Bacterial Regulation of Peripheral Immunity

Mechanistic insights from lactobacilli and *Staphylococcus aureus*

Manuel Mata Forsberg
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Abstract
There is a constant cross-talk between our immune system and the colonizing microbiota. The gut resident bacteria produce a broad range of molecules with regulatory activities in both local and distal tissues. Staphylococcus (S.) aureus is a commensal bacterium with high pathogenic potential due to production of several potent virulence factors including staphylococcal enterotoxins (SEs). These SEs are known to induce overwhelming T cell responses, which can result in a serious condition known as toxic shock syndrome. In contrast, several species of bacteria from the genus Lactobacillus exhibit probiotic features and promote beneficial physiological and immunological effects in its host. The underlying mechanisms behind bacterial activation and regulation of peripheral lymphocytes remain elusive. In this thesis, we explored how secreted factors present in the cell free supernatants (CFS) of cultured S. aureus and lactobacilli mechanistically impact the activation of different types of T cells and NK cells. In paper I, we investigated the influence of S. aureus-CFS and SEA on regulatory T cells and found that despite de novo induction of FOXP3 expression, T<sub>REG</sub> cells also produced pro-inflammatory cytokines, which associated with CD161-expression. In paper II, we could show that S. aureus-CFS and SEA induce proliferation, cytotoxicity and cytokine production in conventional and unconventional T- and NK cells. Moreover, we also showed that the lactobacilli-CFS were able to dampen immune cell activation, which was partly linked to lactobacilli-derived lactate. In paper III, we continued to investigate the mechanism behind Lactobacillus-mediated dampening of induced lymphocyte responses and identified extracellular membrane vesicles to be one of the main components involved in Lactobacillus-mediated regulation of cytokine responses. Other observations made in paper II brought about several questions regarding the ability of SEs to activate unconventional T- and NK cells, which lacks certain receptors known to be required for SE-mediated activation of conventional T cells. In paper IV, we therefore investigated the mechanism behind SE-mediated activation of γδ T-, MAIT- and NK cells and found that SEs indirectly activated γδ T- and NK cells, which required the presence of conventional αβ T cells. In summary, this thesis presents novel insights into how soluble components from bacteria modulate immune cell responses and extends the general understanding of bacterial influence on peripheral immunity.

Keywords: Immune regulation, Cytokines, T cells, gamma-delta T cells, MAIT cells, NK cells, Lactobacillus, Staphylococcus aureus, staphylococcal enterotoxins.
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Mechanistic insights from lactobacilli and Staphylococcus aureus

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"Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less”

– Marie Curie
Populärvetenskaplig sammanfattning


**I studie I** fann vi att lösliga faktorer från *S. aureus* inducerade nyproduktion av en viss sorts T lymfocytter som har en reglerande effekt på övriga immunceller. Vi kunde dock se att dessa celler samtidigt även producerade inflammatoriska signalämnen, vilket skulle kunna tyda på att deras roll är mer komplex än att bara verka dämpande. **I studie II** fortsatte vi att studera hur *S. aureus*, samt dess toxin SEA, aktiverar olika typer av lymfocytter och fann även att laktobaciller effektivt kunde motverka denna aktivering genom att dämpa olika inflammatoriska svar. **I studie II och III** undersökte vi vilka faktorer från laktobacillerna och på vilket sätt dessa faktorer kunde dämpa immunaktivering. Det visade sig delvis vara beroende av att laktobaciller utsöndrar små membranvesiklar som har en reglerande förmåga. **I studie IV** studerade vi hur tre närbesläktade toxiner som produceras av *S. aureus* kan aktivera olika sorters lymfocytter som egentligen saknar de ytsstrukturer som krävs för att aktiveras av toxinerna. Vi såg att aktiveringingen verkar ske indirekt och är helt beroende av närvaro av andra celltyper.

Sammanfattningsvis, har denna avhandling bidragit till nya insikter och en ökad förståelse för hur lösliga faktorer från bakterier påverkar specifika celler i vårt immunförsvar.
Scientific Summary

There is a constant cross-talk between our immune system and the colonizing microbiota. The gut resident bacteria produce a broad range of molecules with regulatory activities in both local and distal tissues. *Staphylococcus* (*S.* aureus) is a commensal bacterium with high pathogenic potential due to production of several potent virulence factors including staphylococcal enterotoxins (SEs). These SEs are known to induce overwhelming T cell responses, which can result in a serious condition known as toxic shock syndrome. In contrast, several species of bacteria from the genus *Lactobacillus* exhibit probiotic features and promote beneficial physiological and immunological effects in its host. The underlying mechanisms behind bacterial activation and regulation of peripheral lymphocytes remain elusive. In this thesis, we explored how secreted factors present in the cell free supernatants (CFS) of cultured *S. aureus* and lactobacilli mechanistically impact the activation of different types of T cells and NK cells.

In paper I, we investigated the influence of *S. aureus*-CFS and SEA on regulatory T cells and found that despite *de novo* induction of FOXP3 expression, T<sub>REG</sub> cells also produced pro-inflammatory cytokines, which associated with CD161-expression. In paper II, we could show that *S. aureus*-CFS and SEA induce proliferation, cytotoxicity and cytokine production in conventional and unconventional T- and NK cells. Moreover, we also showed that the lactobacilli-CFS were able to dampen immune cell activation, which was partly linked to lactobacilli-derived lactate. In paper III, we continued to investigate the mechanism behind *Lactobacillus*-mediated dampening of induced lymphocyte responses and identified extracellular membrane vesicles to be one of the main components involved in *Lactobacillus*-mediated regulation of cytokine responses. Other observations made in paper II brought about several questions regarding the ability of SEs to activate unconventional T- and NK cells, which lacks certain receptors known to be required for SE-mediated activation of conventional T cells. In paper IV, we therefore investigated the mechanism behind SE-mediated activation of γδ T-, MAIT- and NK cells and found that SEs indirectly activated γδ T- and NK cells, which required the presence of conventional αβ T cells.

In summary, this thesis presents novel insights into how soluble components from bacteria modulate immune cell responses and extends the general understanding of bacterial influence on peripheral immunity.
This thesis is based on the following original papers, which will be referred to by their Roman numerals in the text.

*Shared second authorship

*Shared first authorship


*Shared senior authorship
Additional papers not included in this thesis.

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<tr>
<td>AD</td>
<td>Atopic dermatitis</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFS</td>
<td>Cell free supernatant</td>
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<tr>
<td>CLR</td>
<td>C-type lectin receptor</td>
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<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen 4</td>
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<tr>
<td>DAMP</td>
<td>Damage associated molecular pattern</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<td>FOXP3</td>
<td>Forkhead box P3</td>
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<td>Gata binding protein 3</td>
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<tr>
<td>GF</td>
<td>Germ free</td>
</tr>
<tr>
<td>HMBPP</td>
<td>4-hydroxy-3-methyl-but-2-enyl pyrophosphate</td>
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<tr>
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<td>Interferon</td>
</tr>
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<td>Ig</td>
<td>Immunoglobulin</td>
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<td>IL</td>
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<td>Lipoteichoic acid</td>
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<td>MAIT (cell)</td>
<td>Mucosal associated invariant T cell</td>
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<tr>
<td>MAMP</td>
<td>Microbe associated molecular pattern</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MV</td>
<td>Membrane vesicle</td>
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Mw    Molecular weight
NK    Natural killer
NLR   NOD-like receptor
NOD   Nucleotide-binding oligomerization domain
PBMC  Peripheral blood mononuclear cell
PGN   Peptidoglycan
PRR   Pattern recognition receptor
PSM   Phenol soluble modulin
R     Receptor
ra    Receptor antagonist
RLR   RIG-like receptor
RORγt Retinoic acid receptor-related orphan receptor gamma t
S.    Staphylococcus
SCFA  Short-chain fatty acid
SE    Staphylococcal enterotoxin
T-bet T-box expressed in T cells
T_C   Cytotoxic T cell
TCR   T cell receptor
TF    Transcription factor
T_FH  Follicular helper T cell
TGF-β Transforming growth factor β
T_H   Helper T cell
TLR   Toll-like receptor
TNF   Tumour necrosis factor
T_REG Regulatory T cell
TSST  Toxic shock syndrome toxin
Introduction

General Overview of The Immune System

The immune system consists of a highly complex network of tissues, organs, cells and soluble components that cooperate in matters of surveillance, detection and destruction of foreign- and self-threats in order to maintain a protected and well-functioning body. Typically, it is divided into two main branches known as innate and adaptive immunity. Innate immunity is comprised of physiological barriers such as skin and mucosal epithelium as well as a large cohort of innate cells: granulocytes (neutrophils, basophils, eosinophils and mast cells), professional antigen presenting cells (APC) such as monocytes, macrophages and dendritic cells (DC), and the lymphocytes natural killer (NK) cells. These cells are able to mount an immediate response to pathogens and they effectively discriminate self from non-self through sensing of conserved molecular structures and patterns that are shared between large groups of microbes. In contrast, adaptive immunity is represented by T cells and antibody producing B cells, which detect specific antigens through surface receptors generated by somatic recombination of multiple gene fragments\(^1\). The immune system collectively integrates detailed information about the pathogen, whether it is intra- or extracellular, fungal or bacterial, in order to mount a target-specific response with as little collateral damage as possible.

The host’s dependence of a well-functioning immune system is evident considering that even small perturbations of selected parts of the immune
Innate Immunity

The innate immune system is the oldest defence system and can be found in plants, insects and mammals. Innate defences include a plethora of mechanisms to detect and discriminate harmful from harmless antigens, prevent pathogen invasion and - perhaps most importantly - to relay information about the nature of the pathogen to the adaptive immune system. The best way to prevent infection is to stop entry of the pathogen in the first place. Skin and mucosal epithelial cells make up physical barriers that prevent microorganisms from direct access to the host’s body. Moreover, continuous production of mucus, enzymes, antimicrobial peptides and gastric juices by the physical barriers, constitutes additional hurdles that the potential pathogens need to overcome. If both physical and chemical barriers are breached, the innate humoral response consisting of complement proteins, C-reactive protein and lipopolysaccharide (LPS)- and mannose-binding proteins contribute to the elimination of the invading pathogen. Lastly, innate immune cells such as granulocytes (neutrophils, basophils and eosinophils), mast cells, monocytes, macrophages, DCs and innate lymphoid cells (ILCs) continuously patrol tissues and mucosal sites and upon pathogen encounter, they mount an immediate response and orchestrate the subsequent actions of the adaptive immunity.
Pattern Recognition

Innate immune cells rely on conserved pattern recognition receptors (PRRs) that detect microbe-associated molecular patterns (MAMPs) such as microbial cell-wall components, lipids, carbohydrate molecules, nucleic acids and virulence factors such as LPS and other endo- or exotoxins. A pathogenic infection is commonly associated with the release of self-antigens due to tissue injury and cellular damage, so called damage-associated molecular patterns (DAMPs), which are also effectively detected by PRRs. PRRs come in many flavours and are expressed on all innate immune cells and epithelial cells as either surface receptors, intracellular or secreted soluble receptors. Upon binding of its cognate MAMP or DAMP, PRRs evoke an intracellular signalling cascade ultimately affecting the regulation and transcription of thousands of genes.

