Functional and phenotypic profiling of innate immunity during Salmonella infection

Sørensen, Rikke Brandt

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PhD thesis by
Rikke Brandt

Systems Biology of Immune Regulation
Center for Biological Sequence Analysis
Department of Systems Biology
Technical University of Denmark
DK-2800 Kgs. Lyngby
Preface

This PhD thesis is the product of my PhD studies at Center for Biological Sequence Analysis at Systems Biology, Technical University of Denmark in the period between September 2007 and January 2012. It was financed by The Danish Council for Strategic Research through the project entitled “PreGI - Prebiotics for Prevention of Gut Infections”.

First and foremost I wish to thank my main supervisor Susanne Brix for taking me in and for her enthusiastic guidance and support throughout the project. She is incredibly inspiring to work with, encouraging and I am awestruck by the infinite knowledge she has on so many different things.

I also want to deeply thank my co-supervisor Hanne Frøkiær for giving me the opportunity to engage in the PhD study, for her ever-open door, her guidance and support and for being an inspiration.

I am very grateful to past and present members of the Systems Biology of Immune Regulation group and want to thank Jeppe Madura Larsen, Jonas Nørskov Søndergaard, Anna Thysen, Camilla Ingvorsen, Stina Jensen and Janne Marie Laursen for advice, great sparring and encouragement and for always being great company and ready to share a pizza. Very special thanks go to Lisbeth Buus Rosholm for all her help in the lab, her wonderful personality and for being my office mate and friend and the other half of “Mormor & Mormor”.

Many other people deserve my gratitude and acknowledgement for their great work and help; special thanks to Anni Mehlsen, Pernille Wehler Güllich, Kate Vibefelt, Anne Ørngreen and the staff at the animal facility in Mørkhøj and to Ole Lund and Tine Rask Licht for all their help. Supervising Mette Theilgaard Sørensen and Kishwar Musa during their master thesis work and Hanne Holbæk Jensen through her bachelor thesis has been enriching experiences and I am very grateful for these.

Most importantly, I want to thank my family and friends for their never-ending support, and the two men in my life; Christian and Sebastian, without whose love, support (‘Mom, why haven’t you finished your PhD yet?’) and understanding I never would have made it through.

Rikke Brandt
Kgs. Lyngby, January 2012
Summary

Salmonellae are food borne pathogens, typically acquired by the oral ingestion of contaminated food or water, causing disease in both healthy and immunocompromised individuals. To gain insight into early immune regulation events caused by Salmonella as well as inflammatory signatures induced by Salmonella and other bacteria in human monocyte-derived dendritic cells (DC), we examined these properties using in vivo and in vitro experimental settings.

The outcome of infection with Salmonella depends on the host as well as the infecting serovar. Understanding the relative risks associated with and within different serovars is of major importance for public health. Using an established mouse model, we compared the pathogenicity of two S. Typhimurium strains (SL1344 and DT120) and found that the passage through and the ability to proliferate within the host gastrointestinal system determined the pathogenicity of these strains.

Salmonella is a mucosal pathogen, gaining access to host systemic circulation by crossing the gut epithelial barrier and residing intracellularly in DC and Mφ. Until recently focus has been centred on the involvement of Mφ and the conventional antigen-presenting DC (mDC) in bacterial infections, whereas the other major dendritic cell subset, plasmacytoid DC (pDC), plays an important part in antiviral responses, and is less well characterised in regard to antibacterial immunity. Using multi-parametric flow cytometry, we were able to show for the first time that pDC accumulated in Peyer’s patches 24 hours after murine oral Salmonella challenge and while Mφ and mDC exhibited dose-related cellular atrophy, pDC were less susceptible to bacteria-induced cell death, suggesting a role for pDC in early stage Salmonella containment. Furthermore, we identified a number of both DC and Mφ subsets, two of which following infection, accumulated in Peyer’s patches and lamina propria, respectively.

Generally, we tend to set apart pathogenic bacteria from opportunistic pathogens and commensal bacteria based on their abilities to induce disease in different hosts, however, the nature of the inflammatory response they induce in DC that set them apart from commensal bacteria remains largely unclear. In the present study, we developed a system by which we were able to compare the bacteria-induced imprint of important regulatory proteins in DC to bacterial-encoded ligands. We observed that DC responded to six different bacteria in a phyla-specific manner giving rise to similar inflammatory signatures within the groups of proteobacteria, firmicutes and actinobacteria, hence being independent on pathogenic versus non-pathogenic properties, and also on the bacteria-to-cell ratio for most bacteria. The results presented in this thesis add to the current knowledge about innate immunity to Salmonella, suggest new host immune cell subsets important for bacterial containment and provide a basic understanding of bacteria-induced DC inflammatory programs. The two latter could prove important in regard to treatment regimes, as targeted modulation of DC profiles for instance by probiotics, could lead to improved therapy for a number of gut related diseases.
Sammenfatning

Salmonellae er fødevare-bårne patogener, som typisk erhverves ved oral indtagelse af inficerede fødevarer eller vand, og som forårsager sygdom i både raske og immunkompromitterede personer. For at opnå indsigt i tidlig immunregulering forårsaget af Salmonella, såvel som inflammatoriske signaturer induceret af Salmonella og andre bakterier i humane monocytt-afledte dendritiske celler (DC), undersøgte vi disse egenskaber ved brug af in vivo og in vitro eksperimentelle metoder.

Udfaldet af en Salmonella infektion afhænger af værten samt den inficerende serovar. Forståelse for de relative risici associeret med og indenfor forskellige serovar er af afgørende betydning for vores helbred. Ved brug af en etableret muse-model sammenlignede vi patogeniciteten af to S. Typhimurium stammer (SL1344 og DT120) og fandt, at passagen gennem og evnen til at proliferere i værtens fordøjelsessystem, var bestemmende for patogeniciteten af disse stammer.

Salmonella er en mucosal patogen, som får adgang til værtens systemiske cirkulation ved at penetrere tarmbarrieren og residere intracellulært i DC og Mφ. Indtil for nylig har fokus været centreret omkring involveringen af Mφ og den konventionelle antigen-præsenterende DC (mDC) i bakterielle infektioner, mens det andet store DC subset, plasmacytoide DC (pDC), spiller en vigtig rolle i anti-virale responser, men er mindre godt karakteriseret i forhold til anti-bakteriel immunitet. Ved at anvende multi-parametrisk flow cytometri var vi i stand til for første gang at vise, at pDC akkumulerer i Peyer pletter 24 timer efter murin oral Salmonella infektion og mens Mφ og mDC udviste dosis-relateret cellulær atrofi, var pDC mindre påvirkede af bakterie-induceret celledød, hvilket peger på en rolle for pDC i inddæmning af Salmonella tidligt under infektion. Ydermere identificerede vi en del både DC og Mφ subsets, hvoraf to akkumulerede i henholdsvis Peyer pletter og lamina propria efter infektion.

Generelt har vi en tendens til at adskille patogene bakterier fra opportunisteriske patogener og kommensale bakterier baseret på deres evne til at forårsage sygdom in forskellige værter, men typen af det inflammatoriske respons, de inducerer i DC, og som adskiller dem fra kommensale bakterier, er stadig uklart. I dette studie udviklede vi et system som tillod os at sammenligne de bakterier-inducerede præg på vigtige regulatoriske proteiner i DC. Vi observerede at DC responderede på seks forskellige bakterier på en phyla-specifik måde, hvilket gav anledning til inflammatoriske signaturer indenfor grupperne proteobakterier, firmicutes og actinobakterier og dermed uafhængigt af patogene versus non-patogene egenskaber og for de fleste bakterier også af bakterie-til-celle ratioen.

Resultaterne, der præsenteres i denne afhandling udbygger den nuværende viden omkring medfødt immunitet i forhold til Salmonella, indikerer nye værts immun-celle subsets som kan være vigtige for bakterie-inddæmning og giver en basal forståelse af bakterie-inducerede DC inflammatoriske programmer. De to sidste kan vise sig at være vigtige i forhold til behandlingsregimer, eftersom
targeteret modulering af DC profiler, for eksempel ved brug af probiotika, kan lede til forbedrede behandlinger for en del mave- og tarmrelaterede sygdomme.
List of original papers included in this thesis

This thesis entitled “Functional and phenotypic profiling of innate immunity during Salmonella infection” is divided into five chapters. Chapter 1 serves as an introduction to the current status on knowledge and perspectives regarding innate immunity to S. Typhimurium and Chapter 5 summarises and discusses the conclusions of the entire thesis.

The unsubmitted manuscript in Chapter 4 revolves around modulation of the functional phenotypic profile in human monocyte-derived DC phenotypic profiles, depending on the strain of bacteria encountered. Here we have used gut commensals (Bifidobacterium longum, Lactobacillus acidophilus & Escherichia coli Nissle), opportunistic pathogens (Clostridium difficile) and intracellular pathogens (Listeria monocytogenes & S. Typhimurium) and find the functional DC phenotypes to be modulated in a phyla-specific manner rather than determined by the nature of the bacteria (e.g. whether they are commensals or pathogens). This work is not yet finalised, as we also wish to map bacterial genome signatures to the functional DC profile by analysing which bacterial traits, such as virulence traits and/or genes involved in LPS or PGN synthesis that lead to the production of specific cytokines in the DC and thus the resulting DC phenotype.

Chapter 2:

Chapter 3:

Chapter 4:

Appendix:
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ag</td>
<td>Antigen</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
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<tr>
<td>CDP</td>
<td>Dendritic cell-restricted progenitor</td>
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<tr>
<td>CLP</td>
<td>Common lymphoid progenitor</td>
</tr>
<tr>
<td>CMP</td>
<td>Common myeloid progenitor</td>
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<tr>
<td>CCR</td>
<td>Chemotactic cytokine receptor</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FACS</td>
<td>Flow assisted cell sorting</td>
</tr>
<tr>
<td>FAE</td>
<td>Follicle-associated epithelium</td>
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<tr>
<td>Flt3L</td>
<td>FMS-like tyrosine kinase 3 ligand</td>
</tr>
<tr>
<td>GALT</td>
<td>Gut-associated lymphoid tissue</td>
</tr>
<tr>
<td>GC</td>
<td>Germinal centre</td>
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<tr>
<td>GP2</td>
<td>Glycoprotein 2</td>
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<tr>
<td>HEV</td>
<td>High endothelial venules</td>
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<tr>
<td>HSC</td>
<td>Haematopoietic stem cell</td>
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<tr>
<td>iDC</td>
<td>Immature dendritic cell</td>
</tr>
<tr>
<td>IEL</td>
<td>Intraepithelial lymphocyte</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>LP</td>
<td>Lamina propria</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>M cell</td>
<td>Microfold cell</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-associated protein kinase</td>
</tr>
<tr>
<td>mDC</td>
<td>Myeloid dendritic cell</td>
</tr>
<tr>
<td>MDP</td>
<td>Macrophage and dendritic cell precursor</td>
</tr>
<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MLN</td>
<td>Mesenteric lymph node</td>
</tr>
<tr>
<td>Mφ</td>
<td>Macrophage</td>
</tr>
<tr>
<td>NK cell</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>Nramp1</td>
<td>Natural resistance-associated macrophage protein 1</td>
</tr>
<tr>
<td>NTS</td>
<td>Non-typhoidal <em>Salmonella</em></td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PCA</td>
<td>Principal component analysis</td>
</tr>
<tr>
<td>pDC</td>
<td>Plasmacytoid dendritic cell</td>
</tr>
<tr>
<td>PEEC</td>
<td>Pathogen elicited epithelial chemoattractant</td>
</tr>
<tr>
<td>PMN</td>
<td>Polymorphonuclear leukocyte</td>
</tr>
<tr>
<td>PP</td>
<td>Peyer’s patches</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>RA</td>
<td>Retionic acid</td>
</tr>
<tr>
<td>SLC11A1</td>
<td>Solute carrier family of multimembrane spanning protein 1</td>
</tr>
<tr>
<td>SCV</td>
<td>Salmonella containing vacuole</td>
</tr>
<tr>
<td>SED</td>
<td>Sub-epithelial dome</td>
</tr>
<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signalling</td>
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<tr>
<td>SPI</td>
<td><em>Salmonella</em> pathogenicity island</td>
</tr>
<tr>
<td>T1SS</td>
<td>Type 1 secretion system</td>
</tr>
<tr>
<td>T3SS</td>
<td>Type 3 secretion system</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>Tc</td>
<td>Cytotoxic T cell</td>
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<tr>
<td>Tfh</td>
<td>Follicular T helper cell</td>
</tr>
<tr>
<td>TGF</td>
<td>Tranforming growth factor</td>
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<tr>
<td>Th</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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1 Introduction

Dendritic cells
Dendritic cells (DC) are so named because of their long membrane extensions that resemble dendrites of nerve cells, which allow them to intimately interact with antigens and other cells. In their capacity to recognize, process and present foreign antigen they are known as professional antigen presenting cells (APCs) and as such, they are essential mediators of both immunity and tolerance. Because of their unique ability to initiate differentiation of naïve T cells into effector or memory cells, they also serve as an essential link between the innate and adaptive immune system.

![Dendritic Cell Diagram]

**Figure 1.1.** Polarisation of naïve Th cells by DC into Th1, Th2, Th17 or regulatory T cells (Tregs), depending on the specific cytokines produced by the DC upon activation of various PRRs.

Naturally occurring Tregs are not depicted in this figure, as they are educated in the thymus and widely accepted as a separate Th cell lineage. Arrows from the naïve T helper cell towards any Th-type indicates polarisation, whereas cytokines above or below each arrow pertains to cytokines secreted by the DC or present in the local environment. Cytokines in blue writing are required for Th differentiation exclusively in the humans. RA; retinoic acid.

Like other leukocytes, DC are derived from haematopoietic stem cells (HSC) in the bone marrow. Immature DC (iDC) are widely distributed within all tissues, particularly those that interface with the
environment, and in lymphoid organs. They have high phagocytic capabilities and capture antigens by using a range of cell-surface receptors, collectively known as pattern recognition receptors (PRR). Among others, PRR comprise the C-type lectin family and Toll-like receptors (TLR), which recognize pathogen-associated molecular patterns (PAMP) such as specific glycan structures, lipopolysaccharide (LPS) and lipoteichoic acid, stemming from both pathogens and commensal bacteria. iDC also express a variety of chemokine receptors, such as CCR2, CCR5, CCR6, CXCR1 and CXCR2 (31, 122), enabling them to respond to and migrate towards inflammatory chemokines produced at sites of inflammation (35). Upon antigen recognition by PRR, activated iDC migrate to T cell areas in lymph nodes whilst undergoing a maturation process in which the cell is structurally reorganized, leading to loss of antigen-capturing abilities. Maturing DC upregulate CCR7 which directs migration to lymph nodes, major histocompatibility complex (MHC) I and II for antigen presentation, co-stimulatory molecules such as CD80, CD86 and CD40 and start secreting cytokines needed to prompt the appropriate T cell response against the antigen. Depending on the antigen or microbe encountered by the DC, different cytokines and co-stimulatory molecules will be upregulated to ensure that the resulting Th cell responses will be the most optimal for eradication of bacteria, virus, fungi or parasite. This process called Th polarisation by DC is illustrated in Figure 1.1 and is so named, because promoting one Th response will suppress the others. Previously, T cell commitment was believed to be an irreversible, terminal process, however, today the functional plasticity of T cell subsets is gaining recognition, as there is increasing evidence that for instance Th17 cells can convert to Th1 cells (83).

In the presence of an intracellular pathogen, a cytotoxic CD8+ T cell-promoted immune response is warranted in order to kill off infected host cells. This type of immunity is prompted by propagation of enhanced numbers of naïve antigen-specific Th cells into the so-called Th1 cells. Th1 cells secrete interferon (IFN)-γ and help in activation of antigen-specific cytotoxic T cells via CD40L–CD40-feedback activation of DC presenting antigen in the context of MHC class I molecules to the cytotoxic T cells. Moreover, Th1 cells migrate to infected tissues, where they induce activation of macrophages (Mφ) in an antigen-specific manner via CD40L–CD40 and IFN-γ–IFN-γR interactions. The cytotoxic T cells kill infected host cells displaying pathogenic antigen on MHC class I molecules. The infected host cells turn apoptotic and are then removed via phagocytosis by the activated tissue Mφ. Exacerbated and uncontrolled Th1 responses can result in host tissue destruction leading to development of autoreactive CD8+ T cells and establishment of autoimmune reactions.

While activation of Th1 cells are needed to fight intracellular infections, Th2 cells are necessary to eradicate extracellular infections. Th2 cells promote plasma B cell development, class-switching of immunoglobulin (Ig) to IgE, maturation and activation of eosinophils and are necessary in order to induce an antigen-specific antibody response against any extracellular pathogens. As IgE and eosinophils are also involved in allergic inflammation, an unrestrained Th2 bias may lead to development of allergies.
Th17 cells also play a major role in combating extracellular infections. To propagate Th17 differentiation within the lymph node, DC need to produce interleukin (IL)-1β and IL-6 in humans, and transforming growth factor (TGF)-β and IL-6 in the mouse (3, 11). In both mammals, DC-derived IL-23 is required for expansion and survival of Th17 (23, 157). After activation, they migrate into microbial-exposed tissues and are thus primarily found at the interfaces between the external an internal environment, such as the lining of the gastrointestinal tract, the skin and the upper airway, and are important in fighting off especially fungi and bacteria. They are known to exert their effect by inducing the epithelia to secrete defensins and by recruiting neutrophils to the site. Th17 bias is implicated in autoimmune disorders, such as chronic inflammatory bowel disease (152).

A fourth Th subset is constituted by regulatory T cells, or Tregs, and their most prominent function is to maintain self-tolerance and immune homeostasis. Tregs are broadly classified into natural or induced Tregs and the regulatory capacity of both cell types is believed to be conferred by constitutively high levels of the transcription factor Foxp3 (155). Natural Tregs develop in the thymus and induced Treg cells attain their regulatory capacities in the peripheral lymphoid organs. During homeostasis, CD103+ gut DC can induce Tregs via their capacity to release TGF-β and retinoic acid (RA) and upon activation, these Tregs (like natural Tregs) produce IL-10, TGF-β and IL-35 (26, 29). Tregs are also important in re-establishing immune homeostasis following pathogen clearance, as they suppress Th1, Th2 and Th17 responses. Regulatory T cells that lack expression of Foxp3 have also been identified. They are characterised by their high production of IL-10 and termed Tr1 cells (52).

Two additional Th subsets exist, termed follicular Th (Tfh) cells and Th9 cells. Tfh are enriched in the edge of B cell zones, follicular regions and germinal centres (GC). They develop under the influence of IL-6 and autocrine IL-21 (106, 131) and are specialised to regulate antigen-specific B cell immunity (16, 124). Th9 cells appear to be involved in intestinal responses to helminths (75), which were previously believed to be mediated by the Th2 response. Propagation of Th9 cells requires IL-4 and TGF-β and they are characterised by their secretion of IL-9 (144). These two subsets are not described in further details, as they are not relevant to this thesis work.

**Origin of DC**

During haematopoiesis, HSC give rise to common myeloid progenitors (CMP) and common lymphoid progenitors (CLP). Monocytes, Mφ, megakaryocytes, granulocytes and erythrocytes originate from CMP, while T cells, B cells and natural killer cells originate from CLP (4, 77), see Figure 1.2.
Figure 1.2. Origin and differentiation of DC from haematopoietic stem cells (HSCs) in the bone marrow.
Common lymphoid precursor (CLP); common myeloid precursor (CMP); monocyte, macrophage and DC precursor (MDP); DC-restricted progenitor (CDP). Monocytes and pre-DC migrate through the blood into various lymphoid tissues, where they give rise to different subsets. In addition to CMPs, CLPs also have the potential to differentiate into DC, however, their role remains to be firmly established. Coloured boxes represent terminally differentiated cells. Adapted from Kushwah & Hu (79).

DC are traditionally believed to be of myeloid origin; however, quite a few studies indicating that both progenitors can give rise to DC have challenged this view (36, 74, 102, 148, 150). CMPs give rise to a progenitor (MDP) which only differentiates into monocytes, macrophages (Mφ) and DC, and MDP then further differentiates into a DC-restricted progenitor (CDP) (31). CDPs can then differentiate into either plasmacytoid DC (pDC) or pre-DC, which will then migrate into various lymphoid tissues giving rise to the different myeloid DC subsets. pDC in humans express CD123, BDCA-2 and BDCA-4, but unlike other DC, they do not express CD11c. In mice, pDC are positive for B220, PDCA-1 and Siglec-H, low for CD11c and negative for CD11b. In both mice and humans, these cells express TLR7 and TLR9 and upon activation, they produce type I interferons (IFN), which are critical for combating both viruses and bacteria.

Apart from this ‘strict’ DC developmental pathway it was recently discovered that monocytes also can give rise to DC both during inflammation and steady state (79). Murine monocytes are classified into 2 subsets: Ly6C\textsuperscript{hi} and Ly6C\textsuperscript{lo}. The former have been shown to give rise to CD103\textsuperscript{+} DC in the intestinal lamina
propria (LP), while the latter, following phagocytosis of apoptotic thymocytes, could differentiate into immunosuppressive DC in the spleen (12, 110).

The gut associated lymphoid tissue
The gastrointestinal tract, and in particular the mucosa, is the primary route by which we are exposed to antigens. It is the natural habitat of the commensal microbiota, but also the preferred entry site for most pathogens, and as such must be able to mount appropriate protective immunity. The intestinal epithelium, consisting of only a single cell layer, has a vast surface area of the order 400 m² and functions as a selective barrier allowing the passage of nutrients, while preventing the uptake of detrimental agents. Thus, it serves as a first line of defence, reducing the requirement for proinflammatory systemic immunity.

This barrier is part of the largest immune system in the body, collectively known as the gut-associated lymphoid tissue (GALT), which also comprises tonsils, adenoids, Peyer’s patches (PP), lymphoid aggregates in the oesophagus, stomach, appendix and large intestine, intraepithelial lymphocytes (IEL), mesenteric lymph nodes (mLN) and the LP. Because the primary function of GALT is to discriminate between harmful and beneficial microbes entering the tract while tissue destruction needs to be kept at a minimum, the environment within GALT is primarily tolerogenic and the default immune pathway is tolerance induction (99, 149).

The GALT of the small intestine can be divided into loosely organized effector sites, which include LP and IEL, and more organized structures, such as mLN, PP, isolated lymphoid follicles and cryptopatches.

The intestinal epithelium
The mucosa of the small intestine is a highly complex structural network composed of several tissue layers, as shown in Figure 1.3. The luminal surface is completely covered by villi, the core of which is an extension of the LP. At the bases of villi are tubular structures, called intestinal glands or crypts of Lieberkühn.

The intestinal epithelium consists primarily of enterocytes, adhered to one another by tight junctions, and on which the microvilli are coated with a rich glycocalyx of mucous, antimicrobial proteins, secretory IgA and other glycoproteins (90). This structure is also known as the brush border; it provides a large surface area for absorption and contains many of the digestive enzymes and transporter systems involved in metabolism. In addition to enterocytes, the epithelium also entails goblet cells, Paneth cells, entero-endocrine cells, microfold (M) cells and stem cells, the latter giving rise to all the other cell types (17, 24). Stem cells are found at the base of the crypts, and as they mature they move upwards toward the lumen.
Figure 1.3. Tissue layers of the small intestine.
The small intestine in both humans and other placental animals is divided into three structural parts: duodenum, jejunum and ileum. Microscopically these parts are in many ways similar and consist of a columnar epithelium overlying the connective tissue, also known as LP, 2 layers of smooth muscle tissue, followed by connective tissue and finally epithelial tissue which borders the mesentery of the peritoneum. Reprinted from Reece et al. (114) by permission of Pearson Education, Inc., Upper Saddle River, NJ.

Therefore, the differentiated, functional cells are found mainly on the villi and, as they become senescent, are shed into the lumen (14).
Goblet cells are mucin-secreting cells found interspersed among the other cell types, whereas Paneth cells are only found in the crypts. When exposed to bacteria or bacterial antigens, Paneth cells secrete various antimicrobials into the lumen (7), thereby helping to maintain the gut barrier by sequestering gut microbes. A schematic drawing of GALT can be seen in Figure 1.4.

In the following section, focus will be on the GALT of the murine small intestine only, as the human GALT is out of the scope for this thesis work.
Figure 1.4. Micro-architecture of the gut-associated lymphoid tissue.
To the left a villus containing DC (purple), Mφ (blue), plasma cells (green) and intraepithelial lymphocytes (yellow) dispersed in the LP is shown. In the middle a crypt with Paneth cells, stem cells and goblet cells, and on the right a Peyer’s patch with germinal centres and T cell rich areas is shown. The mLN (not drawn to scale) drains both LP and the PP. Inspired by artwork published in Nature Reviews Immunology. © Rikke Brandt, 2011.

