THE NATIONAL FOOD INSTITUTE
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PhD thesis

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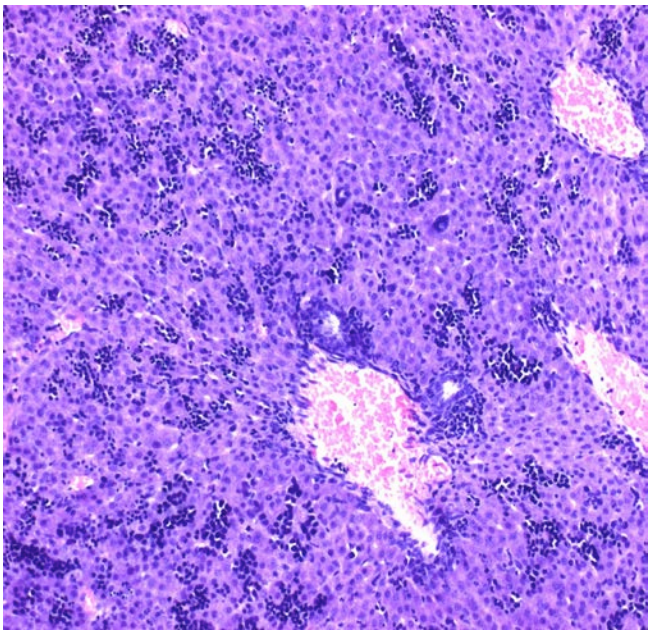
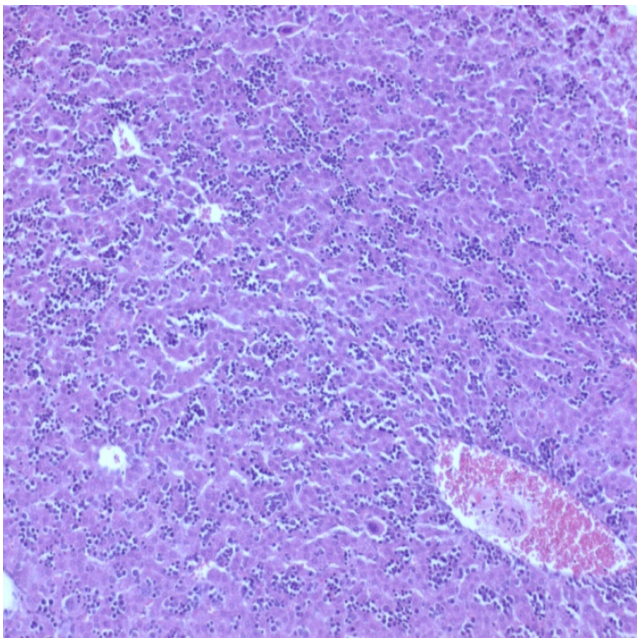
Early Life Microbiota, Neonatal Immune Maturation and Hematopoiesis





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PhD thesis · 2014

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***Front-page figure**: Histology, hematoxylin and eosin (H&E) staining, of tissue sections of murine spleens isolated the day after birth. Liver from a germfree neonate mouse (Left) and liver from a CONV neonate mouse (Right) showing notably less hematopoietic clusters (tissue with groups of cells with darkly stained nuclei) in the liver on the right, from the conventionally colonized mouse. 10x magnification.*

PREFACE

This thesis is submitted to the Technical University of Denmark to obtain the PhD degree. The work presented has been carried out in two groups; The first part at Department of Food Microbiology at The National Food Institute, Technical University of Denmark under supervision of Professor Tine Rask Licht, and the last part in Molecular Immunology Group at Department of Veterinary Disease Biology, Section of Experimental Animal Models, Faculty of Health and Medical Sciences, University of Copenhagen under supervision of Professor Hanne Frøkiær.

All animals were housed and bred at The National Food Institute. Experimental procedures on animals and tissues as well as some cellular analyses were carried out here. All immunologic assays, microscopic analyses and the rest of the cellular analyses were carried out at the Faculty of Health and Medical Sciences at University of Copenhagen.

This study was supported by 'globalization funds' given as a grant to Professor Tine Rask Licht and Professor Hanne Frøkiær in 2007.

