bacterium, *Lactobacillus rhamnosus* GG *in vitro*. *M. capsulatus* induces intermediate phenotypic and functional DC maturation. In a mixed lymphocyte reaction *M. capsulatus*-primed monocyte-derived dendritic cells (MoDCs) enhance T cell expression of CD25, the γ-chain of the high affinity IL-2 receptor, supports cell proliferation, and induce a T cell cytokine profile different from both *E. coli* K12 and *Lactobacillus rhamnosus* GG. *M. capsulatus* Bath thus interacts specifically with MoDC, affecting MoDC maturation, cytokine profile, and subsequent MoDC directed T cell polarization.

Keywords: dendritic cells (DC), old friends hypothesis, immune modulation, environmental bacteria, DC activation, T cell polarization, immunobiotics, soil bacteria

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IMPORTANCE

There has been a growing interest in probiotics for treating both IBD, allergies, and autoimmune diseases, and considerable effort has been invested in identifying novel probiotics aimed for treating immune pathologies. Typically, candidate probiotic bacteria has been of human or animal origin, and a host-associated lifestyle is assumed to be a prerequisite for developing immune-regulatory functions. Here we describe immune modulatory functions of a non-commensal soil bacterium previously shown to exhibit anti-inflammatory effects in a murine colitis model pointing to environmental bacteria as a new and untapped source of bacteria for modulating immune responsiveness.

INTRODUCTION

Although microbes are associated with all epithelial surfaces of animal hosts, the highest number, and most diverse microbial populations are found in the intestines. Some 10–100 trillion microbes colonizes the human gastrointestinal tract, with the highest numbers present in the colon (Turnbaugh et al., 2007). The physiology of these microbes and their hosts is closely connected and mutually regulated (Brown et al., 2013). The host shapes the composition of the intestinal microbiota at species and community levels by supplying nutrients and by producing antimicrobial peptides. The human microbiota in return, adds to the metabolic, and biochemical activities of the host and play essential roles in the development and differentiation of the host intestinal epithelium, the immune system, and in the maintenance of mucosal homeostasis (Nicholson et al., 2012; Sommer and Backhed, 2013).

Only a single layer of epithelial cells separates the luminal contents and microbial community from underlying tissues, and the epithelial barrier therefore provides a possible entry point for opportunistic pathogens into the body. The host must maintain a mutualistic relationship with the commensal microbiome, while retaining protective responsiveness against pathogenic bacteria. To achieve this it must preserve epithelial integrity and regulate pro- and anti-inflammatory signaling, in an appropriate manner. Homeostasis is maintained through continuous and dynamic interactions and communication between the intestinal microbiota, the epithelium, and immune cells in the intestinal mucosa.

The regulatory interactions that exist between multicellular organisms and the microbial world are not necessarily limited to those between commensals and their hosts. The increasing prevalence of inflammatory bowel disease and autoimmune diseases in the western world has been associated with reduced exposure to helminths and environmental microorganisms from soil, water, and fermenting vegetables (Rook, 2007). The "hygiene hypothesis" was forwarded as a result of studies coherent with the idea that postnatal infections may be protective against allergy later in life, and that such protection may be lost in the presence of modern hygiene (Strachan, 1989, 2000). The related "old-friend hypothesis" explains the striking increase in chronic inflammatory disorders as largely being due to reduced contact with microorganisms that we have coevolved with, and therefore depend on, for proper immune development and regulation (Rook, 2010). In this context both pathogenic bacteria, the commensal microbiota, pseudo-commensals, and even the environmental microbiota may be essential regulatory components of the mammalian immune system. An increased mechanistic understanding of how such microbes and microbial products affect immune homeostasis may form a basis for developing novel tools for modulating immune responses in chronic inflammatory disorders.

Recently we demonstrated that a bacterial meal of the Gram-negative soil bacterium, *Methylococcus capsulatus* Bath, ameliorates dextran sulfate sodium (DSS) induced colitis in mice (Kleiveland et al., 2013). The study points to a potential for non-commensal environmental bacteria in protecting against experimental colitis in mammals, but the mechanisms behind these effects have not been identified. Both live and heat-killed probiotic bacteria have previously been shown to protect against experimental colitis (Mileti et al., 2009; Sang et al., 2014; Toumi et al., 2014; Souza et al., 2016). Proposed modes of action include competitive pathogen exclusion, production of antimicrobial substances, gut flora modulation, modulatory effects on epithelial barrier integrity, regulatory effects on innate, and adaptive immunity and effects on epithelial development and survival (Bermudez-Brito et al., 2012). However, direct effects on dendritic cells (DCs) with subsequent effects on cytokine production and T cell development is expected to be a common mode of action for those probiotic strains able to modulate adaptive immunity (Bienenstock et al., 2013).

DCs are professional antigen presenting cells that play a key role in both innate and adaptive immune responses (Steinman, 2012). Intestinal DCs expresses pattern recognizing receptors (PRRs) to recognize various microbial structures and can distinguish between microbe-associated molecular patterns (MAMPs) of even closely related organisms to initiate specific and appropriate response. The capacity of DCs to activate naïve T cells inducing T cell expansion and polarization, position DCs as critical mediators of host immune tolerance, and inflammatory responses (Mann et al., 2013).

The dietary inclusion of *M capsulatus* Bath in DSS-colitis model affected the intestinal epithelium through increased cell proliferation and mucin production, suggesting beneficial effects on gut barrier function. However, direct effects on cells of the immune system was not evaluated in that study. Here, for the first time, we show that *M. capsulatus* Bath, a non-commensal environmental bacterium, specifically and strongly adheres to murine and human DCs, an immune cell type central in regulating both innate and adaptive immunity. We compare the immune modulatory effects of *M. capsulatus* Bath to those of the Gram-negative commensal *Escherichia coli* K12, a non-pathogenic *E. coli* strain originally isolated from stool of a diphtheria patient (Agency USEP, 1997), and the well characterized Gram-positive probiotic bacterium *Lactobacillus rhamnosus* GG. The interaction between DC and *M. capsulatus* leads to functional activation of the DCs, affects DC cytokine profile, improves T cell activation, and proliferation and drive T effector cell polarization *in vitro*.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

M. capsulatus strain (Bath) (NCIMB 11132, Aberdeen, UK) were grown in nitrate mineral salts medium (Whittenbury et al., 1970) with a head-space of 75% air, 23.75% CH_4, and 1.25% CO_2 at 45°C and 200 rpm. *E. coli* strain K12 was kindly provided by Department of Bacteriology, the Norwegian Veterinary Institute, Norway. *E. coli* K12 (Blattner et al., 1997) was grown in LB medium (Oxoid Ltd., Basingstoke, United Kingdom) at 37°C and 200 rpm. *L. rhamnosus* GG was grown in MRS medium (Oxoid Ltd.) anaerobically at 37°C without shaking.

Cells and Culture Conditions

Human erythrocyte- and plasma depleted blood were obtained from healthy volunteers from Ostfold Hospital Trust, Fredrikstad, Norway in accordance with institutional ethical guidelines and with approval from the Regional Committee of Medical and Health Research Ethics with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on a Lymphoprep gradient (Fresenius Kabi). Human T cells were isolated from PBMCs by negative selection using Dynabeads Untouched Human T Cells Kit (Thermo Fisher). $CD14^+$ cells were isolated by positive selection using human CD14 MicroBeads (Miltenyi Biotech). To develop immature monocyte-derived dendritic cells (MoDCs) $CD14^+$ cells were cultivated for 6 days in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum and 25 μg/ml gentamicin sulfate (Lonza), 1 mM sodium pyruvate and 100 μM non-essential amino acids (both from PAA Laboratories), 25 ng/ml interleukin 4 and 50 ng/ml granulocyte macrophage colony stimulating factor (both from ImmunoTools).

Bacterial Stimulation, Cytokine Analysis, and Immune Phenotyping of MoDCs

For immune phenotyping and DC cytokine analysis MoDCs were primed for 24 h by bacteria in a ratio of 1:10 (MoDC: bacteria) or by a maturation cocktail of 15 ng/ml TNF-α (ImmunoTools), 100 ng/ml LPS and 5 μg/ml PGE2 (Sigma-Aldrich). Culture supernatants were harvested and stored at −20°C then analyzed for cytokines by ProcartaPlex Multiplex immunoassay (eBioscience). TGF-β and IL-6 was measured by ELISA kits (eBiosciences and PeproTech respectively). MoDCs were also harvested and stained using PE-conjugated mouse anti-human CD80 antibodies, PE-Cy5 conjugated mouse anti-human CD83, and PE-Cy5 conjugated mouse anti-human CD40 (all from BD Biosciences). For viability testing cells were stained by 1μg/ml PI and analyzed by flow cytometry.

DC-T Cell Co-cultures for Cytokine Analysis and Immunophenotyping

To induce antigen specific T cell responses a modified mixed leukocyte culture system (MLC) were used with MoDC as stimulator cells and purified peripheral blood T cells as responder cells. MoDCs, either unprimed or primed by UV-inactivated bacteria in a ratio of 1:100 (MoDC:bacteria) for 24 h, were co-incubated with allogeneic T cells from two different donors (1:10 ratio between MoDCs and T cells). For cytokine analysis cells were grown in 48 well plates. After 5 days culture supernatants were harvested and T cells phenotyped by flow cytometry using FITC-conjugated anti-human CD4 and APC-conjugated anti-CD25 (both from Miltenyi Biotech). Fluorescence was detected by a MACSQuant flowcytometer and analyzed using the MACSQuantify software (both from Miltenyi Biotech). Cytokine concentrations in culture supernatant were measured by ProcartaPlex Multiplex immunoassay (eBioscience).

T Cell Proliferation Assay

MoDCs were primed for 24 h with UV-inactivated *M. capsulatus* 1:100 (DC:bacteria) in Nunc™ UpCell™ plates (Thermo Fisher). After 24 of stimulation the MoDCs were harvested, washed and co-incubated with allogenic human T cells in 96-well plates in a ratio of 1:10 (DC:T cells). Next day recombinant human IL-2 was added to each well to a final concentration of 10 U/ml. After 96 h of co-culture cells were pulsed by [^{3}H]-thymidine (1 μCi, Perkin Elmer) for 18.5 h. Cells were harvested onto glass-fiber filters and incorporated thymidine determined by liquid scintillation counting using a TopCount NXT™ Luminometer (Packard BioScience Company).

Scanning Electron Microscopy (SEM)

Immature MoDCs were co-cultivated with *M. capsulatus* Bath in 1:100 ratio (cells:bacteria) in medium free of antibiotics for 2–4 h in a humified atmosphere with 5% CO_2. Cells were washed twice by phosphate buffered saline (PAA Laboratories), fixed with 4% PFA and 2.5% glutaraldehyde (1:1) for 20 min at room temperature. Cells were washed again, dehydrated in a graded ethanol series and dried using a critical point dehydrator (CPD030 BalTec). Samples were coated with ∼500 Å Pt in a sputter coater (Polaron SC7640, Quorum technologies) and analyzed using an EVO-50 Zeiss microscope (Carl Zeiss AG).

Confocal Imaging

Immature MoDCs were generated from $CD14^+$ monocytes as described above. 1×10^8/ml *M. capsulatus* Bath were stained by 10 μM CFSE in PBS. CFSE-stained bacteria were co-incubated with immature MoDCs in a ratio of 1: 100 cells-bacteria. Cells were washed, fixated in PBS with 1% formalin then washed twice before coverslip was mounted on object slide with ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific). Samples were scanned under a Zeiss LSM510 META confocal microscope (Carl Zeiss). Confocal stacks were acquired with z-spacing of 0.2 μm.

Statistical Analysis

Data were sampled in hierarchical structure, with multiple measurements per individual. This violates the assumption of independent measurements underlying ANOVA and conventional linear regression. This issue was remedied by analyzing the data using a mixed effects linear model, in which each individual acted as a random effect. Box-Cox analyses were used for finding suitable normalizing transformations. Data were analyzed on the log-scale and subsequently back-transformed for interpretation. All analyses were controlled by residual plots and Shapiro-Wilks test for normality.

RESULTS

M. capsulatus Bath Adheres Specifically to MoDC

A bacterial protein preparation of *M. capsulatus* Bath was previously found to have anti-inflammatory effects in a murine model of colitis (Kleiveland et al., 2013). When studying possible immune modulatory effects on immune cells, we

observed that bacteria clustered around a small subset of cells in peripheral blood mononuclear cell preparations (**Figure 1A**). The appearance and low frequency of the target cells were consistent with the size and expected frequency of DCs among PBMCs. To determine whether the target cells were in fact DCs we incubated *M. capsulatus* Bath with $CD14^+$ monocytes or MoDCs generated from $CD14^+$ monocytes in the presence of IL-4 and GM-CSF. *M. capsulatus* did not bind to $CD14^+$ monocytes (**Figure 1B**), but quickly associated with dendritic cells (**Figure 1C**). The interaction between *M. capsulatus* Bath and MoDCs was further visualized by scanning electron microscopy (SEM) showing *M. capsulatus* Bath in large clusters on MoDCs after 3 h of co-incubation, even after several washes with PBS (**Figure 1D**).

To study binding kinetics we co-incubated CFSE-stained bacteria with MoDCs. Cells were counterstained with DAPI and confocal microscopy was used to visualize interactions over time (**Figure 2**). *M. capsulatus* Bath were found in scattered association with cells after just 30 min of co-incubation, and after 2 h a large number of bacteria associated with most cells. Strikingly, after around 3 h of co-incubation *M. capsulatus* were typically found to cluster around the nucleus of the MoDCs. A large number of bacteria could be seen associated with cells up to 20 h after co-incubation. At 48 h bacteria were cleared from most cells although a few intact bacteria was found associated with cells up to 72 h after co-incubation (**Figure 2**).