Peyer’s patches

PP are mainly localized opposite the mesentery and are important sites for induction of intestinal immune responses (123). In the human and murine small intestine, PP are found both in duodenum and jejunum, but the majority are concentrated in the distal ileum (30, 89, 140). They are not contained within any form of capsule, they do not have sinuses running through, and as they do not lie along the pathway of lymphatic ducts, they cannot be described as lymph nodes. Rather, they are secondary lymphoid organs, consisting of 3 compartments; the follicular area, the interfollicular area and the follicle-associated epithelium (FAE) (103), as shown in Figure 1.5.
Figure 1.5. Peyer’s patches of the small intestine.
As shown in the left hand picture, these structures are visible to the naked eye. Facing the lumen (right hand picture) is the FAE, devoid of microvilli but rich in M cells. Directly underneath are APCs such as DC, followed by the follicular area, consisting of germinal centres rich in B cells, and the interfollicular area containing mostly T cells. Images from [http://www.ppdictionary.com/bacteria/gnbac/enterocolitica.htm & http://www.bu.edu/histology/p/12006ooa.htm].

The follicular area comprise the PP lymphoid follicles consisting of B cell rich zones with germinal centres (GC), while the interfollicular area contains mostly T cells, located around high endothelial venules (HEV) (72). Naïve lymphocytes enter PP via HEV (151) and leave via efferent lymphatics, originating on the serosal side of the PP, to the mLN. The follicle is surrounded by the sub-epithelial dome (SED), a mixed-cell zone harbouring both B and T cells, Mφ and a dense network of DC.

M cells are found in the FAE overlying PP. Instead of microvilli, they have microfolds on their luminal surface, and since they lack the surface glyocalyx, they are well suited for transepithelial transport of various antigens. As such, they are the primary target for many pathogens seeking access to deeper tissues. They take up antigens by endo- or phagocytosis into vesicles that are transported through the interior of the cell (99). The basolateral membrane of M cells is extensively folded (M cell pocket) and surrounds adjacent lymphocytes (78) and APCs in the SED providing direct delivery of luminal antigens, thus enabling initiation of antigen-specific mucosal immune responses (2).

In murine PP, 3 subsets of CD11c⁺ DC have been identified. Adjacent to M cells in the SED, CD11b⁺ DC reside, while CD8α⁻CD11b⁺/⁻ are found within the T cell rich interfollicular regions. The third subset is a double negative (CD8α⁻CD11b⁻) which can be found in both locations. PP DC can also be discriminated in terms of their expression of CX3CR1, CCR6 and CCR7 (104, 121). CX3CR1 are closely associated with the FAE in the steady state, ideally located for bacterial capture; however, the initiation of an immunological response to bacteria is restricted to CCR6⁺ DC (121). These DC largely fall into the CD11b⁺ and double negative subsets and are recruited from the SED to the FAE during bacterial infection. All DC in the interfollicular region express CCR7, while a CD11b⁺ subset in the SED was found to upregulate this receptor and migrate into the interfollicular region after systemic stimulation with a *Toxoplasma gondii* antigen. Incidentally, this CD11b⁺ subset also expresses CCR6 and is involved in
induction of IgA responses to orally administered antigen (28). In terms of T cell polarization, the CD11b+ subset has a higher capacity to produce IL-10 and thereby prime Th2/Treg cells, whereas CD8+ and DN DC produce IL-12p70 and prime towards a Th1 response (66) (see Figure 1.1). Recently, a DC subset expressing the αE(CD103)β7-integrin was also described in GALT, but while these cells are present in the PP, DC in the dome region of solitary intestinal lymphoid tissue are primarily CD103- (67).

pDC are also present in PP and stand apart from ‘classical’ pDC in their lack of IFN production (6, 84). pDC cannot be detected in gut lymphatics draining to the mLN, and thus are not believed to migrate to the mLN from PP (154).

**Lamina propria**

The LP is the layer of connective tissue between the epithelium and the underlying structures (Figure 1.3 & 1.4). It contains large numbers of scattered immune cells, stromal cells, B cell, T cells, DC and Mφ (1); LP lymphocytes are largely IgA-secretting plasma cells and memory T effector cells. The LP is drained by afferent lymphatics that join into the mLN. Cryptopatches are located at the base of crypts and are loosely organized clusters of approximately 1000 cells. They appear to be randomly distributed throughout the small intestine and colon, but are most numerous in the small intestine (73).

Many murine LP DC express CD103 and are very adept in inducing expression of gut homing molecules, such as CCR9 on T cells which allows for access to the intestinal mucosa, and to drive the peripheral generation of Treg cells, as well as CD8+ T cells (67, 70). They are located primarily within the villous core (105), migrate to the mLN and are found to be a non-dividing population which is likely to be continually replenished by blood-derived precursors (67). It has been speculated that LP DC promote non-inflammatory responses as a consequence of constant exposure to epithelial cell derived factors, such as TGF-β, IL-10 and RA, but in vitro CD103+ LP DC are induced to express IL-6, IL-12p40 and IL-12p70 in response to flagellin (TLR5 agonist), implicating that they can indeed be proinflammatory in nature (138). CD103+ LP DC may, based on their TLR5 expression and cytokine secretion be the same cells as Uematsu & Akira identified as being CD11chiCD11bhi. This subset was shown both in vitro and in vivo to induce Th1 as well as Th17 cells from naïve CD4+ T cells (138).

The majority of LP DC are found closely associated with enterocytes and are characterized by their expression of CX3CR1 which enable them to produce trans-epithelial dendrites and sample bacteria and antigens from the lumen directly (104, 116). However, whether the CX3CR1-expressing cells are DC is still being debated as accumulating evidence show that they display markers associated with tissue-resident Mφ (12, 141). This also correlates with the finding, that CX3CR1 cells do not migrate, either in steady
state or after oral stimulation with a TLR agonist (126). In the steady state, these cells are reported to outnumber CD103+ DC by 3-4 fold (111).

Mesenteric lymph nodes
The mLN are the largest lymph nodes in the body. They consist of a sponge-like network of stromal cells, fibres and extracellular matrix. Like the PP, these lymph nodes contain B cell rich zones with GCs and interfollicular regions containing T cells (Figure 1.4). mLN are connected via an intricate network of afferent and efferent lymphoid venules to the GALT and drain the lymph from the entire gut. They are home to populations of CD11c<sup>hi</sup>CD11b<sup>−</sup>CD8α<sup>+</sup>, CD11c<sup>hi</sup>CD11b<sup>−</sup>CD8α<sup>+</sup> and CD11c<sup>hi</sup> double negative DC. The two latter subsets, as well as pDC, are localized in the T cell regions, whereas the former is found primarily outside T cell zones (69). In addition to these subsets, staining of mLN DC with antibodies specific for the β7-integrin and CD8α has identified four subsets; two β7<sup>hi</sup> (CD8α positive and negative) resembling LP DC and two β7<sup>int/lo</sup> (CD8α positive and negative) more similar to PP DC (94). The β7<sup>hi</sup> DC could very well be CD103<sup>+</sup> LP DC, as a proportion of mLN DC express CD103, and numbers of β7<sup>hi</sup> DC as well as CD103 LP DC are very low in the mLN of CCR7<sup>−/−</sup> mice, where DC migration is unattainable (70). Whether PP DC are able to migrate to the mLN is still unclear.

Salmonella
Bacteria belonging to the *Salmonella* genus are Gram-negative, rod-shaped, facultative intracellular anaerobes (Figure 1.6). The genus belongs to the family *Enterobacteriaceae* and based on DNA sequences, the closest related genera are *Escherichia*, *Shigella*, and *Citrobacter*.

Figure 1.6. *Salmonella* Typhimurium invasion of epithelial cells.
Colour-enhanced scanning electron micrograph showing *Salmonella* Typhimurium (red) invading cultured human epithelial cells. Note the characteristic membrane ruffles induced by *Salmonella* effector molecules, facilitating bacterial uptake. Image from [http://www.niaid.nih.gov/SiteCollectionImages/topics/biodefenselated/SALMON_1.JPG](http://www.niaid.nih.gov/SiteCollectionImages/topics/biodefenselated/SALMON_1.JPG). Credit: Rocky Mountain Laboratories, NIAID, NIH.
The taxonomic classification of the genus *Salmonella* has been controversial, since the original taxonomy was not based on DNA relatedness; rather names were given according to clinical considerations, e.g. *Salmonella typhi, Salmonella choleraesuis, Salmonella abortusovis* and so on. Careful analysis of DNA homology later revealed that the genus consists of two species: *Salmonella enterica* and *Salmonella bongori* (81, 115). The latter is a commensal of cold-blooded animals, whereas the former is found in reptiles and warm-blooded animals and divided into six subspecies: (I) *enterica*, (II) *salamae*, (IIIa) *arizonae*, (IIIb) *diarizonae*, (IV) *houtenae*, and (VI) *indica* (80, 115). These subspecies are further classified into more than 50 serogroups based on the O antigen structure, which reflects variation in the exposed part of the LPS, and then further divided into > 2500 serovars based on the variation in flagellin (H antigen) (51). Subspecies I has far more serovars than the others (112) and contains around 60% of known *Salmonella* serovars that inhabit the intestinal tract of humans and warm-blooded animals (5). Notwithstanding this, only a small fraction within subspecies I serovars are enteric pathogens; in fact according to Center for Disease Control and Prevention, United States of America, the 12 most prevalent *Salmonella* serovars have been shown to be responsible for more than 60% of all human *Salmonella* infections (18).

Many *Salmonella* serovars are quite promiscuous as they are not host-specific and can cause disease in a wide range of hosts. Others are highly adapted to a specific host, such as *S. Typhi* and *S. Gallinarum*, which cause systemic illness in humans and poultry, respectively. Most infections result from the ingestion of contaminated food products, such as poultry, eggs and dairy products and, in children and animals, from direct faecal-oral spread. Humans infected with *S. enterica* subspecies I show one of 2 major clinical syndromes: non-typhoidal salmonellosis (gastroenteritis) or typhoid fever (systemic disease).

**Gastroenteritis**

Gastroenteritis is caused by non-typhoidal *Salmonella* serovars (NTS), such as *S. Typhimurium* and *S. Enteritidis*, which are not host-specific, and is localized to the ileum of the small intestine, although inflammation in colon also occurs (120). The pathophysiology is characterized by mucosal oedema and massive recruitment of neutrophils to the site of bacterial entry. Symptoms usually occur between 6 and 72 hours after ingestion, and are marked by acute onset, fever, abdominal pain, nausea, vomiting and diarrhoea with or without blood. Symptoms of this self-limiting diarrhoeal disease usually last between 5 and 7 days and has an untreated case fatality of 0.1% in otherwise healthy individuals (50). Infants, the elderly and immunocompromised patients are more likely to experience complications of *Salmonella* gastroenteritis, such as reactive arthritis, meningitis, bacteraemia and abscesses of spleen, liver and lungs and are often treated with antibiotics to prevent invasion and bacteraemia. An estimate of 93.8 million cases annually of intestinal disease, including 155,000 deaths, has been reported worldwide (87).
Typhoid fever
Typhoid fever is caused by S. Typhi or S. Paratyphi A and B and manifests around one to 2 weeks following bacterial inoculation. Symptoms include fever and general discomfort, abdominal pain with or without other symptoms including headache, muscle pain, nausea, anorexia, and constipation (reviewed by Parry et al. (108)). Diarrhoea occurs occasionally but is typical only of infection in immunocompromised individuals (25). The disease is characterised by intestinal perforation and haemorrhage as well as enlargement of mLN, spleen and liver. In the absence of complications, disease resolves within 4 to 6 weeks. In approximately 5% of cases, a chronic carrier state may follow acute illness, during which bacteria are shed in faeces (96). This state may persist for years and in a minority of patients relapse occurs (25). The case fatality is 10-20% in untreated cases, but reduced to around 1% following treatment with appropriate antibiotics (50); however, resistance to antimicrobials is widespread. The global health burden of typhoid fever is estimated to 21 million cases per year (32).

The murine typhoid model
Animal models have been extensively used to study Salmonella pathogenesis and disclose key bacterial virulence factors, and this has greatly aided in our understanding of the complex host-pathogen interplay. Models commonly used include rhesus macaque monkeys, calves, guinea pigs, rabbits and mice (reviewed by Tsolis et al. (136)), but most of our understanding of the highly specialised pathogenesis of typhoid fever is extrapolated from early volunteer studies in humans (64) and from the mouse model.

Mice infected with S. Typhimurium, a natural mouse pathogen, do not develop gastroenteritis; instead they develop a systemic disease with pathophysiological changes similar to those observed in human typhoid fever patients and high bacterial loads in PP, mLN, spleen and liver (135).

Natural resistance-associated macrophage protein 1 (Nramp1) is an intracellular metal transporter believed to limit availability of divalent cations and therefore to inhibit intracellular bacterial growth (21). A point mutation in this gene, resulting in a non-functional protein, renders certain mouse strains, such as C57BL/6 and BALB/c mice, susceptible to lethal S. Typhimurium infection.

Low cost and the ease of genetic manipulation of both mouse and bacterial strains has facilitated detailed studies of specific genes, cell types and pathways involved in host-pathogen interactions. However, there are also certain shortcomings when using such a model. An obvious weakness is that human infection by S. Typhimurium normally results in gastroenteritis rather than typhoid fever. Moreover, not all pathogenicity genes are shared between S. Typhimurium and S. Typhi; for instance the Vi antigen, a polysaccharide capsule enabling intracellular survival within phagocytes and protection against host immune responses, is present in S. Typhi but absent in S. Typhimurium (119).
*Salmonella* pathogenesis

Typically acquired by oral ingestion of contaminated food or water, *Salmonella* has to survive passage through the gastric acid, evade killing by digestive enzymes, bile salts, opsonisation by secretory IgA, defensins and other antimicrobial peptides as well as other innate immune defence mechanisms to gain access to the underlying epithelium and deeper tissues. The gastric acid would normally reduce the inoculum size significantly, however, *Salmonella* have an adoptive acid-tolerance response, which may increase their survival through the stomach (45).

Although invasion of *Salmonella* serotypes has been extensively studied in both human and murine models, some doubts as to what mechanism is the prevalent exist. *In vivo*, 3 routes of translocation have been demonstrated. One is via M cells, the second involves uptake via para-epithelium extensions of dendrites into gut lumen by CD18+ phagocytes, and the third involves uptake via enterocytes through manipulation of the cytoskeleton, see Figure 1.7.

**Figure 1.7. Schematic representation of *Salmonella* gut invasion routes.**

Bacteria preferentially invade through M cells, leading to uptake by DC and Mφ in the PP and initiation of mucosal immune responses. CD18-expressing phagocytes, such as DC, Mφ and monocytes, may also take up bacteria in the LP and travel to mLN, thereby inducing a mucosal immune response. However, these CD18-positive cells may also move directly into circulation upon phagocytosis, thus by-passing mucosal immunity. The third route of invasion involves epithelial cells. Upon binding to receptors on these cells, *Salmonella* inject virulence effectors, leading to rearrangement of the host cell cytoskeleton, thereby facilitating their own uptake. Inspired by Vazquez-Torres *et al* (142) and artwork published in Nature Reviews Immunology.

**M cell invasion**

Infection models have been used to trace the *Salmonella* route of infection, revealing that invasive species preferentially interact with PP M cells (19, 62, 71). In 1994, by use of the intestinal ligated loop model, Jones *et al.* showed that within 30 minutes of infection, large numbers of invasive *S.*
Typhimurium preferentially bound to and passed through the apical surface of M cells. This was associated with pronounced membrane ruffling (see “Invasion of enterocytes”) followed by M cell destruction (71). During the initial attachment to M cells, no significant invasion of enterocytes was detected; however, following bacterial influx through dead M cells, bacteria invaded both apically and basolaterally adjacent enterocytes, leading to destruction of the epithelium.

M cells are also found in solitary intestinal lymphoid tissues (55) and following oral infection in mice, *Salmonella* were found in these follicles (54), suggesting that these non-PP structures may function as an entry point into the host.

Binding to M cells was thought to be mediated by the lpf fimbrial operon (9), but recently, Hase and associates identified glycoprotein 2 (GP2) on M cells as a transcytotic receptor for mucosal antigens (59). They were able to show that GP2 recognizes FimH, a mannose-binding component of the type I pili on the bacterial outer membrane, in both *E. coli* and *S. Typhimurium*, and that interactions lead to initiations of mucosal immune responses. However, several other adhesins have been described for *S. Typhimurium* and their functions may very well be redundant (for a recent review, see Wagner and Hensel (146)).

**M cell-independent invasion**

Vazquez-Torres *et al.* have demonstrated that *Salmonella* pathogenicity island (SPI) 1-deficient strains, in spite of their inability to invade, were able to cause systemic disease, and that this was due to uptake and transport by CD18-expressing cells. CD18- leukocytes were believed to move directly into circulation upon phagocytosis of attenuated *Salmonella*, as bacteria could be isolated from blood-borne phagocytes within one hour after oral inoculation (142). These cells were proposed to be of the monocyte/macrophage lineage and it was later shown that DC in the LP were in fact able to extend protrusions through the epithelial barrier, by expressing tight junction proteins, and sample non-invasive *Salmonella* from the lumen (116). However, whether these CD18-phagocytes are Mφ, DC or monocytes remains to be elucidated.

**Invasion of enterocytes**

*Salmonella* are able to facilitate uptake in cultured epithelial cells, as well as in vivo, by taking direct control of the host cytoskeleton (40, 132). This is done by injecting a series of virulence effectors into the cytoplasm, which by interaction trigger reorganization of the actin skeleton, resulting in membrane ruffling and engulfment of the bacteria (42, 43). Following bacterial uptake, the cell regains original architecture, while the bacteria resides in a membrane bound vesicle, known as the *Salmonella*
containing vacuole (SCV); an intracellular niche ensuring replication, survival and dissemination (38, 65, 129).

**The mLN and beyond**

Once the bacteria cross the mucosal epithelia, they encounter the immune cell populations of GALT, and these host-pathogen interactions initiate disease (118).

Shortly after oral infection, *Salmonella* is found associated with DC in the SED of PP (63). This area is enriched in CD11c⁺ DC that express CCR6 and belong to the CD8α⁺CD11b⁺ and CD8α⁺CD11b⁻ DC subsets, and CD8α⁻ DC are also the cells taking up *Salmonella* (130). If bacteria traverse the intestinal epithelia by invading enterocytes, they can subsequently be found within LP CX3CR1⁺ DC and non-DC, but if they are taken up by antigen-sampling DC protruding through the epithelial layer, bacteria are only found within CX3CR1⁺ DC (104). What happens to CX3CR1⁺ DC following bacterial uptake is unknown, as these cells have been shown not to migrate to the mLN. The presence of transepithelial dendrites also appears to be mouse strain dependent and thus, this mechanism of antigen-uptake may not be the prevalent (139), however, mice lacking CX3CR1 are more susceptible to *Salmonella* infection (104). If bacteria are invading through M cells, they induce IgA secretion; however, if they are taken up by DC extending dendrites into the lumen, this is not the case (88).

Invading bacteria then reach the mLN; some may be travelling in the lymph extracellularly, but most likely the majority are transported in CD103⁺ DC from the LP and/or PP (12, 133, 145). Some evidence also suggest that neutrophils may play a prominent role in shuttling microbes from tissues to draining lymph nodes (13, 98), albeit, in these studies bacteria were injected into either skin or facial tissue, thus bypassing the interaction with epithelia. From here, *Salmonella* are able to disseminate systemically, and evidence suggest that bacteria leave the mLN as free bacteria (113) and eventually reach the blood via the thoracic duct to the liver and spleen. Here *S. Typhimurium* bacteria reside predominantly within phagocytes, but the SCV enables bacteria to survive within both phagocytic and non-phagocytic cells. As a consequence, during late stages of infection *in vivo*, *Salmonella* can be found within B and T cells (46), Mφ (97), DC (153), neutrophils (22) and hepatocytes (27), however, Mφ are still believed by many to be the primary target cells for *Salmonella* intracellular replication.

**Salmonella** virulence traits in immune activation

Successful infection and intracellular replication within the host require a range of virulence genes, many of which in *Salmonella* species are encoded within large regions of the bacterial chromosome, termed *Salmonella* pathogenicity islands. These SPIs are common attributes of Gram-negative pathogens and in *S. Typhimurium*, several have been identified. SPI-1 and SPI-2 are of great importance for pathogenicity,
as mutations in these genetic regions result in impaired ability to infect host cells (43, 61). SPI-1 and -2 each encode a T3SS (T3SS-1 and T3SS-2), responsible for delivery of effector proteins directly into the host cell, which leads to reprogramming of cellular functions, favouring the uptake and intracellular lifestyle of *S. Typhimurium* (56). The structure of T3SS resembles a molecular needle which links to the host cell forming a direct channel between pathogen and host cell cytoplasm (reviewed by (44)). Although these T3SS are thought to play distinct roles during the course of an infection (T3SS-1 is induced extracellularly promoting invasion of intestinal epithelial cells and inflammation, while T3SS-2 is thought to direct invasion via DC and be required for survival within Mϕ), evidence suggesting co-dependence for efficient functioning exist (34).

Recently, SPI-4 was shown to encode a type 1 secretion system (T1SS) for a large adhesin named SiiE, which mediates adhesion to apical brush border of enterocytes (48). Expression of SPI-1 and SPI-4 genes are under control of the global regulators SirA and HilA, and it was discovered that the concerted regulation of both SPIs is essential for the pathogenesis of *Salmonella* during the infection process (47, 76).

**SPI-1 and gastroenteritis**

The T3SS of SPI-1 secretes 13 proteins of which 6 (SipA, SipC, SopB, SopD, SopE and SopE2) are required for gastroenteritis as well as invasion of M cells and enterocytes. The latter process is also known as bacterial mediated endocytosis. SipC is an actin-binding protein, which is inserted into the host cell plasma membrane (125). It nucleates actin polymerization, leading to filament growth, and mediates actin assembly by bundling and cross-linking existing filaments (60). SipA is translocated into the host cytoplasm where it potentiates SipC activity by decreasing the critical concentration of actin and inhibiting depolymerisation of filaments (156). Both effectors are required for an efficient bacterial internalization by the host cell (92).

While SipA and SipC bind directly to actin, SopB, SopE and SopE2 modulate the actin architecture indirectly by mimicking host-cell regulatory proteins. They activate the host Rho GTPases Cdc42, Rac-1 and RhoG, but whereas SopE is a potent guanine nucleotide exchange factors for both Cdc42 and Rac-1, SopE2 only interacts efficiently with Cdc42 and SopB only stimulates RhoG indirectly through its phosphoinositide phosphatase activity (41, 109). Collectively, these events result in the cellular process of membrane ruffling and bacterial internalization.

Apart from its role in bacterial invasion, SPI-1 also possesses additional functions related to the activation of the innate immune response. Stimulation of Cdc42 by T3SS-1 effectors triggers activation of the mitogen-associated protein kinases (MAPKs) Erk, JNK and p38 (58). This leads to subsequent induction of NF-kB and production of proinflammatory cytokines, such as IL-8. During gastroenteritis, the recruitment of neutrophils to the intestinal epithelium is the histopathological hallmark of disease. Both
in vitro and in vivo, SipA induces intestinal epithelial cells to produce pathogen elicited epithelial chemoattractant (PEEC) and IL-8, which are required to recruit basolateral neutrophils to the apical epithelial membrane (49, 91, 147). SipA, SopB, SopE and SopE2 have also been implicated in the disruption of epithelial tight junctions through their activation of Rho GTPases, which further facilitates the transepithelial migration of neutrophils into the lumen (15).

Salmonella invasion of enterocytes triggers cell-death with characteristic morphological changes of apoptosis, including activation of caspase-3 and caspase-8, but not caspase-1, as well as nuclear condensation and DNA cleavage (37). However, the T3SS-1 effector SipB enhances the inflammatory response by increasing production of IL-1β and IL-18 through binding and activating caspase-1, a process which depending on the level of activation of caspase-1, can lead to rapid inflammatory death (pyroptosis) of Mφ and DC, but not enterocytes (10).

SPI2 and immune evasion by virulent Salmonella

SPI-2 is required for intracellular replication and systemic infection in mice, and has recently been implicated in directing invasion of DC (57, 107). Unlike T3SS-1, the T3SS-2 functions intracellularly by translocating effectors across the phagosomal membrane into the host cytosol (for a recent review on SCV formation, see Bakowski, Braun & Brumell (8)). Although Salmonella induce formation of SCVs in DC, they are unable to replicate in DC, and deletion of SPI-2 or specific genes reduces intracellular DC survival (68, 134). Some T3SS-2 effectors are able to subvert DC function by preventing SCV fusion with lysosomes (128) or increase the resistance to oxidative killing by inhibiting recruitment of NADPH oxidases to the SCV (143), while others, such as SseI, may interfere with DC migration (93).