Toll-like receptors (TLRs) are the most well studied PRRs. In humans, they consist of a family of 10 transmembrane receptors that localize to the surface (TLR-1, -2, -4, -5, -6, -10) or to the endolysosomal compartment (TLR-3, -7, -8, -9) and initiate signalling upon recognition of microbial derived carbohydrate-based structures, proteins and nucleic acids. C-type lectin receptors (CLRs) are mainly, but not excluded to, sugar-binding receptors important for detection of and initiation of immune responses towards bacteria, fungi and viruses. The family of CLRs consist of both activating and inhibitory receptors, such as Dectin-1/2 and myeloid inhibitory C-type lectin receptor (MICL), respectively. In contrast to TLRs and CLRs, RIG-I-like receptors (RLRs) are localised to the cytoplasmic compartment, where they initiate protective responses towards intracellular viruses through detection of viral RNA. Similarly, the intracellular NOD-1 and NOD-2 receptors, belonging to the NOD-like receptor (NLR) family, respond to bacterial peptidoglycan (PGN) components and trigger activation of the caspase-dependent inflammasome.
Antigen Presentation

One of the main roles of innate immunity is to recruit and activate the adaptive immune system. Major histocompatibility complex (MHC) class I and II are surface receptor complexes expressed on all nucleated cells and specialised “professional” APCs, respectively. MHC class I molecules present intracellular-derived peptides to CD8+ T cells and are thus important for generating adaptive immune responses towards intracellular pathogens and tumour cells. MHC class II molecules present extracellular-derived peptides to CD4+ T cells required for efficient elimination of extracellular pathogens. Apart from MHC-mediated presentation of classical peptides, non-classical antigens like glycolipids, phosphoantigens and metabolic derivatives are presented through alternative MHC-like receptors to unconventional NKT cells, γδ T cells and mucosal associated invariant T (MAIT) cells, respectively.

Antigen Presenting Cells

Mononuclear phagocytes, such as monocytes, macrophages and DCs, represent innate cells of the myeloid lineage, which have indispensable roles in immune response initiation, pathogen clearance as well as maintaining tissue homeostasis and repair. Initially, monocytes, which are abundant in circulation, were only considered to be a source of monocyte-derived macrophages and DCs in tissues, however, it has been demonstrated that monocytes also have broad functions in terms of antigen presentation and in anti-microbial defences.

Macrophages generated early during gestation in the foetal liver give rise to long-lived self-renewable tissue-resident macrophages that carry out tissue specific functions. After birth, macrophages in tissues can be replenished by blood-derived monocytes and possess highly versatile abilities and con-
tribute to immune defence, wound repair, angiogenesis and general tissue maintenance\textsuperscript{17}.

DCs are highly specialised antigen presenting cells distributed in tissues and lymphoid organs. Immature DCs express high amounts of PRRs and continuously sample the environment for MAMPs and DAMPs. Upon activation, mature DCs down-regulate PRRs and up-regulate MHC class I and II molecules, chemokine receptors and cytokine production in order to migrate to lymphoid organs to present captured antigens to T- and B-cells. Compared to other APCs, mature DCs excel in antigen presentation due to exceptionally high expression of MHC-class I and II molecules, co-stimulatory receptors (CD40, CD80 and CD86) and the unique ability to cross-present exogenous antigens via the MHC class I pathway\textsuperscript{18,19}.

Natural Killer Cells

ILCs consist of four subtypes designated 1, -2, -3 (distinguished by cytokines and immune functions) and NK cells. ILCs are typically tissue resident cells with important roles in tissue homeostasis, immune responses at mucosal barriers and regulation of adaptive immunity\textsuperscript{20}. NK cells can be found throughout the human body, in circulation and tissues, and take part in the destruction of virus-infected cells or malignant tumour cells. NK cells are not antigen specific but rather respond to infected or malignant cells by a mechanism referred to as “missing or altered-self recognition”\textsuperscript{21}. NK cells display a wide array of activating and inhibiting receptors and the net sum of activating and inhibitory signals received, from the target cell, determines the final outcome. Infected or malignant cells often down-regulate expression of MHC class I (to avoid T cell-mediated killing). As MHC class I expression per se is an inhibitory signal for NK cells, this will tip the balance in favour of activation and the NK cell will subsequently release cytolyltic mediators that lyse the infected target cell in a manner highly similar to CD8\textsuperscript{+} T\textsubscript{C} cells\textsuperscript{21,22} (described below under the section “CD8\textsuperscript{+} T\textsubscript{C} cells”).
Although NK cells are not naïve in the same sense as T cells, NK cell survival and effector responses are significantly enhanced in the presence of inflammatory cytokines. NK cells exposed to interleukin (IL)-12, IL-15 and IL-18 display increased proliferation and cytotoxicity in an IL-2R dependent pathway\textsuperscript{23}. IL-21 was found to have synergistic effects in combination with IL-2 and IL-15 by modifying surface receptor expression and increasing cytotoxic potential\textsuperscript{24}.

**Cytokines**

Cytokines are a broad group of relatively small proteins typically categorized into interleukins and interferons (IFN) but also others, such as transforming growth factor β (TGF-β) and tumour necrosis factor (TNF). Cytokines enable communication between immune cells and tissues to mount and orchestrate a directed and appropriate immune response towards a specific target. The main functions of cytokines involve differentiation, proliferation, activation and regulation of immune cell effector responses\textsuperscript{25}. Below follows a brief description of the main sources and functions of the most relevant cytokines for this thesis.

**Interferon-γ**

IFN-γ is the signature cytokine of CD4\textsuperscript{+} T\textsubscript{Helper} (H) 1 cells, but is also produced by CD8\textsuperscript{+} T\textsubscript{Cytotoxic} (C) cells and NK and NKT cells. The contributions of IFN-γ to the inflammatory response are many, including increased cell recruitment and tissue infiltration, enhanced APC function through up-regulation of co-receptors, MHC class I/II receptors, nitric oxide production, inhibition of viral replication and enhanced NK cell cytotoxicity. Furthermore, IFN-γ induces IL-12 secretion from APCs which directs the differentiation of naïve
T-helper (Th) cells into Th1 cells while simultaneously inhibiting Th2 differentiation\(^{26}\). Deficiency in IFN-\(\gamma\) signalling results in impaired resistance to microbial challenge in mice and increased susceptibility to mycobacterial infections in humans, further highlighting the critical role of IFN-\(\gamma\)^{27,28}. On the other hand, excessive IFN-\(\gamma\) production has been linked to autoimmune diseases such as SLE (Lupus) and under certain condition even suppression of anti-tumour responses, supporting the importance of balancing immune activation\(^{29}\).

**Interleukin 17**

The IL-17 family of cytokines consists of 6 members, IL-17A-F, where IL-17A is the most well studied. The main sources of IL-17A are hepatocytes, Th17, \(\gamma\delta\) T- and NKT cells. In the presence of IL-6, IL-23 and TGF-\(\beta\), naive Th cells differentiate into Th17 cells producing the signature cytokine IL-17A. Macrophages, neutrophils, endothelial cells and fibroblasts are known targets of IL-17A and responds by production of chemokines, cytokines and metalloproteases, which will further increase immune cell recruitment and inflammation\(^{30}\). Although IL-17A is critically important for protective immunity against infections caused by *Listeria monocytogenes*\(^{31}\) and *Staphylococcus (S.) aureus*\(^{32}\), excessive IL-17A signalling is also associated with multiple immune related diseases including the autoimmune diseases rheumatoid arthritis\(^{33}\) and multiple sclerosis\(^{34}\).

**Interleukin 12, -15, -18**

IL-12 production is induced by a large number of diverse microbial factors via TLR signalling in monocytes, macrophages, DCs and neutrophils. The active form of IL-12 is produced as a heterodimer composed by a p35 and a p40 subunit (IL-12p70). Once secreted, IL-12 drives inflammation by triggering IFN-\(\gamma\) production in Th1 cells and NK cells. IFN-\(\gamma\) in turn, acts on both APCs and Th1 cells promoting further IL-12 secretion by the former
and up-regulation of the IL-12R on T cells\textsuperscript{35}. Indeed, loss of functional IL-12 signalling causes impaired Th1-mediated immunity and results in severe mycobacterial and salmonella infections\textsuperscript{36}.

IL-15 is produced by a variety of cell types including keratinocytes, epithelial cells, APCs and CD4\textsuperscript{+} T cells and shares structural and functional similarities with IL-2\textsuperscript{37}. Unlike most cytokines, which are secreted as soluble mediators and binds its cognate receptor expressed on target cells, newly synthesised IL-15 is bound by cytosolic IL-15R\textalpha and is transported to the surface membrane as a complex where it is “presented” to an adjacent target cell\textsuperscript{38}.

IL-18 is an innate derived cytokine released in response to PRR activation on epithelial, endothelial and mononuclear phagocytes. IL-18 is constitutively expressed as an inactive precursor protein known as pro-IL-18, which is cleaved by caspases upon PRR-mediated inflammasome activation. IL-18 synergises with IL-12 or IL-15 to induce IFN-\gamma responses from T- and NK cells, primarily. IL-12 or IL-15 primes target cells to up-regulate IL-18R\textbeta expression required for IL-18 mediated signal transduction\textsuperscript{39}.