M. capsulatus Bath Induces Phenotypic and Functional Maturation of MoDCs

Upon microbial stimulation, DCs undergo a program of maturation that endows them with capacity to activate naïve T cells, induce T cell expansion, and to polarize T cells toward effector subpopulations appropriate to the stimulus encountered. Mature DCs are characterized by expression of co-stimulatory molecules required for efficient T cell activation. We compared the ability of *M. capsulatus* Bath, Gram-positive, and Gram-negative control bacteria to induce MoDC activation as assessed by expression of costimulatory molecules like CD40, CD80, and CD83. MoDCs, either left unprimed or co-incubated with bacteria (*M. capsulatus* Bath, *L. rhamnosus* GG, or *E. coli* K12) were stained for co-stimulatory molecules and maturation markers and analyzed by flow cytometry (**Figure 3**). Cells activated by a maturation cocktail containing TNF-α, LPS, and PGE_2 were used as a positive control. The maturation cocktail, *E. coli* K12, and *M. capsulatus* Bath induced upregulation of CD40, CD83, and CD80 in immature MoDCs. *E. coli* K12 was found to represent the most potent bacterial stimulus for inducing a mature DC phenotype compared to unprimed control cells, and induced expression of all activation markers. *M. capsulatus* Bath showed intermediate ability to induce MoDC maturation and elicited CD40 and CD80 expressions comparable to positive control, but a lower expression of CD83 compared to *E. coli* and the maturation cocktail (**Figure 3**). *L. rhamnosus* GG was a weak inducer of MoDC maturation, and produced a phenotype similar to unprimed cells. Cell viability, determined by PI staining, was similar between treatments suggesting that none of the strains asserted toxic effects on MoDCs (Data not shown).

MoDCs Respond to Bacterial Stimulation by Eliciting Distinct Cytokine Profiles

Depending on the nature of the stimuli, functionally mature DCs release cytokines promoting differentiation of naïve T cells into specific effector cell subsets. Since *M. capsulatus* and *E. coli* induced phenotypic maturation of MoDCs we wanted to see whether the bacteria also resulted in functional maturation characterized by cytokine release. Multiplex immunoassay and enzyme-linked immunosorbent assay (ELISA) was used to measure select cytokines in culture supernatants of MoDCs co-cultivated with bacteria for 24 h (**Figure 4**). In general, and in accordance with the observed phenotypic activation of MoDC, *E. coli* K12 was the most potent inducer of cytokine production, and resulted in increased release of IL-1β, IL-12p70, IL-10, TNF-α, IL-2, IL-23, IFN-γ, and IL-6 compared to unprimed control. *L. rhamnosus* GG in comparison was the least potent inducer of cytokine production in MoDCs of the three tested bacteria (**Figure 4**). Incubation with *M. capsulatus* Bath resulted in intermediate levels of cytokines. Similar to *E. coli*-primed MoDCs, incubation with *M. capsulatus* enhanced the production of IL-12p70, IL-10, TNF-α, IL-2, and IL-23 compared to unprimed MoDCs. However, *M. capsulatus* treatment in general resulted in lower cytokine levels than *E. coli* K12. *M. capsulatus*-primed cells produced substantially less IL-1β, IL-6, IL-10, IL-12p70, IL-23, and TNF-α than *E. coli*-primed cells, but the two bacteria induced similar levels of IL-2 from the MoDCs. TGF-β could not be detected in any of the co-cultures (data not shown). Thus, the interaction between *M. capsulatus* Bath and MoDCs resulted in both quantitative and qualitative differences in cytokine production compared to *E. coli* K12.

M. capsulatus Bath Increases DC-Induced T Cell Activation and Proliferation

Antigen recognition and a co-stimulatory signal through CD28 on T cells is required to induce functional T cell activation and clonal expansion. As the bacteria differently induced expression of DC co-stimulatory molecules, we examined the ability of bacteria-primed MoDCs to activate and induce proliferation in peripheral blood T cells. We co-incubated bacteria-primed MoDCs with allogenic T cells and measured expression of CD25 by flow cytometry. T cells co-cultivated with *M. capsulatus*-primed MoDCs expressed increased levels of CD25 compared to T cells cultivated with unprimed MoDCs or MoDCs primed by any of the control bacteria (**Figure 5A**). To test the ability of bacteria-treated MoDCs to induce proliferation in allogeneic T cells we measured DNA synthesis by $[^3H]$ thymidine incorporation. MoDCs activated by *M. capsulatus* were stronger supporters of T cell proliferation than MoDCs primed by any of the control bacteria (**Figure 5B**).

MoDCs Primed by Different Bacteria Have Different Ability to Drive T Cell Differentiation

Cytokines produced by mature DCs contribute to drive T cell differentiation into specific effector cell subsets. In order to evaluate functional effects of bacteria-treated MoDCs on T cell polarization, activated MoDCs were co-incubated with

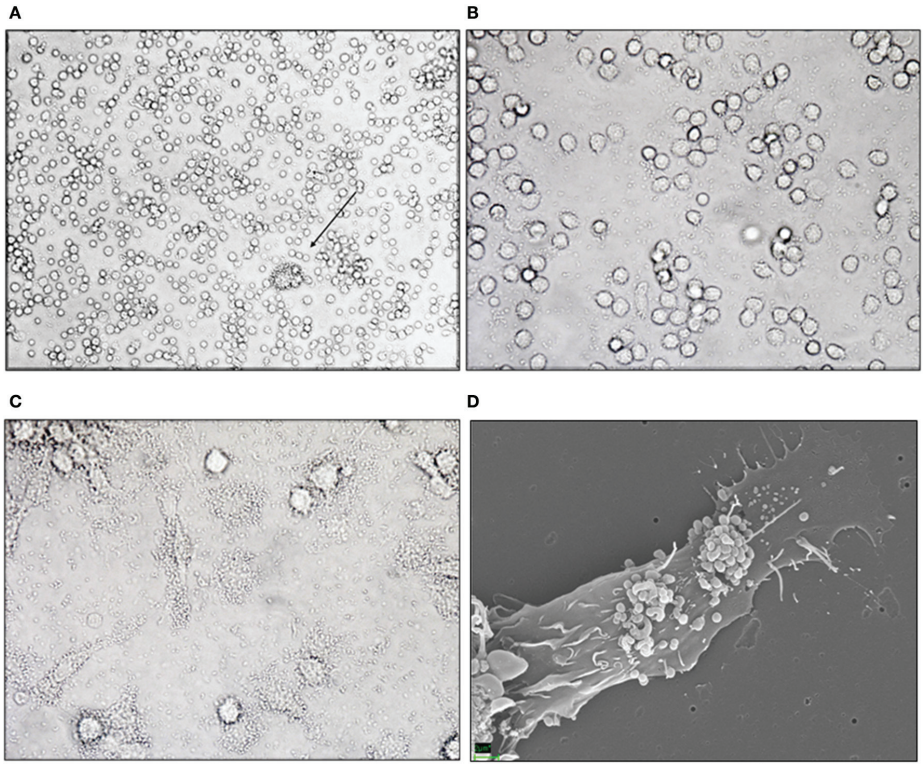

FIGURE 1 | *M. capsulatus* Bath interacts specifically with human MoDCs. (A–C) Light microscopy image of *M. capsulatus* Bath co-incubated (1:100 cells:bacteria) with human PBMC, $CD14^+$ monocytes, or monocyte-derived dendritic cells without washing. *M. capsulatus* Bath clusters around low frequency-cells in PBMC **(C)** (arrow), but not $CD14^+$ monocytes **(B)**. In co-culture with MoDCs bacteria cluster around a majority of cells **(C)**. **(D)** SEM electrograph showing *M. capsulatus* Bath adhering to human MoDCs after 3 h co-incubation.

allogeneic T cells. Culture medium was collected and analyzed for cytokines associated with different effector T cell subsets (**Figure 6**). MoDCs primed by any of the bacteria resulted in markedly reduced levels of typical Th2 cytokines like IL-5 and IL-13. All bacteria further resulted in increased release of the Th1 cytokine IFN-gamma and IL-10, an anti-inflammatory cytokine produced by several effector T cell lineages, compared to the basal level produced by T cells co-incubated with unprimed MoDCs.

Although all bacteria shifted T cells toward a Th1 rather that a Th2 phenotype, a major difference was found between Gram-negative *M. capsulatus* Bath and *E. coli* K12 on the one hand and Gram-positive *L. rhamnosus* GG on the other. Compared to T cells co-cultivated with unprimed MoDCs only *L. rhamnosus*-treated MoDCs resulted in significantly reduced release of IL-18, a proinflammatory cytokine that enhances innate immunity as well as Th1- and Th2-driven immune responses depending on cytokine milieu.

Conversely, only the Gram-negative bacteria *M. capsulatus* Bath and *E. coli* K12 gave significantly higher levels of the proinflammatory cytokines IL-6, TNF-α, IL-1β, and IL-1α. Both bacteria also increased IL-23, a cytokine linked to the generation and maintenance of Th17 cells, Th17 cytokines (IL-17A, IL-21, IL-22), Th22 cytokines (IL-22, TNF-α), and Th9 cytokines (IL-9 and IL-21).

Not all differences could be attributed to dissimilarities between Gram-positive vs. Gram-negative bacteria, however. No significant difference was found between *E. coli* and *M. capsulatus* in their ability to induce Th1, Th22, or Th9 cells, as evaluated by IFN-γ, TNF-α, IL-9, and IL-21, but compared to *E. coli*, *M. capsulatus* resulted in significantly less IL-23, Th17- associated cytokines IL-17A, and IL-22 as well as pro-inflammatory cytokines IL1-α, IL-1β, and IL-6 and the anti-inflammatory cytokine IL-10. Furthermore, reduction in Th2 cytokines IL-5 and IL-13 was lowest for *M. capsulatus* Bath primed co-cultures and *E. coli* and *L. rhamnosus*, but not *M.*

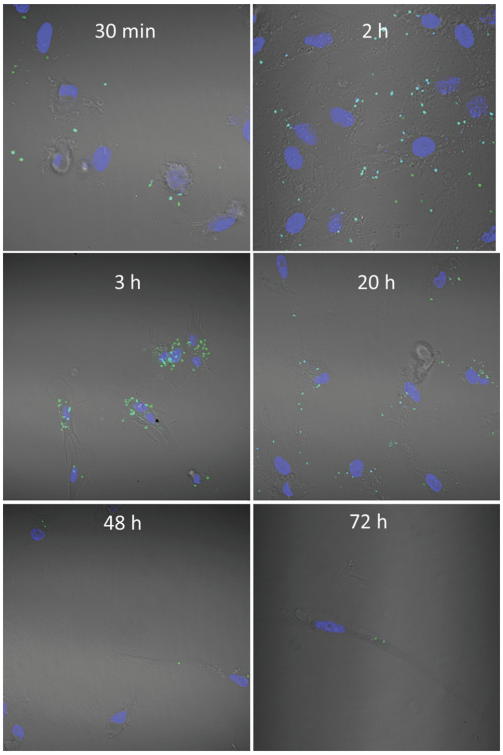

FIGURE 2 | *Methylococcus capsulatus*—DC interaction kinetics. Figure shows CFSE labeled *M. capsulatus* Bath (green) co-incubated with human MoDCs for 30 min to 72 h. MoDC nuclei were counterstained with DAPI (blue) to aid visualization and interactions were visualized by confocal microscopy.

capsulatus Bath, reduced IL-1RA and lymphotoxin-α levels in the cultures. *M. capsulatus* thus induces a T cells response functionally distinct from both *E. coli* K12 and *L. rhamnosus* GG.

DISCUSSION

Previous studies have described protective properties of probiotic bacteria, commensals, and their metabolites against experimental colitis in animal models (Pils et al., 2011; Qiu et al., 2013; Toumi et al., 2014; Souza et al., 2016). Although a connection between chronic intestinal inflammation and a reduced exposure to bacteria from soils and water has been made (Rook, 2007), few studies have focused on immune modulatory effects of non-commensal environmental bacteria. Here we show that a soil bacterium previously shown to reduce symptoms of chemically induced colitis in mice (Kleiveland et al., 2013) specifically targets human dendritic cells *in vitro,* affecting DC maturation, T cell activation, proliferation, and differentiation. *M. capsulatus* Bath was found to adhere specifically to human DCs. To our knowledge, this is the first report of an environmental bacterium to target mammalian DCs to modulate immune function.

The realization that a soil bacterium interacts specifically with human DCs raises some important questions. The significance of the commensal microbiome in health and disease is increasingly recognized, and there is a growing interest in probiotics within the scientific and public community. However, the role of environmental bacteria in immune regulation has been underappreciated for understandable reasons. It is not difficult to imagine that commensals living in close connection with humans are also closely connected to human physiology (Sommer and Backhed, 2013). Similarly, there is a long history of probiotics in fermented food associated with longevity and health. In a modern world of reduced microbial diversity it may be less intuitive to connect environmental bacteria to regulation of human health. However, as emphasized by the "old friends" hypothesis, mammals are evolutionary linked not only to commensals and probiotics, but also to ambient microbes in both soil and water (Rook, 2010).

Not only have mammals evolved from environmental bacteria, but the mammalian immune system has developed in the presence of such microbes. Throughout evolution some of these microbes may have taken on functions for us, some may relay signals necessary for immune development, while others, because of our long evolutionary association, are recognized by the immune system as harmless and have taken on regulatory roles (Rook et al., 2004). For example, chronic exposure to environmental LPS and other bacterial components present in farm dust may protect against asthmatic disease (Schuijs et al., 2015) possibly by reducing the overall reactivity of the immune system.

M. capsulatus Bath is an environmental bacterium that has been isolated from soil, water, sewage, mud, and lake sediments. It does not require a host to survive and may therefore face no obvious selection pressure to express immune modulatory molecules. Nevertheless, as discussed by Casadevall and Pirofski (2007), soil is an extreme environment with rapidly changing conditions, and bacteria living in soils will encounter an enormous number of predators of different types: unicellular amoebas, slime molds, protists, nematodes, snails as well as larger animals. As they are likely to meet ever-changing conditions as well as predatory hosts with different types of receptors and antimicrobial defenses, soil dwellers have to carry a diverse array of characteristics including host cell adhesins and immune modulatory molecules as defense mechanisms against predators. It was beyond the scope of our study to identify the bacterial factors involved in adhesion. However, a computational and experimental analysis of the *M. capsulatus* secretome has previously identified *M. capsulatus* Bath protein homologs of adhesion proteins that are involved in microbe adhesion to host cells in other bacterial species (Indrelid et al., 2014), showing that candidate host interaction proteins are present in *M. capsulatus* Bath and may facilitate adhesion to DC.