SpiC (or SsaB) contributes to avoiding lysosomal fusion with the SCV by targeting host proteins (Hook-3 and TassC), and thereby alters vesicular trafficking (82, 128). In vitro SpiC is involved in activation of MAPK signalling pathways, leading to IL-10 and prostaglandin E2 production, as well as expression of suppressor of cytokine signalling (SOCS)-3, which is involved in the inhibition of IL-6 and IFN-γ signalling (137). IFN-γ production is rapidly upregulated in GALT and spleen upon infection with S. Typhimurium and is important for control of bacterial replication in the early phase of infection, but is not adequate for elimination of bacteria (100, 101).

By avoiding lysosomal fusion with the SCV and impairing endosomal trafficking, Salmonella are able to interfere with antigen presentation both on MHC I and II, and following infection, both Th and Tc mediated immune responses are slow and inefficient in vivo (86, 95). Infection with S. Typhimurium also reduces the amount of MHC molecules expressed on the DC surface, however, suppression of antigen presentation is only restricted to bacterial antigens, since Salmonella infected DC retain their ability to present bystander soluble antigens (53). The T3SS-2 effector proteins involved in inhibition of antigen presentation have been identified as SifA, SspH2, SlrP, PipB2 and SopD2.
Inside the SCV, *Salmonella* alters gene transcription profile resulting in a severely reduced expression of FliC, the major flagella subunit (33). This then renders the FliC-specific Th cells useless in combating intracellular bacteria and allows for *Salmonella* to escape immune surveillance by some antigen-specific Th cells. Some have speculated that although the SPI-1 effector SipC is a target antigen for Th cell recognition in *Salmonella* infected mice, the induction of SipC-Th cells may not be helpful in protecting the host, as T3SS-1 expression and effector secretion has been believed to be downregulated within host cells. Using proteomics, however, this was very recently challenged – bacteria grown under SPI-2 inducing conditions, mimicking the intracellular environment, displayed both SPI2 and SPI1 effectors on their membrane (127).

*Salmonella* are also able to prevent the development of an adaptive immune response by a contact-dependent, but SPI-1 and -2 independent, mechanism. Van der Velden *et al.* were able to show that *Salmonella* directly interfere with the expression of the β-chain of the T cell receptor (TCR), thus abrogating the first step in T cell clonal expansion (158, 159).

Because of the accumulating evidence of virulent *Salmonella* being able to subvert DC functions, these cells are believed to serve as ‘Trojan horses’, aiding in the spread of bacteria. Following oral infection, CD103+ LP DC take up and transport *Salmonella* to mLN (12), and as these DC have also been shown to induce Tregs and tolerance to food antigens, it may be speculated that by targeting these cells, *Salmonella* are able to facilitate their own dissemination as well as suppress intestinal inflammation. However, DC are probably not the cells responsible for the systemic spread from the mLN to liver and spleen since mLN serve to contain bacteria and so far, DC have never been reported to migrate out of a secondary lymphoid organ (145). Rather, other cell types may contribute to bacterial spreading, as *Salmonella* can be found in non-DC following LP invasion (104), in B cells, where they reside in a late endosomal compartment (117) and in B cell precursors in the bone marrow (20).
References


2 Pathogenicity of *Salmonella* Typhimurium strains in a mouse model is coupled to gut invasiveness rather than the ensuing immune response

Brandt, R., Petersen, A., Brix, S., Licht, T. R. and Frøkiær, H.

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Abstract

Salmonella enterica serovar Typhimurium (S. Typhimurium) is a food-borne pathogen, which causes disease in a broad range of hosts. The outcome of disease varies from gastroenteritis to typhoid fever and has been demonstrated to depend upon the infecting serovar. In this study, we compared the pathogenicity of two genetically very similar S. Typhimurium strains; DT120 and SL1344, both causing a typhoid-like illness in a mouse model. Even in a ten-fold lower dose, orally administered SL1344 gave rise to significantly higher bacterial counts in spleen and liver than administration of DT120 at Day 5 post challenge. This was paralleled by massive neutrophil infiltration of the spleen, an increase in neutrophil apoptosis in the bone marrow, and four out of five mice died before Day 8. The 10-fold higher dose of DT120 resulted in the same bacterial counts in cecum as SL1344, a transiently increased bacterial number in spleen as well as a delayed influx of neutrophils. No increase in neutrophil apoptosis was observed and all mice survived until Day 8. Intravenous injection of the same number of the two strains resulted in similar CFU and neutrophil counts in spleen and bone marrow.

Taken together, the study reveals that strain-related differences in pathogenicity within S. Typhimurium serovars are related to their invasiveness including their survival during gut passage rather than to immune recognition.

Introduction

Salmonella are Gram-negative facultative intracellular anaerobes, estimated to cause an annual 1.3 billion cases of disease worldwide (24). Clinical symptoms range from gastroenteritis to severe systemic typhoid fever and bacteremia, the latter two caused by bacterial translocation from the gastrointestinal tract into circulation. A recent retro perspective study of data from cases of human Salmonella infections during a 10 year period revealed that although the identified serotypes were genetically closely related, they differed significantly in pathogenic potentials, as outcome of salmonellosis was serotype-specific (18). Understanding the relative risks associated with and within different serotypes for invasive infection is of major importance for public health.

Direct comparison of the pathogenicity of different Salmonella strains needs to be studied in animal models. Mouse models are widely used to study Salmonella translocation and effects hereon of the immune system. In susceptible mice, oral infection with Salmonella enterica serovar Typhimurium (S. Typhimurium, ST) leads to a systemic typhoid-like illness, and much of the knowledge and understanding of Salmonella pathogenesis and host defense mechanisms has been deduced from these models (29).

Although the invasion and intestinal translocation of Salmonella serotypes associated with systemic illness have been vigorously studied in humans as well as in murine model systems, the precise mechanism and route is still being debated. The current view is that Salmonella cells, after oral ingestion,
are able to reach deeper tissues by two major routes. One port of entry is through microfold (M) cells overlaying Peyer’s patches (PP) and solitary intestinal lymphoid tissues (15, 17, 21), or through villous M cells distributed in the mucosal epithelium (16). The second port of entry involves uptake by CD18-expressing phagocytic cells, which are believed to carry bacteria directly into the bloodstream and lymph, thus by-passing PPs (33). Direct bacterial entry into the blood stream without simultaneous involvement of phagocytes has never been reported, but cannot be excluded. Which of these two mechanisms is the dominating may depend on the number of bacteria present in the gut as well as on the virulence of the specific strain (14), and both may in turn affect the immune response to the bacterium. Previous studies by Rescigno and colleagues (25) demonstrated that specific virulence genes in the different S. Typhimurium strains may affect the route of gut translocation and their translocation capacity. Specifically, oral administration of wild type S. Typhimurium SL1344 resulted in translocation of a higher number of bacteria to PP, mesenteric lymph nodes (mLN) and spleen, whilst administration of a ten-fold higher dose of the less invasive mutant InvA− caused low bacterial counts in PP, but comparable numbers in mLN and spleen (25). Such differences in intestinal translocation properties may impact the type and extent of the primary immune response against the invading bacteria. Phagocytes such as macrophages (Mφs) and neutrophils play an important role in host survival upon Salmonella invasion (8, 32, 35) although some evidence suggests that neutrophils also play a prominent role in shuttling live microbes from tissues to draining lymph nodes, thereby promoting bacterial dissemination (1, 2). Neutrophils are short-lived cells that degranulate and undergo apoptosis upon bacterial phagocytosis (23). Under healthy conditions, the great majority of murine neutrophils reside in the BM (7), while during infection large numbers are released into blood circulation and directed to the periphery, where they control invading pathogens (11). Induction of antibody-mediated neutropenia has revealed the important role of neutrophils in early killing of invading Salmonella, as neutropenic mice show increased bacterial burden on Day 1 post challenge (6). Orally administered lethal doses of Salmonella were previously reported to involve heavy neutrophil infiltration of spleen, mLN and PP 5 days after infection correlating with bacterial burden (6, 19). The role played by neutrophils in the pathogenesis of S. Typhimurium and in relation to the divergent pathogenic potential of different strains is still not clear. Moreover, it remains to be disclosed whether neutrophil influx to infected organs cause increased granulopoiesis or neutrophil depletion in the bone marrow (BM) upon S. Typhimurium infection.

The host defense systems activated in response to invasion by different S. Typhimurium strains may well depend on the route of entrance as well as the number of translocating bacteria and their ability to proliferate in the intestine and within organs. In the present study we found that a number of S. Typhimurium strains translocated differently in a mouse model and aimed to uncover whether the
pathogenicity of these strains was related primarily to their ability to translocate across the intestinal barrier or to the immune response induced in the host.

**Materials & methods**

*Mice and challenge protocol*

Eight weeks old conventional female BALB/c mice were purchased from Taconic Europe (Lille Skensved, Denmark) and housed in standard cages in an environmentally controlled facility with a 12-h light/dark cycle. During the study the temperature was kept at 22 ± 1°C, relative humidity at 55 ± 5% and air was changed 8-10 times per hour. Mice were fed standard chow and water ad libitum. For oral infections, mice were infected with 10⁷ CFU (*S. Typhimurium DT120*) or 10⁶ CFU (*S. Typhimurium SL1344*) by gastric gavage. For intravenous injections, mice were injected with 10⁴ CFU *S. Typhimurium SL1344* or DT120 in the tail vein. Following challenge, mice were observed twice a day. If symptoms of severe disease (ruffled fur, altered behavior) developed, the mice were euthanized immediately, due to ethical considerations. Animal studies were performed under conditions approved by the Danish Animal Experiments Inspectorate (Council for Animal Experimentation) and by the in-house Animal Welfare Committee.

*Salmonella strains and culture*

*S. Typhimurium SL1344*, DT104, DT120 and DT193 strains resistant to nalidixic acid and chloramphenicol were kindly provided by Jens Bo Andersen, The National Food Institute, Technical University of Denmark. *S. Typhimurium* strains were grown in closed 50 ml tubes at 37°C, 200 rpm overnight in 20 ml LB broth supplemented with 10 µg/ml chloramphenicol. For infections, overnight cultures were diluted appropriately in saline. The number of CFU in the inoculums was determined by plating on LB-agar plates supplemented with 10 µg/ml chloramphenicol.

*Bacterial counts*

Bacterial counts were determined in homogenized halves of spleen, in the cecum and in 100 µl flushed BM (5 ml PBS per femur) with serial dilutions plated on LB-agar supplemented with 10 µg/ml chloramphenicol. Detection limits were as follows: Figure 1; 100 CFU/g in spleen, liver and cecum. Figure 2; 20 CFU/g in spleen, liver and MLN. Figure 3; 10 CFU/g in spleen and cecum. Figure 4; 25 CFU/g in spleen and BM. Figure 6; 25 CFU/g in spleen and BM.

*Immunostaining and flow cytometry*

Single-cell suspensions were prepared from flushed BM and halves of spleen and transferred to round-bottomed 96-well polystyrene plates (Nunc, Roskilde, Denmark). Fcγ III/II (3 µg/ml, 25 µg/ml; BD
Biosciences) was added for 10 minutes to block non-specific binding of antibodies. An additional 25 μl/well PBS-Az (phosphate buffered saline containing 1% (v/v) fetal bovine serum and 0.15% (w/v) sodium azide (Sigma-Aldrich)) containing fluorochrome-conjugated antibodies at pre-titrated concentrations was added and the cells were incubated for 45 minutes. The cells were then washed and resuspended in 200 μl/well PBS-Az for flow cytometric analyses. All stainings were carried out at or below 4°C. Antibodies used in this study were against CD11b (FITC, clone M1/70) from eBioscience, CD11c (PE, clone N418) and CD19 (APC-Cy7, clone 6D5) both from BD Biosciences, F4/80 (PE-Cy7, clone BM8), Gr1 (APC-Cy7, clone RB6-8C5), CD3 (PerCP, clone 145-2C11), and CD49b (PE, clone DX5) all from BioLegend. For detection of apoptotic cells, the Vybrant DyeCycle Violet/SYTOX AADvanced Apoptosis Kit (Invitrogen, Denmark) was used according to manufacturer’s instructions. Stained cells were analyzed on a BD FACSCanto™ II flow cytometer (BD Biosciences). Neutrophils were identified as CD11b<sup>hi</sup>Gr1<sup>hi</sup>F4/80<sup>neg</sup>; Mφ as CD11b<sup>hi</sup>CD11c<sup>lo</sup>Gr1loF4/80<sup>hi</sup>; NK cells as CD3<sup>neg</sup>DX5<sup>pos</sup>; monocytes as CD11b<sup>pos</sup>F4/80<sup>lo/pos</sup>; dendritic cells as CD11c<sup>hi</sup>CD11b<sup>lo</sup>F4/80<sup>neg</sup>; B cells as CD19<sup>pos</sup> and T cells as CD3<sup>pos</sup>. Analyses were based on a minimum of 500,000 cells and data were analyzed using FCS Express software (version 3.0, De Novo Software, Los Angeles, CA).

**Statistical analysis**

The statistical significance of variations among experimental groups was analyzed (GraphPad Prism, version 5.01, GraphPad Software, San Diego, CA) using either one-way ANOVA, followed by a Tukey’s multiple comparison test, or Student’s t-test. Statistical significances were accepted at P<0.05. P<0.05 = *, P<0.01 = ** and P<0.001 = ***.

**Results**

*Different strains of S. Typhimurium administered by gavage lead to different bacterial loads in the host.*

The invasive properties of four orally administered S. Typhimurium strains were compared in mice. Based on their physical condition and appearance, infected mice were sacrificed at various time points following infection, and the bacterial loads in cecums, livers and spleens were determined (Figure 2.1A). No statistical differences in mean body weights between the groups were recorded. Generally, infection with strain SL1344 gave high bacterial loads at all 3 sites, while DT193 caused intermediate bacterial loads, and DT104 and DT120 resulted in the lowest bacterial burden. The bacterial counts correlated with the number of days infected mice were able to survive without developing signs of severe disease.
Figure 2.1. Neutrophil influx to spleen and strain-dependent gut translocation of *Salmonella* spp.

(A) Mice (n = 6) were infected by gastric gavage with $10^6$ CFU of strain SL1344, DT104, DT120 or DT193 and subsequently sacrificed between one and fourteen days post challenge and the bacterial load evaluated. The dashed line represents the detection limit. (B) Dose-dependent neutrophil recruitment to the spleen of ST SL1344 infected mice. Mice were infected by gastric gavage and subsequently sacrificed at Day 5 post-infection. Neutrophils were identified by flow cytometry as Gr1$^{hi}$, CD11b$^{pos}$, F4/80$^{neg}$. Viable cells were gated by use of SYTOX® AADVanced™ Dead Cell Stain and exclusion of cell debris (not shown). The dashed line and grey area represent naïve levels ± SD, respectively. Data are mean ± SD (n=6) and represent one of two similar experiments. (C) Bacterial counts in liver, spleen and mesenteric lymph nodes following oral inoculation of BALB/c mice with $10^7$ CFU *S. Typhimurium* DT120 (filled circles) or $10^6$ CFU SL1344 (squares). The dashed line represents detection levels and the horizontal lines represent mean (n=6). Students $t$-test was used for analysis of statistical significance between groups.

As SL1344 and DT120 seemed to differ the most, these strains were chosen for further comparisons of the basis of their pathogenic potential. Strain SL1344 was the most invasive based on bacterial counts in liver and spleen; however, when administered in doses below $10^6$ CFU, no bacteria were detected in the spleen on Day 5 post challenge (data not shown). This correlated well with the observation that only doses at or above $10^6$ CFU led to an increase ($p = 0.027$) in neutrophil levels in the spleen (Figure 2.1B).

To examine if administration of a higher number of bacteria would overcome the seemingly low invasive potential of DT120 at local and systemic sites, a 10-fold higher dose of DT120 ($10^7$ bacteria) was administered. Upon administration of this dose, detectable levels of *Salmonella* were found in mLN and spleen (Figure 2.1C), but in significantly lower numbers than upon administration of $10^6$ SL1344. In
contrast to SL1344, few or no bacteria were also detected in the liver of DT120 challenged mice. This suggested that the higher capacity of SL1344 to translocate across the intestinal barrier is not just a matter of ability to proliferate within the gut. Moreover, it implied that a high number of translocating bacteria is a prerequisite for dissemination, directly or indirectly, from the intestinal lymphatic system into blood circulation.

**S. Typhimurium SL1344, but not DT120, leads to neutrophil infiltration of the spleen five days post-challenge.**

As neutrophil recruitment to sites of inflammation is a hallmark of systemic *Salmonella* infection (6), we evaluated the number of neutrophils present in the spleen five and eight days post challenge in mice orally administered either DT120 or SL1344.

Figure 2.2. The infective potential of *S. Typhimurium* strains depends on the invasiveness of the strain.

(A) Bacterial loads in cecum and spleen five and eight days post challenge. The horizontal line represents mean (n=6) and the dashed line represents the detection limit. (B) Number of viable neutrophils present in spleen five and eight days post challenge. The dashed line represents mean of naïve mice. Data are mean ± SD (n=6). (A)-(B) DT120 (10^7 CFU); black bars/filled circles, SL1344 (10^6 CFU); white bars/squares. Data were analyzed for statistical significance between groups using Student’s t-test.

Because of the differences in invasive potential of the two strains, we administered SL1344 in a 10-fold lower dose than DT120. Despite the differences in administered doses, we observed no variation in the number of bacteria recovered from either cecum or spleen in the two groups at Day 5 post infection (Figure 2.2A). Bacteria were, however, found in more animals infected with SL1344 than with DT120. Infection with SL1344 led to significantly increased neutrophil infiltration in the spleen at Day 5 post challenge (Figure 2.2B), which was not observed in DT120-animals where neutrophil levels did not rise above those in naïve mice. At Day 8, DT120 levels in the cecum remained constant, whereas the levels of SL1344 increased, both compared to DT120 numbers and to Day 5 levels (Figure 2.2A). In the spleen, the
bacterial load in DT120 administered mice decreased as compared to Day 5. In contrast, the bacterial load in SL1344-mice had increased dramatically compared to DT120 numbers and to Day 5 levels, whereas no change in neutrophil numbers from Day 5 to Day 8 could be observed (Figure 2.2A, B). In DT120-animals the splenic bacterial decrease from Day 5 to 8 was accompanied by a fairly large increase in levels of neutrophils. The Day 8 neutrophil level in spleens of DT120 administered mice, however, was still significantly lower than that of SL1344 administered mice (Figure 2.2B). Thus, despite the much higher splenic neutrophil influx in the SL1344 infected mice, these mice were less protected, as evidenced by higher bacterial numbers in spleen and a higher mortality.

Based on these data, we hypothesized that the bacterial pathogenicity of these 2 strains was related to invasiveness, i.e. the translocation, rather than the immune response they induced in the host. To test this, mice were either dosed perorally or i.v. injected with DT120 or SL1344.

**S. Typhimurium SL1344 and DT120 exhibit different capacities for exhausting the host neutrophil pool.**

To characterize neutrophil apoptosis in spleen and BM, cells were stained for condensed chromatin and the percentage of apoptotic neutrophils was determined by flow cytometry. The proportion of apoptotic neutrophils in spleens of S. Typhimurium challenged mice did not differ significantly between the two strains, but was significantly enhanced compared to naïve mice (Figure 2.3A). Despite the high numbers of neutrophils in SL1344 spleens (Figure 2.2B), the bacterial counts in spleens were 100 times higher in SL1344 challenged mice than in DT120 challenged mice (Figure 3B). The number of systemic neutrophils depend on BM turnover (granulopoiesis and/or peripheral outfluxes), and regulation of BM numbers may therefore affect the splenic neutrophil counts.

In BM, the neutrophil numbers did not diverge significantly in the two S. Typhimurium challenged groups, although DT120 exhibited a trend towards increasing the total number (Figure 2.3C). However, the percentage of apoptotic BM neutrophils varied significantly between DT120 and SL1344 challenged mice; with SL1344 challenge resulting in apoptosis in approximately 25% of BM neutrophils (Figure 2.3D). No bacteria were detected in BM of DT120 challenged mice; in contrast, bacteria were found in BM of three out of five SL1344 challenged mice (Figure 2.3E). All mice in the DT120 administered group survived to Day 8, while only a small proportion of mice infected with SL1344 were able to survive this long and presented with high bacterial loads in both spleen and BM, as well as a halt in splenic neutrophil influx (data not shown, but correlated with previously observed levels shown in Figure 2.2A, B).
Figure 2.3. Alterations in neutrophil numbers in spleen and BM five days post challenge.

(A) The percentage of total neutrophils in the spleen displaying condensed chromatin as an indicator of apoptosis was determined using the Vybrant® DyeCycle™ Violet and SYTOX® AADVanced™ Apoptosis Kit. (B) Bacterial loads in spleen. (C) The number of viable neutrophils present in BM five days post oral *Salmonella* challenge. Data are mean ± SD (n=3-5). (D) The percentage of apoptotic neutrophils in the BM. (E) Bacterial loads in BM. (A)-(B)+(D)-(E) Horizontal lines represent mean (n=3-5).

(A)-(F) Naïve mice; grey bars, DT120 (10^7 CFU); black bars/filled circles, SL1344 (10^6 CFU); white bars/squares. One way ANOVA and Tukey’s post hoc test were used to test for statistical significance.

These data suggest that neutrophils recruited to the spleen were able to control pathogen replication in DT120, but not SL1344 challenged mice. The high increase of apoptotic cells in BM together with the cessation in neutrophil influx in the spleen in SL1344 administered mice indicated that a proportion of the newly generated neutrophils were retained in the BM, rather than led into circulation.

Table 2.1. Number of leukocytes in spleen and BM in naïve, DT120- and SL1344-infected mice.

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<thead>
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<th>Spleen</th>
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<td>(x 10^6 cells)</td>
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<tr>
<td>Naïve</td>
<td>25.7 ± 6.6</td>
<td>25.7 ± 6.6</td>
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<tr>
<td>ST DT120</td>
<td>64.6 ± 12.8 □ □</td>
<td>28.2 ± 8.0</td>
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<tr>
<td>ST SL1344</td>
<td>64.8 ± 23.8 *</td>
<td>46 ± 16.1</td>
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*Spleens were cut in two; one half was used for plating and the other for cellular analyses.

*^p = 0.003 as compared to naïve mice.

*^p = 0.0354 as compared to naïve mice.

To further disclose the cellular dynamics during a *S. Typhimurium* infection, the splenic cellular composition in DT120 and SL1344 mice five days post challenge was analyzed (Figure 2.4). SL1344 administration affected not only the number of neutrophils in the spleen, but also increased the number of dendritic cells, Mφs, and total T cell numbers; this increase was paralleled with an increase in total number of leucocytes in the spleens of SL1344 infected mice (Table 2.1). The cellular composition in spleens of DT120 administered mice did not deviate significantly from naïve mice for any cell type (Figure 2.4), thus suggesting less cellular infiltration than in SL1344 infected mice.
Figure 2.4. Numbers of neutrophils, dendritic cells, Mφs and total T cells are increased in the spleen on day 5 of an SL1344 infection.

The cellular composition of infected spleens was analyzed using flow cytometry. The percentage of viable cells is shown. NK cells are defined as CD3neg, DX5pos; monocytes as CD11bpos, F4/80lo/pos; Mφs as CD11c(lo), CD11bhi, F4/80hi; dendritic cells as CD11c hi, CD11blo/pos, F4/80neg; B cells as CD19 pos and T cells as CD3 pos. Naive mice; grey bars, DT120 (10^7 CFU); black bars, SL1344 (10^6 CFU); white bars. Data represent mean ± SD (n=3-5). One-way ANOVA and Tukey’s post hoc test were used to test for statistical significance.

Intravenous injection of SL1344 or DT120 results in comparable neutrophil numbers in spleen and BM.

Although we observed a higher bacterial translocation, more widespread dissemination and increase of neutrophils in the spleen of SL1344 mice as compared to DT120, we could not decipher, based on oral administration alone, if the two bacteria affected the immune system differently, and/or if the two bacteria possessed different abilities to penetrate mucosal tissue or to proliferate within the host. To disclose this, we injected mice i.v. with 10^4 CFU DT120 or SL1344. On Day 3 after administration, comparable elevated numbers of neutrophils were detected in the spleens from both groups (Figure 2.5A), while the neutrophil numbers in BM did not differ from the uninfected control group (Figure 2.5B). In line with this, no difference in bacterial loads in spleen or BM was observed between the two strains (Figure 2.5C). Furthermore, the percentage of apoptotic neutrophils did not differ significantly between the two S. Typhimurium strains in spleen and BM when i.v. injected (Figure 2.5D, E). Taken together, this showed that i.v. administration of moderate doses of the two S. Typhimurium strains did not reveal differences in the systemic proliferation potential nor in the innate immune response to the bacteria.
Figure 2.5. No differences in neutrophil numbers and viability after intravenous injection of SL1344 and DT120.
Mice were intravenously injected with $10^4$ CFU of either SL1344 or DT120 and sacrificed three days post injection. (A)-(B) The number of viable neutrophils present in spleen and BM. Data are mean ± SD (n=3-5). (C) The bacterial counts recovered from spleen and BM. (D)-(E) The percentage of total neutrophils displaying condensed chromatin as an indicator of apoptosis. (C)-(E) The horizontal lines represent mean. For all data, one-way ANOVA and Tukey’s post hoc test was used to test for statistical significance.