**Interleukin 6**

IL-6 is a highly pleiotropic cytokine involved in the regulation of immunity, metabolism, bone and tissue homoeostasis. The main source of IL-6 are mononuclear phagocytes but can also be produced by a number of other cells and tissues as well, e.g. adaptive lymphocytes, the endothelium and liver. The IL-6 signalling complex involves IL-6 bound to the IL-6R, which subsequently forms a complex with the signalling-domain carrying receptor CD130 (also known as gp130). The IL-6R can be either membrane bound (mbIL-6R) or soluble (sIL-6R), which has important consequences for the biological effects of IL-6 signalling. MbIL-6R signalling is associated with anti-inflammatory responses by promoting intestinal epithelial cell proliferation and resolution of inflammation, whereas sIL-6R signalling together with
other cytokines promotes inflammation by recruitment and maintenance of TH17 and TH2 cells\textsuperscript{40}. Dysregulation of IL-6 leading to excessive signalling is common in cancer development and during certain viral infections\textsuperscript{41}.

**Interleukin 1**

The IL-1 family of cytokines now consist of several members (including IL-18) with various roles in immunity of which IL-1\(\alpha\) and IL-1\(\beta\) are the best studied ones. IL-1\(\alpha\) and \(\beta\) have similar biological activities, however, IL-1\(\alpha\) is typically associated with apoptotic and necrotic cells and rarely detected in circulation\textsuperscript{42-44}, which is why this work has mainly been focused on the role of IL-1\(\beta\). Similar to IL-18, IL-1\(\beta\) is stored intracellularly as an inactive pro-peptide in APCs. Upon signals from PRRs, tissue damage or metabolic changes, pro-IL-1\(\beta\) is quickly cleaved, by caspase-1, into its active form and released from the cell. IL-1\(\beta\) signals mainly through the IL-1R1 expressed by most cells including innate and adaptive lymphocytes, with the purpose to amplify inflammation by induction of IFN-\(\gamma\) and IL-17\textsuperscript{45}.

IL-1\(\beta\) is a potent mediator of innate and adaptive immunity, where deficiency or dysregulation of it have been linked to increased susceptibility to intestinal infections and autoinflammatory diseases, respectively\textsuperscript{46}. Thus, the activity of IL-1\(\beta\) is strictly regulated by several mechanisms, including IL-1 receptor antagonist (ra). IL-1ra is structurally homologous to IL-1\(\beta\) and binds to IL-1R1 as well. However, it lacks the ability to recruit the IL-1R accessory protein (IL-1RAcP) required for signal transduction. Moreover, IL-1ra has a higher binding affinity for IL-1R1 compared to IL-1\(\beta\) and therefore inhibits IL-1 signalling through receptor sequestration\textsuperscript{47}. Genetic mutations causing IL-1ra deficiency results in severe systemic autoinflammatory disease\textsuperscript{48}, highlighting the importance of controlling IL-1 signalling.
Interleukin 10

In contrast to most cytokines, whose main role is to enhance and direct inflammatory immune responses, IL-10 is a regulatory cytokine, produced by regulatory T- and B cells and APCs, that limits and suppresses both innate and adaptive immune cell activation\textsuperscript{49}. IL-10 down-regulates TLR signalling, MHC class II receptor expression, co-stimulatory molecules and cytokine production, thus limiting APC function and subsequent T cell responses\textsuperscript{50}. In addition to direct immune suppression, IL-10 promotes maintenance of regulatory T (T\textsubscript{REG}) cells and T cell memory formation\textsuperscript{51,52}. Although blocking IL-10 can result in a temporarily improved pathogen clearance and survival by enhanced adaptive responses, IL-10 knock-out mice suffer from spontaneous mucosal inflammation involving the microbiota and have a higher mortality rate during parasite infections\textsuperscript{53–55}.

\textit{Adaptive Immunity}

Cells of the adaptive immunity originate from hematopoietic precursor cells in the bone marrow. B cells exit the bone marrow as fully functional (but immature) cells, while T cell precursors require further development and maturation in the thymus before being released into circulation. Although innate immunity is very efficient in sensing and limiting pathogen invasion, adaptive immunity ensures pathogen elimination and the generation of a long-lasting immunological memory. While innate immunity depends on a limited number of germ-line encoded PRRs, adaptive immunity relies on an almost unlimited number of antigen specific receptors (“clonal receptors”) generated through randomised recombination of multiple gene-segments.
B Cells

B cells are adaptive lymphocytes responsible for the antibody-based humoral response. Immature B cells develop from progenitor cells in the bone marrow and complete their maturation in the spleen. B cells then circulate through blood to lymph nodes and secondary lymphoid organs to take part in antibody mediated responses. B cells detect foreign antigens with a membrane bound immunoglobulin (Ig) receptor known as the B cell receptor (BCR), which is generated by rearrangement of multiple gene segments to ensure that each clone of B cells expresses a BCR with unique antigen specificity. Activation of B cells occur in germinal centres, which are dispersed throughout secondary lymphoid tissues, with the help of APCs and T_H cells that provide co-stimulatory signals for the generation of antibody secreting plasma cells and memory B cells. Five main classes of antibodies exist, IgM, -D, -G, -E and -A, which can be either membrane bound or secreted, that mediate protection through complement activation, opsonisation and antibody-mediated cell cytotoxicity56.

T Cells

T cells represent a diverse group of lymphocytes that can be subdivided based on T cell receptor (TCR)-chain usage, co-receptor expression and effector functions. The majority of T cells belong to the conventional CD4^+ T_H cells or CD8^+ T_C cells expressing a highly variable αβ TCR and respond to peptide antigens presented through MHC class II and I, respectively. In addition, γδ T-, MAIT- and NKT cells collectively represent the unconventional T cells that express the γδ-chain TCR or semi-variable αβ TCRs, respectively. These cells are numerous in peripheral and mucosal tissues, exhibit both adaptive and innate functional traits and respond to non-peptide antigens such as lipids and metabolic derivatives presented through non-classical MHC-like receptors57.
**CD4⁺ T⁻H Cells**

Upon antigenic stimulation, naïve CD4⁺ T⁻H cells differentiate into one of several effector subtypes, e.g. T⁻H1, T⁻H2, T⁻H9, T⁻H17, T⁻FH or regulatory T⁻REG cells, defined by their respective cytokine profile and effector functions. The local cytokine environment induces lineage-specific transcription factors (TF) that direct the differentiation and maintenance of each lineage (Figure 1). IL-12 and IFN-γ promote T⁻H1 cell differentiation by induction of the TF T⁻bet, which in turn represses the development of other subtypes, ensuring that lineage commitment is maintained⁵⁸. IL-4 together with the TF GATA3 induce and maintain the differentiation of IL-4 producing T⁻H2 cells⁵⁹, while IL-6, IL-23 and TGF-β promote T⁻H17 cell commitment by up-regulation of the TF RORγt⁶⁰. Interestingly, TGF-β together with IL-4 drives T⁻H9 differentiation⁶¹ but together with IL-2 induces FOXP3 expression resulting in T⁻REG cell differentiation instead⁶². Although each T⁻H lineage expresses individual TFs and cytokine profiles, which also antagonistically inhibits other lineages, it is now clear that a significant level of flexibility do exist⁶³. CD4⁺ T⁻H1 memory cells were demonstrated to adopt a T⁻H2 type cytokine response upon reactivation under T⁻H2-polarizing conditions, and vice versa⁶⁴.

*Figure 1.* Schematic representation of CD4⁺ T⁻H cell differentiation. APCs instruct naïve antigen-specific T⁻H cells to differentiate into one of various T⁻H lineages, expressing line-age specific TFs and cytokines. From Russ, BE et al. (2013), Frontiers in Genetics⁶⁵.
CD8$^+$ T$_C$ Cells

In lymph nodes, naïve CD8$^+$ T$_C$ cells respond quickly to peptide antigens presented through MHC class I and undergo extensive proliferation and differentiation into either short-lived effector cells or long-lived memory T cells in a CD4$^+$ T$_H$ cell dependent manner$^{66}$. Upon activation, CD8$^+$ T$_C$ cells develop into cytotoxic T lymphocytes (CTLs) and employ two main ways to kill infected or malignant cells. Engagement of the TCR to peptide-bearing MHC class I molecules on target cells results in the formation of an immunological synapse through which cytolytic granules are released by the CTL. These granules contain multiple effector molecules including perforin and granzyme, which induce apoptosis of the target cell in a caspase-dependent mechanism$^{67}$. In addition to cytolytic enzymes, CTLs can kill their target cells through a membrane receptor mediated pathway that involves delivery of FasL to the Fas receptor expressed on the target cells. The ligation of Fas-FasL results in induction of apoptosis, which is important for elimination of infected or malignant cells but also autoreactive T cells$^{68}$.

The CD161 Receptor

CD161 is a C-type lectin-like receptor and is expressed mainly on NK cells and T cells$^{69}$, where it is a defining marker for the unconventional MAIT cell (described below). The role of CD161 in immunity has proven to be very complex. It clearly associates with pro-inflammatory cytokine producing T cells$^{70-72}$, including T$_{REG}$ cells$^{73}$. However, whether it is simply a phenotypic marker of highly activated/memory T cells or if it is a bona-fide co-receptor with stimulatory activity is not completely known and might be dependent on cell type and context. Indeed, CD161 ligation enhances T cell activation only in the presence of TCR stimulation but not upon cytokine-mediated stimulation$^{74}$. The only known ligand for CD161 is lectin-like transcript 1 (LLT-1), a membrane receptor expressed on a large variety of cells. Resting
immune cells do not express LLT-1 but is up-regulated on APCs upon TLR signalling, while T and B cells up-regulate LLT1 upon TCR and BCR signalling, respectively. The role of CD161 on NK cells appears to be different from that on T cells. Several studies have shown that the interaction of CD161 with LLT-1 inhibits NK cell cytotoxicity and IFN-γ production. In support of this, tumours expressing high levels of LLT-1 are more refractory to NK cell-mediated killing.

Unconventional T Cells

NKT cells are characterized by expression of an αβ TCR, the T cell marker CD3 together with CD56 and CD161 (both usually associated with NK cells) and are defined by their ability to respond to lipid antigens presented through the CD1d complex. NKT cells are further divided into type I and II NKT cells based on TCR-chain usage. In humans, type I NKT cells are low in numbers in the periphery and represent less than 1% of circulating T cells. Still, NKT cells are believed to be important for anti-bacterial, -viral and -tumour responses.