ACKNOWLEDGEMENTS

Present thesis was never completed without the help and encouragements from so many skilled and great people.

Most importantly, I want to thank both my supervisors: Professor Hanne Frøkiær for invaluable scientific guiding and constant inspiration, patience and encouragement. I am grateful for the time I have had in your fantastic group. Professor Tine Rask Licht thanks for listening and guiding me through this work, and for your effort, advices and flexibility that made it possible for me to finish this work.

I also want to express my gratitude for the skilled scientists, I have had the pleasure to work with during my PhD years; Stine Broeng Metzdorff, Lisbeth Nielsen Fink, Louise Hjerrild Zeuthen and Anders Bergström. Thank you for the inspiration, effort and collaboration.

Anni Mehlsen and Marianne Petersen, thank you for your skilled help and fantastic company! I am further very grateful for the technical help from Heidi Letting and Bodil Madsen at DTU FOOD, and for the great help and communication from Maja Danielsen and Anne Ørngreen, and their fellow co-workers in the stable at DTU FOOD, during the first years of my PhD. Thank you for your immense patience with the troubled breeding of germfree mice.

Working in two different groups has given me the opportunity to work with many inspiring and skilled PhD students, masterstudents and Post-docs; Anne Petersen, Tine Ebersbach, Louise Kristine Vigsnæs and Lene Hemmingsen, thank you for the great time we had together in our basement office at DTU FOOD. Dina Silke Malling Damlund, Louise Hemmingsen, Lisbeth Drodz Lund, Eva Fuglsang, Rasmus Baadsgaard Mærkedahl, Gudrun Weiss, Kristina Maetoft Udsen and Ronja Mathiesen thanks for a great time at KU and for sharing the ‘tough months and the fantastic days’ with me.

Additionally I had the pleasure to supervise the master students Christina Kjærgaard and Line Rieck Schmidt during their work at DTU FOOD. I hope you had a good time too.

I also want to thank Professor Fredrik Bäckhed and technician Anna Hállen for valuable help with the germfree mice during my stay at Sahlgrenska Academia, University of Gothenburg in October 2010, and Erwin L. Roggen for giving a very important scientific advice in late 2012 that turned the present work upside down.

Lastly, I want to thank my dear family, especially Sinus, Magnus, Jens and Anna, Mor and Far. Your love and care have been the most important factors through all the years.

Matilde, March 2014

DANSK RESUMÉ

Epidemiologiske data støtter i tiltagende grad hypotesen om at mikrobiotaen hos det nyfødte barn spiller en vigtig rolle i udviklingen af et balanceret immunsystem. Forhold tidligt i livet, som fødselsmåde og spædbarnets ernæring, har vist sig at have indflydelse på udviklingen af immunrelaterede sygdomme senere i livet. Sygdomme som diabetes, astma og inflammatoriske tarmsygdomme er relateret til den tidlige kolonisering af tarmen.

Tarmepitelet udgør en fysisk og kemisk barriere mellem bakterierne i tarm lumen og immuncellerne i det submukosale væv. Tarmens epitelceller danner en meget stort mono-lags overflade og er af stor betydning for den synergistiske sameksistens mellem milliarder af bakterier i tarmen og deres mammale vært. Tarmens epitel celler kommunikerer aktivt med bakterierne i tarmen, og denne sameksistens er med til at etablere en nyttig tolerancetilstand i tarmen. Tarmens homeostase fremmes af en balanceret og kontrolleret kommunikation mellem tarmcellerne og de kommensale bakterier i tarmen.

Hematopoietiske stamceller fra den føtale lever distribueres via blodet til den føtale/neonatale milt og marv i den perinatale fase. I spædbørn og nyfødte mus beskrives en markant øgning af granulære celler i blodet i de første dage efter fødsel. Dette er en naturlig del af den neonatale hematopoiese. Tilstedeværelse af granulære myeloide suppressor celler i humant navlestrengsblod er beskrevet i en helt ny artikel. Disse granulære suppressor celler er tidligere beskrevet i forbindelse med cancer, inflammation og under sepsis. De dannes i forbindelse med den myeloide hematopoiese og flere nyligt beskrevne studier viser en immunregulerende rolle for disse celler i den nyfødte.