Discussion
Few studies address both the invasiveness of various orally administered *Salmonella* strains and the subsequent innate immune response and hence link bacterial translocation to the type and strength of the immune response (6, 34). We demonstrated that *S. Typhimurium* DT120 and SL1344 differ in their capacity to translocate from the gut, and that strong translocation capacity coincided with uncontrollable dissemination of bacteria to systemic organs. Furthermore, we found that neutrophil influx in spleen correlated with splenic bacterial burden and development of end-stage disease, hence supporting previous findings indicating that neutrophil numbers in the spleen is a semi-quantitative indicator of bacterial translocation (6). Thus, the ability to survive passage in the gut and traverse the gut epithelial barrier seem to be key factors in determining the immune response in susceptible mice and hence the likelihood of survival.

Neutrophils play an important role in host survival during the primary response to *Salmonella* infection (6, 8, 32), in part by restricting pathogen replication (9), as well as by stimulating other immune cells
We observed that bacterial loads in the spleen on Day 8 post challenge were significantly increased in the SL1344-group, indicating massive, uncontrollable proliferation. However, this does not necessarily reflect an increased ability to proliferate per se, but rather, as we concomitantly observed a cessation in the influx of splenic neutrophil numbers in this group on Day 8, a failure of the host immune system to contain infection. DT120-infected mice on the other hand, were able to reduce the number of bacteria in the spleen on Day 8 perhaps by a steady and continuous recruitment of neutrophils.

It has been demonstrated that neutrophils may stimulate the production of interferon (IFN)-γ, e.g. from NK cells, but only in collaboration with IL-12 producing cells, such as dendritic cells (30). Moreover, it was recently shown by in vitro experiments that neutrophils may condition dendritic cells to promote the generation of regulatory T cells through the secretion and action of elastase (31). Hence, the role of neutrophils may depend on the presence and interaction of other cells at the site of infection as well as in the draining lymphoid organs, also including the spleen in systemic infections. The involvement of cell-cell interactions as a parameter for bacterial clearance is supported by other previous findings showing that Mφ, dendritic cells, NK and NKT cells all play a significant role in the primary response against Salmonella (4, 22, 28, 36). Alongside the neutrophil influx to the spleen upon oral administration of SL1344, we observed a significant increase in the total numbers of Mφ, dendritic cells and total T cells on Day 5 post-challenge. The levels of Mφ and dendritic cells are in line with previous findings (19, 20).

We did not observe any change in total numbers of Mφ or other cell types in DT120-administered mice on Day 5. On Day 8 we detected an increase in the number of neutrophils present in the spleen of DT120 challenged mice, suggesting that DT120 induces a delayed immune response compared to SL1344. Although purely speculative, this delayed immune response in DT120 infected mice could be explained by fewer bacteria reaching the spleen giving rise to diverse proportions of cellular immune subsets recruited to the spleen, which may lead to activation of different immune responses. In turn, this could lead to a more balanced immune response able to recruit a sufficient number of neutrophils and other cells to contain the bacterial infection without exhausting the immune system. This is supported by Navarini et al. (26), who recently showed that during a high-dose i.v.-induced infection with L. monocytogenes, BM neutrophils are depleted, leading to uncontrollable, systemic bacterial replication and host death. Here we found that peroral administration of SL1344, but not of DT120, resulted in some degree of immune exhaustion, demonstrated by a rise in the percentage of apoptotic BM neutrophils.

Interestingly, even though the BM reservoir had to sustain both massive efflux of neutrophils to the spleen as well as combat invading bacteria in the BM, at Day 5 post challenge no decrease in the number of neutrophils in the BM of SL1344 challenged mice was observed, suggesting a high level of granulopoiesis. Even so, these mice were not able to contain the infection. In DT120 challenged mice, no bacteria were detected in the BM and accordingly, no increase in apoptotic BM neutrophils was
observed. In these animals, a delayed and moderate recruitment of neutrophils to the spleen helps contain the invading pathogen.

*S. Typhimurium* has previously been demonstrated to colonize primarily the distal ileum and cecum (5). Our data suggest that SL1344 rapidly establishes a replicative niche and is more proficient at proliferating in the cecum than DT120. The specific conditions in the gut may affect growth ability as well as the ability to translocate. We have recently reported that feeding certain prebiotics, such as apple pectin, to BALB/c mice increased the number of SL1344 in the ileum, whereas fructo- or xylo-oligosaccharides increased translocation of SL1344 to liver and spleen (27). This illustrates that not only are number and type of bacteria as well as the innate immune status of the host important, but environmental factors such as the diet may play important roles in determining the outcome of an infection.

The virulence factors involved in the bacterial translocation cannot be deduced from the present study. For bacteria, the harsh acidity of the stomach is a challenging barrier to infection, and even though *Salmonella* species are capable of mounting an acid tolerance response, thereby enhancing resistance to extreme acidity (12, 13), the production of host reactive nitrogen species (RNS) in the gastric lumen has been found to exert potent antimicrobial activity against enteric bacteria, including *Salmonella* (3, 10). In contrast to other studies addressing the primary immune response upon oral administration of high doses of *Salmonella* (19, 25), we did not neutralize the stomach acidity prior to administration and despite the 10 fold higher administration of DT120, we found comparable CFU in cecum at Day 5 post challenge in the two groups. Thus, differences in acid resistance between the two strains are likely but not proven. If more SL1344 than DT120 bacteria are able to survive passage through the gastric lumen, more bacteria are able to replicate in the gut and this could lead to faster and increased loads of translocating bacteria. However, virulence factors directly affecting the translocation ability also seem to be in play as the same levels of SL1344 and DT120 were found in cecum, yet SL1344 exhibited a higher translocation. In support of this, the bacterial loads in spleen, and in particular the splenic influx of neutrophils as reporters of bacterial presence, were both higher in the SL1344 group. Hence, our data indicate that the different bacterial strains possess different capabilities to trespass the gut barrier, but this is concentration dependent and thus also dependent on resistance towards the gut environment and the capability to proliferate in the gut.

In conclusion, we have in the present study provided evidence that differences between highly similar strains exist which, due to different invasive capabilities, may have profound impact on the innate immune response and, as a consequence, on host survival.

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References


3 *Salmonella* Typhimurium-induced accumulation and regulation of plasmacytoid dendritic cells, myeloid dendritic cells and macrophages in gut-associated tissue during early-stage infection
Brandt, R., Licht, T.R. and Brix, S.

(Manuscript in preparation for *Mucosal Immunology*)
Abstract
The key to sufficient eradication of microbial pathogenic intruders is to evoke ideal timing of mucosal cellular influx and activation by elaborate control of the cellular network of signals conferring immunity versus tolerance in tissues. To examine early regulation events within the innate Ag-presenting cell compartment in infected gut mucosa, mice were orally dosed with 10^3 CFU or 10^7 CFU of *S. Typhimurium* SL1344. At 24 hours post challenge, the regulation of pDC, mDC, Mφ and subsets thereof, in ileal Peyer’s patches + subepithelial dome regions (PP-SED), LP and mLN was studied. Notably, pDC and Mφ accumulated in PP-SED after exposure to *Salmonella*, and while Mφ and mDC exhibited dose-related cellular atrophy, pDC were less susceptible to bacteria-induced cell death. CD40 expression was dose-independently reduced by *Salmonella* in PP-SED mDC and pDC. In PP-SED, a mixed myeloid lineage cell type (CD11c<sup>int</sup>CD103<sup>hi</sup>CD11b<sup>hi</sup>F4/80<sup>hi</sup>CX3CR1<sup>hi</sup>) was augmented, and in LP a subset displaying mDC markers (CD11c<sup>hi</sup>CD103<sup>hi</sup>CD11b<sup>lo</sup>F4/80<sup>lo</sup>) accumulated due to *Salmonella* infection. Collectively, our data demonstrate explicit regulations of innate cells in *Salmonella*-exposed gut tissue, and point to pDC taking part in early stage *Salmonella* containment.

Introduction
During the last decades there has been a collective effort to understand the innate trails that drives development of protective immunity to food-borne *Salmonella* infections with focus on the possible role of Mφ, and more recently DC in initiating these events. *Salmonella* is an intracellular pathogen, known to infect both mDC and Mφ (6, 16) upon gut invasion, and a combination of innate and adaptive immune responses are necessary in controlling *Salmonella enterica* serovar Typhimurium (hereafter *S. Typhimurium*) infection in mice (17). Until recently focus has been centred on the involvement of the conventional Ag-presenting DC, i.e. mDC, in bacterial infections, whereas the other major DC subset, pDC, plays an important part in antiviral responses, and is less well characterised in regard to antibacterial immunity. Both mDC and pDC subsets are now recognised to be critical at the interface between innate and adaptive immunity due to their activation of naive T cells in LN, and the ensuing induction of T cell-dependent B cell activation (7, 14). During steady-state conditions, mDC are found in the intestinal epithelial tissue, e.g. the SED region overlying PP and the lamina propria, and migrate to PP or mLN, respectively upon maturation (reviewed by Milling et al. (25)). Conversely, pDC are absent in intestinal epithelium tissue in the steady-state, and are directly recruited to intestinal LN from the blood (13). Mφ, on the other hand, are important tissue phagocytes presenting Ag to and activating effector T cells in tissues. They play a critical role in innate immunity to *Salmonella* by controlling bacterial growth in the early phases of infection, but historically they have also been considered the prime target for intracellular replication of virulent *Salmonella* (6, 26, 30, 34, 36), thus playing ambiguous roles in host survival (41).
During gut infection the general concept is that virulent *Salmonella* preferentially invades through the ileum of the small intestine by using microfold (M) cells overlying PP (19, 21), solitary intestinal lymphoid tissue (15), and/or by intestinal DC protruding dendrites through the epithelial barrier (32) as access points. The outcome of salmonellosis differs substantially by serotype (20) and previous work from our lab has demonstrated that also the dose of administered *S. Typhimurium* in mice influences whether bacteria are cleared (up to $10^4$ CFU) or if septicaemia (above $10^6$ CFU) develops 5-7 days post challenge (5). This dose-dependent survival effect may rely on alterations in the way the immune system handles bacteria; a smaller dose is cleared whereas the larger dose causes a burden which cannot be appropriately dealt with by all infected mice. As the causal mechanism is of importance for proper treatment of acute *Salmonella*-induced gut infections, the present study looked into the immune modulation in selected tissues and LN directly interacting with *S. Typhimurium* at an early stage following oral challenge. Here, we used multi-parametric flow cytometry to examine the degree of involvement of pDC, mDC as well as Mφ in intestinal tissue and LNs isolated 24h post challenge with two oral doses of *S. Typhimurium* SL1344 differing by 1000-fold in numbers. Moreover, we determined the accumulation of functionally different DC and Mφ subsets into the three gut locations. The goal was to study the contribution of the mere presence of *Salmonella* versus dosing implications on innate immune cell regulation in the gut mucosa at this time point.

Briefly, our data demonstrated a dose-dependent enhancement of pDC numbers along with impaired viability of mDC and Mφ, thus implying a previously undefined role for pDC in host control of *Salmonella*. Moreover, we defined two myeloid subsets being enhanced in PP-SED, and the LP, respectively at 24h upon *Salmonella* challenge.

**Materials & methods**

*Bacterial cultivation*

*Salmonella* Typhimurium SL1344 resistant to nalidixic acid and chloramphenicol was kindly provided by Jens Bo Andersen, The National Food Institute, Technical University of Denmark. Bacteria were grown in closed 50 ml tubes at 37°C, 200 rpm overnight in 20 ml Luria-Bertani broth (Merck, Darmstadt, Germany) supplemented with 10 μg/ml chloramphenicol. For *in vivo* infections, cultures were diluted appropriately in saline. For *in vitro* infections, cultures were harvested, washed twice in sterile phosphate-buffered saline (PBS, Lonza, Basel, Switzerland) and resuspended in pre-warmed cell culture medium (RPMI 1640 supplemented with 2 mM L-glutamine, 10% (v/v) heat-inactivated fetal calf serum (FCS) (both from Lonza) and 50 μM 2-mercaptoethanol (Invitrogen, Carlsbad, CA)). The number of CFU in inoculums was determined by plating on LB-agar plates supplemented with 10 μg/ml chloramphenicol.
Mice and challenge protocol

8 week-old conventional female BALB/c mice were purchased from Taconic Europe (Lille Skensved, Denmark) and housed in standard cages in an environmentally controlled facility with a 12 hour light/dark cycle. During the study, the temperature was kept at 22 ± 1°C, relative humidity at 55 ± 5% and air was changed 8-10 times per hour. Mice were fed standard chow and water ad libitum. Mice were infected with $10^7$ or $10^3$ CFU of *S. Typhimurium* SL1344 or saline (negative controls) by gastric gavage. Following challenge, mice were observed twice a day. If symptoms of severe disease (ruffled fur, altered behavior) developed, the mice were euthanized immediately due to ethical considerations. No statistical differences in mean body weights between the groups were recorded. Animal experiments were carried out under the supervision of the Danish National Agency for Protection of Experimental Animals.

Isolation of cells from mouse tissues

Mice were killed by cervical dislocation. Mesenteric LN were removed, cells mechanically released by squeezing in Hank’s balanced salt solution, calcium and magnesium free (CMF-H; Lonza), and the cell suspension passed through a 100 μm cell strainer. Ileum was excised and the contents expelled. PP were excised, mechanically disrupted in CMF-H and passed through a 100 μm cell strainer to obtain single-cell suspensions. PP-SED from each mouse were pooled. The remaining ileum was cut open longitudinally and washed thoroughly in PBS. Using a scalpel, the submucosa was separated from the muscularis layer, which was discarded. The mucosa and submucosa was cut into small pieces and digested for 45 minutes at 37°C in CMF-H with 20% FCS, 100 U/ml collagenase D and 30 μg/ml DNase (both from Roche Diagnostics), after which the suspension was passed through a 100 μm cell strainer.

In vitro stimulation of cells from PP-SED

Single-cell suspensions from PP-SED, harvested as described above, were seeded in culture plates (Nunc, Roskilde, Denmark) at $2 \times 10^5$ cells/100 μl/well in culture medium and cultured with viable *S. Typhimurium* at a multiplicity of infection (MOI) of 1, 10 or 25. Cells to which only medium was added were used as uninfected controls. Cells were incubated for 1 hour at 37°C in a 5% CO₂ humidified atmosphere before gentamycin was added in a final concentration of 50 μg/ml/well to kill extracellular bacteria. Cells were then incubated for an additional 19 hours and subsequently harvested for flow cytometric analysis.

Immunostaining and flow cytometry

Single-cell suspensions from freshly harvested PP-SED, LP and mLN or in vitro stimulated cells were transferred to round-bottomed 96-well polystyrene plates (Nunc) and added ice cold PBS-Az (PBS containing 1% (v/v) fetal bovine serum and 0.15% (w/v) sodium azide (Sigma-Aldrich)) to prevent
internalization of surface markers during subsequent handling of the cells. To block non-specific binding of antibodies, cells were incubated for 10 minutes with Fcγ III/II (BD Biosciences) before addition of various concentrations of fluorochrome-conjugated antibodies. After staining for 45 minutes, cells were washed twice in PBS-Az and analyzed on a BD FACSCanto™ II flow cytometer (BD Bioscience). All stainings were carried out at or below 4°C. Antibodies used in this study were against CD11b (BD Horizon V500, clone M1/70), CD11c (APC-Cy7, clone HL3), CD103 (PE, clone M290), CD40 (FITC, clone HM40-3), CCR7 (PerCP-Cy5.5, clone 4B12), all from BD Biosciences; F4/80 (PE-Cy7, clone BM8), CD86 (APC, clone GL1) and PDCA-1 (eFluor450, clone eBio927), both from eBioscience; and CCR6 (PE, clone 29-2L17) from BioLegend. Polyclonal CX3CR1 was purchased from eBioscience and conjugated to AF647 using the APEX™ Alexa Fluor® 647 Antibody Labeling Kit (Molecular Probes, Invitrogen, Denmark) according to manufacturer’s instructions. Analysis of in vivo data was based on a minimum of 300,000 cells and in vitro data on a minimum of 50,000 cells. Data were analyzed using FCS Express Plus software (version 4.0, De Novo Software, Los Angeles, CA).

Statistical analysis
The statistical significance of variations among experimental groups was analyzed by one-way ANOVA followed by a Tukey’s multiple comparison test using GraphPad Prism software (version 5.01, GraphPad Software, San Diego, CA). Statistical significances were accepted at $p<0.05$ within a 95% confidence interval. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Results
Early-stage Salmonella infection and dose-dependent recruitment of gut-associated dendritic cells and macrophages.
To evaluate regulations and dose-dependent effects induced by Salmonella during early stage infection, BALB/c mice were orally inoculated with $10^3$ or $10^7$ CFU of S. Typhimurium SL1344, or left uninfected (designated naive).

The presence of mDC, pDC and Mφ in PP-SED, LP and mLN were determined by multi-parametric flow cytometry at 24h post challenge to follow modifications in cell numbers and viability at different gut tissue locations (Figure 3.1). In PP-SED, pDCs were found to accumulate upon dosing with $10^3$ CFU Salmonella resulting in a 9-fold increase in the number of pDCs as compared to uninfected mice (Figure 3.1a, PP-SED). Mφ were likewise enhanced by 1.5-fold. The percentages of viable mDC present in PP-SED at 24h post challenge were not affected by S. Typhimurium presence and/or dose, although a tendency to a dose-dependent decline was observed (Figure 3.1a, PP-SED). Improved viability of pDC and Mφ upon Salmonella exposure could not account for their increase in numbers in PP-SED, as no or only slightly enhanced viability levels were detected in contrast to naive mice (Figure 3.1b (PP-SED); mDC: $p=0.28,$
pDC: \( p = 0.057 \), Mφ: \( p = 0.048 \) by 1-way ANOVA). The changes in pDC and Mφ numbers in PP-SED in *Salmonella*-challenged mice may therefore be based on enhanced influx of pDC (most significantly for \( 10^3 \) CFU) and Mφ (for both CFU) to PP-SED.

Contrarily to PP-SED, the number of pDC, mDC and Mφ in LP and mLN remained unchanged (Figure 3.1a, LP, mLN), however, in LP the viability of pDC was significantly enhanced by *Salmonella* at both doses (Figure 3.1b; \( p = 0.032 \)), whereas in mLN, \( 10^7 \) CFU *Salmonella* resulted in an impaired viability of both mDC (\( p = 0.0084 \)) and Mφ (\( p = 0.0050 \)) (Figure 3.1b, LP and mLN). This significant decrease in mDC and Mφ viability in mLN was not seen at \( 10^3 \) CFU, implicating an early effect on phagocytes in the gut-draining LN that depends on the infectious dose of *Salmonella*.

Based on these data, it appears that *Salmonella* differentially modulates both the numbers and the viability of DC and Mφ at different gut locations already in the early hours upon infection. Besides the regulation of DC and Mφ influx to infected tissue and LN, the Ag-presenting function of DC and Mφ is of importance for bacterial clearance. We therefore measured the level of expression of CD40 and CD86 on all three cell subsets, as one of the mechanisms by which virulent *Salmonella* is known to be able to evade immune recognition is by interfering with the ability of Ag-presenting cells to activate naive and effector T cells (23, 38, 43). MHC class II was not included in this study as its expression tends to be modified on murine mDC upon cellular handling.
Figure 3.1. Regulation of gut-associated dendritic cells and macrophages 24 h upon oral infection with S. Typhimurium.

(a) Cellular profile showing the percentage of viable pDC, mDC and MΦ in ileal PP-SED, LP and in the mLN 24 h post infection with S. Typhimurium SL1344 at 10^3 or 10^7 CFU, compared to naive mice. Data are obtained by flow cytometric analysis using the gating strategy shown in (c). (b) The percentage of viable (dark grey) versus dead (light grey) pDC, mDC and MΦ in different locations at 24 h post challenge. (c) Representative dot plots of cellular gating. Viable cells were gated by use of SYTOX Green, and exclusion of cell debris (not shown). mDCs were gated from R1 representing F4/80^lo/int, and then on CD11chi, PDCA-1^lo. pDCs were selected as PDCA-1^hi (R2), and CD11b^neg. MΦ were defined as F4/80^hi, CD11c^lo/int. (a-c) Data is mean±sd, n=4 mice per group, and represents one of two similar experiments. One-way ANOVA and Tukey’s post hoc test was used for statistical analysis.
Alterations in activation status of dendritic cells and macrophages in gut tissue during oral Salmonella challenge.

The expression of CD40 and CD86 by pDC, mDC and Mφ in PP-SED, LP and mLN was determined by multi-parametric flow cytometry following both high and low dose *Salmonella* administration (Figure 3.2a-e). The percentage of viable cells with CD40 and/or CD86 expression is shown as percent positive, and the level of CD40 and CD86 expression is based on expression levels on the positive cells.

In naive mice, the steady state percentages and expression levels of CD40 and CD86 on pDCs, mDCs and Mφ were found to be independent of the tissue location (PP-SED, LP or mLN), whereas it varied in-between the three cell types. Specifically, less than 10% of pDCs were found positive for CD40 and CD86, and with most being positive for CD86, while 60-80% of mDCs and Mφ displayed CD40, and 20-40% expressed CD86.

In PP-SED, mDCs exposed to both high and low dose *Salmonella* showed a slightly, although statistically significant reduction in CD40 expression (1.2-fold) compared to naive mice (Figure 3.2a), whereas no effect on the percentage of CD40-positive mDC was observed. Conversely, the CD86 expression in mDC was not modified by *Salmonella* at any of the doses (Figure 3.2d). In relation to pDC in PP-SED, no significant regulation of the percentages of positive cells was induced by *Salmonella* at 24h post infection. However, as for mDC, the level of CD40 expression on the CD40-positive pDC in PP-SED was significantly reduced by two-fold in a *Salmonella* dose-independent manner, whereas the CD86 expression remained unaffected (Figure 3.2a,d). Neither the percentage of positives nor the level of CD40 and CD86 expression on Mφ from PP-SED changed at 24h post challenge (Figure 3.2a,d). In LP, *Salmonella* did not affect the percentage nor MFI of CD40 or CD86 on mDC, pDC or Mφ (Fig 3.2b,e). Remarkably, no LP pDC were found to express CD40 (Figure 3.2b). In mLN, no significant changes of CD40 was observed 24h post challenge with *Salmonella* (Figure 3.2c), but the level of CD86 expression on mDC was reduced by both doses (Figure 3.2f).

All together these data show that already at 24h post challenge, *Salmonella* causes regulation of the functionality of pDC and mDC by reducing the level of CD40 expression on these cells in the PP-SED compartment, and the levels of CD86 on mLN-located mDC, perhaps due to migration.

In order to increase the insight into the migratory abilities of mDC present in PP-SED, LP and mLN, we measured the level of expression of CCR6 and CCR7. On mDC, CCR6 is preferentially expressed by immature, blood-circulating cells, whereas CCR7 is enhanced on mDCs upon activation, thereby enabling migration from peripheral tissues to LN.
Figure 3.2. Distribution of activation markers on pDC, mDC and Mφ in PP-SED of S. Typhimurium challenged mice. Expression of CD40 and CD86 on pDC, mDC and Mφ in PP-SED (a, d), LP (b, e) and mLN (c, f), 24 h upon oral challenge with S. Typhimurium SL1344 at $10^3$ or $10^7$ CFU, as compared to naïve mice. The mean fluorescence intensity is given in the right hand panels, and the percent positive cells in the left hand panels. For all data, n=4 mice per group, representing data from one of two experiments. Data represent mean±sd. *, P<0.05, **, P<0.01 as tested by one-way-ANOVA and Tukey’s post hoc test.
In naive mice, CCR6-positive cells in PP-SED made up approximately 20% of the mDC population, and CCR7 was found on approximately 16% of mDC (Figure 3.3a). In LP, CCR6 was only found on 1% of mDC, whereas CCR7 was expressed on roughly 8% of mDCs. These expression dynamics were different from mLN mDC, where CCR6 was expressed on just about 20% of mDC, but only 2% expressed CCR7. CCR6 and CCR7 expression was solely found on pDC located in the lymphoid tissues, i.e. PP and mLN, with up to 1% of cells expressing the chemokine receptors (data not shown). Expression levels of CCR6 differed considerably between the three gut tissue locations (Figure 3.3b). CCR6 levels were 3 times higher on PP-SED and LP mDC than on mLN-located mDC, and CCR7 levels were similar in naive mDC from all locations.