γδ T cells develop in the thymus, similar to conventional T cells, however, they express a TCR from a limited repertoire of γ- and δ-chains. γδ T cells exhibit diverse functions ranging from direct anti-bacterial and antiviral responses to indirect activities involving recruitment of both innate and adaptive immune cells, antigen presentation and wound healing. The number of γ- and δ-chains are rather limited in comparison to the classical αβ TCR. Furthermore, the pairing of specific γ- and δ-chains is also biased in the sense that particular γ-chains tend to pair with specific δ-chains, e.g. the γ9 chain usually pairs with the δ2-chain. The chain-usage is also linked to tissue distribution and effector function. In humans, the δ2-chain is mostly expressed by γδ T cells found in circulation that respond to the phosphoantigen 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP) generated during isoprenoid biosynthesis, whereas the δ1 and δ3 chains are more associat-
ed with mucosal sites and are activated by viral and stress-induced antigens presented via CD1\(^{80}\). HMBPP activation of \(\delta^{2+}\) T cells is induced via an alternative receptor known as butyrophilin 3A1 (BTN3A1) expressed on APCs\(^ {13}\). Interestingly, unlike conventional antigen presentation, BTN3A1 was found to bind phosphorylated antigens via its intracellular domain\(^ {81}\) (Figure 2).

MAIT cells are found in large numbers at peripheral sites and mediate important protective functions in mucosal immunity. The MAIT cell TCR consist of a fixed V\(\alpha7.2\) (TRAV1-2)-chain paired with a limited number of V\(\beta\)-chains. Phenotypically, MAIT cells are characterized by high expression of CD161, the IL-18R\(\alpha\) and the skin/mucosal homing chemokine receptor CCR6. MAIT cells respond to bacterial infections by recognition of reduced metabolic derivatives of the riboflavin pathway presented through the MHC class I-related molecule MR1 (Figure 2). This enables MAIT cells to effectively engage a wide variety of both Gram-positive and Gram-negative bacterial species. Upon activation, MAIT cells produce cytokines such as IFN-\(\gamma\), IL-17 and TNF and have cytotoxic properties via perforin and granzyme production\(^ {82}\). A distinctive feature of unconventional T cells, including MAIT cells, is the ability to become activated indirectly in the presence of inflammatory cytokines such as IL-12 and IL-18. Indeed, MAIT cells express high amounts of the IL18R\(\alpha\) and are potently activated in the presence of IL-12 and/or IL-18, even in the absence of TCR stimulation\(^ {83,84}\).
Figure 2. Antigen presentation and activation of γδ T and MAIT cells. (a) APCs internalise HMBPP or HMBPP-producing bacteria, which binds to the cytosolic domain of the membrane receptor BTN3A1 and subsequently activates Vγ9Vδ2+ T cells. (b) APCs internalise microbial metabolites derived form vitamin B2 biosynthesis, which are then presented to MAIT cells through the MHC class I-like receptor complex MR1. From Liuzzi, AR et al. (2015), Current Opinion in Immunology.

Balancing The Adaptive Immune Response

The ability to mount an adaptive inflammatory response is critically important for host survival. This becomes most evident in the case of X-linked severe combined immune-deficiency (X-SCID), a disease where patients lack functional T and B cells and thus succumb to aggressive respiratory and intestinal infections in early infancy if left untreated. However, excessive immune activation can also be dangerous where failure in regulation of the intensity or duration of inflammation may result in detrimental tissue damage, hypersensitivity reactions and autoimmunity. The immune system holds a number of intrinsic mechanisms to adequately balance activation through inhibitory- or decoy receptors, antagonistic ligands, regulatory cells and cytokines such as IL-10 and IL-1ra.
T\textsubscript{REG} cells represent one of the main strategies of the immune system to down-regulate inflammation. Identification of phenotypic markers for these cells has been proven difficult, however, a commonly accepted phenotype is CD4\textsuperscript{+}CD25\textsuperscript{high}CD127\textsuperscript{low}FOXP3\textsuperscript{+} T\textsubscript{REG} cells\textsuperscript{87}. T\textsubscript{REG} cells are generated in the thymus, or can be induced peripherally upon activation, and suppress both innate and adaptive immune responses\textsuperscript{88}. Extensive efforts have been made towards finding unique markers for thymus- and peripheral-derived T\textsubscript{REG} cells. The TF HELIOS have historically been suggested to be a marker for thymic origin\textsuperscript{89}, however, a more recently described marker for thymus-derived T\textsubscript{REG} cells is neuropilin-1, which have been shown to differentiate between thymic and peripheral origins\textsuperscript{90,91}.

Several mechanisms of T\textsubscript{REG}-mediated suppression have been identified, such as IL-10 secretion, which down-regulate co-stimulatory receptors and pro-inflammatory cytokines of effector cells. Interestingly, several inflammatory T effector cells can also acquire the ability to down-regulate inflammation by receiving local signals that induce IL-10 production. This may represent an intrinsic mechanism to prevent excessive tissue inflammation by highly activated T effector cells\textsuperscript{92}. In addition to IL-10 production, T\textsubscript{REG} cells constitutively express high amounts of the inhibitory surface receptor cytotoxic T-lymphocyte associated protein 4 (CTLA-4), which binds to and competes with the ligand for the co-stimulatory receptor CD28\textsuperscript{93}. CTLA-4 signalling regulates migration, differentiation and effector responses to control and limit inflammatory damage\textsuperscript{94–96}. The importance of establishing an appropriate immune response is further demonstrated by the fact that effector cells themselves up-regulate inhibitory receptors, such as CTLA-4, upon activation\textsuperscript{97,98}. The main role of T\textsubscript{REG} cells is clearly to down-regulate inflammation, however, under certain conditions T\textsubscript{REG} cells themselves produce pro-inflammatory cytokines\textsuperscript{99,100}, which has been associated with CD161 expression\textsuperscript{73}. 

18
The Gut Microbiota

At birth, the human infant experiences an enormous microbial colonization of body and mucosal surfaces. The gut colonization is a continuous process, highly influenced by environmental factors such as mode of delivery, breastfeeding and antibiotic administration. The multitude of factors shaping the early colonization is reflected by the high degree of individual variation over time in the microbiota composition that exists in infants, whereas both microbiota richness and stability tend to stabilize towards adulthood. Co-evolution has resulted in a mutualistic relationship between the gut microbiota and its host, where tissue- and immune development and maturation are greatly influenced by the former. Germ free (GF) mice, raised in a sterile environment, display an altered intestinal morphology and are more susceptible to immune related diseases. Several immune defects have been observed in GF mice such as lack of MAIT cells, aberrant NKT cell responses in the lamina propria resulting in inflammatory bowel disease (IBD) and dysfunctional TREG cells accompanied with excessive inflammation. Changes in the composition of the microbiota in humans, termed dysbiosis, have also been associated with improper immune function and autoimmune disease. IBD, diabetes, asthma and allergy are examples of immune mediated diseases that associate with intestinal dysbiosis. The relationship between microbiota diversity and dysbiosis is reciprocal, still some bacterial groups and species have been identified as being either positively or negatively associated with risk of developing disease. Children developing allergies or atopic dermatitis (AD) have an increased prevalence of clostridia spp, Escherichia coli and S. aureus or Faecalibacterium prausnitzii, respectively, while bacteria such as lactobacilli and bifidobacteria have been found to infer protection against allergy.
**Lactobacillus**

Lactobacilli represent a highly diverse genus of gram-positive commensal bacteria typically colonizing the human urogenital- and gastrointestinal tract. A key characteristic feature of lactobacilli is their ability to ferment carbohydrates into short-chain fatty acids (SCFAs) and lactic acid; thus, they are frequently found or used in fermented dairy products. Moreover, as human colonizers they have been shown to promote beneficial immune development, maturation and mucosal homeostasis. In the gut, lactobacilli promote homeostasis by pathogen exclusion mechanisms, induction of mucin production and tolerogenic immune responses\textsuperscript{120,121}. Interestingly, gut-associated enrichment of *Lactobacillus (L.) johnsonii* protects against both viral and allergen induced respiratory pathology by modulation of T cell responses\textsuperscript{122}, demonstrating the ability of lactobacilli to indirect affect distal tissues. In terms of immune development and maturation, lactobacilli colonization in young children associates with reduced risks of developing immune related diseases later in life\textsuperscript{118}, despite genetic predispositions\textsuperscript{119}. Furthermore, the potential use of lactobacilli in probiotic supplementation therapies is being evaluated for numerous different immunological and non-immunological conditions including liver disease\textsuperscript{123}, diabetes\textsuperscript{124}, atherosclerosis\textsuperscript{125}, neurological disorders\textsuperscript{126} and cancer\textsuperscript{127}.

**Lactobacillus and The Intestinal Epithelium**

Already at an early age, human infants are colonized by a broad range of *Lactobacillus* spp. For efficient long-term colonization, lactobacilli express several adhesive molecules with which they can bind to and retain themselves from the expulsing forces generated by peristaltic movements and luminal flow. Surface hydrophobicity is suggested to play a role in initial adhesion, while cell-wall associated proteins including mucus binding pro-
The pilus protein SpaC and GAPDH have been found to be important for efficient adhesion to intestinal epithelial cells. Lactobacilli contribute to intestinal health through various mechanisms including upregulation of tight junction proteins, stimulation of mucus production by goblet cells, promotion of intestinal stem cell regeneration and modulation of cytokine and chemokine responses.