I nærværende studie viste vi tilstedeværelsen af en dominerende gruppe af $CD11b^{+}Gr\text{-}1^{+}$ celler in den nyfødte musemilt. Tilstedeværelse af disse celler var mikrobiota afhængig, idet kimfrie mus havde markant færre af disse celler i milten i den første leveuge. Mikroskopidata indikerede at disse celler stammede fra hematopoietisk væv i den nyfødte mus, og at mobilisering og aktivering af dette væv blev fremmet af en konventionel kolonisering af tarmen. Regulering af tarmens barriere var påvirket af mikrobiotaen, idet ekspressionsstudier af tight junction protein gener viste en hurtigere og strammere regulering af disse gener i nyfødte mus med en konventionel mikrobiota end i den kimfrie mus. Den komplekse mikrobiota fremmer desuden ekspression af gener involveret i mucin sekretion, TLR signalering og cytokin produktion i tarmen, mens den nedregulerer gener for chemokin ekspression samme sted. Ny viden indikerer at den dominerende $CD11b^{+}Gr\text{-}1^{+}$ cellegruppe kan være immunregulerende celler af betydning for den tidlige etablering af tolerance i den nyfødte, men det er ikke bevist i dette studie.

ABSTRACT

Emerging epidemiologic data supports the hypothesis that early life colonization is a key player in development of a balanced immune system. Events in early life, as birth mode and infant diet, are shown to influence development of immune related diseases, like asthma, diabetes and inflammatory bowl disease, later in life.

The intestinal epithelium makes up a physical and biochemical barrier between the bacteria in the gut lumen and the immune cells in the submocusal tissue. This monolayer of intestinal epithelial cells (IEC) makes up an extremely large surface and is highly important for the synergistic coexistence between trillions of bacteria in the gastrointestinal tract and their mammalian hosts. The IEC actively communicate with the microbiota of the gut lumen and tolerance establishment in the intestine is induced as a result of a balanced and controlled communication between IEC and the commensals in the gut.

Hematopoietic stem cells from the fetal liver seed the fetal spleen and bone marrow in perinatal phase. Granulocytosis in neonate mice and man just after birth is a natural event of early life hematopoiesis and likely contributes to elevated counts of neutrophil-like cells in the peripheral blood of newborns. Granular myeloid derived suppressor cells (MDSC) have recently been described in human cord blood. MDSC are potential immunosuppressive cells often described in cancer, inflammation and during sepsis. They evolve from immature myeloid cells during hematopoiesis. Several recent studies show a role for various myeloid derived and immune suppressive cellular subsets in the newborn.

In the present work we showed the presence of a prominent group of $CD11b^{+}Gr\text{-}1^{+}$ cells in the neonate murine spleen. The presence of these cells were dependent on the colonizing microbiota, as germfree neonate mice held notably fewer of these cells in the spleen. Microscopy of spleens and livers indicated that these cells derived from hematopoietic tissue in the liver of the neonate mouse, and that mobilization and activation of the hematopoietic tissue is promoted by the presence of colonizing microbes.

The regulation of epithelial barrier integrity was influenced by the nature of the microbiota, as expression of tight junction (TJ) protein encoding genes showed a faster and more tightly regulated rate in the murine ileum of conventionally colonized mice compared to the GF ileum. The conventional microbiota furthermore promotes the expression of genes involved in mucin secretion, TLR signaling pathways and cytokine production in the intestine, while downregulating genes encoding chemokines in the epithelial tissue.

Newly published studies indicate that the prominent $CD11b^{+}Gr\text{-}1^{+}$ cell group may have a role in early life immune regulation. This is however not proven by the data of present study.

LIST OF ARTICLES

Paper 1

Kristensen M. B., Metzdorff S. B., Bergström A., Damlund D. S. M., Fink L. N., Licht T. R. and Frøkiær H. Neonatal microbial colonization in mice promotes prolonged dominance of $CD11b^{+}Gr\text{-}1^{+}$ cells and establishment of the $CD4^{+}$ T cell population in the spleen. Submitted to American Journal of Physiology – Gastrointestinal and Liver Physiology (February 2014), under review.