Upon Salmonella challenge, CCR6 levels on PP-SED mDC were decreased in mice dosed with $10^7$ CFU S. Typhimurium (Figure 3.3b), while the percentage of CCR6-positive mDC at $10^7$ CFU remained unchanged.
from that of naive mice (Figure 3.3a). Contrarily, the percentage of CCR6-positive mDC was significantly lower with $10^3$ CFU, suggestive of fewer immature cells amongst the mDC populating PP-SED upon challenge with $10^3$ CFU *S. Typhimurium*. The levels of CCR6 and CCR7-positive mDC in LP were not significantly changed by *Salmonella* administration, but CCR6 expression levels on mDC were significantly enhanced by $10^3$ CFU. In mLN, no effects on CCR6 or CCR7 of *Salmonella* dosing were observed at 24h post infection. Collectively, these data showed that subtle differences in CCR6 expression exist between the two dosing groups. No *Salmonella*-induced effects on CCR7 expression on mDC were found in any of the gut tissue locations.

Taken together, the combined activation status of pDC, mDC and Mφ suggest that *Salmonella* at 24h post infection actively modifies the immune response from these three APCs in the local gut mucosa. The factors giving rise to the most significant dose-induced differences in mice orally infected with *S. Typhimurium* SL1344 seem to relate to the modulation of pDC numbers in PP-SED, concomitantly with a reduced viability of mDC and Mφ in mLN by high dose administration. Diverse subsets of functionally distinct mDC and Mφ exist within the gut mucosa (11, 27), and we therefore also studied if these subsets are differentially regulated during early stage *Salmonella* infection thereby influencing host survival in a dose-dependent manner.

**Characterization of *Salmonella*-induced regulation of subpopulations within the myeloid compartments during early-stage infection.**

Identification of mDC subpopulations were based on their expression of CD11b, CD11c and CD103, resulting in classification of 3 distinct CD11c⁺-subpopulations, defined as: CD11b⁹⁺CD103⁺ cells (designated DC1); CD11b⁺⁺CD103⁺ cells (DC2), and CD11b⁹⁺⁺CD103⁻ cells (proinflammatory DC; proDC) (Figure 3.4a).

All F4/80⁺ cells were characterised as Mφ or mixed-lineage populations, and were not counted as mDC. We found that in both PP-SED and LP of naïve mice, the proDCs were the dominating population (Figure 3.4b), whereas they made up about 25% in mLN during homeostatic conditions. On the other hand, DC2 cells were hardly detectable in LP, only 3% were found in PP-SED, and about 25% were identified in mLN of naïve mice, underscoring that DC2 can be identified in gut tissue, but that other mDC subsets dominate in PP-SED and ileal LP. In mLN, DC1 were slightly more prominent than DC2 and proDC, but a more balanced distribution between the three subsets were found in mLN than in PP-SED and LP, and collectively more than 50% of MLN DC were CD103 positive (DC1 and DC2, Figure 3.4b).

At 24h post challenge, *Salmonella* enhanced the DC2 numbers in LP, whereas no changes were observed in PP-SED and mLN (Figure 3.4c). Minor variations in the percentages of the three subpopulations were identified between the two *Salmonella* doses (Figure 3.4c), but at this time point these were only subtle, non-significant differences.
Figure 3.4. Modification of cellular subpopulations within the myeloid dendritic cell compartment upon oral infection with *S. Typhimurium*.

(a) mDCs (CD11c<sup>hi</sup>, F4/80<sup>lo/int</sup>, PDCA-1<sup>lo</sup>) were gated into three subgroups, DC1, DC2 and proDC. Cells defined as CD11b<sup>hi</sup>, CD103<sup>int/hi</sup> were named DC1. CD11b<sup>lo</sup>, CD103<sup>hi</sup> were identified as DC2, and proDC as CD11b<sup>hi</sup>, CD103<sup>lo</sup>. (b) Distribution of cellular subpopulations within the mDC subsets in PP-SED, LP and mLN from naive mice. (c) Heatmap displaying the cellular profiles from (b) as the ratios in infected (10<sup>3</sup> or 10<sup>7</sup> CFU) versus naïve mice. One of two representative experiments is shown. Data represent mean±sd, n=4 mice per group.

Mφ, named due to their F4/80<sup>hi</sup> expression, were divided into 2 subpopulations termed M1 and M2, and identified as CD11c<sup>lo</sup>, CD11b<sup>hi</sup>, CD103<sup>lo/int</sup> for cells named M1, while M2 cells were CD11c<sup>int</sup>, CD11b<sup>hi</sup>, CD103<sup>hi</sup> (Figure 3.5a). CD103 has previously been identified as a discriminative marker for gut DC, and as such Mφ should not display CD103 on their surface (4, 28, 37), however in our hands we found CD103 expression on the M2 subpopulation (Figure 3.5b). CX3CR1 was also detected on M2, whereas it was low/absent on M1 (Figure 3.5b). In the gut tissue, the M1 and M2 subpopulations did not make up all Mφ (Figure 3.5c). The remaining Mφ were termed ‘undefined’, and were CD11c<sup>lo</sup>, CD11b<sup>lo</sup>, CD103<sup>lo</sup>. In naïve mice, the M2 subpopulation was found to dominate in both PP-SED and LP, while M1 and M2 were of equal sizes in mLN (Figure 3.5c). In both PP-SED and mLN, the undefined Mφ comprised half or more than half of the Mφ. An up-regulation of M2 numbers in PP-SED was found to dominate upon *Salmonella*-challenge (Figure 3.5d). When focusing on dose-dependent modifications of M1 and M2, the
most prominent effect was on M1 cells. In PP-SED and mLN, M1 numbers increased in low dose animals, but decreased in high dose animals, suggesting a reversed dose-dependency that may contribute to the host ability to eradicate *Salmonella*. No effect of doses was evident in LP Mφ subsets. Altogether, we found no statistically significant regulations of the mDC and Mφ subpopulations in the gut tissue locations at 24h post challenge that could account for the dose-dependent differences in host mortality found in our previous studies to be effective at Day 8 upon oral *Salmonella* administration, but the mere presence of *Salmonella* in the gut lumen enhanced the numbers of specifically LP-located DC2, and PP-SED M2. However, it seems conceivable that bacteria-to-cell load at time of entry may play an important role in salmonellosis due to the dose-dependent mortalities previously observed by us at Day 8, and the effect on cellular atrophy in mLN mDC and Mφ for $10^7$ CFU at 24h post challenge. Because *in vivo* studies make it difficult to examine the direct bacteria-induced modification of immune cells, due to continued inflammation-induced influx of new, immature cells from blood into lymphoid tissues, we went on to study by *in vitro* experiments how direct bacteria-to-cell ratios affect the number, viability, as well of expression of CD40, and CD86 on pDC, mDC and Mφ from murine gut tissue.
Figure 3.5. Modification of cellular subpopulations within the Mφ compartment upon oral infection with S. Typhimurium.

(a) Macrophages (F4/80^hi^) were divided into two subsets: M1 and M2. M1: CD11c^lo^, CD11b^hi^, CD103^int^. M2: CD11c^int^, CD11b^hi^, CD103^lo/int^.
(b) Histograms displaying CX3CR1 and CD103 expression levels on M1 and M2 Mφ.
(c) Distribution of cellular subpopulations within the Mφ subsets in PP-SED, LP and mLN from naive mice.
(d) Heatmap displaying the cellular profiles from (c) as the ratios in infected (10^3 or 10^7 CFU) versus naive mice. One of two representative experiments is shown. Data represent mean±sd (n=4).

**Dose-dependent modification of dendritic cells and macrophages by Salmonella in short-term in vitro cultures.**

Cells were exposed to Salmonella at bacteria-to-total cell ratios of 1:1, 10:1, and 25:1, here referred to as multiplicity of infection (MOI), and the direct effect on pDC, mDC and Mφ from PP-SED was evaluated after 20h.
By use of multi-parametric flow cytometry, we found that the abundance of viable pDC, mDC and Mφ changed depending on the cell type when exposed to the same MOI (Figure 3.6a). In particular, with MOI 1, the number of mDC increased, while it remained constant for pDC and dropped in Mφ. For MOI 10, pDC and mDC numbers remained constant, and Mφ numbers were further decreased. MOI 25 resulted in fewer than initial numbers of all three cell types (Figure 3.6a). When focusing on the viability of the cells, it appeared that mDC and Mφ had significantly impaired viability upon exposure to increasing doses of *Salmonella*, whereas pDC were less refractory to bacteria-induced killing (Figure 3.6b). Combining the decrease in viability of mDC along with increased MOI to the rise in numbers of mDC at MOI 1 (Figure 3.6a), it seems likely that the increase in numbers of mDC must be based on recruitment from a DC precursor population. The level of regulation of pDC and Mφ numbers correlates well with the dose-dependent reduction in cell viability for these two subsets (Figure 3.6a,b).

Similar to what we observed in the *in vivo* studies, we also found an effect on the cellular activation profile in the viable fraction upon *in vitro* exposure to *Salmonella* (Figure 3.6c). Both CD40- and CD86-expression on viable mDC increased slightly at MOI 1 and 10, but plummeted by 50% at MOI 25. Mφ showed a MOI-dependent decrease in expression of both CD40 and CD86, indicating that although the cells selected for in the flow cytometric analysis were still alive, they were affected phenotypically by the increase in bacterial dose.

The level of regulation of CD40 and CD86 expression in pDC differs from that of mDC and Mφ with a specific threshold of MOI, around 10, giving rise to enhanced CD40 and CD86, indicative of activation. More or less bacteria (MOI 1 or 25) resulted in only slight or no activation (Figure 3.6c). Identical results were seen in all gut tissue locations (PP-SED, LP, mLN), and also in cells derived from the spleen (data not shown). These *in vitro* data signifies that all three cell types, mDC, pDC and Mφ, are affected considerably by increasing doses of *Salmonella*.

Combining *in vivo* data with those from our *in vitro* studies suggests that during early stages of *Salmonella* challenge, the numbers of pDC, mDC and Mφ are dose-dependently modified with increased influx of all cell types into PP-SED when taking into account the critical *Salmonella*-induced reduction in viability of mDC and Mφ. At bacteria-to-cell ratios above 10:1, the CD40 and CD86 expression was impaired in all three cell types, hence implying dose-dependent regulations of APC function, also in pDC, by *Salmonella*. 

Figure 3.6. *S. Typhimurium* dose-dependently impairs viability and activation profile of mDC, pDC and Mφ *in vitro*. Cells isolated from PP-SED were *in vitro* incubated with *S. Typhimurium* at varying cell-to-bacteria ratios for 20 h, and then subjected to flow cytometric evaluations. (a) Dose-dependent modifications in prevalence of pDC, mDC and Mφ given as ratios to uninfected cells. (b) The viability of pDC, mDC and Mφ presented as the ratio to uninfected cells. (c) Expression of CD40 and CD86 on pDC, mDC and Mφ. Data are mean (n=3).

Discussion
To gain insight into the processes of initial events in the Ag-presenting immune compartment taking place following mucosal infection with *S. Typhimurium*, this study focused on evaluating dynamical regulations of the major innate immune cell compartment made up by pDC, mDC and Mφ, and minor subsets of the two latter, in ileal-associated intestinal tissue (PP-SED and LP), as well as in the intestinal draining LN (mLN). Immune recognition by these innate immune cells plays a major part in early stages of an infection to enable eradication of intruders by phagocytosis and activation of adaptive immunity (1).
We here for the first time demonstrate that pDC accumulates in a dose-dependent manner in PP-SED at 24h post challenge with S. Typhimurium, reaching the levels of mDCs in total numbers. Involvement of pDC in bacterial infections is for most parts undefined, but our findings are consistent with a few recent reports where pDC are observed to play a role in infections with *Listeria monocytogenes*, *Aspergillus fumigatus*, *Legionella pneumophila* and *Chlamydia* spp. in murine models (2, 12, 39), and/or in human model systems (29). pDC are characterised by their secretion of high amounts of type I IFN in response to viruses and have been identified in PP-SED, interfollicular regions of PP and LP as well as mLN in mice (3, 10, 40). Since pDC numbers do not increase in a way similar to mDC during the present *in vitro* studies, it appears that the rise in PP-SED pDC observed during *in vivo* *Salmonella* exposure is based on influx of blood-derived pDC or pDC precursors into PP-SED rather than on renewal from the DC precursor pool (42). Such blood-derived pDC may be phenotypically distinct from pDCs generated in PP-SED due to the PP microenvironment that seems to prime to IL-17-T-cell polarizing pDCs (22), but further studies are warranted to address the specific functional phenotype of PP-SED pDCs in early stage *Salmonella* infection. The main role of pDC in the immune process against *Salmonella* may be to boost immunity rather than to be modified to induce tolerance (31). This assumption is based on the fact that the highest pDC numbers were found to be present in low dose animals generally outliving the infection. However, we cannot exclude that the infectious kinetics (i.e. the time point for measurement) play a part in this. Indeed the lower levels of CD40 on pDC from both dosing groups suggest that *Salmonella* also modifies pDC immunity in a similar manner as reported for mDC (8). Since the pDC regulation by *Salmonella* was not the main focus of the present study, we did not measure levels of type I IFN in PP-SED/intestinal tissue nor in the *in vitro* culture setup.

In increases in pDC and Mφ in PP-SED upon challenge with a high dose (10⁷ CFU) of S. Typhimurium were not as apparent as for low dose administration, although the cell viabilities at both doses were similar. By combining data from our *in vivo* and *in vitro* experiments, we assumed it may be possible to extract important information concerning the dynamical regulation of the CD86 and CD40 expression levels by *Salmonella* on PP-SED-derived APCs. For CD40, the *in vivo* decrease in expression levels on pDC and mDC upon *Salmonella* dosing was comparable to the decrease above approx. MOI 10 during *in vitro* experiments. It, however, appears peculiar that we find the same decrease in CD40 levels by the two orally administered *Salmonella* doses differing by a factor of 1000 in bacterial numbers. This observation suggests that the mere presence of S. Typhimurium SL1344 is more definable for the CD40 expression levels on mDC and pDC in PP-SED, than the actual number of bacteria. *In vivo*-induced activation cues in *Salmonella* could be a factor, and it may be relevant to consider this in future studies. On the other hand, we observed quite different expression patterns *in vivo* and *in vitro* for CD86 on all three APC subsets as *in vivo* *Salmonella* exposure did not appear to change the level of expression, contrasting to the *in vitro*
dose-dependent effects observed on all three subsets. Presently we cannot definitely conclude on these differences.

In LP, the enhanced viability of pDC, and the indication of increased numbers of pDC for both bacteria doses illustrate that also the ileal LP compartment is affected by the *Salmonella* challenge, but in a dose-independent manner. An impairment of mDC and Mφ viability at $10^7$ CFU *S. Typhimurium* in mLN, along with unchanged numbers of mDC and Mφ, point to replacement by recruitment from the blood to mLN of these phagocytes, but more importantly it signifies that *Salmonella* modifies the viability of these phagocytes during early stages of infection, and implies that the bacterial dose plays a part in (down) regulation of immunity. Whether mDCs are infected with *Salmonella* in LP or PP-SED and migrates to mLN where they undergo cell death, or whether they are directly infected in mLN, we cannot decipher based on the current study. However, as the viability of mLN-resident Mφ is also impaired, and as Mφ are known not to migrate from LP/PP to mLN, it seems plausible that *Salmonella* can directly affect blood-derived phagocytes inside mLN. How *Salmonella* gets access to mLN is not apparent from the present study, but since the impaired viability in mLN mDC and Mφ was observed for $10^7$ CFU only, it seems possible the cellular injury is linked to the infectious dose of *Salmonella*. Indeed, as revealed in the *in vitro* experiments, the viability of mDC and Mφ, but not of pDCs, was dependent on *Salmonella* doses. The dose-dependent differences in numbers and viabilities of pDC, mDC and Mφ in PP-SED and mLN could be caused by inappropriately controlled influx of *Salmonella* bacteria carried within CD11c$^+$-cells from PP to mLN, as earlier described by MacPherson and Uhr (24). Collectively, based on our data focusing on dose-dependent changes, we speculate that *in vivo* increments of pDC in PP-SED may be of significance for intracellular bacterial containment in PP-SED by enhancing phagocytotic potential of mDC and Mφ (in a type I IFN-dependent manner), hence hampering bacterial spread of viable *Salmonella* to mLN (31).

In the present study, we focused on a careful delineation of the involvement of specific functional subsets within the mDC and Mφ lineages in the early stage host response against *Salmonella*. Specific attention was centred at *Salmonella*-induced regulations in numbers of three subpopulations of mDCs and two Mφ subsets. At present, increasing appreciation of the functional roles of these subsets as well as their differential presence in different tissues is developing, but their characterization is still in its infancy, and contradictory results appear due to divergent markers used for identifications. Several reports discriminate mDC populations based on CD8α, CD11b and CD11c expression levels. The mDCs identified here represent CD8α$^{neg}$CD11b$^+$ mDC (DC1), and DC2 make up the CD8α$^{+}$CD11b$^{neg}$ mDC, both of which we find to express CD103 at similar levels. CD103 is generally recognized to be a useful marker for identifying mDC that migrate from LP to mLN, being consistent with our data where approximately 50% of CD103$^+$-cells (DC1+DC2) are found in mLN during steady-state conditions. More surprisingly was our identification of the relative percentages of viable DC1, DC2 and proDC in PP-SED and LP, as proDC was
observed to make up a considerable part of the mDC populations at both sites, and CD103^+mDCs were barely seen in LP. The almost complete absence of CD103^+ mDCs in LP seems notoriously inconceivable due to their regulatory function in LP (18), and it warrants attention as it may relate to handling issues. Based on our data it is, however, conceivable that the M2 subset make up the CD103^+ population in LP, but as they do not express CCR7 they are unlikely to represent the mLN migrating subset. More detailed studies are necessary to uncover this dichotomy.

The *Salmonella*-induced increase in DC2 numbers in LP, and also modestly in PP-SED is of interest since it points to the contribution of a mDC subset (CD11c^{hi}CD103^{hi}CD11b^{lo}F4/80^{lo}) that contributes to *Salmonella* sensing in LP (28, 32), and presumably also in SED. Due to the low numbers of DC2 and variation in kinetics within animals, it is difficult to characterise the identity and function of these by flow cytometry, and more detailed analyses are needed, now they are identified here to be involved. Our finding of the M2 subset, displaying mixed myeloid lineage markers of mDC and MΦ, in PP-SED of *Salmonella*-exposed animals suggests that this cell type could be involved in sampling of *Salmonella* in the SED/gut lumen region. In the present study, M1 and M2 were identified based on F4/80, CD11c and CD11b expression, and were then further analyzed for CX3CR1 and CD103 expression. We found M2 to express high levels of CX3CR1 simultaneously with CD103, whereas M1 had intermediate expression of both. This finding adds to a growing body of evidence that CX3CR1^+ MΦ cells can sample Ag in the gut lumen (9, 28). Since M2 also exhibits CD11c expression, it is not surprising that divergent results exist about the role of DC (CD11c^+) versus MΦ in paracellular sampling of gut lumen material. Based on our current collected data on mDC and MΦ subsets derived from gut tissue, and their subset-individual expression levels, it points towards MΦ, and explicitly M2, as being the CX3CR1^+ cell type sampling the gut in PP-SED (35). The M2 increment observed in PP-SED of *Salmonella*-exposed animals indicate a process were the M2 cells could locate in SED and be the cell type involved in *Salmonella* trans-epithelial sampling in the intestinal lumen. Our novel finding of M2 being enhanced in PP-SED, and of DC2 increases primarily in LP points to a collaborative influence of both cell types in sensing of *Salmonella*. Their specific involvement in mucosal immunity remains unclear, but requires further attention as both cell types are accumulating upon *Salmonella* exposure.

Collectively, we demonstrate a role for pDC in immunity against *Salmonella*, and our data suggest that the accumulated number and their activation status may be important for *Salmonella* containment, as cellular atrophy, and decreases in CD40/CD86 surface levels is amplified in mDC and MΦ in a dose-dependent manner. Notably, we also identified a *Salmonella*-induced enhancement of two myeloid subsets in PP-SED and LP regions, respectively. Altogether our present findings emphasize that a delicate interplay between the innate Ag-presenting immune cell compartments takes place during early phases of a *Salmonella* infection, and entails that interventions to progress pDC accumulation and/or functionality at gut mucosal sites may improve host immunity to *Salmonella*.
References


sensitization is controlled by regulatory T-cells and plasmacytoid dendritic cells. PloS One 6:e20784.


Integration of functional and genomic signatures from gut-associated bacteria reveals phyla-specific entities encoding immune inflammatory abilities

In collaboration with Janne Marie Laursen, Ole Lund, Jonas Nørskov Søndergaard, Hanne Frøkiær and Susanne Brix.
Abstract
Mutualistic interplays between intestinal bacteria and the host is appearing to significantly influence host health and to protect against local and systemic diseases principally due to regulation of inflammation. Gut-associated dendritic cells (DC) enforce sensing of microbial products, and represent a main driver and regulator of sustained inflammatory processes. Through their interaction with the gut content, DC gets into contact with various microbes, pathogenic or not, that induces or modifies their inflammatory response profile. To enhance our basic insight into divergent bacteria-induced profiles in DC, we here characterized the pro-inflammatory signature imparted in DC by six viable bacteria recognized as pathogens (Salmonella enterica serovar Typhimurium SL1344, Listeria monocytogenes EGD-e), opportunistic pathogens (Clostridium difficile 630), or commensals (Lactobacillus acidophilus NCFM, Bifidobacterium longum DSM20088, and Escherichia coli Nissle 1917).
DC responded to these bacteria in a phyla-specific manner giving rise to similar inflammatory signatures within the groups of proteobacteria (S. Typhimurium and E. coli Nissle 1917), firmicutes (L. monocytogenes, C. difficile, L. acidophilus), and actinobacteria (B. longum), hence being independent on pathogenic versus non-pathogenic properties, and also on the bacteria-to-cell ratio for most bacteria. For S. Typhimurium, however, a dose-dependent increase in inflammatory-induced cell death (pyroptosis) was effectuated underscoring a specific pathogenic trait of S. Typhimurium that was not identified for L. monocytogenes, or any of the gut commensals.

Introduction
Interactions between bacteria and gut-associated DC mediate activation of explicit inflammatory cues that differ in strength and nature depending on the combined bacterial molecular composition, and the recognition of it by germ-line encoded pattern recognition receptors (PRR) present in DC (26, 45). Generally, we tend to segregate disease-causing bacteria, i.e. the pathogenic bacteria, from opportunistic pathogens, and commensal bacteria based on their abilities to provoke disease in different hosts, but the nature of the inflammatory response they induce in DC that set them apart from commensal bacteria remains largely unclear. Recent technological advances have made it possible to compare gut bacterial compositions in various individuals, hence enabling us to pin-point which bacteria species that could play a part in health versus specific disease settings (2, 41). Based on these results, it is now generally recognized that some gut bacteria, being part of the resident microbiota in healthy individuals, may confer protection against development of diverse inflammation-related diseases due to modifications of the gut inflammatory status and thereby seemingly taking part in adjustment of systemic inflammatory trails. Presently, however, we are largely unaware of which bacterial genome-encoded components that are employed by the diverse gut-associated bacteria to exert their specific actions. The genetic composition of the bacteria unambiguously code for expression of molecular
entities of which some can be turned on/off dependent on the growth conditions, whereas others are permanently expressed or silent. Since genomic profiles of bacteria are becoming widely accessible, it may be of great relevance to pursue a track that enables identification of genome-encoded single entities or enzymatic pathways in bacteria that can be directly linked to a functional inflammatory profile, or the absence of it, in DC. Such bacterial genome-to-host functional linkage analysis would allow us to develop tools to predict the inflammatory status in the gut based on the collective gut microbiome. As a key immunoregulatory cell type within the body, the DC is central for host immunity (19), and as such it is well fit for use in controlled studies of specific bacterial-induced host inflammatory signatures. DC are located just beneath the simple columnar gut epithelium and are able to protrude dendrites into the gut lumen, making them among the first immune cells to sense and phagocytose invading or commensal bacteria (21, 44). Upon capture of bacterial-derived materials, DC undergo phenotypic changes and migrate to lymph nodes where they prime T cells to an appropriate response (26). The phenotypic changes in DC rely on activation of individual PRR that turn on diverse signal transduction pathways, collectively resulting in imprinting of various inflammatory or tolerance-inducing signatures depending on the molecular bacterial units being recognized. Based on their specific regulatory functions within the gut environment, we find DC suitable to use as a model system for studying the influence of bacterial composition and genetics on programming of host inflammatory profiles.

In the present study, we developed a system by which we were able to compare the bacteria-induced imprint of important regulatory proteins in DC to bacterial-encoded PRR-ligands. To test the applicability of the system, different viable gut-associated bacteria were added at different bacteria-to-cell doses (referred to as multiplicity of infection (MOI)), followed by the analysis of 10 secreted proteins, and 4 surface proteins of known biological function. We used the following bacteria, that were all genome sequenced: Pathogenic: *Salmonella Typhimurium* (ST), *Listeria monocytogenes* (LM), opportunistic pathogenic: *Clostridium difficile* (CD), and gut commensals: *Lactobacillus acidophilus* (LA), *Bifidobacterium longum* (BL) and *Escherichia coli* (EcN). At the current status we are in the progress of combining the functional data from the DC to the genome-encoded molecular signatures of these bacteria, and therefore we here report on the bacteria-induced DC profiles based on comparisons to well-known bacterial taxonomies. Overall, we find that the bacteria induce phyla-specific inflammatory programs in DC, and that this is independent of MOI, and whether bacteria were classified as commensals, opportunistic pathogens or intracellular pathogens.