**Figure 3.** Schematic representation of known modes of interaction between *Lactobacillus* and the intestinal epithelium. (a) *L. plantarum* modulates tight junction complexes and induce NF-κB nuclear translocation via TLR2 and 6 resulting in cytokine release into the sub-mucosa. *L. rhamnosus* GG secretes two proteins, p40 and p75, which induce phosphorylation of epidermal growth factor receptor (EGFR) leading to activation of intracellular signalling cascades via phosphoinositide 3-kinase (PI3K)-Akt and protein kinase C (PKC)-extracellular signalkinase 3 (ERK3) resulting in strengthening of the intestinal barrier and release of mainly TH1-type cytokines. (c) *L. acidophilus* NCFM-derived cell wall components, such as lipoteichoic acid (LTA) and surface layer protein A (slpA), modulate cytokine responses via epithelial cells and via luminal sampling DCs expressing the CLR DC-specific ICAM3-grabbing non-integrin (DC-SIGN) receptor. (d) Cell wall components of Gram-positive bacteria can be internalised by intestinal epithelial cells, activate cytoplasmic or endosomal PRRs and induce downstream gene transcription modulating immune cell derived cytokine responses. Reprinted with permission from Springer Nature, Nature Reviews Microbiology.
Lactobacillus and Innate Immunity

Studies on innate immune cells reveal that all stages of activation including differentiation, maturation and effector responses are heavily influenced by lactobacilli. An important role of monocytes is to differentiate into functionally mature macrophages and DC in tissues. Lactobacilli were found to induce phenotypic and functional characteristics of Langerhans cells (skin-resident DCs) in monocytes\textsuperscript{140}. \textit{Lactobacillus} treatment of bone marrow derived DCs results in up-regulation of co-stimulatory molecules (CD40, CD80 and CD86) and antigen presentation (MHC class II). Moreover, lactobacilli induced production of a range of pro-inflammatory cytokines such as IFN-β, IL-12 and TNF as well as regulatory cytokines like IL-10\textsuperscript{141–143}. An important effector function of APCs is phagocytic killing of encountered pathogens, a process that requires production of reactive oxygen and nitrogen species (ROS/NOS). Interestingly, macrophages pre-treated with lactobacilli developed enhanced phagocytosis and intracellular ROS-mediated killing of pathogenic bacteria\textsuperscript{144}. Gut-associated lactobacilli can also have systemic effects. In a mouse model of allergic airway inflammation, feeding mice with \textit{L. gasseri} resulted in reduced inflammation and allergic symptoms by activation of the peroxisome proliferator-activated receptor gamma (PPARγ)-pathway in DCs\textsuperscript{145}.

Lactobacillus and Adaptive Immunity

The immunomodulatory capacity of lactobacilli extends beyond innate immunity to the adaptive immune compartment as well. B cell production of IgA is important for gut homeostasis by inhibiting pathogen entry, preventing overgrowth of commensal bacteria and promoting sampling of luminal contents by innate immune cells. Oral administration of \textit{L. paracasei} induces antibody class switching in B cells to IgA production resulting in increased total and antigen-specific IgA titres\textsuperscript{146}. Furthermore, \textit{L. brevis} was shown to
inhibit production of total and antigen-specific IgE, an effect that was linked to induction of IL-12 and promotion of Th1 immunity\(^\text{147}\). Others have also reported lactobacilli-mediated skewing of Th cell differentiation in favour of a Th1 type response over a Th2, which is thought to be an underlying mechanism behind the allergy-protective effects associated with *Lactobacillus*-colonization\(^\text{148,149}\). Although lactobacilli promote Th1 differentiation, T cell-mediated effector responses such as proliferation, cytokine production and cytotoxicity is at the same time down-regulated by lactobacilli\(^\text{150,151}\). The suppressive activity of lactobacilli is often attributed to induction of IL-10 and increase in T\(_{\text{REG}}\) cell numbers and suppressive capacity\(^\text{152,153}\).

**Mechanisms of Immunomodulation by Lactobacilli**

Due to their probiotic potential for a wide range of diseases, extensive research has been carried out in order to characterize the mechanisms involved in the cross-talk between lactobacilli and the immune system. So far, several components of lactobacilli with immune regulatory activity have been described\(^\text{154}\). Cell wall components such as lipoteichoic acid (LTA), surface polysaccharides and PGN are classical MAMPs recognized efficiently by PRRs expressed on epithelial cells and innate immune cells. Indeed, *Lactobacillus*-derived LTA interact with the TLR-2/-6 heterodimer inducing NF-κB signaling\(^\text{155}\) and lactobacilli-PGN ameliorates experimental colitis in a NOD-2 dependent mechanism\(^\text{156}\). The resulting outcome of LTA and PGN stimulation can vary heavily depending on species or strain, which have been linked to chemical and structural differences, such as sugar composition and polymer length, between species\(^\text{157}\). Microbial DNA and RNA is detected by intracellular or endosomal PRRs and *Lactobacillus* is no exception. Chromosomal DNA as well as a specific CpG motif isolated from *Lactobacillus rhamnosus* GG (LGG) reduce both NF-κB signalling and loss of trans-epithelial resistance in TNF-stimulated polarized intestinal epithelial cells\(^\text{158}\). Others, using the same CpG motif from LGG, observed increased B
cell proliferation and suppression of allergen-specific IgE production in a mouse allergy model\textsuperscript{159,160}. Isolated RNA from \textit{L. gasseri} have anti-proliferative effects on CD4\textsuperscript{+} T cells, which was found to be dependent on an intrinsic T cell mechanism involving the signalling adaptor molecule MyD88\textsuperscript{161}.

As viable and heat-killed lactobacilli tend to trigger different types of immune responses, a contribution of the lactobacilli-secretome in host interactions is also likely\textsuperscript{162}. Indeed, the cell free supernatant (CFS) of LGG was shown to have anti-apoptotic effects in epithelial cells that was later linked to the presence of two proteins named p75 and p40\textsuperscript{163}. Subsequent \textit{in vivo} studies using purified p40 confirmed additional protective effects in a murine colitis model by reducing tissue damage and inflammation through binding to the epidermal growth factor receptor (EGFR)\textsuperscript{164}. Other proteins including LGG specific pilin and a secreted protease from \textit{L. paracasei} was shown to regulate IL-8 production and degrade the inflammatory chemokine IP-10, respectively\textsuperscript{165,166}. Apart from proteins, the lactobacilli secretome also contains a range of metabolic end products including SCFA and lactic acid. Lactic acid has been shown to act immunosuppressive by inhibiting NK cell anti-tumour activity as well as inducing regulatory immune functions\textsuperscript{167}. Furthermore, bacteria-derived lactic acid is suggested to promote mucosal homeostasis by reducing pH levels and susceptibility to infections\textsuperscript{168}. Indeed, lactobacilli-derived lactic acid was shown to inhibit \textit{Streptococcus pyogenes} induced cell cytotoxicity by degradation of its LTA\textsuperscript{169}.

Production and secretion of small extracellular membrane vesicles (MVs) was initially only reported from studies on Gram-negative bacteria and was believed to not exist in Gram-positive bacteria due to the PGN-layer enclosing Gram-positive bacterial cells. However, it is now well established that Gram-positive bacteria produce MVs with broad functional roles in bacterial defence, communication and interaction with host immunity\textsuperscript{170}. \textit{Lactobacillus}-derived MVs are beginning to emerge as important immunomodulatory
factors with significant contribution to its host-beneficial effects. Lactobacilli-derived MVs range from 10-300 nm in diameter and contain both nucleic acids and proteins\textsuperscript{171,172}. Based on a detailed proteomics analysis, mostly secretory and membrane-associated proteins were found in the isolated MVs, although no evidence was found that would imply an active sorting mechanism\textsuperscript{171}. On the other hand, proteins with known probiotic effects have been identified within \textit{Lactobacillus}-derived MVs\textsuperscript{173} and studies using isolated MVs have reported induction of IgA\textsuperscript{174}, inhibition of hepatic cancer growth\textsuperscript{175}, protection against AD\textsuperscript{176} and promoting anti-microbial immune responses\textsuperscript{177}. Similar to microbial metabolites, bacterial MVs are present in the circulatory system and was found to disseminate rapidly throughout the body\textsuperscript{178,179}. Interestingly, the composition of gut-derived MVs in the blood associate with neurodegenerative disease\textsuperscript{178} and MVs derived from \textit{L. plantarum} stimulate gene expression in neuronal cells and protect against depression-like behaviour in mice\textsuperscript{180}.

\textbf{Staphylococcus aureus}

\textit{S. aureus} is a Gram-positive bacterium that is typically found in the human nasopharynx, skin, throat and gastrointestinal tract. Although it is a frequent member of the human commensal flora, \textit{S. aureus} harbours several virulence factors such as hemolysins, phenol soluble modulins (PSMs) and a wide range of enterotoxins as well as potent immune evasion strategies making it a potential pathogenic bacterium. \textit{S. aureus} is a common cause of food poisoning and toxic shock syndrome. Multi-drug resistant strains such as methicillin resistant \textit{S. aureus}, better known as MRSA, frequently cause severe nosocomial infections world-wide\textsuperscript{181}. Furthermore, \textit{S. aureus} and its enterotoxins have been implicated in several hypersensitivity/allergic disorders such as AD and asthma. Indeed, children who develop allergy are more fre-
quently colonized with *S. aureus*\(^{115,182}\). In patients with AD, both *S. aureus* colonization and abundance correlates with disease severity and elimination of *S. aureus* results in amelioration of disease symptoms\(^{183}\).

**Staphylococcus aureus** and Immunity

*S. aureus* has evolved multiple strategies to compete for colonization, escape immune surveillance and elimination. Most staphylococci produce a range of antimicrobial molecules that target other members of the commensal flora\(^{184}\). Interestingly, as *S. aureus* are particularly resistant to host defence mechanisms mediated by antimicrobial peptides and proteins such as lipocalin and lysozyme, it has been suggested that *S. aureus* intentionally induce local inflammation in order to outcompete less resistant commensal bacteria\(^{185}\). Furthermore, expression of a polysaccharide capsule and secretion of staphylococcal surface protein A, which binds specifically the Fc portion of antibodies, enables the bacterium to mitigate complement mediated lysis and elimination by immune cells\(^{186,187}\).

Epithelial recognition of *S. aureus* is mediated through PRR binding of MAMPs such as PGN and LTA, which result in activation of both innate and adaptive immune responses. DCs play a key role in optimal clearance of *S. aureus* infections by secretion of IL-12 and IL-23 resulting in activation of effective T\(_{H1}\) and T\(_{H17}\) responses, respectively\(^{188,189}\). Although B and T cell responses have been suggested to be obsolete\(^{190}\), both cell types are heavily activated upon *S. aureus* encounter, resulting in antibody production and memory formation\(^{181,191}\). *S. aureus* is most often associated with potent pro-inflammatory responses; however, cell wall associated molecules have been shown to induce regulatory responses as well. *S. aureus*-activated DCs induce proliferation and IL-10 production in B cells\(^{192}\) and priming of DCs with PSMs, in the presence of TLR-2 ligands, promote the differentiation of T\(_{REG}\) cells and enhance their suppressive activity\(^{193,194}\). Moreover, *S. aureus*-mediated suppression of T cell responses during chronic infection in mice
was found to be mainly dependent on a robust expansion of myeloid-derived suppressor cells (MDSCs) rather than T<sub>REG</sub> cells<sup>195</sup>, showing that this bacterium have multiple strategies to escape host immunity.