Paper 2

Bergström A., **Kristensen M. B.**, Bahl M. I., Metzdorff S. B., Fink L. N., Frøkiær H. and Licht T. R. (2012). Nature of bacterial colonization influences transcription of mucin genes in mice during the first week of life. *BMC Research Notes.* 5:402

Paper 3

Fink L. N., Metzdorff S. B., Zeuthen L. H., Nellemann C., **Kristensen M. B.**, Licht T. R. and Frøkiær H. (2012): Establishment of tolerance to commensal bacteria requires a complex microbiota and is accompanied by decreased intestinal chemokine expression. *American Journal of Physiology – Gastrointestinal and Liver Physiology*. 302: G55-G65

Paper 4

Zeuthen L. H., Fink L. N., Metzdorff S. B., **Kristensen M. B.**, Licht T. R., Nellemann C. and Frøkiær H (2010). *Lactobacillus acidophilus* induces a slow but more sustained chemokine and cytokine response in naïve foetal enterocytes compared to commensal *Escherichia coli. BMC Immunology. 11:2*

SCIENTIFIC PRESENTATIONS

The scientific work conducted was presented at the following conferences:

Oral presentations

"Impact of first bacterial colonizers on immune system development and homeostasis"
INRA-RRI Symposium on Gut Microbiome, Functionality and Interaction with host and Impact on the environment, Clermont-Ferrand, France, June 2008

"Impact of first bacterial colonizers on immune system development and homeostasis"
Workshop on Nutritional Immunology, Elsinore, Denmark, September 2008

"The Early Life Microbiota Influences Recruitment of Immune Cells and Establishment of Intestinal Integrity"
9th Symposium on Food Microbiology, LMC, Elsinore, Denmark, May 2011

Poster presentations

Kristensen M.K., Fink L.N., Metzdorff S.B., Frøkiær H. and Licht T.R.
"Impact of first bacterial colonizers on immune system development – presentation of a new project"
Danish Conference on Molecular Biology and Biotechnology: Functional Foods and Nutrigenomics, Vejle, Denmark, May 2008

Kristensen M.K., Fink L. N., Kærgaard C., Frøkiær H. and Licht T.R.
"Lactobacillus acidophilus NCFM induces a fast and early maturation of T-cells in mono-associated mouse pups"
14th International Congress of Mucosal Immunology, Boston, MA, USA, July 2009

Kristensen M.K, Licht T. R., Bergström A., Metzdorff S.B. and Frøkiær H.
"The complexity of the murine microbiota influences recruitment of immune cells in early life"
15th International Congress of Mucosal Immunology, Paris, France, July 2011

ABBREVIATIONS

Ab	antibody
AMP	antimicrobial peptides
Arg1	arginase 1
BCG	bacillus Calmette-Guerin
BM	bone marrow
CONV	conventionally colonized
CD	cluster of differentiation
Cdh1	E-cadherin
CRAMP	cathelicidin-related antimicrobial peptide
CS	caesarean section
DC	dendritic cells
FSC	forward scatter
GF	germfree
GI	gastrointestinal
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
G-MDSC	granulocytic myeloid derived suppressor cells
H&E	hematoxylin and eosin
HSC	hematopoietic stem cells
HPG	hematopoietic progenitor cells
IBD	inflammatory bowel disease
IEC	intestinal epithelial cells
Ig	immunoglobulin
IL	interleukin
IFN	interferon
IMC	immature myeloid cells
iNOS	inducible nitric oxide synthase
IRAK	IL-1 receptor associated kinase

JAM	Junctional adhesion molecule
KC	keratinocyte chemoattractant
LPS	lipopolysaccharide
mAb	monoclonal antibody
M-cell	microfold cell
MC	mono colonized
MDSC	myeloid derived suppressor cells
MIP-2	macrophage inflammatory protein 2
miR	microRNA
MOI	multiplicity of infection
MO-MDSC	monocytic myeloid derived suppressor cells
NK	natural killer
NO	nitric oxide
pIgR	polymeric Ig receptor
PND	post-natal-day
PRR	pattern recognition receptors
Reg3γ	C-type lectin regenerating islet-derived protein 3γ
ROS	reactive oxygen species
SCF	stem cell factor
SSC	side scatter
T1D	type 1 diabetes
T_H1	T helper 1
TJ	tight junction
TLR	toll like receptors
TNF	tumor necrosis factor
Tjp	Tight junction protein
Treg	regulatory T cell