Materials & methods
In vitro generation of human DC from monocytes
DC were generated from human peripheral blood mononuclear cells (PBMC) as described by Zhou and Tedder (55) with minor modifications. Briefly, PBMCs were obtained after Ficoll-Paque (GE Healthcare...
Biosciences, Uppsala, Sweden) density-gradient centrifugation of buffy coats from healthy donors. Monocytes were isolated by magnetic activated cell sorting with human CD14+ microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and were cultured at a density of $6 \times 10^6$ cells/3ml/well in six-well suspension culture plates (Nunc, Roskilde, Denmark) for 6 days in culture medium (RPMI 1640 supplemented with 2 mM L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Lonza, Verviers, Belgium) and 50 μM 2-mercaptoethanol (Invitrogen, Carlsbad, CA) containing 30 ng/ml recombinant human IL-4 and 20 ng/ml recombinant human GM-CSF (both from CellGenix, Freiburg, Germany). On day 3, fresh media containing full doses of IL-4 and GM-CSF was added to the cells. After 6 days, the CD14+ cells had differentiated into non-adherent iDC, characterised by their expression of CD1a.

**Bacterial strains and culture**

The strains used in this study are listed in Table 4.1. *Salmonella Typhimurium* SL1344 and *Escherichia coli* Nissle 1917 were grown at 200 rpm in Luria-Bertani broth (Merck, Darmstadt, Germany), *Clostridium difficile* 630 and *Listeria monocytogenes* EGD-e in brain-heart infusion broth (Oxoid, Thermo Fisher Scientific, Denmark), and *Lactobacillus acidophilus* NCFM and *Bifidobacterium longum* DSM 20088 in Man, Rogosa and Sharpe broth (Merck, Darmstadt, Germany) at 37°C, anaerobically, overnight. The cultures were then diluted and grown until reaching log-phase, harvested, washed twice in sterile phosphate-buffered saline (PBS, Lonza) and resuspended at appropriate concentrations in culture medium. MOI was controlled by plating serial dilutions on BHI plates and counting colonies after 24h incubation at 37°C. ST was kindly provided by Jens Bo Andersen, National Food Institute, Technical University of Denmark. EcN was obtained from Statens Serum Institute, Denmark. CD was kindly provided by Peter Mullany, UCL Eastman Dental Institute, United Kingdom, LM was kindly provided by Lone Gram, Technical University of Denmark and BL was obtained from Danisco, Denmark.

**Table 4.1. Strains used in this study.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Salmonella Typhimurium</em> SL1344</td>
<td>Calf-virulent isolate</td>
</tr>
<tr>
<td><em>Escherichia coli</em> Nissle 1917</td>
<td>Adult, faeces</td>
</tr>
<tr>
<td><em>Clostridium difficile</em> 630</td>
<td>Adult patient</td>
</tr>
<tr>
<td><em>Listeria monocytogenes</em> EGD-e</td>
<td>Rabbit</td>
</tr>
<tr>
<td><em>Lactobacillus acidophilus</em> NCFM</td>
<td>Adult, faeces</td>
</tr>
<tr>
<td><em>Bifidobacterium longum</em> DSM 20088</td>
<td>Intestine of infant</td>
</tr>
</tbody>
</table>

**In vitro stimulation of human DC**
On day 6, iDC were harvested and reseeded in 48-well suspension culture plates (Nunc) at $6 \times 10^5$ cells/500 μl/well in culture medium. DC were cultured with bacteria (MOI 1, 10, 25 or 50) or LPS from S. Typhimurium (Sigma-Aldrich) at a final concentration of 1 μg/ml. Cells to which only medium was added were used as negative controls. Cells were incubated for 1 hour at 37°C in a 5% CO₂ humidified atmosphere before gentamycin was added in a final concentration of 50 μg/ml/well to kill extracellular bacteria. Cells were then incubated for an additional 19 hours. Culture supernatants were collected and stored at -80°C until cytokine analysis, and cells were harvested for flow cytometric analysis.

**Cytokine quantification in culture supernatants**

The production of IL-1β, IL-6, IL-10, IL-12p70, IL-27, TGF-β, TNF-α were analysed used commercially available ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. IL-18 and IL-23p19 productions were analysed using a commercial ELISA kit (eBioscience, San Diego, CA), and IFN-β production was analysed using the Human IFN-beta Tissue Culture Kit from Meso Scale Discovery, and read on the Sector Imager 2400A (Meso Scale Discovery, Gaithersburg, MD). All assays were conducted according to standard manufacturer’s protocols. The lower levels for detection of cytokines were as follows: IL-1β = 18 pg/ml, IL-6 = 17 pg/ml, IL-10 = 12 pg/ml, IL-12p70 = 25 pg/ml, IL-18 = 80 pg/ml, IL-23p19 = 30 pg/ml, IL-27 = 18 pg/ml, TNF-α = 8 pg/ml and IFN-β = 3 pg/ml.

**Immunostaining and flow cytometry**

After collecting supernatants, ice cold PBS-Az (DPBS containing 1% (v/v) fetal bovine serum and 0.15% (w/v) sodium azide (Sigma-Aldrich)) was added to prevent internalization of surface markers during later handling of the cells. Non-adherent cells were harvested by gentle pipetting and transferred to 96-well plates (Nunc). To block non-specific binding of antibodies, cells were incubated with 2% heat-inactivated human AB serum (Copenhagen University Hospital, Denmark) before addition of fluorochrome-conjugated antibodies. After staining, cells were washed twice in PBS-Az and analyzed on a BD FACSCanto™ II flow cytometer (BD Bioscience). Antibodies used in this study were against CD1a (PE, clone HI149), HLA-DR (BD Horizon™ V500, clone G46-6), CD86 (BD Horizon™ V450, clone 2331(FUN-1)) (all from BD Biosciences), CD40 (PerCP-Cy5.5, clone 5C3) (from BioLegend), and CCR7 (APC-eFluor780, clone 3D12) (from eBioscience). For viability staining, a LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Invitrogen Life Science, Paisley, UK) was used. Analyses were based on 50,000 cells and gated on viable cells. Data were analysed using FlowJo software (Version 7.6.3, Tree Star, Inc., Ashland, OR).

**Statistical and principal component analysis**

The statistical significance of variations among experimental groups was analysed by one-way ANOVA followed by a Tukey’s multiple comparison test using GraphPad Prism software (version 5.01, GraphPad...
Software, San Diego, CA). Statistical significances were accepted at $p<0.05$ within a 95% confidence interval. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Principal component analysis (PCA) was performed in R (Foundation for Statistical Computing, Vienna, Austria, 2011) (42) using the function `prcomp`. `prcomp` uses singular value decomposition on the covariance matrix for the PCA computations. In order to include the relative expression level of the surface markers from flow cytometry, data were included from three donors for all bacteria at MOI 1 and 10, LPS and iDC. The dataset included the concentrations of IL-1β, IL-6, IL-10, IL-12p70, IL-18, IL-27, IFN-γ, TNF-α and TGF-β (measured by ELISA) and mean fluorescence intensity (MFI) of HLA-DR, CD40, CD86 and CCR7 (determined by flow cytometry). The values were centred and scaled for each donor before appending the three datasets and performing the PCA.

**Results**

The bacteria used in this study were intracellular pathogens (ST and LM), opportunistic pathogens (CD) and gut commensals (LA, BL and EcN). LM, CD and LA belong to the firmicutes phylum, BL to the actinobacteria phylum and ST and EcN to the proteobacteria phylum.

ST is a food-borne Gram-negative, facultative intracellular bacterium, causing disease in a wide range of hosts. In humans ingestion typically leads to gastroenteritis but in children, old people and immunocompromised persons it may lead to systemic enteric fever and septicemia (35).

LM, a Gram-positive, facultative intracellular bacterium, is the etiologic agent of listeriosis, a severe food-borne disease. Clinical symptoms range from gastroenteritis to sepsis or meningo-encephalitis, and although the incidence of human listeriosis is relatively low, the disease has a high lethality rate in immunocompromised individuals, neonates, and foetuses (52).

CD is a Gram-positive, spore-forming anaerobic bacterium, often found in the healthy gut, and a leading cause of infectious diarrhoea among patients in hospitals worldwide. The pathogen is frequently associated with antibiotic treatment and causes diseases ranging from antibiotic-associated diarrhoea to life-threatening pseudomembranous colitis (23).

LA and BL are Gram-positive rods, termed as probiotics and natural inhabitants of the human gastrointestinal tract. They both ferment a wide variety of carbohydrates into lactic acid and thus lower pH and inhibit growth of other organisms.

EcN is a Gram-negative bacterium, which due to its absence of virulence factors and production of antimicrobial compounds is used as a probiotic (51).
**Bacteria number and type of organism differentially affects programming of the functional DC phenotype**

We compared the bacteria- and MOI-induced pattern of DC-derived cytokines of importance for differentiation and expansion of naïve T helper cells into the specific functional subsets; Th1, Th17 and iTreg. The levels of cytokines produced from bacteria-stimulated DC were compared to levels from DC stimulated with a single TLR-ligand, namely *S. Typhimurium*-derived LPS (Fig. 4.1). Statistically significant differences between bacteria for each cytokine and MOI are listed in Table 2. The viability of DC was not affected by exposure to LM, CD, LA, BL or EcN, but ST at MOI 10 led to a DC-mortality of more than 50% (data not shown).

**Figure 4.1. Bacteria-induced programming of the DC cytokine pattern.**

Cytokine secretion from DC stimulated for 20 hours with different bacteria at MOI 1 or MOI 10 (designated as 1 and 10 in the figure). Gentamycin was added after 1 hour to kill extracellular bacteria, and DC were stimulated for an additional 19 hours. The dashed line represents LPS levels. Cytokine secretion from iDC were as follows: IL-1β=18 pg/ml, IL-6=1814 pg/ml, IL-10=470 pg/ml, IL-12p70=25 pg/ml, IL-18=80 pg/ml, IL-23p19=44 pg/ml, IL-27=1039 pg/ml, IFN-β=5 pg/ml, TNF-α=1650 pg/ml and TGF-β=1322 pg/ml. Data are means derived from 6 donors. One-way ANOVA and Tukey’s post-hoc test was used for statistical analysis (result presented in Table 2).

Th1 differentiation is driven primarily by IL-12p70 production from the APC, and as shown in Figure 4.1, all bacteria with the exception of BL, induced IL-12p70 above the levels induced by LPS alone. EcN at both MOI 1 and 10 led to higher amounts of IL-12p70 compared to all other bacteria. As sketched in Figure 4.2, IL-18, IL-27 and IFN-β may also contribute to Th1-polarisation by inducing NK cells and Th cells to produce IFN-γ. Although our data showed dose-dependent IL-18 secretion from DC by ST, CD, EcN and LA, only ST levels were significantly different from any other bacteria, except EcN (Table 4.2). None of
the bacteria induced statistically significantly higher levels of IL-27 or IFN-β than LPS. LM, CD and LA increased IL-27 with increasing MOI, whereas ST, EcN and BL decreased IL-27 production from DC with increasing MOI. IL-27 levels from ST at MOI 1 differed from those of LM, LA and BL (also at MOI 1), whereas at MOI 10, CD and LA were different (Table 2). EcN differed from all other bacteria than ST, but only at MOI 1 (Table 4.2).

**Table 4.2.** Comparison of cytokine levels produced by bacteria-stimulated DC.

<table>
<thead>
<tr>
<th>Strain comparisons</th>
<th>IL-12p70</th>
<th>IL-27</th>
<th>IL-18</th>
<th>IFN-β</th>
<th>TNF-α</th>
<th>IL-23p19</th>
<th>IL-1β</th>
<th>IL-6</th>
<th>IL-10</th>
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<tbody>
<tr>
<td>ST vs LM</td>
<td>ns</td>
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<td>ns</td>
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<td>ST vs CD</td>
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<td>ST vs EcN</td>
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<td>ST vs LA</td>
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<td>ST vs BL</td>
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<tr>
<td>EcN vs LM</td>
<td>***</td>
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<td>ns</td>
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<tr>
<td>EcN vs CD</td>
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<td>EcN vs LA</td>
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<tr>
<td>EcN vs BL</td>
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<td>**</td>
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</table>

**MOI 10**

| ST vs LM           | ns       | ns    | ***   | ns     | ns     | ns      | *      | ns   | ns    |
| ST vs CD           | *        | **    | **    | ns     | ns     | ns      | *      | ns   | ns    |
| ST vs EcN          | **       | ns    | **    | ns     | ns     | ns      | ns     | ns   | ns    |
| ST vs LA           | ns       | *     | ***   | ns     | ns     | ns      | ns     | ns   | ns    |
| ST vs BL           | **       | ns    | *     | ns     | ns     | *       | ns     | ns   | ns    |
| EcN vs LM          | **       | ns    | ns    | *      | *      | *       | ns     | ns   | ns    |
| EcN vs CD          | ***      | ns    | ns    | **     | ns     | *       | ns     | *    |    |
| EcN vs LA          | ***      | ns    | ns    | *      | ns     | *       | ns     | ns   | ns    |
| EcN vs BL          | ***      | ns    | ns    | **     | *      | **      | ns     | *    |    |

* Only strain combinations for which statistically significant differences were observed are listed.

**TGF-β** is omitted from this table, as no differences were observed.

* *, p<0.05; **, p<0.01; ***, p<0.001; ns, not significant.
In relation to TNF-α levels, only EcN was found to enhance the production as compared to the other bacteria (Figure 4.1 and Table 4.2). Again, BL showed an inverse correlation between MOI and cytokine level. Taken together, the expression of Th1 cytokines by DC was overall most potent in DC stimulated with ST followed by EcN (Table 4.2).

Cytokines important for orchestrating Th17-responses in the human system are IL-1β, IL-6 and IL-23 (Figure 5.2). As this response is recognised as important for clearing extracellular pathogens, especially in the mucosal environment, we evaluated the ability of the different bacteria to prime to a Th17 profile. All bacteria induced IL-1β at or above LPS-levels, but only ST and EcN were significantly different from the majority of compared bacteria (Table 4.2). These two bacteria also induced significantly elevated levels of IL-23p19 at MOI 1, but not of IL-6 at MOI 1. With the exception of BL at MOI 10, IL-6 secretion did not change significantly between bacteria (Table 4.2). Thus, like for Th1, the strongest inducers of Th17 responses of the bacteria tested here were ST and EcN.

As some bacterial pathogens have evolved mechanisms by which they may induce tolerogenic responses and thus evade both innate and adaptive immunity, we examined the cytokine potential of bacteria-stimulated DC to produce TGF-β and IL-10 (Figure 4.2).

Figure 4.2. DC cytokines relevant for Th polarisation and inflammasome-derived mediators.

Th1 responses are driven primarily by DC-derived IL-12p70, but IL-27, IL-18 and IFN-β may also augment this polarisation. Th17 cells are induced by IL-6 and IL-1β and sustained by IL-23 (1). Inducible regulatory T cells can be propagated in a TGF-β, RA and IL-10 dominated environment (6). Both inflammasome activation and pyroptosis leads to secretion of IL-1β and IL-18 by DC (4, 14).

We found no differences in TGF-β levels induced by the six bacteria (Figure 4.1 and Table 4.2). We did, however, observe a significant upregulation of IL-10 secretion from DC stimulated with ST at MOI 1 compared to LM, CD and LA, as well as from EcN-stimulated DC compared to LM, CD, LA and BL at MOI 1. At MOI 10, ST, EcN and BL all led to reduced IL-10 levels (compared to MOI 1 levels). For most bacteria, a
dose-dependent increase in cytokines was observed; however, BL stood out by displaying a dose-
dependent decrease of most cytokines.

![Figure 4.3. Bacteria-induced cytokine profiles in DC.](image)

DC were stimulated with the indicated bacteria at MOI 1 and 10, LPS or medium for 20 hours as shown in figure 5.1, followed by cytokine quantification by ELISA. MOI 1 is shown in the upper panel, shaded light gray; MOI 10 is shown in the lower panel, shaded dark grey. Values for each cytokine are relative to LPS values within each donor to adjust for donor variation. Data are means from 6 donors.

Figure 4.3 displays the functional phenotypic pattern of bacteria-stimulated DC. Considering that ST and LM are both intracellular pathogens employing some of the same virulence mechanisms, the functional phenotypes they induced in human monocyte-derived DC were quite different. In fact, apart from IL-18 levels, the functional phenotype induced by ST mostly resembled that of the genetically closely related EcN (both proteobacteria), whereas the LM phenotype was most alike that of LA (both belonging to the firmicutes phyla). It is interesting to note that also the other firmicutes, CD and the LA-induced phenotypic pattern were very similar. The phenotype of ST-stimulated DC was dominated by IL-12p70, IL-18 and IL-1β, thus suggesting that the Th1 polarisation ability dominate over Th17 and Treg responses. Apart from their roles in inducing Th1 and/or Th17 responses, the proinflammatory cytokines IL-1β and IL-18 are also released from cells in which the inflammasome has been activated or cells undergoing pyroptosis, an inflammatory programmed cell death (Figure 4.2, (14)). Interestingly, no other bacteria than ST provoked secretion of high amounts of IL-18.

The three bacteria classified here as commensals also induced heterogeneous DC phenotypic patterns. Whereas LA and EcN promoted IL-12p70 production, BL did not, and generally BL provoked very low secretion of all examined cytokines as compared to the other five bacteria.
Taken together, these data reveal phyla-specific modulation of the functional cytokine pattern in DC rather than segregation of DC responses based on known pathogenic/non-pathogenic properties of the bacteria.

**Modification of surface molecule profile in DC and its relation to the DC phenotypic profile**

One of the immune escape mechanisms employed by some pathogens is to interfere with APC antigen presentation on MHC class I and II, resulting in slow and inefficient Th and Tc responses (33). Here, however, following 20 hours of bacterial stimulation we observed upregulation of surface HLA-DR expression by ST and all other bacteria, compared to LPS and unstimulated DC (Figure 4.4). Although not significant, we did find HLA-DR expression slightly reduced when DC were exposed to MOI 10 compared to MOI 1. Apart from ST and EcN, all bacteria induced CD40 and CD86 in a dose-dependent fashion. ST-induced CD86 expression did not change with MOI, but CD40 was reduced at MOI 10. EcN-induced CD40 and CD86 expressions were lower for MOI 10 than MOI 1. CCR7 expression levels, of importance for the DC migratory ability, were not enhanced by all bacteria as the firmicutes, LA, CD, and also LM, tended not to differ from unstimulated DC. Contrarily, the LPS-containing bacteria, ST and EcN, and also BL induced up-regulation of CCR7, and only for ST and EcN dose-dependent changes were observed (Fig 4.4).
Figure 4.4. Surface molecule profile of bacteria-stimulated DC.

Histograms showing the expression of maturation markers by DC stimulated with six different viable bacteria. After 1 hour of stimulation, extracellular bacteria were killed by gentamycin, and DC were stimulated for an additional 19 hours. Data were obtained by flow cytometric analysis, followed by gating on viable cells. Histograms display 50,000 DC from one donor representative of six experiments. Mean fluorescence intensities (MFI) are given in upper left corners, where numbers in bold are MOI 10 data.
A principal component analysis (PCA) of the collected DC data was found to segregate the bacteria-induced DC profiles mainly based on levels of IL-12p70, TNF-α, IFN-γ, and CD86 in PC1, whereas PC2 primarily separated based on IL-18 and IL-27 levels.

The PCA-clustering of data made it apparent that the pattern of expression by bacteria-exposed DC is related to the bacterial phyla (Figure 4.5). Specifically, EcN and ST clustered together at MOI 1 and also at 10, and differed in collective DC profile from the other bacteria that were much more alike. LA and LM at MOI 10 resembled the profile induced by LPS the most. CD at MOI 10 promoted a DC profile similar to
LA, LM and BL at MOI 1, whereas at MOI 1, the CD-induced profile resembled mostly that of iDC e.g. characterised by very low levels of virtually all cytokines measured.

Salmonella induces dissimilar MOI-dependent modulation of the functional DC phenotype.

Based on our data, it was clear that ST and EcN profiles differed from those of the other bacteria at both MOI 1 and 10 with the IL-18 and IL-1β levels being the primary clustering was associated to MOI or to bacterial species, phyla and/or virulence factors, we analysed the functional profile of DC exposed to ST, LM, EcN and LA at MOI, 1, 10, 25 and 50 (Figure 4.6A), and compared to the viability of DC at these MOIs (Figure 4.6B).

Figure 4.6. Dose-dependent cytokine induction in DC by gut-associated bacteria.

(A) DC cytokine profiles induced by intracellular pathogens (ST, LM) or gut commensals (EcN, LA) at increasing MOI (1, 10, 25, 50). Cytokine levels are presented relative to mean LPS levels per donor and plots are coloured according to the DC viability after 20 hours of stimulation, as shown in (B). (B) DC viability following bacterial exposure relative to viability of DC stimulated with LPS (dashed line). Data is (A) mean or (B) mean ± SD from 6 donors. One-way ANOVA and Tukey’s post-hoc test was used for statistical analysis.

ST mediated significant MOI-dependent changes in IL-12p70, IL-18 and IL-1β levels from DC that differed from the three other bacteria where DC profiles where generally more MOI-independent (Fig. 4.6A). More intriguingly, however, was the differences in DC atrophy levels induced by the bacteria (Fig. 4.6B).
ST induced a significant reduction in viability of DC already at MOI 10 were DC viability was reduced with more than 50% compared to LPS-stimulated DC and at MOI 50 only 5% of DC were alive after 20 hours. Contrarily, the three other bacteria did not significantly affect the DC viability up to MOI 50. For ST the decrease in viability coincided with an increase in IL-18 secretion (Figure 4.6A), signifying that not only did ST stimulation lead to inflammasome activation in DC, but also induction of pyroptosis. The viability of EcN-stimulated DC was affected at MOI 50, but did not differ significantly from the viability of LPS-stimulated DC (Figure 4.6B). At this MOI, a slight increase in IL-18 secretion was also observed (Figure 4.6A), suggesting some degree of inflammasome activation. Interestingly, no IL-18 secretion was detected from LM- and LA-stimulated DC up to MOI 50.

These data further support the induction of phyla-specific DC phenotypes also at higher cell-to-bacteria ratios, and stresses a MOI-dependent killing of DC by ST. Particularly, our results also show that despite ST and LM share common intracellular replicative traits, they exert quite different effects on DC, suggesting divergent pathogenic trails.

Discussion

Bacteria notoriously differ in their ability to cause disease or to propagate host health upon interactions with the host and its residual microbiota. At present, we lack insight into definable factors that determine the differences between the two outcomes, as most information either lack in vivo comparisons between more bacteria species, or is based on in vitro studies comparing effects of non-viable bacteria (47, 54), hence excluding specific pathogenic-related factors that come into play when bacteria breach the mucosal barrier and interact directly with innate immune cells. Here, by studying the functional phenotype induced in the key immunoregulatory immune cell, the DC, upon interaction with six viable bacteria differing in pathogenicity properties, we found that DC respond to these bacteria in a phyla-specific manner based on their classification as proteobacteria, firmicutes or actinobacteria, and not dependent on their known pathogenic traits. Generally, the response profiles were MOI-independent, however, for ST, a MOI-dependent increase in inflammatory-induced cell death, also known as pyroptosis, was initiated in DC at less than MOI 10, and as such ST stands out from the other proteobacterium, EcN, as well as the other bacteria phyla studied here.

The indigenous microbiota plays a major role in host well-being by fermenting various unused energy substrates, preventing growth of pathogenic bacteria and educating the gut immune system (17). It has been suggested that pathogenic and commensal microbes may activate DC differently (31, 32). Here, we did not observe any notable differences in the functional DC phenotypes induced by commensals as compared to pathogenic bacteria when comparing within phyla, suggesting that whole, viable bacteria that are taxonomically alike, and display similar endogenous molecules, are able to activate the same PRR giving rise to quite identical inflammatory profiles, that are independent of the bacterial presence or
absence of pathogenic factors. The only exception from this conclusion relates to ST-induced IL-18 that relies on cytotoxicity more than immunomodulating capabilities, but is very relevant to take into account in this model system, as it is indicative of specific pathogenic characteristics of some bacteria.

The rationale behind this study was to advance towards development of a functional model that can be used to predict inflammatory abilities amongst gut bacteria based on their expression of genome-encoded PRR ligands. We here report on the functional data from DC exposure only, and are in the progress of combining the functional and bacterial genomic fingerprints. Notably our data showed explicit inflammatory-based groupings of bacteria corresponding to phylogenetic classifications. The present delineations are, however, based on few bacteria strains, and only three phyla, and needs to be extended to include more. Nevertheless, several of our current observations need specific attention: i) Use of viable bacteria vs UV-inactivated, ii) MOI-dependency, iii) identification of the functional DC protein array.