Staphylococcal Enterotoxins

The ability of <i>S. aureus</i> to engage and activate T cells is mainly dependent on production and secretion of staphylococcal enterotoxins (SEs)<sup>196</sup>. The family of SEs have grown extensively since their first discovery and now consists of 26 different members, including several SE-like (SEl) proteins, and are located in genomic pathogenicity islands, plasmids and prophages (latent bacterial viruses). SEs are relatively small (19 to 29 kDa) proteins with low sequence homology but share a highly conserved 3D-structure<sup>197,198</sup>. The large number of different SEs and the fact that human clinical isolates often carry as many as 5 different SE genes suggest a non-redundant and important role for <i>S. aureus</i> colonization and survival<sup>199</sup>. Indeed, SE-production have been implicated in regulation of bacterial density during nasal colonization<sup>200</sup> and is critically important for the ability to cause infectious disease such as sepsis, endocarditis and acute kidney injury<sup>201</sup>.

T cells are the main targets of SEs, which induce activation by cross-linking the TCR Vβ-chain with the MHC class II receptor expressed on APCs. All SEs have a weak affinity binding site for the MHC class II receptor α chain, while some (e.g. SEA) have an additional high affinity binding site for the β chain<sup>198</sup>. SEH is the exception that proves the rule and binds the TCR Vα chain and the MHC class II receptor β chain<sup>202</sup>. Normally, any given antigen only activates a fraction of the T cell repertoire due to the requirement of antigen specificity. However, the cross-linking of the TCR and MHC receptors by SEs circumvents the need for antigen specificity and results in activation of up to 30% of the total T cell pool. Such a massive non-specific activation may result in the life-threatening condition known as a
“cytokine storm” followed by T cell exhaustion, which is proposed to be an immune evasion strategy\textsuperscript{203}.

Accumulating evidence now suggest that SEs also interact with additional host receptors other than the TCR and MHC class II. CD28 is a co-stimulatory receptor expressed on T cells that enhance TCR stimulation upon binding to CD86 on APCs. Interestingly, multiple SEs were found to specifically bind CD28 and induce T\textsubscript{H}1 cytokines. Further structural analyses revealed that the CD28 binding site was located outside of those mediating TCR and MHC binding, suggesting that SEs can simultaneously interact with CD28, TCR and MHC molecules\textsuperscript{204}. LAMA2 is a subunit of the laminin receptor expressed on the surface of both T cells and APCs. A recent study reported a direct binding of SEs to LAMA2, which also resulted in T cell activation despite the absence of functional LCK, a tyrosine kinase normally required for TCR-mediated activation of T cells\textsuperscript{205}. Finally, SEA was observed to bind to adipocytes via the IL-6 co-receptor CD130, which resulted in altered insulin signalling\textsuperscript{206}. In support of this, chronic low-dose SE exposure in rabbits also caused impaired glucose tolerance\textsuperscript{207}. 

Present Study

Objectives

The general aim of this study was to investigate the underlying mechanisms behind bacterial induction and regulation of peripheral lymphocyte responses, with a special focus on the commensal and human colonizing bacteria *Staphylococcus aureus* and *Lactobacillus*.

Specific aims

- To investigate how *S. aureus* activate T- and NK cells and to what extent and through what mechanism(s) staphylococcal enterotoxins contribute (*paper I, II and IV*)

- To investigate by what mechanism(s) soluble factors produced by lactobacilli modulate T cell activation (*paper II and III*)

- To isolate and characterize immune-regulatory factors derived from lactobacilli (*paper III*)
Material and Methods

The studies included in this thesis were performed on human peripheral blood mononuclear cells (PBMC) isolated from anonymous healthy adult blood donors. Studies were approved by the Regional Ethic’s Committee at the Karolinska Institute, Stockholm, Sweden {Dnr 04-106/1 and 2014/2052-32} and all study subjects gave their informed written consent.

The main bacterial species used were S. aureus 161:2 (food isolate expressing genes for SEA and SEH), S. aureus 139:3 (enterotoxin-negative), S. carnosus TM300 (non-pathogenic isolated from food), S. epidermidis Kx293A1 (unpublished isolate), L. rhamnosus GG (ATCC 53103) and L. reuteri DSM 17938 (a kind gift from BioGaia AB). All bacteria were grown at 37°C with 5% CO₂ atmosphere and cell free supernatants (CFS) were collected from 72h still cultures (for staphylococci) or 48h still cultures (for lactobacilli) by centrifugation and 0.2 µm sterile filtration. The sterile CFS was aliquoted and stored at -20°C until used in assays. In addition to bacterial CFS, the following staphylococcal enterotoxins were used: SEA (Sigma-Aldrich and in-house made), SEH (in-house made) and TSST-1 (Toxin Technology, Inc.).

For detailed descriptions of specific experimental procedures, the reader is referred to the material and methods within each paper included in this thesis.
Results and Discussion

Paper I

FOXP3 expressing CD4⁺ T cells have been shown to exhibit high plasticity in terms of function and cytokine production. Normally, CD4⁺FOXP3⁺ T cells (often called T<sub>REG</sub> cells) associate with a regulatory function through various immune suppressive mechanisms including IL-10 production. However, observations of T<sub>REG</sub> cell-derived pro-inflammatory cytokine production and their involvement in exacerbation of SE-induced toxic shock suggest diverse functional roles of these cells<sup>208</sup>. In paper I, we investigated the influence of soluble factors derived from <i>S. aureus</i> on FOXP3 expression and cytokine responses in CD4⁺ T cells baring phenotypic markers associated with T<sub>REG</sub> cells. In agreement with current literature, <i>S. aureus</i> stimulation increased the proportion of CD25⁺FOXP3⁺CD127<sup>low</sup> cells among CD4⁺ T cells<sup>209</sup>. Further, stimulating CD4⁺CD25-depleted PBMC with <i>S. aureus</i>-CFS revealed that FOXP3 expression was in fact induced <i>de novo</i>. Interestingly, the ability of <i>S. aureus</i> to induce this population of cells correlated with enterotoxin-production since the same results could be observed using purified SEA while CFS from SE-negative staphyloccoci failed to influence the proportion of these cells or to induce cytokine production in CD4⁺ T cells. A plausible explanation for this could be the fact that FOXP3 expression is partly regulated by TCR signal transduction pathways<sup>210,211</sup>, which are typically induced upon SE-binding to the TCR<sup>212</sup>. Still, the fact that <i>S. aureus</i>-derived PSMs have been shown to induce T<sub>REG</sub> cells via DCs<sup>193,194</sup> and UV-irradiated <i>S. aureus</i> bacteria can promote generation of T<sub>REG</sub> cells in neonates<sup>209</sup>, indicates that also other - non-SE-mediated - pathways to induce T<sub>REG</sub> cells exist. However, optimal induction of peripheral T<sub>REG</sub> cells requires cytokines such as IL-2 and TGFβ<sup>213</sup> and we did not, unfortunately, assess the production of these cytokines. It is plausible that since the APCs in PBMC
cultures consist mostly of monocytes, rather than DCs, TCR engagement by SEs might be necessary for induction of FOXP3. Induction of T_{REG} cells by S. aureus is suggested to be an immune evasion strategy by increasing host immunosuppressive pathways, which favours S. aureus survival and persistence. However, based on the observations in paper I, FOXP3^{+}CD4^{+} T_{REG}-like cells can also contribute to inflammation by production of IFN-\gamma and IL-17A. It is tempting to speculate that this could be, at least short-term, a host-adaptation to prevent microbial exploitation of its own immune regulatory pathways. It has also been reported that T_{REG} cells adopt the phenotype of the target T_{H} cell population. FOXP3^{+} T cells expressing the T_{H}17 lineage specific TF ROR\gamma_{t} were shown to exhibit an enhanced suppressive activity during gut-inflammation. Interestingly, the expression of ROR\gamma_{t} in FOXP3-expressing T cells appeared to be driven by the gut microbiota^{214}. Unfortunately, we were not able to investigate the suppressive capacity of the S. aureus-induced FOXP3^{+}CD25^{+}CD127^{low} T_{REG}-like cells but we did observe potent IL-10 production by these cells.

HELIOS expression is suggested to be a marker for thymic derived T_{REG} cells and to be associated with a more stable phenotype that does not convert into inflammatory effector cells^{215}. Indeed, HELIOS expression correlated with low-responding cells in terms of all cytokines investigated within the FOXP3^{+} cell population. Instead, cytokine expression correlated better with CD161 expression, including IL-10 expression. CD161 is a C-type lectin-like receptor that binds its cognate ligand LLT-1, expressed on many cell types upon activation^{216,217}. The function of CD161 signalling remains controversial and is likely to have cell type specific roles (This is further discussed in paper IV). We observed strong up-regulation of CD161 expression within FOXP3^{+} cells in response to S. aureus and CD161 expression correlated strongly with cytokine expression. Interestingly, CD161^{+} T_{REG} cells were recently characterized as a high cytokine producing population with concomitant suppressive activity and having an important role in intes-
tinal wound repair. This highlights the complexity of immune regulation and caution should be taken when assessing functional roles based on limited parameters, such as cytokine production alone.

Paper II

Exposure of conventional CD4$^+$ and CD8$^+$ T cells to *S. aureus* or its enterotoxins results in a proliferative response and induction of cytokine production. On the other hand, less is known regarding responses of unconventional T cells and NK cells to *S. aureus*. In paper II, we evaluated IFN-$\gamma$ responses and indirectly measured cytotoxic granule release by surface translocation of CD107a (a membrane integrated marker present on cytotoxic granules) upon stimulation with either *S. aureus*-CFS or SEA. Activation of IFN-$\gamma$ producing conventional CD4$^+$ and CD8$^+$ T cells, by *S. aureus*-CFS and SEA was expected, as this is the hallmark cytokine of T$_{H1}$ cells, commonly observed during *S. aureus* infections. Interestingly, we also observed potent activation of $\gamma\delta$ T-, MAIT- and NK cells, which is further explored and discussed in paper IV. *S. aureus*-induced degranulation of T cells was intriguing since cytotoxic responses usually target intracellular pathogens while *S. aureus* has historically been considered to be a typical extracellular bacterium. On the other hand, *S. aureus* has also been shown to escape lysosomal degradation and persist within phagocytes and a broad range of non-phagocytosing cells such as fibroblasts, osteoblasts, keratinocytes and epithelial cells. Moreover, the intracellular lifestyle of *S. aureus* involves a marked change in phenotype, reduced expression of virulence factors and up-regulation of adhesion molecules connected to long-term persistence *in vivo*. Activation of cytotoxic T cell responses might thus serve as an important complementing strategy to combat intracellularly persistent *S. aureus* and prevent the establishment of recurrent chronic infections.