CONTENT

OBJECTIVES OF STUDY

Since Strachan in 1989 defined the 'Hygiene hypothesis' as a possible explanation of the increased incidences of allergic and autoimmune diseases in western countries[1], evidence has emerged for the importance of the intestinal microbiota in balancing immune responses and intestinal homeostasis. Several immune related diseases have been linked to alterations in the bacterial communities at the mucosal surfaces, and during the past decades the adult human microbiota has been extensively studied.

Development of various animal models has made it possible to more specifically study the interaction between microbe and man, and the use of the germfree (GF) animal model has opened new opportunities for elucidating the synergistic relationship between the mammals and their intestinal inhabitants. In present study we used the GF mouse as animal model. The presented study aimed at describing how the very early encounter between the first pioneering gut microbes and their mammalian host influences establishment of intestinal homeostasis and the maturing neonate immune system.

We hypothesized that

- The intestinal barrier of the neonate is permeable to bacteria at the time of birth
- This permeability enables pioneering bacteria of the intestine to cross the epithelial layer
- The resulting postnatal bacterial influx is of very short duration.
- The pioneer bacteria of the intestinal mucosa are indispensible for an efficient establishment of integrity in the immature intestine.
- The early life influx of bacteria may play a key role in priming and calibrating the immature immune system.

The work of present thesis resulted in four papers:

In Paper 1 the age and colonization dependent development of various immune cells in the neonate mouse is described. Furthermore hematopoietic events are shown to be highly influenced by the nature of the colonization in early life, which additionally was shown to influence the establishment of the intestinal epithelial tight junction complexes.

Paper 2 shows how the nature of the gut microbiota influences expression of various mucin genes in the intestinal mucosa of neonate mice.

Paper 3 and Paper 4 contain *in vivo* and *in vitro* gene expression studies, respectively, of the effect of colonization on genes encoding chemokines, cytokines and genes involved in antigen presentation.

INTRODUCTION

The aim of the introduction is to introduce the reader to the immune system and hematopoiesis of the neonate. An introduction to microbial colonization of the neonate gut, the establishment of intestinal homeostasis and the maturing immune system is given. Lastly the dynamic character of the cellular development in the hematopoietic organs of the neonate mouse is reviewed, with a particular focus on development of myeloid and granulocytic cells in early life.

EARLY LIFE MICROBIOTA

The bacteria colonizing humans have historically been seen primarily in terms of a problematic battle between the bacteria and their hosts, and the large numbers of bacteria that inhabit the gastrointestinal (GI) tract of humans were principally ignored as they caused no obvious disadvantage. The past 20 years we have, however, seen constantly growing evidence that the microbiota plays a crucial role in development of the GI tract and the maturation of a balanced mammalian immune system [2, 3].