The maturation state and cytokine profile of DCs is functionally important. Although DCs represent a heterogeneous group of antigen-presenting cells, they have traditionally been divided into immature and mature differentiation stages

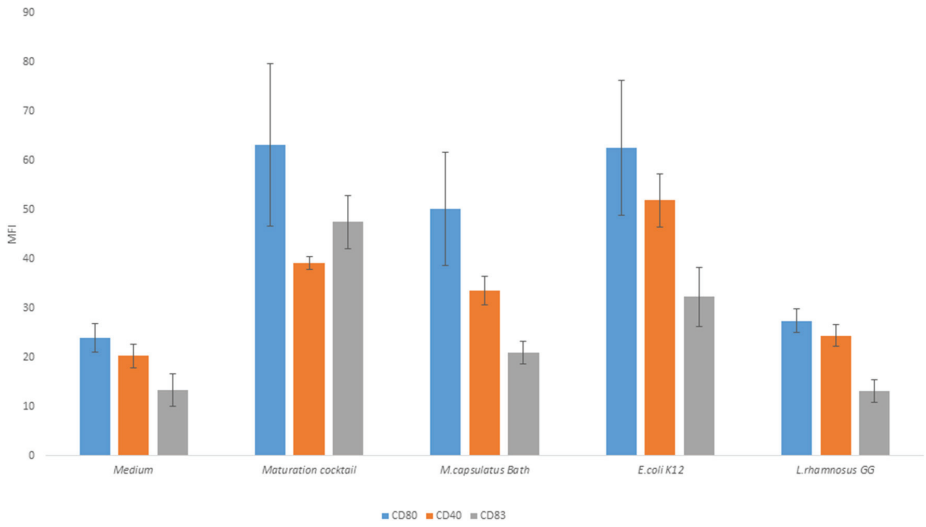

FIGURE 3 | *M. capsulatus* Bath and *E. coli* K12 induce maturation of MoDCs. Human MoDCs were either activated by a maturation cocktail of TNF-α, PGE2, and LPS or co-incubated with bacteria (*M. capsulatus* Bath, *E. coli* K12, or *L. rhamnosus* GG) for 24 h. Cells were stained for CD80, CD83, and CD40 and analyzed by flow cytometry in this figure. Median fluorescence intensity (MFI) is reported. Error bars indicate standard error on median fluorescence intensity values from 6 different donors.

(Reis e Sousa, 2006). Immature DCs are characterized by low surface expression of major histocompatibility complex (MHC) class II molecules and co-stimulatory molecules (e.g., CD80, CD86, and CD40). However, when DCs encounter microbes, pattern-recognition receptors (PRRs) are triggered by microbe-associated molecular patterns resulting in major changes in gene expression and acquisition of a number of functional properties: antigen processing and presentation, migration, and T-cell co-stimulation (Dalod et al., 2014).

It has been proposed that pathogen, probiotic, and commensal bacteria can be divided into three distinct classes based on the extent of host response by DCs and other PRR expressing cells. MAMPs of pathogenic microorganisms tend to induce a strong host response, probiotics induce an intermediate response whereas commensal bacteria exhibit homeostatic control of the response (Lebeer et al., 2010). In the present study the non-commensal, non-pathogenic *M. capsulatus* Bath induced a DC response intermediate between the Gram-positive probiotic *Lactobacillus rhamnosus* GG and the commensal Gram-negative *E. coli* K12. Substantial differences were found between *M. capsulatus* Bath, *L. rhamnosus* GG and the *E. coli* K12 in their ability to induce phenotypical and functional maturation of monocyte-derived DCs. Toll like receptor 4 is expressed on MoDCs and recognize lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria (Schreibelt et al., 2010). LPS represents a strong stimulatory signal for inducing expression of co-stimulatory molecules and cytokine production in DCs (Verhasselt et al., 1997). Concordantly, *E. coli* K12 and *M. capsulatus* Bath were found to be stronger inducers of DC maturation and cytokine release compared to the Gram-positive *L. rhamnosus*. It has been suggested that probiotic bacteria modulate immune response by controlling the maturation of DCs and thereby the release of proinflammatory cytokines (Foligne et al., 2007). Both the Gram-negative bacteria tested in our study induced phenotypical and functional maturation. However, *M. capsulatus* Bath produced a less mature phenotype and substantially lower cytokine levels than *E. coli* K12. The fact that the Gram-negative *M. capsulatus* Bath results in a less mature phenotype and low levels of proinflammatory cytokines, suggests that similarly to probiotic bacteria *M. capsulatus* may modulate immunity through directing the maturation of DCs.

Interestingly, although priming with *M. capsulatus* resulted in a less mature MoDC phenotype than *E. coli* K12, it was found more efficient than both *E. coli* K12 and *L. rhamnosus* GG bacteria in inducing T cell activation and proliferation in the presence of interleukin 2, a growth factor necessary for cell cycle progression and clonal expansion (Smith, 1988). *M. capsulatus*-primed MoDCs enhanced T cell expression of CD25, the α-chain of the trimeric high affinity IL-2 receptor explaining the increased proliferative T cell response compared to the other bacteria.

Whereas, co-stimulatory molecules on DCs and T cells are necessary for T cell activation and proliferation, DC cytokines are central in polarizing effector T cell development. In addition to antigen presentation and signaling through co-stimulatory molecules, cytokines provide a third signal for activation and differentiation of naïve T cells to effector cells. The nature of signal 3 depends on the triggering of particular PRRs by MAMPs

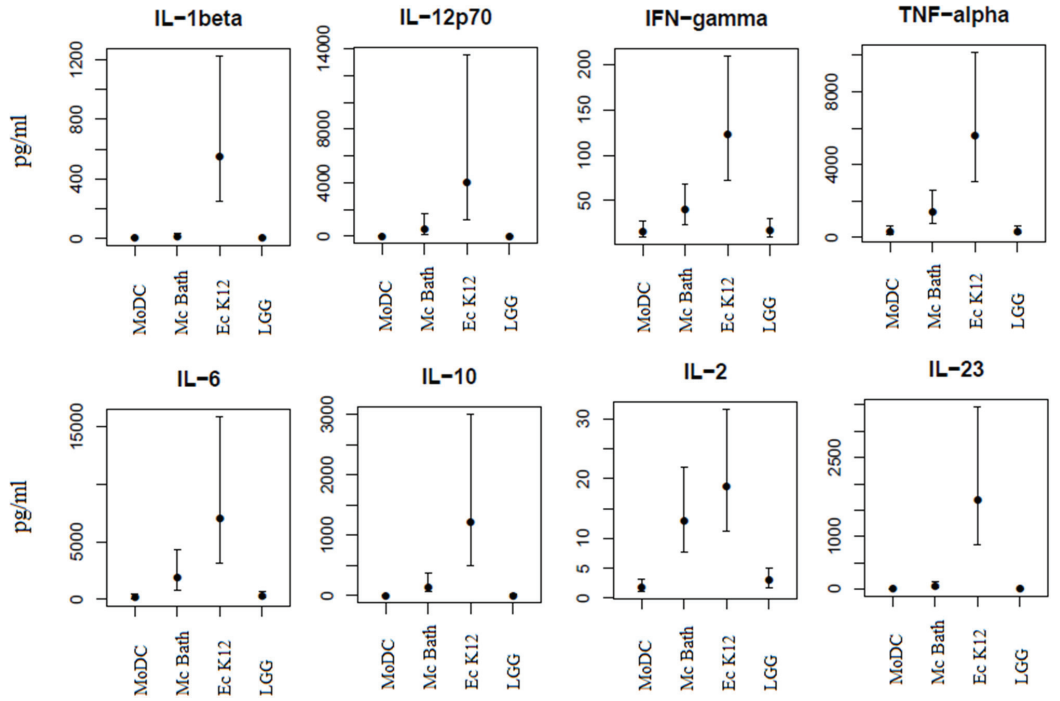

FIGURE 4 | MoDCs produce distinct cytokine profiles in response to different bacteria. MoDCs were incubated with bacteria *(M. capsulatus* Bath, *E. coli* K12, *L. rhamnosus* GG) for 24 h in a ration of 1:10 (DC: bacteria). Culture supernatants were collected and analyzed for cytokines by multiplex immunoassay or ELISA. Cytokine concentrations are given in picogram/milliliter. Bars represents 95% confidence intervals on cytokine concentrations from 4 different donors.

specific to the microbe encountered (Kapsenberg, 2003). Several DC-derived cytokines are important for T cell polarization into specific T cell subsets, e.g., IFNγ and IL-12p70 are known to be important for Th1 polarization, IL-4 is essential for the Th2 differentiation process, and TGF-β to TH17 and Tregs. Although *M. capsulatus* behaved more similar to *E. coli* than *L. rhamnosus* in its ability to induce cytokine production from MoDCs, both the magnitude and cytokine profiles of the two strains were different. Both strains for example induced similar levels of IL-2, but *E. coli* induced considerably higher levels of IL-23, a cytokine linked to the generation and maintenance of Th17 functions. *M. capsulatus* induced negligible IL-1β, and compared to *E. coli* substantially less of Th1 polarizing factors IL12p70 and IFN-γ as well as reduced IL-6 levels. IL-6 is a cytokine that plays a role in proliferation and survival of both Th1 and Th2 cells, is important for the commitment of $CD4^+$ cells to the Th17 and Th22 lineages and has an inhibitory role in Treg development (Hunter and Jones, 2015).

Bacteria-induced differences in MoDC cytokine production were also functionally reflected in different T cell polarizing ability in MoDC-T cell co-cultures. In response to bacteria-primed MoDCs, T cells produced increased levels of the anti-inflammatory cytokine IL-10. IL-10 plays important roles both in preventing inflammatory responses to intestinal microbiota under steady state conditions, and in limiting T cell-driven inflammation in pathogen clearance (Maynard and Weaver, 2008). Notably, MoDC-priming with all three bacteria significantly increased concentration of the Th1 signature cytokine IFN-γ and reduced the levels of typical Th2 cytokines IL-13 and IL-5. Th2 development has previously been suggested to be a "default pathway" in the absence of IL-12 (Moser and Murphy, 2000). In agreement with that, in our experiments unprimed MoDCs tended to induce Th2 cell responses compared to MoDCs primed by bacteria. The observation that even the Gram-positive *L. rhamnosus* drives Th1 development suggest that LPS is not a critical factor in bacteria driven DC-mediated Th1 development, in support of previous reports (Smits et al., 2004).

Coherent with results from DC cytokine analysis, the cytokine profile of T cells co-incubated with MoDCs primed by Gram-negative bacteria was markedly different from that of T cells activated by MoDCs treated with the Gram-positive *L. rhamnosus*. Again *L. rhamnosus* resulted in lower levels of most of the cytokines measured, a reduction in the pro-inflammatory

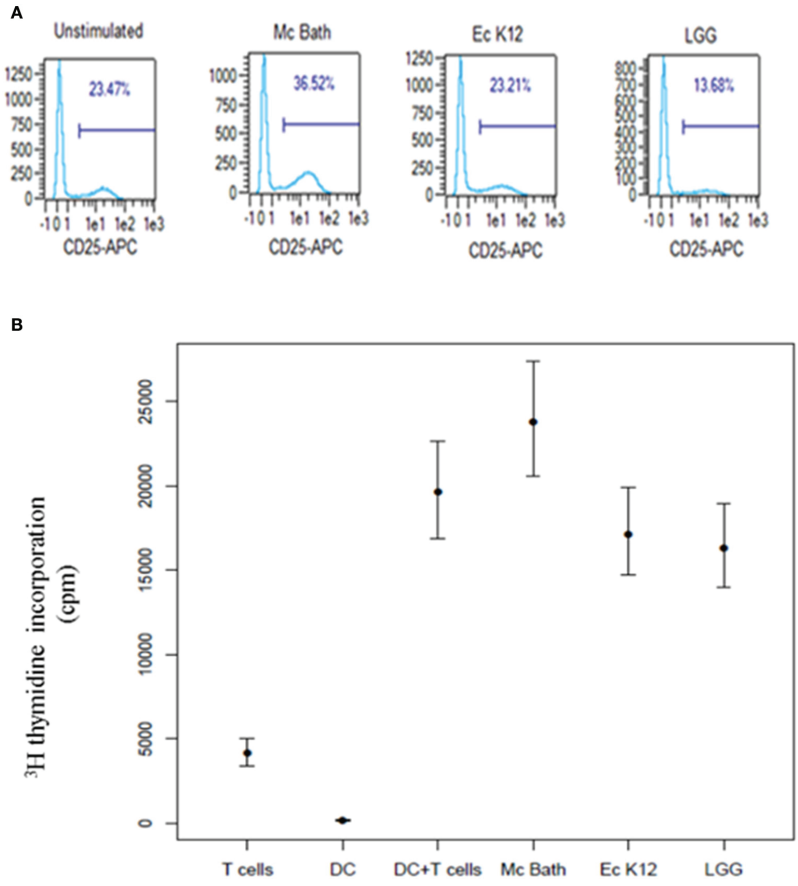

FIGURE 5 | *M. capsulatus* primed MoDCs efficiently induce T cell activation and proliferation. (A) Immature MoDCs were primed by UV inactivated bacteria for 24 h in a ratio of 1:100 (DC: bacteria). Primed MoDCs were co-incubated with allogenic T cells in the presence of IL-2. After 5 days of co-culture cells were harvested, stained for CD4 and CD25 surface protein and analyzed by flow cytometry. Cells were gated on CD4-FITC expression and the percentage of $CD4^+$ cells expressing CD25 are shown. Plots represent results from 4 different MoDC/T cell donor combinations. **(B)** MoDCs primed by either UV-inactivated *M. capsulatus* Bath, *E. coli* K12, or *Lactobacillus rhamnosus* GG for 24 h were co-incubated with allogenic T cells from two different donors. After 96 h cells were pulsed by 1 μCi [^{3}H] thymidine. Thymidine incorporation was determined by liquid scintillation counting 18.5 h after pulsing. The amount of incorporated thymidine is reported as counts per minute (cpm). Bars indicate 95% confidence interval on values from 5 different donor combinations.