Probiotic bacteria, such as lactobacilli, have diverse beneficial effects on host physiology and immunity. However, detailed mechanistic studies re-
garding the ability of lactobacilli to modulate responses of peripheral immune cells are limited. We investigated the ability of two different probiotic strains of lactobacilli, LGG and \textit{L. reuteri} DSM 17938, to regulate activated T- and NK cell responses. The CFS of both species of lactobacilli was able to dampen cytokine expression, proliferation and degranulation in all cell types investigated, regardless of the stimuli (αCD3/CD28, SEA, PMA/IO or \textit{S. aureus}-CFS) used. Certain species of lactobacilli have been shown to secrete factors that actively degrade either pathogen- or host-derived inflammatory mediators\textsuperscript{166,169}, which could potentially result in reduced inflammatory responses. However, pre-incubation of PBMC with lactobacilli-CFS followed by extensive washing prior to subsequent stimulations resulted in equally potent IFN-γ dampening. This suggests that instead of active degradation of inflammatory mediators, host- or pathogen-derived, lactobacilli seem to affect the responsiveness of T- and NK cells to subsequent activation. This would be in agreement with the ability of lactobacilli to induce IL-10 production or increase the T\textsubscript{REG} cell population, which has been frequently observed\textsuperscript{153,157,223–226}. However, antibody-mediated neutralisation of IL-10 had no effect on the ability of lactobacilli to dampen IFN-γ, nor did \textit{L. reuteri} affect the proportion of T\textsubscript{REG} cells or FOXP3 expression. In support of this, not all species and strains of lactobacilli induce IL-10, but are still able to dampen IFN-γ and IL-17A secretion (unpublished observations).

A characteristic feature of lactobacilli is the production of lactic acid, which has several reported host-beneficial effects including reduced bacterial vaginosis\textsuperscript{168} and increased epithelial development\textsuperscript{135}. Upon stimulation of PBMC in the presence of physiologically relevant levels of lactate, we observed a significant reduction of IFN-γ expression in unconventional T- and NK cells but not in conventional T cells. Lactic acid-mediated inhibition of CD8\textsuperscript{+} T\textsubscript{C} cell effector responses was found to be dependent on reduced intracellular pH\textsuperscript{227}. The use of lactate in our setting, which does not influence pH, supports our observations of a lack of effect on conventional T cells. T cell
activation requires robust metabolic reprogramming, which also differ between naïve, effector, regulatory and memory T cells\textsuperscript{228–230}. Extracellular lactate have also been shown to influence monocyte-derived cytokine responses\textsuperscript{231}, which could have differential effects on unconventional T cells and NK cells compared with conventional T cells during PBMC stimulations. Indeed, neutralisation of IL-12 during \textit{S. aureus} or SEA stimulation tended to affect the unconventional T cells and NK cells more than the conventional T cells, although this was not statistically evaluated. In the end, stimulation of PBMC in the presence of the lactobacilli-CFS effectively dampen activation of conventional T cells as well, implying that additional immune regulatory mechanisms are indeed involved, which require further investigation.

**Paper III**

Several questions arose from \textbf{paper II} regarding the underlying mechanisms and the factors secreted by lactobacilli that were mediating the immunoregulatory effects. Pure lactate could only partially explain the effects observed with the CFS. Although IL-10 induction is suggested to be a regulatory mechanism employed by lactobacilli, we still observed potent cytokine-dampening activity in the absence of IL-10. In \textbf{paper III}, we aimed to characterize the immune regulatory activity of the lactobacilli-CFS in more detail. Lactobacilli can modulate adaptive immunity through both direct and indirect mechanisms. CD4\textsuperscript{+} T cell proliferation is reduced in a direct manner through a T cell intrinsic mechanism\textsuperscript{161}. In addition, lactobacilli induce or modify APC-derived cytokine and chemokine responses, which indirectly affects subsequent T cell responses\textsuperscript{232,233}. Therefore, we investigated whether or not the IFN-\(\gamma\) dampening activity occurred through a direct or indirect mechanism. LGG was not able to dampen IFN-\(\gamma\) in monocyte-depleted PBMC or in isolated T cell cultures. This clearly supported an indirect mechanism where LGG dampened IFN-\(\gamma\) production via monocytes. There
are mainly two ways in which monocytes regulate T cell responses. Monocytes and other APCs express surface-bound co-stimulatory (e.g. CD80 and CD40L) or inhibitory receptors (e.g. galectin-9 and PD1) that shape the subsequent T cell response\textsuperscript{234}, which naturally require cell-to-cell contact. In addition, secretion of pro- and anti-inflammatory cytokines by activated APCs will also regulate T cell responses, however, independent of cell-to-cell contact. Further experiments showed that the dampening activity could be transferred via LGG-primed monocyte conditioned medium suggesting that LGG induced the secretion of a monocyte-derived soluble inhibitor. Since there are several secreted negative inhibitors, we subjected the LGG-primed monocyte conditioned medium to a proteomic array analysis. Interestingly, in addition to a number of chemokines, only two candidate molecules likely to be involved in dampening of cytokine responses were detected, namely IL-10 and IL-1ra. Since we already confirmed an IL-10 independent mechanism in paper II, we focused our attention on IL-1ra. IL-1ra is an antagonistic ligand that competes for IL-1R binding with IL-1α/β. The indication of a possible role for IL-1 signalling in IFN-γ and IL-17 responses is reasonable and supported by other studies\textsuperscript{235–238}. More importantly, we detected IL-1β production upon stimulation with \textit{S. aureus} and addition of recombinant IL-1ra to \textit{S. aureus}-stimulated PBMC resulted in a dose-dependent dampening of IFN-γ. Although \textit{S. aureus} also induced secretion of IL-1ra, the ratio of IL-1ra/IL-beta was significantly higher in response to LGG compared to \textit{S. aureus}. Interestingly, anti-CD3/CD28\textsuperscript{239} stimulation as well as SEs\textsuperscript{240} have been shown to induce IL-1β production, which further supports that lactobacilli-dampening involves regulation of IL-1 signalling. It should, however, also be noted that although the proteomic array included 105 cytokines and chemokines, several other known secreted inhibitory molecules, such as IL-18Bp, IL-36ra, IL-37 and IL-38 was not included in the array. Furthermore, we cannot completely exclude the possibility that lacto-
bacilli-mediated dampening also involves surface receptor, such as down- or up-regulation of co-stimulatory and inhibitory receptors, respectively.

Extensive efforts have been made to identify immune regulatory factors produced by lactobacilli in order to better understand the mechanisms behind the effects of probiotic supplementation. Crude size fractionation of the CFS using centrifugal columns with filters of different pore sizes revealed that the cytokine-dampening activity was present in two separate fractions, the smallest (less than 3 kDa) and the largest (equal to or larger than 100 kDa) fractions. The dampening activity of the low molecular weight (Mw) fraction was expected as we already confirmed, in paper II, IFN-γ-dampening activity using pure lactate, which should be present in the lowest Mw fraction. Moreover, the low Mw fraction was able to reduce IFN-γ expression in conventional T cells, as opposed to pure lactate, confirming that additional low Mw factors with cytokine regulatory activity are indeed produced by LGG. Lactobacilli ferment complex carbohydrates into various SCFAs, such as butyrate, propionate and acetate, with documented effects on host metabolism and immune functions. These SCFAs inhibit NFκB-mediated activation of TNF production and butyrate and propionate positively influence the generation of peripheral TREG cells. Monocytes stimulated with S. aureus in the presence of butyrate produce less IL-12 and more IL-10. Interestingly, the down-regulation of IL-12 was found to be independent of IL-10 induction. Whether or not SCFAs are produced by our strains of lactobacilli, under our growth conditions, remains to be confirmed. However, the proportion of CD25^+FOXP3^+CD127^low among CD4^+ T cells or the level of FOXP3 expression was not affected by treatment with L. reuteri DSM 17983 (paper II), suggesting that the levels of butyrate or propionate are low or not being produced at all under our growth conditions. Lactobacillus-derived histamine has been shown to have immunomodulatory effects, however we have previously ruled out the involvement of histamine in the lactobacilli-mediated regulation of IFN-γ by our strains. Further, another study using
histamine-mutant strain of *Lactobacillus* confirmed the ability of lactobacilli to dampen IL-1β-driven IL-8 production in the absence of histamine\textsuperscript{246}.