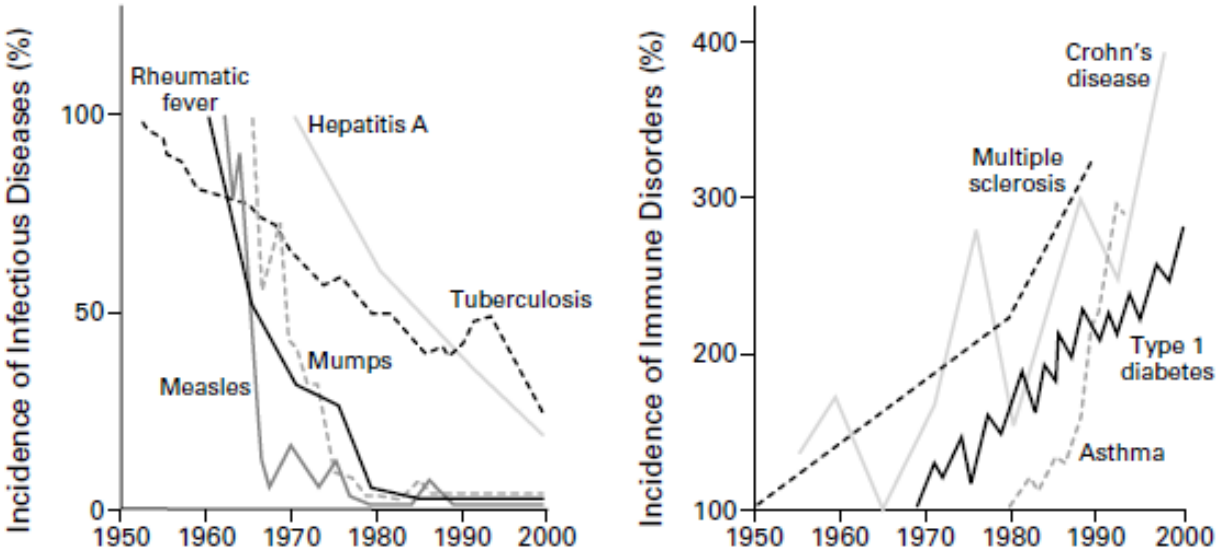

Figure 1. **The inverse relation between the decrease in typical infectious diseases and the steadily increase in the incidence of allergic and autoimmune diseases.** Estimated incidences of tuberculosis, rheumatic fever, measles, hepatitis A and mumps in the United States and France over a 50-year period, and the corresponding estimated incidences of various allergic and autoimmune diseases in northern Europe during the same period of time (Figure copied from [4])

In 1989 Strachan [1] defined the 'Hygiene hypothesis' suggesting that the increasing incidences of allergic and autoimmune diseases, documented in the western world since the 1950es, can be explained by modern hygiene and medical practices, which increased the use of vaccines and antibiotics and limited the exposure to pathogens in early life [1, 4, 5] and, as one of several consequences, influenced the composition of the establishing microbiota in early life. An inverse

relation exists between the distinct decrease in typical infectious diseases and the increasing prevalence of autoimmune and allergic diseases in the industrialized countries from the 1950es to year 2000, as depicted in Figure 1. This further suggests that the limited exposure to pathogens can be a major factor in the increasing prevalence of these disorders [4]. Despite several revisions of the 'Hygiene hypothesis', it is by now generally accepted that the microbiota is a key player in the development of these diseases [5-8], and recent studies, reviewed below, point out events in the early microbial colonization as being highly influential on subsequent development of a homeostatic immune system.

COLONIZATION OF THE NEONATE

The colonization of the neonatal GI tract is a sequential process, considered to take its beginning at birth, when the infant, on its way through the birth canal, is exposed to various bacteria of maternal vaginal and fecal microbiota. The diversity of the gut microbial colonization is limited during the first days of neonatal life comprising a few bacterial species; these are typically facultative aerobe or microaerophilic bacteria like Enterococci, Lactobacilli, Streptococci and members of the Enterobacteriaceae. These bacteria metabolically reduce the local oxygen concentration and thereby fertilize the environment for a subsequent colonization by strict anaerobes such as *Bifidobacteria, Clostridium* spp. and *Bacteroides* spp.. The numbers of anaerobic bacteria expand in the maturing gut and the environmental changes brought about by these bacteria drive the facultative bacteria of the very early gut out of competition. Facultatives represent less than 1% of the adult human intestinal microbiota. The diversity of the microbiota increases with time, undergoing distinct changes at the time of weaning and when diet is markedly changed [9-11]. In humans the neonatal microbiota is dynamically changing with changes in diet or antibiotic treatments. By one year of age, however, the microbiota of the baby has converged into a microbiota profile characteristic to that of the adult GI tract. Despite this general stereotyping of the microbiota into an adult profile, it is known that interindividual differences observed in the neonate gut microbiota persist even in the adult bacterial community [12]. The human adult GI tract harbors approximately 10^{14} bacteria, and a large cohort study on fecal samples of 124 adult Europeans, the adult microbiota was described to comprise more than 1000 bacterial species and each individual to harbor at least 160 of such species. Though the microbiota can be periodically influenced by use of antibiotics, diseases and changes of diet, the bacterial composition remains relatively stable throughout life [13, 14].