IL-18 and no increase of IL-1α, IL1-β, IL-6 compared to negative control. Neither did it induce cytokines typically associated with Th17/Th9/Th22 cells (IL-23, IL-17A, IL-21, IL-22, IL-9, TNF-α) compared to a control of T cells stimulated by unprimed DC. The low T cell-levels of cytokines in response to *L. rhamnosus* is in agreement with a previous report showing that *L. rhamnosus*-primed MoDCs induce hyporesponsive T cells in DC-T cell co-cultures (Braat et al., 2004).

In contrast to *L. rhamnosus M. capsulatus* Bath, and *E. coli* K12 induced proinflammatory cytokines IL-6, IL-1β, and IL-1α as well as cytokines associated with generation and maintenance of the Th17 subset (IL-23, IL-17A, IL-21, IL-22), Th22 cytokines (IL-22, TNF-α) and Th9 cytokines (IL-9 and IL-21). However, *M. capsulatus* induced significantly less pro-inflammatory cytokines IL1-α, IL-1β, and IL-6 and anti-inflammatory IL-10. There was no significant difference in the Th1 signature cytokine IFN-γ or Th9 cytokines IL-9 and IL-21. However, significantly less IL-23, IL-17A, and IL-22 was produced in response to *M. capsulatus* than to *E. coli*. The cytokine profile thus indicated that different effector cells dominate in response to the two Gram-negative bacteria. *E. coli* is a stronger inducer of the Th17 subset whereas *M. capsulatus* induce Th1/T9 effector cells over Th17

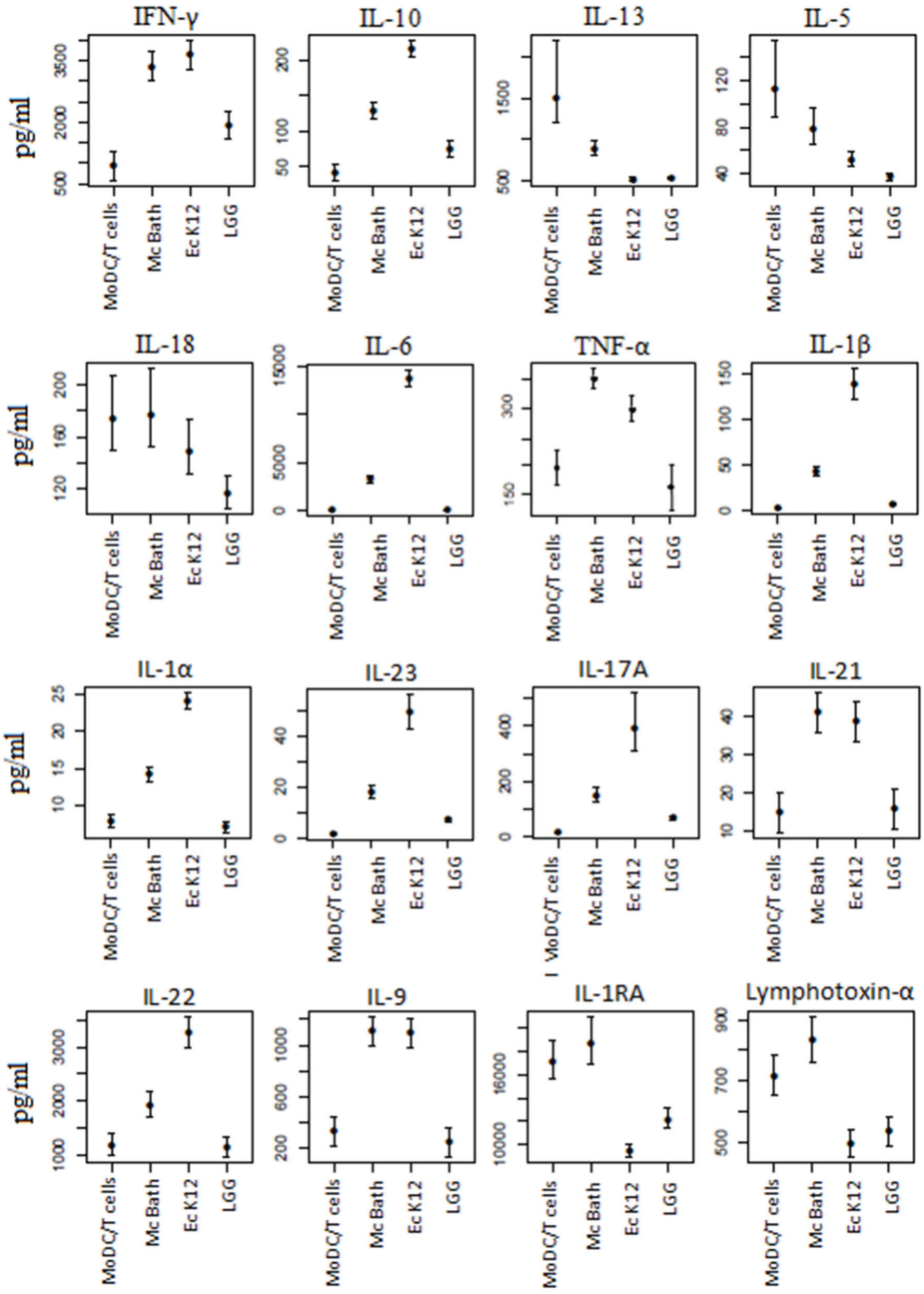

FIGURE 6 | Bacterial stimulation results in different effector T cell profiles. Unprimed MoDCs or MoDCs primed by *M. capsulatus* or control bacteria were co-incubated with allogenic T cells for 5 days. Growth medium was collected and analyzed for cytokines by multiplex immunoassay. Bars represent 95% confidence intervals on cytokine concentrations from 4 donor combinations.

cells *in vitro*. Some probiotics have been reported to induce Foxp3+ regulatory T cells (Kwon et al., 2010). It has been suggested that peripherally-induced Treg develop from naïve, CD4+ cells exposed to antigens under tolerogenic conditions (e.g., by immature DCs with low levels of co-stimulation) with an essential requirement for TGF-β signaling (Marie et al., 2005; Johnston et al., 2016). We did not find detectable levels of TGF-β released from MoDC stimulated by *M. capsulatus*. Neither did we find significantly increased expression of Foxp3 in T cell co-cultures with bacteria stimulated MoDC (data not shown).

E. coli and *L. rhamnosus*, but not *M. capsulatus* Bath, reduced lymphotoxin-α and IL-1RA in culture supernatants. Lymphotoxin-α is important for lymphoid organ development, regulates T cell homing and IgA production in the gut and contributes to shaping the gut microbiome (Ruddle, 2014). The balance between IL-1 and IL-1RA in local tissues plays an important role in the susceptibility and severity of a number of diseases, including IBD (Arend, 2002). For example, significant decrease in the intestinal mucosal IL-1RA/IL-1 ratio has been found in freshly isolated intestinal mucosal cells, and in mucosal biopsies obtained from both Crohn's disease and ulcerative colitis patients as compared to control subjects (Casini-Raggi et al., 1995). The observation that IL-1α and IL-1β is reduced, while IL-1RA is kept high in *M. capsulatus* primed DC-T cell co-cultures is interesting in the light of *M. capsulatus* anti-inflammatory effects in a murine enteritis model (Kleiveland et al., 2013). Screening for cytokine profiles associated with specific T effector cell populations may be a useful first step to identify strains with potential pro- or anti-inflammatory properties e.g., for further mechanistic investigation (Papadimitriou et al., 2015). It is important however to notice the limitations of *in vitro* systems on making *in vivo* predictions. Although the bacteria tested here induced different effects in T cells *in vitro*, caution should be exercised in drawing conclusions both about the direction of T cell polarization by these bacteria and the functional relevance *in vivo*. T cell differentiation occurs in a finely tuned manner dependent on a variety of tissue factors and cytokines, and *in vitro* systems cannot necessarily reflect the complex cytokine environment of the gut. For example, TGF-β a cytokine abundant in the intestines, was not detected in our MoDC supernatants. TGF-β is not only involved in development of Tregs, Th9 and Th17 effector cells, but it also suppresses Th1 and Th2 cell differentiation (Zheng, 2013). TGF-β is produced by $CD103^+$ DC (Coombes et al., 2007) a DC subset common in the intestines and is expected to play a prominent role in regulating mucosal immunity (Ruane and Lavelle, 2011). The results of bacterial priming *in vitro* may thus be expected to have different outcomes in an *in vivo* situation. The impact of immune modulatory effects of *M. capsulatus* on DC in maintaining intestinal homeostasis thus remains to be determined (study in preparation).

CONCLUDING REMARKS

Environmental bacteria, although immensely numerous and diverse, have remained largely unexplored for their immunomodulatory properties. Our results demonstrate the direct binding and functional effects of a soil bacterium on human monocyte-derived dendritic cells. The same bacterium has recently been shown to possess anti-inflammatory properties in a murine colitis model. The identification of anti-inflammatory and immunomodulatory properties of this bacterium was serendipitous. In fact, such properties may not be a rare trait of this particular soil bacterium, but rather a common feature of many environmental bacteria. Our study thus emphasizes the need to scrutinize, identify, and understand possible physiological consequences of environmental microbe-host interactions, and we suggests that bacteria from soil and water should receive increased attention for their potential health benefits.

AUTHOR CONTRIBUTIONS

SI contributed to design of the work, acquisition, analysis, and interpretation of data and drafted the work. TL and CK contributed to design of the work, interpretation of data and revising work critically for important intellectual content. RH contributed to data analysis and revising work critically for important intellectual content. MJ contributed to interpretation of data and revising work critically for important intellectual content. All authors approved final version and agreed to be accountable for all aspects of the work.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

PAPER III

The Non-commensal Soil Bacterium *M. capsulatus* Bath and the Probiotic Bacteria *L. rhamnosus* GG and *E.coli* Nissle 1917 Differentially Modulate Gene Expression in Human Monocyte Derived DCs

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Abstract

The non-commensal environmental bacterium *Methylococcus capsulatus* Bath has been shown to possess anti-inflammatory effects in both a salmonid and a murine model of intestinal inflammation. However, the mechanisms responsible for the observed anti-inflammatory effects *in vivo* have not been identified. Recently we showed that the same bacterium specifically interacts with, and adheres to, human monocyte-derived dendritic cells (MoDC). This interaction affects MoDC cell maturation, cytokine production and DC ability to drive T cell activation and differentiation. To gain more detailed knowledge about functional consequences of the interaction between *M. capsulatus* Bath and MoDC we employed a full genome transcriptomic and bioinformatic approach. The dendritic cell-modulatory effects of *M. capsulatus* Bath was compared to those of two well established probiotics, *Lactobacillus rhamnosus* GG and *E.coli* Nissle 1917, both previously shown to be protective in animal models of colitis and to have modulatory potential. All bacterial treatments profoundly affected dendritic cell gene expression, and all bacteria induced elevated expression of cytokine genes known to be involved in promoting regulatory T cell development. *M. capsulatus* Bath induced dendritic cell maturation intermediate between the two probiotics, increased expression of galactin-10, an S-type lectin involved in the generation of regulatory T cells and reduced expression of CD70, a co-stimulatory molecule implicated in auto-immunity and suggested as a promising target for antibody-directed immunotherapy in chronic inflammatory diseases. Key cytokines involved in the generation of regulatory T cells were upregulated in response to all bateria. However, interleukin-10 and leukemia inhibitory factor was only upregulated in response to the Gram-negative *M. capsulatus* Bath and *E.coli* Nissle and TGF-β was only upregulated in response to the Gram-positive *Lactobacillus rhamnosus* GG, suggesting MoDC preferentially induce different subsets of Tregs in response to Gram-negative and Gram-positive bacteria. Finally, the two established probiotics *E. coli* Nissle and *Lactobacillus rhamnosus* were found to have very different and partly opposing effects on DC maturation, cytokine and chemokine production, innate and adaptive immune activation. *Lactobacillus rhamnosus* induced an immature phenotype and a gene expression pattern indicative of high potential for antigen sampling but reduced potential for antigen presentation and co-stimulation. *E. coli* on the opposite, was a strong stimulus for dendritic cell maturation and induced cells expected to have high capacity T cell activation and instruction and high potential for promoting antimicrobial/antiviral responses.

Introduction

Studies of germ-free (GF) animals have highlighted the importance of microbes in immune system development and function. GF animals display developmental defects in the intestinal mucosa such as fewer/smaller Peyer's patches and mesenteric lymph nodes, and reduced numbers of $CD4^+$T cells and IgA-producing plasma cells (Belkaid & Hand, 2014; Round & Mazmanian, 2010; Smith, McCoy, & Macpherson, 2007). In addition to providing stimuli necessary for full immune system development, a role of commensal microbiota in providing tonic regulatory signals necessary for a normal immune function is well established (Round & Mazmanian, 2010). Gut commensals, their metabolites and secreted proteins have been reported to regulate the differentiation and expansion of several T effector subsets in animal models (Furusawa et al., 2013; Ivanov et al., 2009). Intestinal bacteria influence the expression and localization of pattern recognition receptors in the gut, and GF animals are deficient in important antimicrobial substances and more susceptible to infections than conventional mice (Round & Mazmanian, 2010)

Not only colonizing commensals, but also transiently associated probiotic bacteria have been reported to provide health benefits to the host and have been suggested for therapeutic use in both allergic and chronic inflammatory diseases (Ritchie & Romanuk, 2012). However, little attention has been given to the role of environmental bacteria in modulating immunity.

In 2011 (Romarheim, Overland, Mydland, Skrede, & Landsverk) showed that dietary inclusion of BioProtein®, a feed product based on bacterial protein, in which the soil bacterium *Methylococcus capsulatus* Bath (McB) is the main ingredient, could prevent soybean-induced enteritis in Atlantic salmon (*Salmo salar*). Furthermore, in 2013 Kleiveland et al. reported that dietary BioProtein® could also abrogate inflammation in a murine dextran sulfate sodium (DSS)-induced colitis model. These results were reproduced using a bacterial meal consisting of McB only, without the additional bacteria present in BioProtein® proving that McB represents the anti-inflammatory principle in BioProtein® (Kleiveland et al., 2013).