Much focus has been on deducing TLR recognition of bacterial ligands and the resulting immune response, however, insight into how whole, viable bacteria modulates the functional phenotype of DC is still lacking. In many studies UV irradiated, heat-inactivated or freeze-dried bacteria have been used to determine the different types of Th responses induced by bacteria-activated DC (15, 22, 50), however, increasing evidence suggests quite different immunostimulating potentials of inactivated compared to viable bacteria. MacDonald and Speert showed that viable, but not heat-killed, *Burkholderia* bacteria were able to induce necrosis in human monocyte-derived DC (moDC) (34). In murine bone-marrow derived DC, *Chlamydia trachomatis* was found to induce distinct phenotypic and immunologic profiles, depending on whether viable or UV-irradiated elementary bodies were used, and only DC exposed to viable elementary bodies were able to confer protective immunity (45). In our lab, we have also observed that UV-inactivated bacteria are insufficient in inducing certain cytokines such as IL-18 in DC, thus underscoring that insight into specific regulatory patterns may be missed when effects of non-viable bacteria are studied. Generally, however, we have observed that the pattern of expression of the common Th1, Th17 and Treg promoter cytokines are regulated likewise by UV-inactivated and viable bacteria (data not shown), suggesting that PRR activation plays a major role in driving the DC inflammatory profile. Here we specifically showed that the functional phenotypes resulting from DC exposure to ST and EcN were very much alike and generally induced a pattern of cytokines in DC associated with Th1 and Th17 polarising properties. This is an interesting finding, as EcN is non-pathogenic and non-invasive and therefore the high levels of cytokines induced by this phylum do not reflect the inherent pathogenicity of bacteria, underscoring that the general functional phenotype of the DC is determined by PRR recognition of bacterial ligands.
Probably the most dominating component setting these six bacteria apart is LPS. Firmicutes and actinobacteria do not contain LPS, whereas proteobacteria do. LPS, which is sensed by TLR4 together with CD14 and the adaptor protein MD-2, induces IL-12p70 in DC (40). In 2005, a study by Napolitani and colleagues revealed that triggering of TLR3, TLR4 and TLR8 leads to synergistic induction of IL-12p70 and co-stimulatory molecules (38). In this study, both firmicutes and proteobacteria led to IL-12p70 levels in DC which were above those induced by LPS alone, suggesting multiple TLR triggering. Peptidoglycan, which is a constituent of the cell wall in both Gram-negative and Gram-positive bacteria, is sensed by both TLR2 and the intracellular PRR nucleotide oligomerisation domain 2 (NOD2) (16). TLR2 agonists do not induce IL-12p70 in human DC (43) and the NOD2 agonist, muramylide, induces DC to secrete IL-1 and IL-23, thus suggesting promotion of Th17 immune responses (3). The two proteobacteria induced high levels of IL-23p19, probably via synergistic effects of TLR4 and NOD2 triggering, as TLR4 signaling upregulates NOD2 (49). LA has been reported to induce high amounts of IFN-β in bone-marrow derived DC, leading to an increase in IL-12 production and upregulation of TLR3, which is dependent on TLR2 (53). Here we observed high levels of both IFN-β and IL-12p70 from DC exposed to ST, CD or EcN, indicating that this mechanism may also be in play here, but we were not able to detect high levels of IFN-β from LA-stimulated DC, which may reflect the distinct differences between viable versus UV-inactivated bacteria.

Seeing as the firmicutes used here do not contain LPS, the IL-12p70 they induce must stem from triggering other TLR, and indeed, most TLR ligands have been found to stimulate APC to produce Th1-inducing cytokines, such as IL-12 (24).

Inflammasomes are important innate immune activators, regulating host responses against both exogenous- and endogenous-derived stimuli. They are multiprotein complexes containing PRR and the protease caspase-1, which cleaves the inactive precursors of IL-1β and IL-18 (10). Together with IL-12p70, bioactive IL-18 promotes IFN-γ production from T and NK cells, which then activates the microbicidal activity of macrophages by inducing nitric oxide production (8). IL-1β in its active form induces rapid recruitment of neutrophils to inflammatory sites, activates endothelial adhesion molecules, as well as induces production of cytokines and chemokines (7). ST is detected by at least two inflammasome PRR (5, 36), and here, we observed that exposure to ST induced inflammasome activation in human monocyte-derived DC, determined as IL-1β and IL-18 secretion. Interestingly, of all the bacteria tested, only ST was able to induce both IL-1β and IL-18 secretion, whereas all bacteria, except BL, induced IL-1β. It has been demonstrated, that although processing of IL-18 and IL-1β is catalysed most efficiently by caspase-1, under particular circumstances other proteases may also process IL-1β (18), which could be in play for the bacteria studied here.

In addition to ST, LM and CD are also reported to enable inflammasome activation via their toxins (9, 39). CD inflammasome activation was shown in Mφ, however, by use of purified toxins and not whole
bacteria. Our present data indicate that the inflammasome is not activated by viable CD at MOI 1 and 10, nor at 25 or 50 (data not shown), suggesting that either the toxins were not produced at sufficient amounts or that viable bacteria may somehow amend inflammasome activation by CD toxins. We used bacteria grown to mid-exponential phase, and since CD toxins are primarily expressed during the late exponential and the stationary phase (25), thereby accumulating in the growth environment, this difference in study setup is a likely reason for our lack of CD-mediated inflammasome activation. Others have shown IL-18 production from monocyte-derived DC stimulated with LM at MOI 10 (30), however, we were not able to reproduce this finding in this study. As the expression of the LM toxin, listeriolyisin o, is relatively low during exponential growth but increases during the stationary phase (48), differences in growth conditions may explain the disparate results. However, LM also activates inflammasome sensors by their expression of flagellin and due to presence of DNA in the cytosol (29), both being largely unaffected by exponential or stationary growth. It has also previously been reported by others that type I IFN signalling is required for strong LM-stimulated IL-1β and IL-18 production (20), something which we were not able to detect here either, implying that growth conditions may affect the pathogenicity of LM.

As previously mentioned, inflammasome activation may also lead to pyroptosis and recent evidence suggest that pyroptosis is an innate defence mechanism, serving to restrict bacterial replication in vivo (37). It is mediated by caspase-1 activation, and characterised by plasma membrane rupture of host cells resulting in release of proinflammatory intracellular contents (12), thus making it morphologically and mechanistically distinct from other forms of cell death (11). *Salmonella* is well known to induce pyroptosis in infected APC (13) and our findings are in line with prior data, showing a dose-dependent decrease in viability of infected DC. Although ST and LM are both foodborne intracellular pathogens, some of the differences observed in the functional DC phenotypes could be due to differences in intracellular life cycles. After infecting a host, both bacteria are taken up by host APC by phagocytosis, however, because of a pore-forming toxin (listeriolyisin o), a fraction of LM are able to escape the phagosome, whilst ST remains trapped (28). This allows for LM replication both in the cytosol and phagosome, ultimately leading to Ag-presentation on both MHC I and II molecules and activation of both Th and Tc responses, whereas ST antigens are presented preferentially on MHC II molecules (27).

All together, our comparisons of the functional data revealed novel linkages between bacterial taxonomies and the inflammatory response profile in DCs against viable bacteria. We expect that incorporation of the bacterial genome-encoded signatures of PRR ligands into this data set will underscore the importance of specific bacterial PAMP defining the differences in inflammatory properties amongst the three representative phyla studied here.
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5 Summarising discussion

This thesis deals with aspects of innate immunity towards the intracellular pathogen *S.* Typhimurium, as well as bacteria-induced imprint of important regulatory proteins in DC, and the work presented here has led to the following novel findings:

- The passage through and ability to proliferate in the host gastrointestinal system determines the pathogenicity of different *S.* Typhimurium strains in a mouse model.
- Plasmacytoid dendritic cells play a role in immunity against early *S.* Typhimurium infection *in vivo*.
- Selected bacteria induce phyla-specific inflammatory programs in human monocyte-derived DC, irrespectively of whether bacteria are classified as commensals, opportunistic pathogens or intracellular pathogens.

In Chapter 2 we find that following peroral dosing, *S.* Typhimurium SL1344 is much more pathogenic than DT120. As this is not observed when bacteria are administered intravenously, we argue that the passage through the gastrointestinal system increases the pathogenicity of *S.* Typhimurium SL1344. Although the majority of bacteria are killed when passing through the gastric acid, *Salmonella* are able to turn on acid tolerance genes, which enable the surviving bacteria to overcome subsequent severe acid stress (2), like the harsh intracellular environment of phagocytes. Whether SL1344 and DT120 retain different capacities for induction of their acid tolerance responses, we cannot conclude from this study. What we can deduce though, is that following passage through the gut, SL1344 is able to rapidly establish a replicative niche, resulting in higher bacterial loads within the cecum at Day 5 post challenge compared to DT120. As we did not examine the direct interaction of bacteria and epithelium, we do not know whether and how the epithelial barrier is affected by the two bacteria. In the case of SL1344 infection, however, it is reasonable to assume that the more bacteria present in the intestine will lead to more bacteria crossing the barrier, probably by damaging both M and epithelial cells (3).

The enhanced replicative abilities of SL1344, coupled with the inability of neutrophils to contain the infection within the spleen ultimately led to most mice dying before Day 8. Why the recruited neutrophils were unable to contain the infection is still unclear, however, it could be speculated that the cytotoxicity of SL1344, which kills off both mDC and Mφ *in vivo* (Chapter 3), also affects neutrophils. How this is brought about, we cannot say and of course this needs further investigation. The lack of Day 8 measurements is inopportune, as they would have provided us with more detailed information regarding the further fate of neutrophils in both spleen and BM.

In Chapter 3 we show that pDC are involved in the early stage of *S.* Typhimurium infection and are not affected by bacterial cytotoxicity, as pDC viability *in vitro* is not altered drastically by increasing doses.
Although we did find dose-dependent modifications of pDC, mDC and Mφ in vivo, administration of doses differing by 1000-fold gave rise to a number of comparable responses, e.g. reduction in CD40 expression on pDC and mDC in PP-SED as well as reduction in CD86 expression on mLN mDC, suggesting that the presence of bacteria, rather than the amount of bacteria, defines the resulting innate immune response.

We found that both mDC and Mφ are involved in early-stage infection and are differently modulated at the examined tissue locations. To our knowledge this is the first time this amount of mDC and Mφ subsets has been identified using multi-parametric flow cytometry. CX3CR1-positive phagocytes (in Chapter 3 designated as M2) in PP has only been reported by one other group, finding them closely associated with the FAE (7). As close proximity to the epithelial layer may predispose DC to induce Tregs rather than immunity (6), it is speculated that the function of PP CX3CR1 phagocytes may be to down-modulate immune responses (4). Here we find an increase in the M2 subset in PP-SED response to S. Typhimurium infection, but whether they serve to initiate immunity or assist virulent bacteria in evading the immune system, remains to be elucidated. The observed increase of the subset M2 in PP-SED is not dose-dependent, which probably has to do with the fact that Salmonella preferentially invade through M cells in PP, resulting in interactions between PP phagocytes and bacteria from both doses. We speculate that the number of orally administered bacteria affects the site of adhesion on gut epithelium with a high dose resulting in physical limitations around M cells, thereby facilitating adhesion to other epithelial locations, such as LP. This provides an explanation for the dose-dependent increase in DC2 in LP, as the low dose most likely confines adhesion to M cells and therefore bacteria will interact much less with LP compared to the high dose.

Chapter 4 deals with bacteria-induced imprints on human monocyte-derived DC and reveals that the inflammatory response induced is phyla-specific, rather than bacteria- or strain-specific. We observed that the bacteria-induced fingerprints were dose-independent, except for S. Typhimurium which also led to massive IL-18 production, thus emphasising the pathogenic potential of this bacterium.

Of the six bacteria tested in our system, B. longum induced a unique DC phenotype, defined by a low production of the cytokines assayed, which was found to be inversely correlated to bacteria-to-DC ratio. This functional phenotype could be the result of anti-inflammatory compounds secreted by the bacteria; however, an increase in IL-10 would then be expected. Bifidobacteria are obligate anaerobes and as such, the lack of cytokine production from exposed DC most likely is because the bacteria die or are attenuated in the aerobic environment during stimulation.

Since all bacteria activate and mature DC, the surface molecules analysed for do not give any bacteria- or phyla-specific information, leading to the conclusion that the bacteria-induced cytokines define the functional phenotypes. When studying the DC response to various inactivated bacteria, and in particular
probiotics, it is quite customary to use the IL-10/IL-12 ratio to determine, whether bacteria are immunogenic or tolerogenic. As we found nearly all bacteria to induce high amounts of IL-12p70, this ratio does not suffice in describing the inherent nature of these viable bacteria. Instead we find that the cumulative production of pro-inflammatory cytokines (IL-1β, IL-6, IL-18 and TNF-α) nicely captures the essence of the phyla-specific segregation.

Collectively, the results presented in this thesis show the involvement of mDC, MΦ and pDC early during infection with S. Typhimurium SL1344, with pDC seemingly playing a host protective role. Both mDC and MΦ are killed off by virulent SL1344 and the large dose-dependent IL-18 secretion from human monocyte-derived DC points toward pyroptotic cell death. Ideally, pyroptosis should serve to limit bacterial spread by exposing intracellular bacteria to phagocytes such as neutrophils; however, as we report in Chapter 2, the gut passage may enhance the pathogenicity or cytotoxicity of SL1344, thereby leading to inefficient containment of bacteria by neutrophils, and ultimately pyroptosis proves detrimental to the host.

The implications of these findings when thinking in terms of prophylactic therapy may be I) to enhance the presence/recruitment of pDC in the gut and II) to enhance the presence of certain phyla in the gut microbiota, which could then induce functional DC profiles more suited to handle a pathogen such as S. Typhimurium.

Production of type I IFN in the spleen by pDC has been shown to confer protection of especially MΦ and mDC (5) and enhancement of pDC presence could be achieved using FMS-like tyrosine kinase 3 receptor ligand (flt3L), which is required for their steady-state development (as well as mDC development). Whether PP pDC are equally capable of conferring protection has been questioned, as the presence of IL-10, TGF-β and prostaglandin E2 in PP seem to inhibit pDC type I IFN production ex vivo (1). Whether this holds true in vivo still remains to be elucidated. As we find pDC not to be affected by the cytotoxicity of S. Typhimurium SL1344 in vivo, we speculate that the type I IFN production may be secondary and rather that pDC exert their seemingly protective effect by augmenting IL-12p70 production in mDC which increases NK cell-mediated cytotoxicity and promotes Th1 responses.
References


Appendix – Some putative prebiotics increase the severity of *Salmonella enterica* serovar Typhimurium infection in mice


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Some putative prebiotics increase the severity of Salmonella enterica serovar Typhimurium infection in mice

Anne Petersen\textsuperscript{1}, Peter MH Heegaard\textsuperscript{2}, Anna L. Pedersen\textsuperscript{1}, Jens B Andersen\textsuperscript{1}, Rikke B Sørensen\textsuperscript{3}, Hanne Frekiaer\textsuperscript{4}, Sampo J Lahtinen\textsuperscript{5}, Arthur C Ouweland\textsuperscript{5}, Morten Poulsen\textsuperscript{1} and Tine R Licht* \textsuperscript{1}

Address: \textsuperscript{1}The National Food Institute, Department of Microbiology and Risk Assessment, Technical University of Denmark, Moesgård Bygade 9, DK-2600 Søborg, Denmark, \textsuperscript{2}The National Veterinary Institute, Department of Veterinary Diagnostics and Research, Technical University of Denmark, Bülowsvej 27, DK-1892 Copenhagen V, Denmark, \textsuperscript{3}Department of Systems Biology, Center for Biological Sequence Analysis, Technical University of Denmark, Søltofts Plads, Building 221, DK-2800 Lyngby, Denmark, \textsuperscript{4}Department of Basic Sciences and Environment Faculty of Life Sciences Copenhagen University, Thurovsensgade 46, DK-1871 Frederiksberg C, Denmark and \textsuperscript{5}Danisco Health & Nutrition, Solvalla 20, 02400 Kemijärvi, Finland.

Email: Anne Petersen - apet@food.dtu.dk, Peter MH Heegaard - pmbh@food.dtu.dk, Anna L. Pedersen - annalopmaa@yahoo.dk, Jens B Andersen - jban@food.dtu.dk, Rikke B Sørensen - rbs@bio.dtu.dk, Hanne Frekiaer - hanha@life.ku.dk, Sampo J Lahtinen - sampo.lahtinen@danisco.com, Arthur C Ouweland - arthur.ouweland@danisco.com, Morten Poulsen - mo@food.dtu.dk, Tine R Licht* - tfl@food.dtu.dk

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Abstract

Background: Prebiotics are non-digestible food ingredients believed to beneficially affect host health by selectively stimulating the growth of the beneficial bacteria residing in the gut. Such beneficial bacteria have been reported to protect against pathogenic infections. However, contradicting results on prevention of Salmonella infections with prebiotics have been published. The aim of the present study was to examine whether S. Typhimurium SL344 infection in mice could be prevented by administration of dietary carbohydrates with different structures and digestibility profiles. BALB/c mice were fed a diet containing 10% of either of the following carbohydrates: inulin, fructo-oligosaccharide, xylo-oligosaccharide, galacto-oligosaccharide, apple pectin, polydextrose or beta-glucan for three weeks prior to oral Salmonella challenge (10^7 CFU) and compared to mice fed a cornstarch-based control diet.

Results: The mice fed with diets containing fructo-oligosaccharide (FOS) or xylo-oligosaccharide (XOS) had significantly higher (P < 0.01 and P < 0.05) numbers of S. Typhimurium SL344 in liver, spleen and mesenteric lymph nodes when compared to the mice fed with the cornstarch-based control diet. Significantly increased amounts (P < 0.01) of Salmonella were detected in ileal and fecal contents of mice fed with diets supplemented with apple pectin, however these mice did not show significantly higher numbers of S. Typhimurium in liver, spleen and lymph nodes than animals from the control group (P > 0.20).

The acute-phase protein haptoglobin was a good marker for translocation of S. Typhimurium in mice. In accordance with the increased counts of Salmonella in the organs, serum concentrations of haptoglobin were significantly increased in the mice fed with FOS or XOS (P < 0.001). Caecum weight was increased in the mice fed with FOS (P < 0.01), XOS (P < 0.01), or polydextrose (P < 0.01).
0.001), and caecal pH was reduced in the mice fed with polydextrose (P < 0.001). In vitro fermentation in monocultures revealed that S. Typhimurium SL1344 is capable of fermenting FOS, beta-glucan and GOS with a corresponding decline in pH. **Conclusion:** Supplementing a cornstarch-based rodent diet with 10% FOS or XOS was found to increase the translocation of S. Typhimurium SL1344 to internal organs in mice, while 10% apple pectin was found to increase the numbers of S. Typhimurium in intestinal content and feces.

**Background**

One of the basic physiological functions of the resident microbiota is that it functions as a microbial barrier against pathogens [1]. A healthy, balanced microbiota has been suggested to be predominantly saccharolytic, with significant numbers of bifidobacteria and lactobacilli [2]. The use of pre- and probiotics has thus been suggested as approaches to prevent *Salmonella* infections and infections by enteric pathogens in general [3,5].

Prebiotics were originally defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health" [6]. The main candidates that meet the required criteria for classification of a food ingredient as a prebiotic are fructo-oligosaccharides, including inulin, galacto-oligosaccharides and lactulose [7]. Numerous studies have shown that prebiotics stimulate the growth of bifidobacteria and lactobacilli in vivo [8-12] and specific strains from these genera have been shown to suppress bacterial infections including those caused by ingestion of *Salmonella enterica* serovar Typhimurium (S. Typhimurium) [13-17]. Mechanisms proposed to explain the enhanced resistance to pathogens induced by lactobacilli and bifidobacteria include (i) competitive inhibition of the epithelial and mucus adherence of pathogens, (ii) production of antimicrobial substances, (iii) immune modulation, and (iv) production of short chain fatty acids which can reduce the growth of acid-sensitive pathogens like *Salmonella* [1,8,18,19].

*Salmonella* infections are a global problem with *Salmonella enterica* serovar Typhi (S. Typhi) and serovar Paratyphi (S. Paratyphi) causing epidemics of severe systemic infections in developing countries [20,21]. S. Typhi and S. Paratyphi do not cause systemic infections in other mammalian hosts than humans, but the BALB/c mouse model used in the present study provides a murine model of human typhoid fever [22]. In the EU, *Salmonella enterica* serovar Enteritidis (S. Enteritidis) and S. Typhimurium are the most frequently reported serovars causing human salmonellosis. A total number of 160,649 cases of human salmonellosis were reported in the EU in 2006 [23].

Despite the promising effects of prebiotics on the prevention of *Salmonella* infections in mice [1,4,14,17,24], studies with prebiotics have shown conflicting results. Inulin has been found to reduce the mortality of mice challenged with S. Typhimurium [25] and in rats fed an inulin-oligosaccharide diet, numbers of S. Typhimurium in the content of ileum and caecum were reduced [26]. Additionally, increased resistance to S. Typhimurium infection in mice was reported with combined administration of bifidobacteria and galacto-oligosaccharides [15]. Finally, a recent study showed that oral administration of galacto-oligosaccharides to mice immediately prior to S. Typhimurium SL1344 infection reduced the clinical signs of infection, significantly reduced the organ counts of S. Typhimurium, and reduced the pathology associated with murine salmonellosis [27]. In contrast to these findings, a number of papers reporting an increased translocation of S. Enteritidis in rats fed inulin, fructo-oligosaccharides or lactulose have been published by one group of investigators [28-31]. However, these studies were all based on low calcium-diets and the adverse effect could be reversed by oral administration of calcium [31].

The aim of the present study was to examine if mouse susceptibility to S. Typhimurium SL1344 infection was affected by ingestion of carbohydrates with different structures and digestibility profiles. Effects of diets containing inulin, fructo-oligosaccharide (FOS), xylo-oligosaccharide (XOS), galacto-oligosaccharide (GOS), apple pectin, polydextrose or beta-glucan on murine S. Typhimurium infection were compared to a cornstarch-based control diet. This is, to our knowledge, the first study comparing the effects of non-digestible carbohydrates with different structures on *Salmonella* infection.

**Results**

**Body weight and euthanisation**

To monitor the effect of feeding with different potentially prebiotic carbohydrates on the susceptibility to infection with S. Typhimurium, groups of mice were fed with diets containing either of the seven abovementioned carbohydrates for three weeks prior to challenge with *Salmonella*.

During the three weeks of feeding on the experimental diets, no significant differences in mean body weights were recorded between the dietary groups. Following the
Salmonella challenge, the mice were monitored and euthanised before schedule in case of adverse signs of infection due to ethical considerations. Only mice euthanised as scheduled on Day 5 were included in the analysis. Those constituted five mice in the group fed polydextrose, six mice in the groups fed apple pectin, beta-glucan and COS, seven mice in the groups fed XOS and control diet (study B), and all mice in the remaining groups (inulin, FOS and control diet in study A-C).

**Caecum weight and pH**
The weight of caecum was significantly increased in mice fed diets containing FOS (P < 0.01), XOS (P < 0.01) or polydextrose (P < 0.001) when compared to groups fed the control diet (Table 1). Polydextrose ingestion was found to decrease (P < 0.001) the caecal pH (Table 1).

**Salmonella cultivated from faecal samples and distal part of ileum**
There was a trend (Figure 1), though not statistically significant, indicating that faecal counts of S. Typhimurium cultivated from faecal samples were higher on Day 3 after challenge in the groups fed FOS (P = 0.068) and XOS (P = 0.066) when compared to the group fed the control diet. (Data not shown). In mice fed apple pectin, faecal counts of S. Typhimurium were significantly higher on Day 3 (P < 0.01) and Day 5 (P < 0.01) (Figure 1C). The increased faecal counts in the apple pectin group corresponded to a significantly higher number of S. Typhimurium in the content of the distal part of ileum at euthanisation on Day 5 (P < 0.01). Also in the FOS and XOS group, there was a trend that ileal *Salmonella counts were elevated* (P = 0.182 and P = 0.242, respectively), though this was not statistically significant (Figure 1A).