The dampening activity of the high Mw fraction was intriguing; especially since further fractionation using high performance liquid chromatography (HPLC) suggested a very large and broad size distribution of the activity. Extracellular polysaccharides (EPS) typically form large polymers ranging in size up to 1.6 MDa and would thus be consistent with the HPLC fractionation\textsuperscript{247,248}. Furthermore, *Lactobacillus*-derived EPS have been shown to regulate inflammatory pathways in mice\textsuperscript{249}. However, based on the protein absorbance chromatogram, generated during HPLC fractionation, we also identified five main protein peaks within the high Mw fraction in total. Interestingly, the first one was distinctly smaller in the growth medium control and correlated well with the fractions exhibiting cytokine-dampening activity. Few proteins secreted by lactobacilli have a molecular mass larger than 100 kDa\textsuperscript{250,251} including the secreted probiotic proteins p40 and p75. Therefore, we subjected the high Mw fraction to heat treatment or proteinase K-mediated digestion prior to stimulations. This revealed that the active factor was resistant to enzymatic proteolysis while still susceptible to heat inactivation. This, seemingly contradictory results, lead us to explore the involvement of extracellular MVs. MVs are spherically shaped exosome-like vesicles enclosed by a lipid membrane and have been shown to have diverse functions in bacterial communication, virulence and host-interactions\textsuperscript{252}. Furthermore, *Lactobacillus*-derived MVs have been shown to contain proteins\textsuperscript{171,172}, which due to the membrane enclosure should be protected from enzymatic digestion while still susceptible to heat inactivation. Indeed, we found that depletion of lipids resulted in a complete loss of dampening activity and isolated *L. reuteri*-derived MVs recapitulated the cytokine-dampening activity. Several studies have reported immunoregulatory and stimulatory activities with isolated *Lactobacillus*-derived MVs, e.g. promoting IgA production\textsuperscript{174}, amelioration of *S. aureus*-induced AD\textsuperscript{176} and induc-
tion of apoptosis in hepatic tumour cells. The immunomodulatory mechanism(s) of bacterial MVs remain elusive but some studies have reported intracellular uptake of MVs. In mast cells, MVs were shown to be internalized by a phagocytosis-independent manner and in epithelial cells via lipid rafts in vitro. We were unfortunately not able to investigate the mechanism of how MVs dampen IFN-γ and IL-17A in our settings; however, increased production of IgA by L. sakei-derived MVs was mediated through TLR-2. Indeed, it has been shown that Lactobacillus-derived MVs contain exposed LTA on the surface, a known TLR-2 ligand, and it is thus likely that the MV-dampening effects we observed are dependent on PRR expressing monocytes.

The relevance of this finding is highlighted by reports linking gut-derived microbial MVs, found in circulation, to neurodegenerative disease and further supports the systemic effects exerted by gut-resident microbes, including lactobacilli.

Paper IV

In paper II, we observed activation of unconventional T cells and NK cells towards S. aureus-CFS. This raised a number of questions considering that S. aureus does not produce either of the two microbial antigens that typically activate MAIT cells and the majority of γδ T cells found in PBMC. In addition, pure SEA induced a similar response in unconventional T and NK cells while the SE-negative strain S. aureus 139:3 was unable to activate these cells (unpublished data). This indicates that, as for conventional T cells, SEs are the main stimulatory factors for unconventional T cells and NK cells as well. This is particularly interesting since NK cells lack expression of a TCR and although an alternative binding site for SEA on the γδ TCR has been reported, the relevance of this binding site is far from clear. In paper IV, we explored the underlying mechanisms of activation of these particular cell types using three different SEs (SEA, SEH and TSST-1). SEs share a high
level of homology in their tertiary structure but bind to unique Vβ chains, or Vα for SEH, of the TCR. If and how this affect the type of response induced by different SEs is not well known. We observed a striking similarity, despite differences in binding specificities, between different SEs with regards to induction of cytokine and cytotoxic responses. The only differences observed, which is consistent throughout all experiments and responses analysed so far, is that SEA tends to induce a stronger response with higher levels of cytokines and cytotoxic compounds, while SEH always results in weaker responses. This might reflect the difference in the number of potential TCR chains the SE can engage, where SEA is more promiscuous and binds to multiple chains, and can thus potentially engage a larger repertoire of T cells leading to increased cytokine responses. Furthermore, unlike TSST-1 and SEH, SEA has two binding sites for MHC class II and consequently has the ability to cross-link multiple MHC class II molecules, which has been suggested to enhance T cell activation\textsuperscript{257}. SEs have also been shown to induced MyD88-dependent signalling in monocytes with subsequent induction of cytokines upon binding to MHC class II\textsuperscript{258}. The relevance of this in regards to the higher potency of SEA to activate immune cells is uncertain, however, transcriptional activation of IL-6 and IL-12 was far greater in response to SEA compared with the two other enterotoxins.

Innate derived cytokines are important for optimal responses of both T and NK cells. We already confirmed the involvement of IL-12 in activation of IFN-γ by S. aureus-CFS and SEA. In paper IV, we decided to explore the possibility that other cytokines might be important as well. MAIT cells express high amounts of the IL-18R, however, blocking IL-18 or IL-15 did not significantly hamper the ability of SEs to induce IFN-γ in any cell type investigated, including MAIT cells. This was further supported by the fact that only IL-12 was transcriptionally activated, while no effect on IL-15 transcription was observed and IL-18 seemed to be down-regulated compared to
unstimulated cells. This is not unlikely since IL-18 is constitutently transcribed and stored intracellularly as an inactive form in resting monocytes.

MAIT cells and γδ T cells are typically activated by antigens presented through alternative MHC-like complexes, however, as SEs normally bypass the requirement for antigen specificity in conventional T cells, it is reasonable to assume that activation of unconventional T cells might also bypass MHC restriction. Research support the fact that SE-mediated activation of MAIT cells occur through a MR1-independent, but an MHC class II-dependent mechanism despite that MAIT cells are typically MR1 restricted. Furthermore, although MAIT cells have a fixed Vα chain, they do express a Vβ chain that could potentially be recognized by SEs that would use MHC class II for cross-linking and activation. Indeed, MAIT cells expressing the Vβ13.2 chain, which is the target chain for SEB, was shown to be required for SEB-induced activation of MAIT cells. We also explored whether or not γδ T and NK cells are activated in a direct manner or solely indirectly. Interestingly, when we co-cultured SE-primed monocytes with isolated γδ T cells or NK cells, we observed no activation in the absence of other T cells. This suggests that SE-mediated activation of these cells occur through an indirect mechanism. This is also supported by an in vivo study showing that γδ T cell responses to SEs is abolished in the absence of conventional αβ T cells. Our findings are in contrast with a study from Morita et al. that provided evidence for a specific binding site on the Vγ2-chain of γδ T cells, suggesting a direct activation of γδ T cells by SEA. However, the SEA concentration required for activation was orders of magnitude higher compared with conventional T cells and far higher than the concentrations we use in our stimulations.

The role of CD161 in immunity has been debated extensively. On T cells, it is suggested to be a co-stimulatory receptor, which also associates with pro-inflammatory T\textsubscript{REG} cells. Similar to paper I, we observed a significantly higher proportion of IFN-γ\textsuperscript{+} T cells within the CD161 expressing popula-
tion in response to all SEs investigated. However, in NK cells, there was an inverse correlation between IFN-γ and CD161 expression. CD161 signalling in NK cells have been linked to reduced NK cell activity and LLT-1-expressing tumours is associated with inhibition of NK cell-mediated anti-tumour activity\textsuperscript{78,261}. However, a recent study identified CD161\textsuperscript{+} NK cells as being a highly proliferative and cytokine producing cell population in response to IL-12 and IL-18 stimulation\textsuperscript{262}. This is in contrast to what we observed and intriguing considering that SE-induced activation of NK cells is most probably cytokine-mediated. However, the ligand for CD161, LLT-1, is induced on most APCs, B cells and T cells upon TLR and TCR activation\textsuperscript{75,263}, which might not be the case for IL-12 and IL-18 stimulation. This could be an explanation to the discrepancies observed. Furthermore, the relative amount of NK cell-expressed CD161 and LLT-1 may also be of importance. LLT-1 was shown to induce NK cell-derived IFN-γ production, when expressed on NK cells\textsuperscript{264}, but when LLT-1 is expressed on NK-target cells, it inhibits NK cell activity\textsuperscript{77}. Thus, it would be highly relevant to investigate LLT-1 expression in our experimental set-up and its potential role in SE-mediated activation of NK cells.
General Conclusions

In this thesis, we have investigated the mechanistic pathways involved in bacterial induction and regulation of peripheral immune responses using two rather different bacterial model organisms, *S. aureus* and *Lactobacillus*. Collectively, we have shown that *S. aureus*, and in particular its enterotoxins, elicits a broad and potent inflammatory response in diverse subsets of T cells (including T cells with a regulatory phenotype) and NK cells, which occur in a direct manner in conventional T cells but indirectly in γδ T- and NK cells. It is also important to remember that these observations are derived from studies performed *in vitro*, using mostly one particular strain of *S. aureus*, which does not entirely reflect the conditions of a living host. Still, this work underlines the complexity of *S. aureus*, its enterotoxins and their capacity to interact with specific immune cells and extends the general understanding of how superantigens are able to evoke such a ferocious and systemic immune activation.

Numerous studies over the years have collectively yielded an image of probiotic lactobacilli as potent immune modulators with diverse abilities to interact with and modify the behaviour of host cells. The results provided here, further supports the many different factors and pathways involved in lactobacilli-mediated regulation of immunity. In addition to low molecular weight compounds such as lactate, we also identified lactobacilli-derived MVs as a significant contributor to the regulation of pro-inflammatory cytokine production. To our knowledge, this represents a novel pathway in which lactobacilli can counteract induction of IFN-γ and IL-17 and thus expands the general understanding of bacterial mechanisms of immune regulation.

The work presented in this thesis highlights the complex interactions between bacteria and the immune system and offers a stepping-stone for the direction of future studies regarding bacterial regulation of immunity.
Future Perspectives

Through the work presented in this thesis, we have gained much knowledge regarding important pathways involved in bacterial activation and modulation of peripheral immune responses. Many questions have been answered, but of course, many more still remain.

The role of CD161 expression in T and NK cell activation, especially in relation to *S. aureus*, deserves further attention. Is CD161 simply a marker for highly activated T cells or is CD161-mediated signalling a requirement for optimal cytokine production in our context? The only documented ligand for CD161 is LLT-1, which is known to be up-regulated on APCs, T- and NK cells upon activation. Therefore, it would be interesting to investigate the expression patterns of LLT-1 in PBMC cultures after *S. aureus* or SE stimulation. Blocking of CD161-LLT-1 interaction during stimulations would also provide additional insights into whether or not active signalling is important for the differential association of CD161 in activation of T- and NK cells.

In paper II and III, we investigated the molecular mechanisms behind the immune-dampening activity of soluble factors derived from two different species of lactobacilli. In both projects, we identified low molecular weight compounds, including lactate, to exert broad immune-dampening activity on T- and NK cells. However, our findings also suggest that small compounds other than lactate are involved. What these are and how they interact with immune cells needs further investigation. Moreover, lactobacilli-derived MVs clearly dampen pro-inflammatory cytokine production, however, by what mechanism remains to be solved. For example, what role does monocyte-expressed surface receptors play? MVs also contain several immunostimulatory components including DNA and proteins, if and how these molecules are involved would be relevant to investigate further.
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