The anti-inflammatory effects of *M. capsulatus* (Bath) in salmonid and murine models of IBD, suggest interactions with cells involved in maintaining mucosal homeostasis *in vivo*. Recently, setting out to investigate effects of *M. capsulatus* Bath on human peripheral blood mononuclear cells, we found that the bacterium adhered specifically to a small subset of cells amongst peripheral blood mononuclear cells (PBMCs), and identified this subset as dendritic cells (DCs) (Indrelid, Kleiveland, Holst, Jacobsen, & Lea, 2017).

DCs play fundamental roles in the initiation of both innate and adaptive immunity. They are the prototypic antigen-presenting cells and direct adaptive immunity by translating environmental cues into modulatory signals to activate and shape effector T cell responses. However, DCs are not only potent mediators of adaptive immune responses, they also have important functions in limiting

undesired responses against self- or innocuous antigens such as food components and commensals (Manicassamy & Pulendran, 2011; Steinman & Nussenzweig, 2002). The ability of DCs to maintain peripheral tolerance and a homeostatic immune status depends on their ability to induce T cell deletion or anergy, shape the development of distinct T helper cell (Th1/ Th2/ Th17/ Th22) and regulatory T cell populations (Manicassamy & Pulendran, 2011; Steinman & Nussenzweig, 2002).

Commensals and probiotic bacteria interact with dendritic cells (DCs) via various surface molecules and secreted factors (Lebeer, Vanderleyden, & De Keersmaecker, 2010), and these interactions likely play a key role in commensal and probiotic modulation of innate and adaptive immunity (Drakes, Blanchard, & Czinn, 2004; Foligne et al., 2007; Hart et al., 2004; Smits et al., 2005; Smits et al., 2004). For example, lactobacilli differentially modulate cytokine secretion, surface expression of maturation surface markers and chemokine production in murine DCs, possibly favoring development of different Th cell subsets (Christensen, Frokiaer, & Pestka, 2002). Strains of lactobacilli has further been shown to prime MoDCs to drive the development of IL-10-producing, regulatory T cells by a mechanism involving the C-type lectin DC-SIGN (Smits et al., 2005) and *Lactobacillus rhamnosus* GG (LGG) has been reported to induce hyporesponsive $CD4^+$ T cells via modulation of DC function (Braat et al., 2004).

It is expected that the interaction between probiotic or commensal bacteria and DCs results in the induction of intracellular signalling cascades characteristic of each microorganism (Lebeer et al., 2010). Our previous study, showing that McB specifically adheres to human monocyte-derived dendritic cells (MoDCs) and controls T cell activation and effector cell development, suggests a potential even for environmental bacteria in affecting immune homeostasis by modulating DC function. However, whereas the cytokine secretion pattern and surface- expressed maturation markers on DCs are commonly used, few studies have addressed how individual species of non-pathogenic bacteria induce different gene expression profiles, and functionally different DCs. Moreover, to our knowledge, no one has studied how an environmental bacterium modulates DC production of cytokines, chemokines, other immunoregulatory and co-stimulatory molecules at the level of mRNA expression. In this study, searching for mechanisms involved in *M. capsulatus* Bath-mediated immunomodulation, we examined how *M. capsulatus* Bath affected global gene expression in MoDCs. Its effects were compared to those of two established probiotic species, the Gram-positive *L. rhamnosus* GG and the Gram-negative *E. coli* Nissle, both previously reported to have DC modulatory functions (Adam et al., 2010; Braat et al., 2004; Vlasova et al., 2016).

Here we show that McB profoundly modulates MoDC gene expression and induces a state of DC maturation, cytokine- and chemokine production intermediate between LGG-primed and EcN-primed MoDCs. McB upregulated the expression of genes for galectin-10, an S-type lectin involved in the generation of regulatory T cells, genes for interleukin-10 (IL-10) and leukemia inhibitory factor (LIF), cytokines involved in the generation of regulatory T cells, and reduced the expression of CD70, a promising target for antibody-directed immunotherapy. Furthermore, the probiotics, LGG and EcN,

have very different, and partly opposing effects on genes important for immunogenic and tolerogenic MoDC functions. LGG maintained an immature MoDC phenotype and reduced the expression of genes necessary for migration and antigen presentation, co-stimulation and cytokine production, but increased the expression of the gene for TGF-β, a cytokine that is involved in generation of both Th9 and Th17 subsets, and is a key regulator of Tregs. In contrast, EcN appears to be a strong inducer of MoDC maturation, and produce MoDCs expected to have high capacity for promoting antimicrobial/antiviral responses.

Our data thus support a potential even for environmental bacteria in immune modulation, pointing to soil and water as novel sources of microbes and microbial components that can be exploited for therapeutic purposes. Furthermore, different strains of probiotic bacteria intended for therapeutic use can be expected to have very different functional effects, highlighting the need to match the right treatment to the right disease, and stage of disease, based on the individual functional properties of each species.

Materials and Methods

Bacterial strains and culture conditions

M. capsulatus (Bath) NCIMB11132 (GenBank accession number AE017282) was cultivated in nitrate mineral salts medium (Whittenbury, Phillips, & Wilkinson, 1970) with a head-space of 75% air, 23.75% CH_4 and 1.25% CO_2. Bacteria were grown in 350 ml flasks at 45°C and 200 rpm. *Lactobacillus rhamnosus* GG was cultivated in MRS medium (Oxoid) at 37° without agitation. *Escherichia coli* Nissle 1917 (Mutaflor, DSM 6601, serotype O6:K5:H1), was kindly provided by Ardeypharm GmbH, Herdecke, Germany and grown in Luria-Bertani Broth (Oxoid, UK) at 37 °C, 200 rpm. All bacteria were UV inactivated for 60 minutes prior to co-cultivation with MoDCs.

Cells and culture conditions

Buffy coats from healthy volunteers were obtained from Ostfold Hospital Trust, Kalnes, Norway, in accordance with institutional ethical guidelines and with approval from the Regional Committee of Medical and Health Research Ethics. All subjects gave written informed consent in accordance with the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation on a Lymphoprep gradient (Fresenius Kabi). $CD14^+$ cells were isolated from PBMC by positive selection using human CD14 MicroBeads (Miltenyi Biotech). Immature monocyte-derived dendritic cells (MoDCs) were prepared from $CD14^+$ cells by cultivation for 6 days in RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum, 25 μg/ml gentamicin sulfate (Lonza), 1 mM sodium pyruvate and 100 μM non-essential amino acids (both from PAA

Laboratories), 25 ng/ml interleukin 4 and 50 ng/ml granulocyte macrophage colony stimulating factor (both from ImmunoTools).

Bacterial stimulation RNA extraction, amplification and labeling

MoDCs were primed for 24 h by UV-inactivated bacteria in a ratio of 1:100 (MoDC: bacteria) or with a maturation cocktail of 15 ng/ml TNF-α (ImmunoTools), 100 ng/ml LPS and 5μg/ml PGE2 (Sigma-Aldrich). Cells were harvested and RNA isolated by Maxwell RSC simplyRNA Cells Kit using the automated Maxwell RSsystem. Quantification was performed using a spectrophotometer (NanoDrop Technologies) and RNA quality was assessed by Agilent Bioanalyzer 2100. All RIN values were above 8.4. RNA was prepared for sequencing using the Strand-specific TruSeqTM RNA-seq kit and single read (unpaired) 75-basepair sequencing was performed using the HiSeq platform (Illumina Inc., San Diego, California, USA) at The Norwegian Sequencing Centre.

Analysis of RNA-Seq data

The strand-specific RNA-Seq data were analyzed using the Cufflinks protocol (Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Trapnell et al. Nature Biotechnology, 2010). Briefly, reads were mapped to the human genome (hg19) using TopHat (version 2.1.1), and Cufflinks (version 2.2.1) was used for transcript assembly. Transcript databases were merged using the cuffmerge script and differentially expressed genes (DEGs) where identified using the Cuffdiff program with default parameters.

Immune phenotyping of stimulated MoDCs

MoDCs were phenotyped by flow cytometry using mouse monoclonal antibodies against human CD303 (BDCA-2)-FITC, HLA-DR,DP,DQ-PE, CD40-VioBrightFITC, CD83-APC, CD80-PE (all from Miltenyi Biotech, Germany) and CD11c-APC, CD14-FITC, CD1c-APC (all from eBioscience). Non-specific Fc receptor-mediated antibody binding was blocked by FcR Blocking Reagents (Miltenyi) prior to staining.

Fluorescence was monitored using a MACSQuant flow cytometer, and data was analyzed using the MACSQuantify software (Miltenyi).

Functional annotation and enrichment analysis

Pathway enrichment analysis was performed using the gene set enrichment analysis tool Enrichr (http://amp.pharm.mssm.edu/Enrichr) (E. Y. Chen et al., 2013; Kuleshov et al., 2016). The PANTHER (protein analysis through evolutionary relationship) classification system was used to

assign functional classification to DEGs (Mi et al., 2017; Mi, Muruganujan, Casagrande, & Thomas, 2013). The PANTHER statistical overrepresentation test (version 12.0 Released 2017-07-10) (http://pantherdb.org) was used to identify overrepresented biological processes among DEGs up- or down-regulated in response to bacterial stimulations using the gene ontology (GO) biological process terms (Thomas et al., 2006). Only the most specific GO subclasses from a group of all related parent classes in ontology were considered. The PANTHER default Bonferroni corrected p-value (P-value) was used. GO terms with $p \leq 0.05$ after Bonferroni correction were considered significant.

Results

Phenotype of MoDCs

Human dendritic cells represent a heterogeneous group of cells and can be divided into conventional (cDCs) and plasmacytoid cells (pDCs) based on phenotype and functional properties, and into immature (imDCs) or mature DCs (mDCs) according to developmental stage (Ezzelarab & Thomson, 2011).

DCs in peripheral blood can be categorized into two populations, lineage^{neg} CD11c^{+} myeloid DCs (mDC) and lineage^{neg} CD11^{-}CD123^{+}CD303^{+}CD304^{+} plasmacytoid DCs. To generate cells that maintain the antigen capturing- and processing capacity characteristic of immature dendritic cells (imDCs) we cultured CD14^{+} blood mononuclear cells in the presence of colony-stimulating factor (GM-CSF) and interleukin 4. Cells were phenotyped for monocyte- and DC markers. Whereas freshly isolated monocytes were CD14^{+}/ HLA class II^{+} (data not shown), CD14 expression was lost upon differentiation. On day 6 cells were CD14^{-} HLAII^{+} CD11c^{+} CD1c^{+} CD40^{+} CD80^{+}CD303^{-}CD83^{-} (figure 1A) demonstrating more phenotypic overlap with mDCs than pDCs. Cells were loosely adherent, with a large cytoplasm and dendritic projections. Morphology and phenotype was thus consistent with the MoDCs reported by others (Grassi et al., 1998; O'Neill, Adams, & Bhardwaj, 2004; Sallusto & Lanzavecchia, 1994).

To assess the ability of McB, EcN and LGG to induce phenotypic MoDC maturation, immature MoDCs were primed either by bacteria or a maturation cocktail before staining for expression of co-stimulatory molecules CD80, CD83 and HLA-DR. The potential of DCs to induce tolerogenic or inflammatory responses is directly related to their maturation status (Li & Shi, 2015; Manicassamy & Pulendran, 2011). Antigen presentation by mature DCs expressing high levels of HLA class II and co-stimulatory molecules is expected to induce CD4^{+} T cell activation and differentiation. On the contrary, in the absence of maturation stimuli, immature DCs with low levels of MHC II and co-stimulatory molecules are thought to promote T-cell anergy, deletion or the development of regulatory cells leading to tolerance (Barratt-Boyes & Thomson, 2005).

The three bacteria showed highly different ability to induce MoDC maturation as evaluated by maturation markers (Figure 1B). EcN was a potent stimulus for MoDC maturation and upregulated all markers comparable to, or higher than, the maturation cocktail. McB demonstrated moderate potential to induce phenotypic MoDC maturation. McB-primed MoDCs upregulated all markers compared to negative control, and similar levels of CD40, CD83 and HLA II, but reduced the levels of the co-stimulatory molecule CD80 compared to EcN. LGG was the weakest inducer of phenotypic MoDC maturation. LGG-priming only weakly increased the surface expression of CD83 compared to unstimulated control cells, suggesting that LGG only partially maturates MoDCs. However, LGG induced similar expression of HLA class II, CD80 and CD40 compared to negative control, but reduced expression levels of all maturation markers compared to maturation cocktail and the Gram-negative bacteria.

Also at the mRNA level LGG reduced the expression of *CD40*, *CD80* and *CD83*. No significant differences were found between McB or EcN and control cells in the expression of *CD40*, *CD80* or *CD83*. Surprisingly, however, although flow cytometric analysis showed that DCs primed by the Gram-negative bacteria increased surface expression of HLA class II molecules, at the gene expression level both EcN and McB reduced *HLA-DRA* expression. Similar results have been reported by others (Jin et al., 2010). A likely explanation is that surface-expressed HLA class II molecules are dominated by the HLA-DP or -DQ isotypes, or both.

Transcriptome assembly and identification of differentially expressed genes

The Illumina sequencing gave an average of 21 367 319 reads per sample (median 20 697 477) and after cuffemerge, a total of 1 085 341 candidate exons could be defined. Three pairwise comparisons were done to look for differentially expressed genes; McB vs Ecn, EcN vs. LGG and McB vs LGG. 2526 DEGs were regulated in response to LGG, 520 in response to EcN and 343 in response to McB, respectively. No transcripts were scored as differentially spliced and no promotor regions were listed as differentially involved when looking at the output from CuffDiff for three pairwise comparisons.

Bacterial stimulation induce dramatic change in gene expression

To identify individual functional effects of McB and the probiotic bacteria EcN and LGG, we performed whole transcriptome shotgun sequencing of bacteria-primed cells in six biological replicates. The transcript profiles of cells stimulated by McB, EcN or LGG were evaluated against a background of mature MoDCs. Genes with significantly different expression levels between the maturation control and bacterial treatments were defined as differentially expressed genes (DEGs) induced by the bacteria, and were further analyzed.