<table>
<thead>
<tr>
<th>Study A:</th>
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<tbody>
<tr>
<td><strong>Control</strong></td>
<td>7</td>
<td>196.96 ± 41.15</td>
</tr>
<tr>
<td><strong>FOS</strong></td>
<td>10</td>
<td>355.32 ± 32.09**</td>
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<tr>
<td><strong>XOS</strong></td>
<td>7</td>
<td>335.74 ± 44.66***</td>
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<tr>
<td><strong>Control</strong></td>
<td>7</td>
<td>181.70 ± 10.60</td>
</tr>
<tr>
<td><strong>Beta-glucan</strong></td>
<td>6</td>
<td>206.40 ± 76.03</td>
</tr>
<tr>
<td><strong>GOS</strong></td>
<td>6</td>
<td>174.03 ± 38.35</td>
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<tr>
<td><strong>Control</strong></td>
<td>8</td>
<td>205.36 ± 20.93</td>
</tr>
<tr>
<td><strong>Inulin</strong></td>
<td>8</td>
<td>263.24 ± 24.05</td>
</tr>
<tr>
<td><strong>Apple pectin</strong></td>
<td>6</td>
<td>216.68 ± 18.30</td>
</tr>
<tr>
<td><strong>Polydextrose</strong></td>
<td>5</td>
<td>637.74 ± 61.1</td>
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*Values represent means ± SD. **Group size on Day 5 post challenge. One mouse died during the acclimatisation period in the control group in study A. *P < 0.01; **P < 0.001. 
Figure 1

*Salmonella* counts in organs, distal ileum, and faeces. Enumeration of *S. Typhimurium* SL1344 from the liver, spleen, mesenteric lymph nodes, distal part of ileum and faeces from mice five days post challenge. A: Control, FOS and XOS. B: Control, beta-glucan and GOS; C: Control, inulin, apple pectin and polydextrose. Values represent means ± SEM. Prevalences of mice with detectable numbers of *Salmonella* in the organs are shown on the columns. *P* < 0.05; **P** < 0.01.
Feeding with beta-glucan and GOS did not significantly affect the ileal and fecal numbers of *Salmonella* when compared to the control (Figure 1B).

**Salmonella cultured from liver, spleen and mesenteric lymph nodes**

Numbers of *S. Typhimurium* cultured from the liver, spleen and mesenteric lymph nodes were significantly higher in mice fed FOS (P < 0.01) or XOS (P < 0.05) with an increase in the mean CFU counts of approximately 1.6 and 3.8 logs (Figure 1A). In animals fed with apple pectin, a similar trend showing increased counts of *Salmonella* in liver (P = 0.154) and spleen (P = 0.198) was observed.

Feeding with beta-glucan and GOS did not significantly affect the numbers of *Salmonella* in the investigated organs when compared to the control (Figure 1B).

**Serum levels of haptoglobin**

In all dietary groups the concentration of serum haptoglobin was markedly and significantly elevated by *Salmonella* challenge (Table 2). The mean haptoglobin concentration was between 1 and 25 μg/ml for all groups before infection. By contrast infection caused haptoglobin concentrations to rise to between approximately 500 to 2500 μg/ml at Day 5 post infection, which was a significant (P < 0.05) increase for all infected groups with the exception of the control group in study C, where only a trend was observed (P = 0.112).

When comparing infected groups fed putative prebiotics with infected control groups, it was seen that for mice fed FOS and XOS, serum haptoglobin concentrations were significantly higher, P < 0.01 and P < 0.05 respectively, when compared to the control group. In the other parts of the study, it was also seen that prebiotic groups generally did not cause a lower and in most cases caused a higher haptoglobin concentration after infection compared to the control group, with the notable exception of GOS where the trend was a lower level.

**Cellular Composition of the Spleen of mice from Study C**

To further explore the action of the immune system on *Salmonella* infection in Study C, the composition of immune cells (CD4+ and CD8+ T cells, NK and NKT cells, B cells, dendritic cells and neutrophils) within the spleen of non-infected as well as infected mice was analysed by flow cytometry. No significant effects of the different prebiotic feeds were demonstrated, however, a significant increase in the percentage of neutrophils (P < 0.01) within the spleen of infected mice was found, compared to non-infected controls (Figure 2A). This increase positively correlated with the numbers of *S. Typhimurium* cultured five days post challenge from liver (P < 0.001), spleen (P < 0.001) and mesenteric lymph nodes (P < 0.01) (Figure 2B), but not from ileum (data not shown). Furthermore, a positive correlation between the percentages of CD4+ T cells within the spleen of infected mice and the numbers of *S. Typhimurium* cultured from liver (P < 0.05), spleen (P < 0.05) and mesenteric lymph nodes (P < 0.05) five days post challenge was established (Figure 2C), although the increase in CD4+ T cells in infected mice was not significant.

**In vitro fermentation study**

By *in vitro* fermentation using monocultures of *S. Typhimurium*, this strain was seen to utilise FOS (P < 0.01), beta-glucan (P < 0.05) and GOS (P < 0.001), but not XOS, inulin, apple pectin or polydextrose. In accordance with these results, a lowering of the culture pH was seen after fermentation with XOS (P < 0.01), beta-glucan (P < 0.001), and GOS (P < 0.001). A significant decrease in the pH was also recorded in the culture with polydextrose (P < 0.001) even though this carbohydrate was not found to support growth of the *Salmonella* strain (data not shown).

<p>| Table 2: Serum haptoglobin concentrations (μg/ml) in mice before and after <em>Salmonella</em> challenge* |</p>
<table>
<thead>
<tr>
<th>Study A:</th>
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<tbody>
<tr>
<td>Control</td>
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<tr>
<td>FOS</td>
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<td>XOS</td>
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<td>Study B:</td>
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<tr>
<td>Control</td>
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<tr>
<td>Beta-glucan</td>
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<tr>
<td>GOS</td>
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<tr>
<td>Study C:</td>
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<tr>
<td>Control</td>
</tr>
<tr>
<td>Inulin</td>
</tr>
<tr>
<td>Apple pectin</td>
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<tr>
<td>Polydextrose</td>
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</table>

*Values represent means ± SEM. *Numbers of mice where serum haptoglobin was measured in uninfected and infected mice.

*Significantly different from the corresponding concentration measured in uninfected mice; P < 0.05; **P < 0.01; ***P < 0.001.

*Significantly different from the concentration measured in infected mice fed the control diet; P < 0.05; **P < 0.01.
Discussion

In the present study we report for the first time that changes in the carbohydrate composition of the diet impair the resistance of BALB/c mice to severe S. Typhimurium SL1344 challenge. Mice fed with a diet containing 10% FOS or XOS had significantly higher numbers of S. Typhimurium in liver (P = 0.006 and P = 0.023, respectively), spleen (P = 0.010 and P = 0.025, respectively) and mesenteric lymph nodes (P = 0.009 and P = 0.017, respectively) when compared to mice fed with the control diet. Additionally, a similar trend was observed for the mice fed with apple pectin, which also had elevated numbers of Salmonella in liver (P = 0.154) and spleen (P = 0.198).

The haptoglobin concentrations seen in the infected mice were closely correlated with the degree of translocation of Salmonella, scored as the numbers of CFU of Salmonella in liver, spleen and mesenteric lymph nodes in the dietary groups of each of the three experiments. Thus in Study A, the significantly increased number of Salmonella in the organs of the FOS and XOS groups compared to the group fed the control diet (Figure 1) correlated with haptoglobin concentrations that were significantly increased in the same groups compared to the control group (Table 2). In Study B and C, no statistically significant differences after infection were detected in either haptoglobin concentration or organ counts between the dietary groups and the control group of each experiments. Still, there was a trend for correlation between high haptoglobin concentrations and high organ counts, as seen for example for the apple pectin group of Study C (in which the haptoglobin level was most significantly increased compared to the level observed before infection) while low haptoglobin levels correlated with low organ counts as observed for the GOS group in study B.

To further explore the mechanism behind the increase in haptoglobin concentration observed post challenge with Salmonella in study A and B, in study C we included flow cytometric analysis of the cellular composition of the spleen. Of all the cell subsets analysed, only the proportions of neutrophils were significantly increased upon infection. We also found a positive correlation between the number of neutrophils in the spleen and the CFU of Salmonella in the organs of the infected mice, but not the CFU of Salmonella in the ileum, indicating that the neutrophil number and thus the haptoglobin concentration reflects an immune response towards the bacteria translocated to the organs rather than the Salmonella present in the gastrointestinal tract. This is in accordance with earlier findings demonstrating that neutrophils are important for host survival during the primary response to Salmonella infection, primarily due to control of bacterial replication [32]. Other investigators have reported changes in other cell subsets in the spleen post infection, e.g. a decrease in T, NK and NKT cells [33], but although there was a posi-
rative correlation between organ CFU and T cell numbers, we did not find other significant changes in the cell numbers of the different cell populations analysed.

Studies reporting adverse effects of FOS and inulin on S. Enteritidis infections in rats have been published [28-31]. In these studies it is hypothesised that the increased translocation of S. Enteritidis, measured as increased urinary excretion of nitrates and nitrates, is caused by fermentation of the prebiotics producing high concentrations of lactic acid and short chain fatty acids. This was found to impair the mucosal barrier, measured as local mucin excretion [28-31]. However, the studies were all based on low calcium diets (0.80-1.20 g Ca/kg) and the adverse effect could be reversed by oral administration of calcium [31]. Acidification of the gut content has been shown to be counteracted by dietary calcium, suggesting that the increased translocation could be connected to low pH [34,35]. However, the diets used in our study contained the amount of calcium recommended for rodents (5 g/kg) [36], and our results thus contradict that the observed increased translocation occurs only when the diet is low in calcium. Additionally, our results contradict that acidification per se should mediate the increased translocation, since no drop in cecal pH was observed in animals fed with FOS or XOS in the present study (Table 1).

The major effects of prebiotic fermentation are typically seen in the large intestine, however according to the refined definition of prebiotics [7], as well as the results presented here, the effects are not restricted to the colon. Salmonella translocates primarily through M cells located in the ileal Peyer’s patches [37], and an increased concentration would be likely to result in an increased number of phagocytosed S. Typhimurium. However, even though the trends in our data indicated that a high ileal content of the pathogen was accompanied by a high amount of Salmonella in internal organs (Figure 1), it should be noted that consumption FOS and XOS, leading to significantly increased amounts of Salmonella in liver and spleen was not accompanied by significantly increased ileal counts of the pathogen (P > 0.20), and that apple pectin, which significantly increased ileal Salmonella counts did not lead to significantly increased numbers of this pathogen in the internal organs (P = 0.154 and P = 0.138, respectively).

With the notable exception of GOS, our data suggest that small-molecule prebiotics increase Salmonella translocation more than larger molecules (Figure 1). Ten Bruggencate et al. [31] studied the effect of FOS and inulin on S. Enteritidis infection in rats and reported an increase in S. Enteritidis translocation in rats fed a low calcium diet with FOS as well as with inulin. However, in the present study, no increased translocation of S. Typhimurium was observed in mice fed inulin (Figure 1C). We speculate that the effect of prebiotics on bacterial translocation may be different in rats and mice, and may also depend on the Salmonella serovar used, and on other dietary or environmental factors than calcium.

A recent study demonstrated that oral administration of a mixture of GOS can reduce numbers of S. Typhimurium SL1344 in the liver and spleen of BALB/c mice when given just prior to infection [27]. This is in contradiction to the results reported in the present paper, which show no protective effect of GOS against Salmonella (Figure 1). The differences may be explained by the fact that oral delivery of GOS (2500 mg/kg) was given to mice just 30 minutes prior to Salmonella challenge [27], as opposed to the approach chosen in the present study, which was designed to mimic how continuous ingestion of non-digestible carbohydrates (e.g. as part of a regular diet) affects susceptibility to infection.

Our findings of increased caecal weight (Table 1) in mice fed FOS, XOS or polydextrose indicate increased fermentation in caecum. However, the increase was only accompanied by a decline in cecal pH in the group fed polydextrose. In accordance with our findings, polydextrose has been reported to increase the weight of caecal dry matter, to decrease cecal pH and to change the composition of the caecal microbial community in rats [38]. Similar changes have been reported for FOS and XOS in rats with increased numbers of caecal bifidobacteria [11].

Our in vitro fermentation experiment showed that S. Typhimurium SL1344 is capable of fermenting FOS, beta-glucan, GOS and glucose with a corresponding decline in pH. Polydextrose was not found to support growth of the Salmonella strain, but a significant reduction in pH was recorded, indicating metabolic activity. In accordance with our observation, Ten Bruggencate et al. [29] stated that Salmonella can use FOS as a substrate for growth. Additionally, Fooks & Gibson [18] reported growth of S. Enteritidis on inulin, FOS and XOS, however generally with a lower specific growth rate than selected probiotic strains. In co-culture with probiotics growth of the Salmonella strains was significantly reduced by FOS and XOS.

The results obtained from the in vitro studies did not explain our in vivo observations. While e.g. apple pectin was not fermented by Salmonella in vitro, highly increased levels of ileal S. Typhimurium was observed in animals fed with this carbohydrate (Figure 1C). This may reflect the growth of Salmonella on by-products from fermentation of apple pectin or XOS by other gut bacteria. Additionally, in vivo, Salmonella competes for nutrients with the resident microbiota, of which some bacteria may be more efficient in fermenting the various carbohydrate.
Conclusion
Based on the results presented within this study we conclude that changes in the carbohydrate composition of diets fed to mice altered the resistance to S. Typhimurium infections. This raises important doubts about the potential use of certain prebiotics for prevention of Salmonella infections. However, it should be kept in mind that our observations do not contradict the proposed beneficial effects of prebiotics in prevention of life-style related diseases such as colon cancer, inflammatory bowel disease and cardiovascular disease, which are likely to be affected by completely different mechanisms than those important for protection against pathogens.

Methods
Animals and housing
4 week-old conventional male BALB/c mice were purchased from Taconic Europe (Lille Skensved, Denmark) and housed individually in standard cages in an environmentally controlled facility with a 12-h light/dark cycle. During the study the temperature was kept at 22 ± 1 °C, relative humidity at 55 ± 5% and air was changed 8-10 times per hour. Animal experiments were carried out under the supervision of the Danish National Agency for Protection of Experimental Animals.

Salmonella strain
A gfp+ tagged S. Typhimurium SL1344 strain resistant to nalidixic acid and chloramphenicol was constructed and used throughout this study in order to facilitate enumeration and verification of Salmonella in unsterile samples. To construct this strain, a spontaneous nalidixic acid resistant mutant of S. Typhimurium SL1344 (designated IB371) was initially selected. Next, the genetic element PrpsA+ gfp+ cat of strain IB3016 [33] was introduced into the chromosomeally located papA region of strain IB371 by P22 transduction using a P22 lysate of strain IB3016 (kindly provided by Isabelle Hautoft, Norwich, UK). The resulting gfp+ tagged S. Typhimurium SL1344 strain resistant to nalidixic acid and chloramphenicol was designated IB400 (designated S. Typhimurium throughout the paper).

Dietary Carbohydrates
Inulin, DP 2-60 (Orafi ST-Gel, Beneo-Orafti, Tienen, Belgium) and FOS, DP 2-8 (Orafi P95, Beneo-Orafti, Tienen, Switzerland) were purchased from Orafti in Stirpe, Bilirker, Denmark. XOS, DP 2-6, COS, DP 2-6, and polydextrose with an average DP of 12 were kindly provided by Danisco Health & Nutrition, Kanta, Finland. Apple pectin was purchased from Bühlmann AG, Bischofszell, Switzerland and beta-glucan (Glucagel® 75) was purchased from GraceLine Limited, Christchurch, New Zealand.

Challenge protocol
S. Typhimurium SL1344 was grown in closed 50 ml tubes at 37°C, 200 rpm overnight in 20 ml LB broth supplemented with 10 µg/ml chloramphenicol. Overnight cultures were diluted to 10³ CFU/ml in saline and animals were orally inoculated with 0.1 ml (10⁵ CFU) by gastric gavage. The number of CFU in the inoculum was determined by plating on LB agar plates supplemented with 10 µg/ml chloramphenicol. The inoculum size was chosen based on a series of pilot experiments determining the dose response of this particular strain in the animal model.

Diets and experimental design
For an acclimatisation period of 1-2 weeks prior to commencement of the feeding experiments the mice were fed a standard mouse diet produced in house as previously described [39] based on the rodent diet AIN-93 [36] containing cornstarch as the major carbohydrate source. Subsequently, the mice were randomised to 8 dietary groups with 8 mice per group (10 in the IOS group). The experimental diets based on AIN-93 were supplemented with 10% of either of the following carbohydrates: fructo-oligosaccharide (FOS), xylo-oligosaccharide (XOS), beta-glucan, galacto-oligosaccharide (GOS), inulin, apple pectin or polydextrose in place of an equal amount (w/w) of cornstarch. Three independent studies were carried out with a cornstarch-based diet as control: Study A: Control, FOS and XOS; study B: Control, beta-glucan and GOS; study C: Control, inulin, apple pectin and polydextrose. Diets and water acidified with citric acid to pH 3.0 to prevent growth of microorganisms were provided ad libitum.

Mice were fed the respective diets for three weeks prior to Salmonella challenge and body weight was recorded weekly. Following the three weeks all mice were challenged with 10⁷ CFU S. Typhimurium SL1344 and scheduled for euthanasia on Day 5 after challenge. The mice were kept on their respective diets and observed twice a day. If symptoms of severe disease (ruffled fur, changed behaviour) developed the mice were euthanised immediately due to ethical considerations.

On the day of euthanasia the mice were dissected and S. Typhimurium SL1344 was cultivated from the liver, spleen, mesenteric lymph nodes and content of the distal part of ileum. The weight (with content) and pH of caecum were recorded for each mouse. In the study with IOS
Salmonella cultured from organs, content of distal ileum and faecal samples
Liver, spleen, mesenteric lymph nodes and content of the distal part of ileum were 10-fold diluted in saline and homogenised. Serial dilutions of the homogenates were plated on LB-agar plates containing 10 μg/ml chloramphenicol. The plates were incubated aerobically at 37°C overnight. Faecal samples (wet weight) were collected from mice on Days 1, 3 and 5 after Salmonella challenge and cultured as described for the organ samples.

Measurement of serum haptoglobin concentrations
Blood samples were taken from all mice one week prior to Salmonella challenge and on the day of euthanasia for analysis of the acute phase protein haptoglobin. Haptoglobin has been described as a highly reactive acute phase protein in mice [40] whereas for example C-reactive protein is not a prominent acute phase protein in the mouse [41]. The samples were stored overnight at 4°C, centrifuged at 3000 rpm for 20 minutes for isolation of serum. Serum samples were stored at -20°C. Buffers used for the haptoglobin determination were PBS/T (0.05% v/v Tween 20 in PBS) and PBS/T/BSA (0.05% v/v Tween 20 in PBS, 1% BSA (Sigma-Aldrich A2153)). All chemicals were from Sigma-Aldrich, all incubation volumes were 100 μl/well and incubations were at room temperature, unless otherwise indicated. ELISA plates (NUNC MaxiSorp) were coated with rabbit anti human haptoglobin (DAKO A030) diluted 1:10000 in 0.1 M sodium hydroxide carbonate pH 9.6 and stored overnight at 5°C. Plates were washed four times in PBS/T blocked with PBS/T/BSA (200 μl/well) and incubated for 30 minutes. Plates were then washed as before and loaded with a mouse haptoglobin standard (BS-300H/L, Gentaur Molecular Products, Belgium) diluted 1:2000 in PBS/T/BSA and applied in six 2-fold dilutions (each dilution applied in two wells). Serum samples were also determined in duplicate, and diluted in PBS/T/BSA. After incubation for one hour, plates were washed as above and then incubated with biotinylated Ab30 diluted in PBS/T/BSA for one hour followed by washing as before. Ab30 was biotinylated by incubation at pH 8.2 with biotin-N-hydroxysuccinimide (approximately 100 μg/mg immunoglobulin), followed by dialysis against PBS. Finally, plates were incubated with peroxidase-conjugated streptavidin (DAKO P997) diluted 1:5000 in PBS/T/BSA for one hour, washed as before and stained with tetramethyl benzidine/peroxide substrate (TMB PLUS from Kem-Lab, Denmark). The reaction was stopped by adding 100 μl 0.5 M H₂SO₄ to each well and the optical density at 450 nm was recorded using a dedicated ELISA reader (Thermo Multiskan Ex spectrophotometer, Thermo Scientific, Waltham, MA, USA). All samples including standards were determined in duplicate. Sample values were calculated from the curve fitted to the readings of the standard (using Ascent software v. 2.6, Thermo Scientific). The detection limit of the assay was 0.5 μg/ml.

Immuno cytostaining and Flow Cytometry
Single-cell suspensions were prepared from spleens and transferred to round-bottomed 96-well polystyrene plates (NUNC, Roskilde, Denmark) with 3 x 10⁶ cells/well. For III/II (1 μg/ml, 50 μg/ml (BD Biosciences)) was added for 10 minutes to block non-specific binding of antibodies. An additional 50 μl/well PBS-Az containing fluorescent-conjugated antibodies at various concentrations was added and the cells were incubated for 45 minutes. The cells were then washed and resuspended in 200 μl well PBS-Az containing 2% formaldehyde for flow cytometric analysis. All stainings were carried out at or below 4°C. The antibodies used in this study were APC-conjugated anti-mouse CD4, clone RM4-5 (rat IgG2aκ), PE-conjugated anti-mouse CD3e, clone 145-2C11 (Armenian hamster IgG); APC-conjugated anti-mouse CD8α (1G7), clone 53-6.7 (rat IgG2aκ); APC-conjugated anti-mouse CD49b, clone DX5 (rat IgG2bκ); PE-conjugated anti-mouse CD19, clone 1D3 (rat IgG2aκ); APC-conjugated anti-mouse CD11c, clone N418 (Armenian hamster IgG); APC-conjugated anti-mouse Ly6-G (Gr-1), clone RB6-8C5 (rat IgG2bκ) and isotype controls for rat IgG2aκ; rat IgG2bκ; Armenian hamster IgG1, clone eBio239Arm; rat IgG2bκ, all purchased from eBioscience. Stained cells were analysed on a BD FACSAria flow cytometer (BD Biosciences) and data was analysed using FC Studio software (De Novo Software, CA).

In vitro fermentation of non-digestible dietary carbohydrates
The fermentation study was performed using a basal medium containing: peptone water (2 g/L, Oxoid), yeast extract (2 g/L, Oxoid), NaCl (0.1 g/L, Merck), KH₂PO₄ (0.04 g/L, Merck), KH₂PO₄ (0.04 g/L, Merck), MgSO₄ 17H₂O (0.01 g/L, Merck), CaCl₂ 2H₂O (0.01 g/L, Sigma-Aldrich), NaHCO₃ (0.01 g/L, Merck), haemin (0.005 g/L, Sigma-Aldrich), L-cystein HCl (0.5 g/L, Sigma-Aldrich), bacto salts (0.5 g/L, Oxoid), Tween 80 (2 ml/L Merck), vitamin K₃ (10 μL/L Sigma-Aldrich), resazurin (0.001 g/L Sigma-Aldrich) and 1% (w/v) test carbohydrate (inulin, FOS, XOS, COS, beta-glucan, apple pectin, polysaccharide and glucose) [42].

Stock solutions of peptone water, NaCl, KH₂PO₄, KH₂PO₄, CaCl₂ 2H₂O, MgSO₄ 17H₂O and NaHCO₃ were prepared and autoclaved (121°C, 15 min). Appropriate volumes of the stock solutions were mixed, autoclaved and supplemented with sterile filtered (0.2 μm) solutions.
of bile salts, L-cystein HCl, resazurin and yeast extract. Furthermore, haemin, Tween 80 and vitamin K1 were added. Stock solutions of the test carbohydrates were prepared by autoclaving (XOS, beta-glucan and apple pectin) or by sterile filtration (inulin, FOS, GOS, polydextrose and glucose).

An overnight culture of S. Typhimurium SL1344 (cultivated in 20 ml LB broth supplemented with 100 µg/ml nalidixic acid) was centrifuged at 1500 g for 30 minutes at 5°C and re-suspended in basal medium. The culture was inoculated in basal medium supplemented with test carbohydrates to an initial OD600 of 0.01. The fermentation study was performed under anaerobic conditions at 37°C, 200 rpm for 24 hours with recording of the initial and 24 h OD600 and pH values. A positive control (glucose) and a blank control with no additional carbon source added were included in the study. The sterility of the basal medium and carbohydrates was tested by incubation without bacterial inoculation, pH was measured before and after fermentation. Growth on a given carbohydrate was defined as significant difference from the OD600 measured in the blank sample after fermentation. All fermentations were performed in triplicate.

Statistical analysis
All parameters were analysed using a one-way analysis of variance (ANOVA). Where ANOVA indicated a significant difference, Student’s t-test was used to compare dietary groups with control. All statistical analyses were carried out using SAS JMP 6.0.2. P values of < 0.05 were considered statistically significant.

Authors’ contributions
All authors were part of a research group, which continuously followed and discussed the progress of the experiments. AP designed and carried out the animal studies, performed the statistical analysis and drafted the manuscript. TRRI and NIF conceived of the study and participated in its design and coordination as well as in the preparation of the manuscript. AP carried out the in vitro fermentation study. PMH carried out the histopathological determination. JBA performed the fluorescent tagging of the Salmonella strain. RBG performed the immunocyto- staining and flow cytometry, and MP contributed to feed design and statistical analysis. SJF and AO contributed significantly to the interpretation of data and the preparation of the manuscript. All authors read and approved the final manuscript.

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