MoDCs responded to bacterial stimulation by dramatic changes in gene expression. 2732 genes were differentially expressed after stimulation by any of the bacteria compared to the

maturation control. Gene-set enrichment/overrepresentation (GO) tools allow the comparison of a list of genes to functional classifications in a database, to help identify shared biological functions or pathways among genes in the list. All over- and under-expressed DEGs regulated in response to bacteria were analyzed by the PANTHER GO statistical overrepresentation tool (Mi et al., 2017; Mi et al., 2013) and the gene set enrichment analysis tool, Enrichr (Chen et al., 2013; Kuleshov et al., 2016). The PANTHER statistical overrepresentation test showed that a large proportion of DEGs (618 of 2732 coding genes) were involved in immune system processes. As expected, Enrichr pathway enrichment analysis showed that bacterial stimulation triggers MoDC functions important for microbe recognition, cell survival, and activation of innate and adaptive immune responses (table 1)

MoDCs respond to microbial stimulation by general and microbe-specific responses

MoDCs responded to bacterial stimulation by both general and microbe-specific responses. McB and the two control bacteria each regulated a unique number of DEGs. Several different protocols have been used to mature MoDCs, and each one generates DCs showing different phenotypes and stimulatory abilities (Castiello et al., 2011). To simulate an inflammatory environment we matured the DCs with a cocktail containing LPS, TNF-α and PGE2, all typical components of a complex inflammatory environment. PGE_2 has been shown to enhance the TNF-α induced maturation of DCs as shown by expression of co-stimulatory molecules, production of pro-inflammatory cytokines and ability to stimulate $CD4^+$ T cells in an allogeneic mixed leukocyte reaction (Steinbrink et al., 2000). The presence of LPS, a component of cell walls of Gram-negative bacteria, resulted in considerably more DEGs identified after stimulation by LGG than the two Gram-negative bacteria (figure 2). However this strategy allow better separation of DEGs regulated by the two Gram-negative bacteria.

A core set of 152 DEGs were regulated in response to all bacteria (figure 2). Functional annotations suggested these DEGs had roles in response to external stimuli, leukocyte activation and differentiation, reorganization of the cytoskeleton and cell adhesion, and may represent a general response to bacteria that prepare MoDCs for immune activation. A similar number of genes (166) was found by (Huang et al., 2001) to be differentially expressed in DCs in response to bacteria, fungi and virus and was described as part of a core DC response to microbes, whereas our results define genes involved in DC responses to bacteria in particular.

In addition to DEGs regulated in response to all treatments, sets of genes were regulated in response to only two of the bacteria or exclusively in response to one species (figure 2). There was a general tendency for genes differentially expressed by two or more of the bacteria to be regulated in the same direction (figure 3) suggesting that common surface receptors and downstream signaling pathways are triggered by the different microbes. However, some DEGs were oppositely regulated thus defining genes regulated by microbe characteristics.

Relatively few DEGs were oppositely regulated in response to McB, EcN or LGG (figures 1A and 1B). However, McB reduced, whereas EcN increased, the expression of epiregulin (*EREG*), the

gene for a secreted peptide hormone involved in inflammation, wound healing and cell proliferation. The expression of two colony stimulating factors (CSFs) were elevated in response to both Gram-negative bacteria, but reduced in MoDCs primed by the Gram-positive LGG. CSFs play roles in hematopoiesis, i.e. in the generation, differentiation and function of granulocytes, macrophages and DCs, but they also regulate innate antimicrobial defenses in infectious diseases (Ballinger et al., 2006; Dale, Liles, Summer, & Nelson, 1995; Hamilton, 2008; Jenkins & Hume, 2014). Thus, elevated expression of these cytokines may be part of the host response to Gram-negative bacteria.

Strikingly, several DEGs were oppositely regulated in response to EcN and LGG (Figure 3C). 36 genes were upregulated in response to the EcN but downregulated in response to LGG and a high proportion of these were related to cytokine-mediated signaling pathways. Oppositely, among 15 genes upregulated by LGG, but downregulated by EcN, were several surface receptor genes as well as genes coding for proteins related to peptide or lipid antigen presentation to T cells (*HLA-DMA, HLA-DMB, CD1A*). The MHC II homolog, HLA-DM, do not directly function in antigen-specific activation of $CD4^+$ T cells, but regulate the loading of self and foreign peptides onto HLA class II molecules in endosomal compartments (Mellins & Stern, 2014). *CD1A* codes for a transmembrane glycoprotein structurally related to the MHC proteins and is involved in the presentation of lipid antigens to T cells (de Jong, 2015). Our data thus suggest that although these probiotic bacteria are often employed in the same disease models they have markedly different potential to stimulate MoDC functions important for the activation of innate and adaptive immunity.

Genes differentially regulated in response to individual species provide ques to species-specific immune modulatory properties.

DEGs regulated only in response to one of the bacteria can provide cues to different immunomodulatory properties of individual strains. Among only 23 DEGs regulated exclusively in response to McB were *CLC*, the gene for galectin-10, also known as the Charcot-Leyden crystal protein (CLC). Galectins are S-type lectins with pleiotropic roles in microbe-host interactions and in both innate and adaptive immune responses. They have been reported to regulate DC function and promote T cell tolerance (Baum, Garner, Schaefer, & Lee, 2014; Manicassamy & Pulendran, 2011; Rabinovich & Toscano, 2009; Hanyu Zeng, Rong Zhang, Boquan Jin, & Lihua Chen, 2015). Although originally considered an eosinophil/basophil-specific protein, galectin-10 is emerging as an immune regulatory molecule expressed also by other immune cells. Kubach et al. (2007) compared protein expression in $CD4^+CD25^+$ Tregs and $CD4^+CD25^-$ cells. Galectin-10 was the most differentially expressed protein between the two cell types and was shown to be critical for the suppressive function of Tregs. Furthermore, galectin-10 expressed by a newly described regulatory subset of eosinophils, was found to be key to the T cell suppressive activity of such cells (Lingblom, Andersson, Andersson, & Wenneras, 2017). To our knowledge galectin-10 expression and function in DCs has not been

previously described. However, based on functions of galectin-10 in other cell types, McB-primed DCs may be involved in regulating T cell activation and differentiation.

LGG increased the expression of the gene for another galectin with established roles in tolerance induction, *LGALS9*. Galectin-9, acts as a ligand for the co-inhibitory receptor TIM3 expressed by T cells, and have been described to induce Th1 apoptosis, promote Treg formation and suppress Th17 differentiation (Sehrawat, Suryawanshi, Hirashima, & Rouse, 2009; Seki et al., 2008; Zhu et al., 2005). Interestingly, the administration of a symbiotic combination of *Bifidobacterium breve* and prebiotic galacto- and fructo-oligosaccharides have been shown to increase galectin-9 expression by intestinal epithelial cells in a murine model of cow's milk allergy (de Kivit et al., 2012). Moreover, a double-blind, placebo-controlled multicenter trial showed elevated levels of galectin-9 in the sera of infants with atopic dermatitis receiving the *B. breve*, and increased levels of galectin-9 correlated with reduced allergic symptoms (de Kivit et al., 2012). The same study showed that that galectin-9 was able to drive Th1 and Treg development, resulting in increased IFN-γ and IL-10 secretion, and reduced IL-17 secretion by activated PBMCs.

The observation that McB and LGG each modulate the expression of galectins with reported roles in tolerance induction suggests a microbial potential to tune T cell development through affecting MoDC galectin expression.

ImDCs express a variety of cell surface receptors involved in microbe recognition and uptake, but PRRs like toll like receptors (TLRs) and C-type lectin receptors (CLRs) are typically downregulated upon DC maturation (van Kooyk, 2008). The expression of PRRs was differentially up and down-regulated in response to all three bacteria (table 2). LGG increased the expression of several genes for surface-expressed TLRs and CLRs, and reduced the expression of a high number of chemokines, cytokines and co-stimulatory molecules (Table 2) Furthermore, chemokine receptors were only differentially expressed in response to LGG. MoDCs stimulated by LGG expressed higher levels of genes for the chemokine receptors CCR1, CCR5, CXCR1 and CXCR2 typically expressed by immature DCs and involved in DC recruitment to inflamed tissues, and reduced expression of *CCR7*, *CXCR4* and *CXCR5* guiding mature DCs to T cell rich areas of lymph nodes for antigen-presentation (McColl, 2002; Sallusto et al., 1999) (table 2). MoDCs primed by LGG thus express a gene expression pattern consistent with an imMoDC phenotype suggesting a potential for LGG to promote host tolerance by inhibiting DC maturation and T cell activation.

Analysis of 150 DEGs regulated only in response to EcN also provides clues to its probiotic functionality. *E.coli* Nissle protects gnotobiotic pigs against human rotavirus by modulating pDC- and NK-cell responses and increase production of pro-inflammatory cytokines (Vlasova et al., 2016). EcN alone induced upregulation of nine genes for interferon-induced proteins. Type I interferon signaling has several functions in innate and adaptive immunity (Ivashkiv & Donlin, 2014). Overrepresentation analysis of all DEGs upregulated in response to EcN confirmed overrepresentation of genes involved in interferon signaling pathways (p value 1.98E-11), negative regulation of viral genome replication

(p value 1.28E-08) and anti-viral responses (p value 2.31E-12). Oppositely, EcN exclusively downregulated *CD1B, CD1C and CD1E,* genes coding for MHC homologs involved in presentation of lipid and glycolipid antigen to T cells and NKT cells (de Jong, 2015), suggesting reduced ability for activation of CD1 restricted T cells and NKT cells.

MoDC regulates expression of genes for inflammatory chemokines in a microbe specific manner

Mature DCs produce inflammatory cytokines and chemokines recruiting appropriate leucocyte populations to the site of pathogen entry (Lebre et al., 2005). Whereas it is well known that stimulation of DC PRRs leads to production of a wide range of chemokines, it has been unclear whether production of inflammatory chemokines is a general feature or specific to certain types of microbes (Griffith, Sokol, & Luster, 2014). In our study, the Gram-negative McB and EcN increased the expression of *CCL5/RANTES,* and all bacteria increased the expression of *CCL3* and *CCL4* compared to maturation control (table 1). CCL3/MIP-1 CCL4/MIP-1 and CCL5/RANTES are all produced at early stages of DC maturation and are likely to sustain the recruitment of circulating imDC, DC precursors, and T cells to inflamed tissue (Lebre et al., 2005) and may define a general response to bacterial stimulation.

In addition to this general chemokine response, MoDCs responded to EcN and LGG in a specific manner by regulating the expression of several chemokine genes (table 2). EcN increased *CCL20* expression, a chemokine implicated in recruitment of DCs from blood to peripheral tissues, and genes for the neutrophil-recruiting chemokines CXCL1 and CXCL2 suggesting that EcN stimulation enhances the ability of DCs to support neutrophil-mediated pathogen clearance. In contrast, LGG stimulation reduced the expression of genes for several chemokines involved in neutrophil trafficking and the migration of T cells, monocytes, NKT and ILCs suggesting reduced antimicrobial potential of MoDCs stimulated by LGG and confirming that DCs do respond to non-pathogenic microbes by distinct chemokine profiles.

Differential expression of pro- and anti-inflammatory cytokines in response to McB, EcN and LGG

Although DC tolerogenicity has been attributed to imDCs, antigen presentation by mature DCs do not always induce a productive immune response. Lutz and Schuler (2002) described phenotypically mature DCs, characterized by high expression of MHC class II and co-stimulatory molecules and low, or absent, production of pro-inflammatory cytokines. These DCs were semi-mature, and did not induce T effector cells, but rather antigen specific $CD4^+$ Tregs. Furthermore, cytokines released by mature DCs are important in regulating the balance between different T effector cell subsets (Th1／Th2／Th17) and in maintaining homeostasis.

We have previously shown that MoDCs respond to LGG, McB and a non-pathogenic *E. coli* by distinct cytokine profiles and T cell polarizing ability (Indrelid et al., 2017). The present gene expression analysis confirms that MoDCs differentially express genes for pro- and anti-inflammatory cytokines in response to the three different bacteria (table 2). The Gram-negative bacteria both induced elevated expression of genes for colony-stimulating factors and the prototypical pro-inflammatory cytokine TNF-α. TNF-α is critical for the pathogenesis of inflammatory bowel disease (IBD) and TNF-α blocking has become a mainstay in the treatment of IBD (Pache, Rogler, & Felley, 2009). The observation that *M. capsulatus* and *E.coli* Nissle, two bacteria shown to have anti-inflammatory potential in models of IBD both upregulates expression of TNF-α in MoDCs suggests that protective effects of these bacteria are not a result of counterbalancing proinflammatory cytokines as was suggested for a probiotic species of Lactobacillus (Christensen et al., 2002). However, EcN induced increased expression of a higher number of DEGs for immune stimulatory cytokines than McB.

An important mechanism for DCs in maintaining peripheral tolerance is by induction of regulatory T cells. Several types of regulatory T cells exists, but $FoxP3^+$ Tregs and $FoxP3^-$ $IL\text{-}10^+$ Tr1s are the most well characterized subtypes. The DC-derived signals that drive their generation is better understood than for other types of regulatory T cells. DC-produced cytokines like IL-10, IL-27 and TGF-β have pleiotropic, context-dependent roles in immune regulation, but play particularly important roles in development of regulatory T cells (Ezzelarab & Thomson, 2011; Kushwah & Hu, 2011; Pletinckx, Dohler, Pavlovic, & Lutz, 2011). McB, EcN and LGG all stimulated MoDC production of cytokines involved in the generation of regulatory T cells (table 2). Both McB and EcN increased *IL10* expression, the gene for a cytokine required for induction of Tr1 cells, and leukemia inhibitory factor (LIF), a cytokine reported to regulate the Treg/Th17 axis favouring Treg development (Kushwah & Hu, 2011; Metcalfe, 2011). Our data thus suggest a potential for both EcN and McB to develop regulatory T cells and to inhibit development of inflammatory Th17 effector cells. LGG, in contrast, did not significantly affect *IL10* expression, and strongly reduced expression of EBI3, the β subunit of IL27, a cytokine involved in driving Tr1 development (H. Zeng, R. Zhang, B. Jin, & L. Chen, 2015). LGG did, however, up-regulate the expression of TGF-β, a cytokine important for the induction of $Foxp3^+$ Tregs (Johnston, Smyth, Dresser, & Maizels, 2016).

Differential expression of co-stimulatory molecules

Although co-stimulatory signaling is commonly represented by the binding of CD28 to CD80/86 molecules on DCs, co-stimulation is more likely to involve a number of different DC ligands interacting with various co-stimulatory or co-inhibitory receptors on T cells (Bakdash, Sittig, van Dijk, Figdor, & de Vries, 2013; L. Chen & Flies, 2013; Pletinckx et al., 2011). Co-stimulatory and co-

inhibitory pathways are promising targets for immunotherapies in transplantation settings, cancer and autoimmune diseases (Zhang & Vignali, 2016).

A high number of genes for co-stimulatory molecules were regulated in response to bacterial stimulation and different sets of co-stimulatory molecules were up- or down-regulated in response to the three bacteria (table 2). Interestingly, McB and LGG both reduced expression of the gene for CD70. Binding of CD70 to its receptor, CD27 is important in priming, differentiation and memory formation of T-cells and (Boursalian T.E., 2009). Several reports implicate the CD70/CD27 pathway in autoimmunity, and blocking the CD70/CD27 pathway ameliorateed inflammation in a murine model of IBD (Manocha et al., 2009). CD70 expression is mostly restricted to activated lymphocytes and DCs under physiological conditions. Given the overexpression of CD70 in chronic inflammatory diseases as well as its restricted expression pattern, it has been proposed as a promising target for antibody-directed immunotherapy in such diseases (Boursalian T.E., 2009).

Several other DEGs for molecules involved in both co-stimulatory and co-inhibitory pathways were downregulated in response to LGG, including the clinically relevant molecules 4-1BB, CD40, PDL1 and PDL2. EcN and LGG, but not McB, also downregulated ICOSLG (B7-H2). Co-stimulation via the ICOS-L/ICOS pathway supports proliferation and production of cytokines in activated T cells (Hutloff et al., 1999), and is likely to play roles in both T cell activation and tolerance complicating the exploitation of this pathway for therapeutic purposes (Bakdash et al., 2013).

As the balance between all co-inhibitory and co-stimulatory signaling is expected to be decisive for induction of T cell tolerance vs. immunogenic T cell responses, the net effect of the DEGs induced by each species remains unsure. However, the observed ability of probiotic bacteria to modulate DC expression of clinically relevant co-stimulatory/ co-inhibitory molecules may be one mechanism behind probiotic functionality that could potentially be exploited for therapeutic purposes.

Variable modes of action of immune modulatory bacteria

The different MoDC gene expression profiles induced by the three bacterial strains are likely to be reflected in different MoDC functions. Understanding the functional consequences of these differences are vital to take advantage of individual species/strains for therapeutic purposes. Surprisingly, the same probiotic strains are claimed to have beneficial effects in the same disease models such as allergy, infectious diseases and chronic inflammation, disregarding the fact that these diseases are driven by different, and partially opposing etiological mechanisms. Furthermore, the activity of the different species/strains clearly depends on the disease model used, as reviewed by (Mileti, Matteoli, Iliev, & Rescigno, 2009). LGG for example had protective effect in an iodoacetamide-induced colitis model, no protective effect in a dinitrobenzene sulfonic acid (DNBS) (Shibolet et al., 2002) and detrimental effect in the DSS-model of colitis (Geier, Butler, Giffard, &

Howarth, 2007). The wide array of effects and variable efficacy of immunomodulatory bacteria *in vivo* highlight the need for improved knowledge about individual microbes immunomodulatory mode of action and emphasize the importance of matching the right strain, to each disease and stage of the disease, based on the functional properties of each species.

Conclusion

While a role for environmental microbes from soil and water in immune regulation has been suggested, few studies have focused on immunomodulatory properties of environmental bacteria. However, supporting a role for environmental bacteria in toning intestinal homeostasis, dietary inclusion of the non-commensal soil bacterium McB has been shown to abolish inflammation in salmonids and in a murine model of IBD. Recently we showed that the same bacterium, targets and adheres specifically to MoDCs, key regulators of innate and adaptive immunity, affecting cytokine production, and MoDC ability for T cell activation and instruction. However, the mechanisms involved have not been identified.

Here we have employed global transcript and bioinformatic analysis to gain insights into how McB influences MoDC gene expression and function. DC modulatory effects were compared to those of two well characterized immunomodulatory probiotic bacteria. Results show that this soil bacterium profoundly modulates MoDC gene expression distinct from both EcN and LGG, and induces a state of DC maturation, cytokine and chemokine production intermediate between LGG- and EcN-primed MoDCs. Furthermore, McB induces expression of immunosuppressive/immune regulatory cytokines, galectin-10, an S-type lectin involved in the generation of regulatory T cells, and reduces expression of CD70, a promising target for antibody-directed immunotherapy.

Furthermore, the two established probiotic species, LGG and EcN, were found to have very different and partly opposing effects on genes important for immunogenic and tolerogenic MoDC functions. LGG maintained an imMoDC phenotype and reduced the expression of genes involved in migration and antigen presentation, co-stimulation and cytokine production, but increased the expression of TGF-β, a cytokine enhancing Treg generation. In contrast, EcN appears to be a strong inducer of MoDC activation, and generate MoDCs expected to have high capacity for promoting antimicrobial/antiviral responses. EcN promotes expression of genes necessary for migration and T cell activation, but also induces increased expression levels of genes for immune suppressive regulatory cytokines like IL-10 and LIF. In conclusion, the current study defines functional properties of three bacteria with demonstrated potential for therapeutic use and may form a basis to make educated decisions about their appropriate applications.

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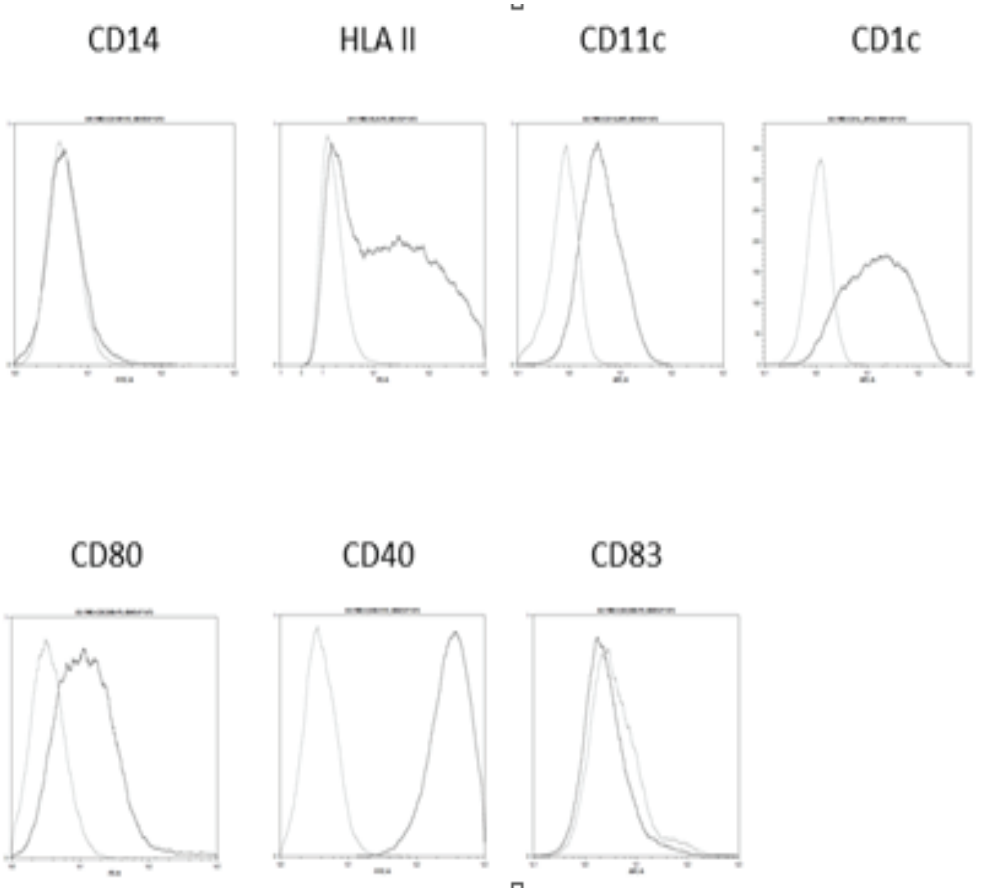
FIGURES AND LEGENDS PAPER III

1A



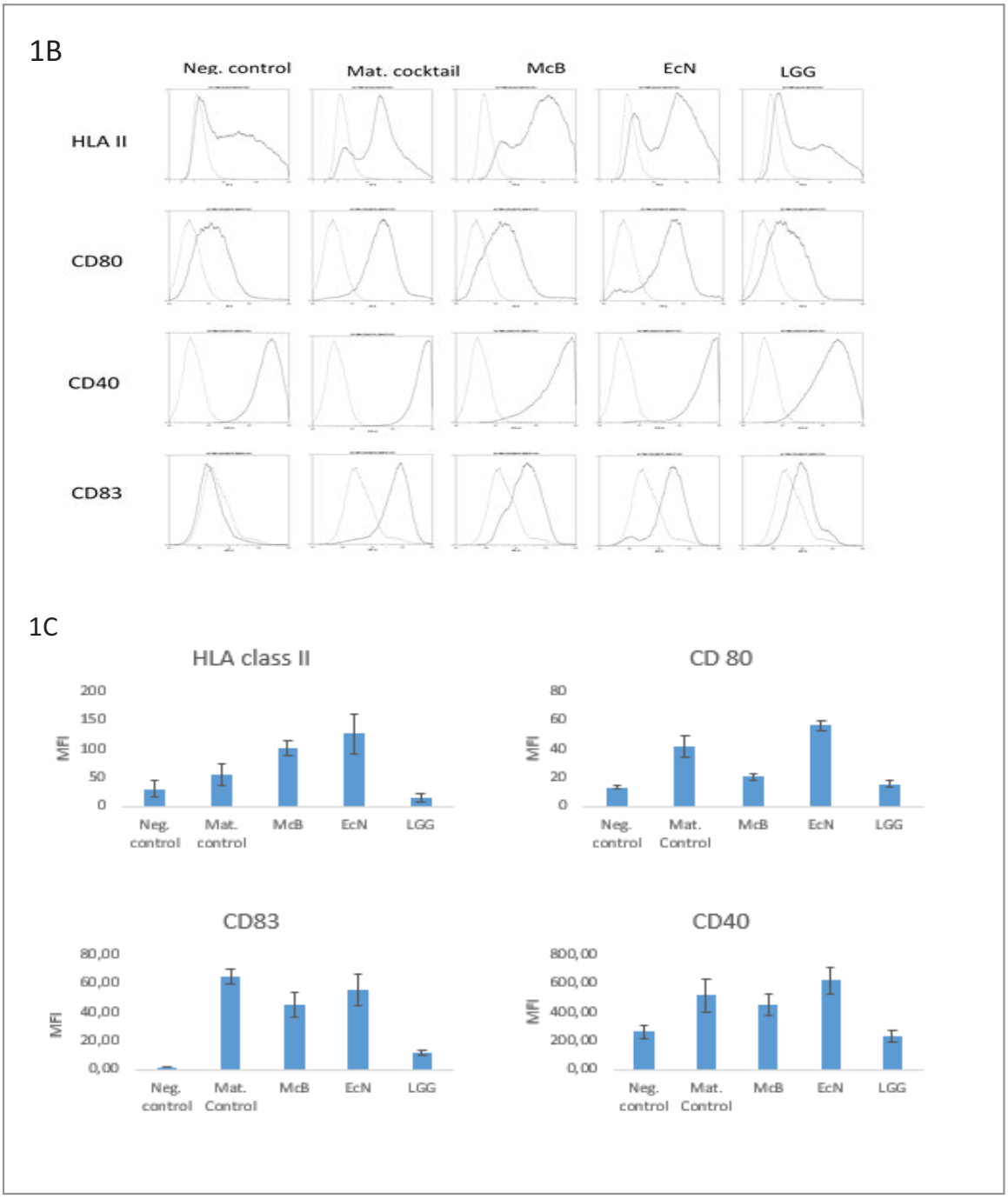


Figure 1. Phenotype of immature monocyte-derived DCs (MoDC) and MoDCs after bacterial stimulation. **1A:** $CD14^+$ monocytes were isolated and cultivated for 6 days in the presence of GM-CSF and IL-4, generating CD14-, HLA+ $CD11^+$ $CD1^+$ $CD80^+$ $CD40^+$ and $CD83^-$ imDCs. Plots show representative results from four different donors. Fluorescence minus one control in light gray. Stained cells in dark gray.

1B and 1C: Immature monocyte derived dendritic cells were either activated by a maturation cocktail of TNF-α, PGE2, and LPS or co-incubated with bacteria for 24 h. Cells were stained for HLA class II, CD80, CD83, and CD40 and analyzed by flow cytometry. 1B: Plots show representative results from four different donors. Fluorescence minus one control in light gray. Stained cells in dark gray. 1C: Median fluorescence intensity (MFI) is reported. Error bars indicate standard error on median fluorescence intensity values from 4 different donors. Medium only, without bacteria was added to negative control.

Table 1. Table shows results of Enricr pathway enrichment analysis of all bacteria regulated DEGs against the PANTHER database. Results with adjusted P value <0.01 are listed.

Term	P-value	Adjusted p-value	Z-score	Combined score
CCKR signaling map ST_Homo sapiens_P06959	7.196e-12	7.412e-10	-1.81	46.47
Interleukin signaling pathway_Homo sapiens_P00036	3.715e-8	0.000001913	-1.51	25.91
Apoptosis signaling pathway_Homo sapiens_P00006	0.000009012	0.0002425	-1.49	17.35
Inflammation mediated by chemokine and cytokine signaling pathway_Homo sapiens_P00031	0.000009419	0.0002425	-1.53	17.65
Integrin signalling pathway_Homo sapiens_P00034	0.00001963	0.0004044	-1.40	15.16
B cell activation_Homo sapiens_P00010	0.00003542	0.0006081	-1.15	11.76
Toll receptor signaling pathway_Homo sapiens_P00054	0.0001576	0.002132	-0.80	7.01
JAK/STAT signaling pathway_Homo sapiens_P00038	0.0001656	0.002132	1.25	-10.90
2-arachidonoylglycerol biosynthesis_Homo sapiens_P05726	0.0002508	0.002870	2.18	-18.06
PDGF signaling pathway_Homo sapiens_P00047	0.0003994	0.004114	-0.79	6.19
T cell activation_Homo sapiens_P00053	0.0005503	0.005153	-0.61	4.54
Pyrimidine Metabolism_Homo sapiens_P02771	0.0008228	0.007063	2.55	-18.10

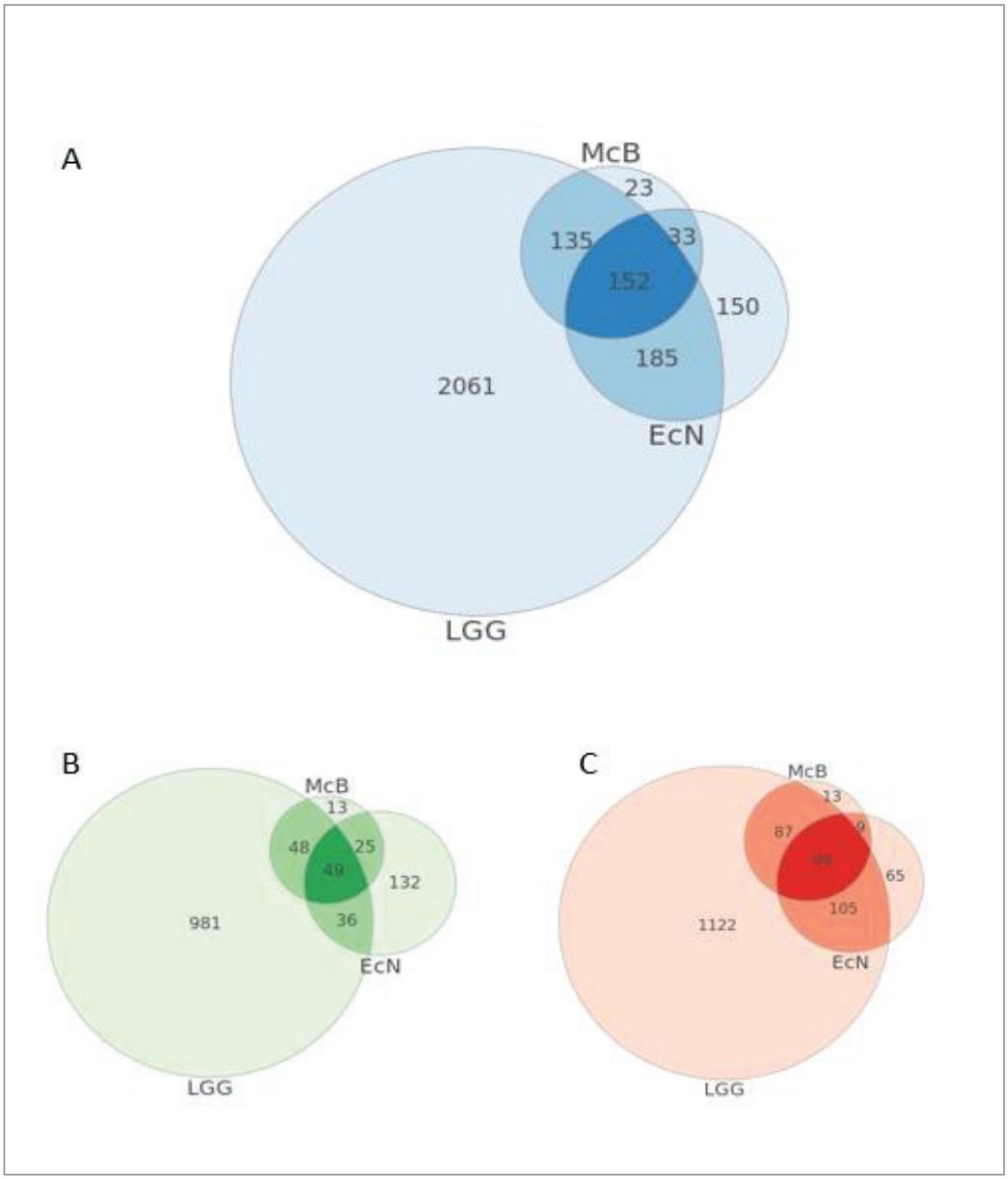


Figure 2. DC respond to microbial stimulation by general and microbe-specific responses
2A: Diagram shows the relationship between DEGs expressed in response to LGG, McB and EcN. A core set of 152 DEGs was regulated in response to all bacteria. 2061 DEGs were regulated only in response to LGG, 150 only in response to EcN and 23 in response to McB. 2B: Relationship between DEGs upregulated in response to each stimulation displayed in green 2C: Relationship between DEGs downregulated DEGs displayed in red.

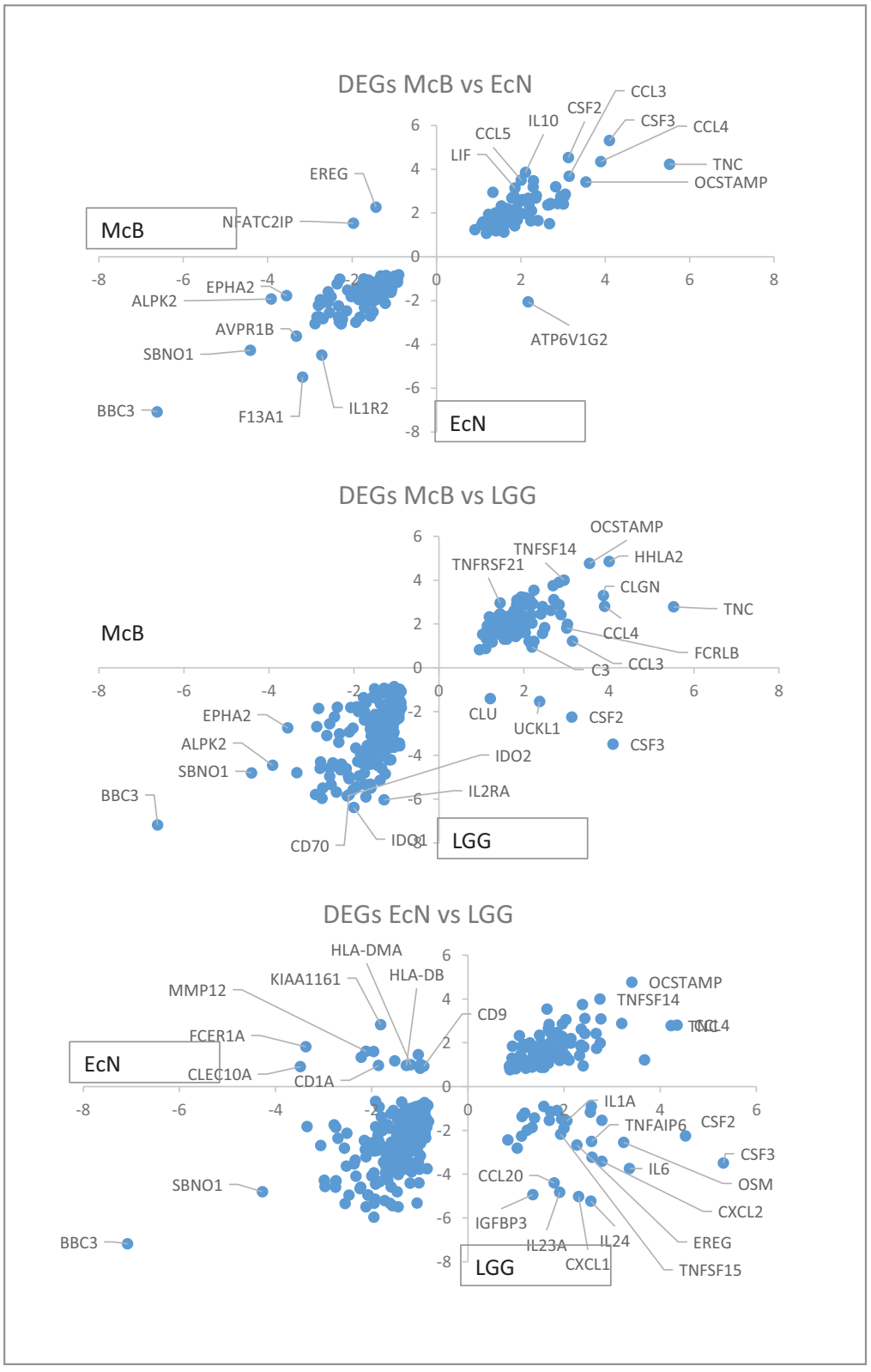


Figure 3. Relationship between DEGs regulated in response to two or more of the bacterial treatments.

Table 2. Genes for pattern recognising receptors and chemokine receptors differentially expressed after bacterial simulations vs control. Upregulated genes indicated in green, downregulated genes indicated in red. Change in exspression given as log2 (fold change).

Pattern recognising receptors				
		log2 (fold change)		
Gene	Description	McB vs A	EcN vs A	LGG vs A
CD207	C-type lectin domain family 4 member K	-2,78518	-2,73964	-4,29641
CD209	CD209 antigen			2,44373
CLEC10A	C-type lectin domain family 10 member A		-3,48479	0,905812
CLEC12A	C-type lectin domain family 12 member A	2,43936		2,79635
CLEC4D	C-Type lectin domain family 4 member D			2,69441
CLEC4E	C-type lectin domain family 4 member E		3,09877	
CLEC7A	C-type lectin domain family 7 member A			1,40263
COLEC12	Collectin-12	-0,92292		-3,57101
TLR1	Toll-like receptor 1			2,00256
TLR4	Toll-like receptor 4			2,19238
TLR6	Toll-like receptor 6			1,62131
TLR8	Toll-like receptor 8		1,18309	-1,21526

Chemokine receptors				
		log2 (fold change)		
Gene	Description	McB vs A	EcN vs A	LGG vs A
Chemokine receptors				
CCR1	C-C chemokine receptor type 1			2,33102
CCR5	C-C chemokine receptor type 5			2,11441
CCR7	C-C chemokine receptor type 7			-4,8929
CXCR1	C-X-C chemokine receptor type 1			1,66573
CXCR2	C-X-C chemokine receptor type 2			1,61803
CXCR4	C-X-C chemokine receptor type 4			-2,69692
CXCR5	C-X-C chemokine receptor type 5			-5,69466

Table 2. continued. Genes for co-stimulatory/ coinhibitory molecules differentially expressed after bacterial simulations vs control. Upregulated genes indicated in green, downregulated genes indicated in red. Change in exspression given as log2 (fold change).

Co-stimulatory / coinhibitory molecules				
		log2 (fold change)		
Gene	**Description**	**McB vs A**	**EcN vs A**	**LGG vs A**
CD274 (PDL1)	Programmed cell death 1 ligand 1			-2,54446
CD276 (B7-H3)	CD276 antigen			1,4891
CD40	Tumor necrosis factor receptor superfamily member 5			-1,62296
CD70	CD70 antigen	-2,11236		-5,8044
CD80 (B7-1)	T-lymphocyte activation antigen CD80			-3,11493
CD84	SLAM family member 5		-1,84118	-0,92928
CD86 (B7-2)	T-lymphocyte activation antigen CD86			-0,95237
ICOSLG (B7-H2)	ICOS ligand		-1,07004	-1,73617
PDCD1LG2 (PD-L2)	Programmed cell death 1 ligand 2			-2,37254
SLAMF1	Signaling lymphocytic activation molecule			-3,20468
SLAMF7	SLAM family member 7			-2,68603
TNFSF14 (LIGHT)	Tumor necrosis factor ligand superfamily member 14	2,94341	2,74688	4,00003
TNFSF4 (OX4OL)	Tumor necrosis factor ligand superfamily member 4			-3,21962
TNFSF9 (4-1BBL)	Tumor necrosis factor ligand superfamily member 9			-3,13798

Table 2. continued. Genes for cytokines/chemokines differentially expressed after bacterial simulations vs control.

Upregulated genes indicated in green, downregulated genes indicated in red.

Cytokines/chemokines

		log2 (fold change)		
Gene	**Description**	**McB vs A**	**EcN vs A**	**LGG vs A**
CCL14	C-C motif chemokine 14			
CCL17	C-C motif chemokine 17			-3,97068
CCL20/MIP-3α/LARC	C-C motif chemokine 20		1,79715	-4,40095
CCL3/MIP-1-α	C-C motif chemokine 3	3,14206	3,6739	1,21409
CCL4/MIP-1β	C-C motif chemokine 4	3,89618	4,35183	2,80628
CCL5/RANTES	C-C motif chemokine 5	2,00845	3,50874	
CX3CL1	Fractalkine			-1,95051
CXCL1/GROα	Growth-regulated alpha protein		2,30482	-5,02427
CXCL16	C-X-C motif chemokine 16			-1,65611
CXCL2/GROβ	C-X-C motif chemokine 2		2,79049	-3,40948
CXCL5	C-X-C motif chemokine 5			-5,05601
CXCL6	C-X-C motif chemokine 6			*
CXCL8/IL-8	Interleukin-8			-3,06431
CSF1	Macrophage colony-stimulating factor 1	1,85434	1,41607	
CSF2	Granulocyte-macrophage colony-stimulating factor	3,12543	4,52773	-2,25126
CSF3	Granulocyte colony-stimulating factor	4,09516	5,31405	-3,4888
EBI3	Interleukin-27 subunit beta			-5,27561
IL10	Interleukin-10	2,11055	3,85238	
IL12B	Interleukin-12 subunit beta			-6,71954
IL15	Interleukin-15		-1,44843	-3,43871
IL16	Pro-interleukin-16			1,53441
IL18	Interleukin-18			-1,08646
IL1A	Interleukin-1 alpha		2,05933	-1,56404
IL1B	Interleukin-1 beta		2,55892	-4,33294
IL23A	Interleukin-23 subunit alpha		1,91244	-4,82522
IL24	Interleukin 24		2,55999	-5,22985
IL6	Interleukin-6		3,36202	-3,7391
IL7	Interleukin-7			-3,16192
LIF	Leukemia Inhibitory Factor	1,85991	3,14322	
OSM	Oncostatin-M		3,23986	-2,55429
TGFB1	Transforming growth factor beta-1			2,28505
TNF	Tumor necrosis factor	2,03665	2,60246	