Contradictory to the traditional point of view on the human fetus as developing in a completely sterile environment, protected and bathed in sterile amniotic fluid, recent studies report that the fetus may incorporate an initial microbiota already during prenatal life [15]. Studies made on healthy and term born infants have characterized bacteria in cord blood [16], amniotic fluid [17] and in fetal membranes [17, 18] of healthy neonates. Furthermore a diverse bacterial community has been found in the meconium of both neonate mice and human infants delivered either vaginally or

by Caesarean section (CS), which further contradicts the general understanding that what the fetus swallows in the womb is sterile. Importantly, the authors of the murine study controlled for bacterial cross-contamination between skin and meconium, excluding the exterior surfaces as the source of microbial colonization [19, 20]. These studies as a whole indicate a plausible presence of maternally transferred pioneer microbiota to the fetus in uterus.

Factors influencing neonate colonization

Colonization of the infant GI tract is influenced by a variety of factors including gestational age, family hygiene, mode of delivery and diet. In several recent studies these factors have, in addition to their influence on the pattern of colonization, been shown to effect disease development later in life.

Mode of delivery

The mode of delivery has a major influence on the composition of the mucosal colonization in early life. Infants delivered vaginally are colonized with microorganisms of maternal vaginal and maternal intestinal origin, while those born by CS are colonized with environmental microorganisms. The vaginally delivered babies additionally acquire bacterial communities similar to their mother's vaginal and intestinal microbiota, while the CS infants obtain microbial communities with bacteria typically originating from the skin surface, which show no closer relation to the microbial population of their mother's skin, than to any other person [15, 21]. CS-delivered infants have also been shown to undergo a delayed colonization, and obtain an altered microbiota more often containing *Clostridium difficile* and with lowered numbers of *Bifidobacterium* and *Bacteroides,* compared to the vaginally delivered infants. Differences in the microbial composition in infancy are furthermore still clearly mirrored in the bacterial composition of CS-delivered compared to the vaginally born infants by one year of age [9, 21-23].

Epidemiologic data indicate long-term effects of CS delivery on health status later in life; A meta-study from 2008 concludes that delivery by CS significantly increases the risk of developing childhood-onset type 1 diabetes (T1D), and another similar meta-study by the same authors further concludes that CS-delivered children have a 20% increased risk of developing asthma later in life [24, 25]. Children born via CS are in addition more prone to develop asthma and allergic rhinitis [26], celiac disease [27] and a study reports that rates of inflammatory bowel disease (IBD) are moderately increased in CS delivered children [28], while another study reports no significant correlation between CS and IBD [27].

The influence of CS delivery on several immune mediated diseases carry conviction, and it can be speculated that the higher rates of autoimmune or allergic diseases in children born by CS can be related to the lack of maternally transferred bacteria, which may in the vaginally delivered infant favor a balanced immune maturation.

Neonatal Feeding

Human milk has traditionally been considered sterile, but several studies have the past 10 years revealed that breast milk and colostrum are rich sources of commensals and potential probiotic bacteria [29-33]. Breast milk of healthy women has been shown to contain a great diversity of bacterial species [34], and the bacterial community in the milk to change considerably during lactation. The milk close to labor contains more *Lactobacillus, Streptococcus, Staphylococcus* and *Lactococcus*, while the breast milk at 1 and 6 months of age harbors increased levels of bacteria typically found in the oral cavity like *Prevotella* and *Veillonella* [15, 33]. With an estimated 800 ml/day consumption of breast milk the human infant will ingest 8×10^4 to 8×10^6 bacteria each day [35], and the human milk is, thus, a major source of bacterial consumption to the infant. As may seem a natural consequence of the high bacterial intake during lactation, the gut microbiota of breast fed infants has been shown to be closely related to that of their mother's breast milk [36]. Formula fed infants are more often colonized with *Escherichia coli, C. difficile and Bacteroides fragilis* than the breast fed infants, whereas the prevalence of *Bifidobacterium* in the gastrointestinal microbiota is the same among both groups. The breast fed group is however reported to harbor a more complex community of *Bifidobacterium* than the formula fed babies [23, 37, 38]. Several studies find lactobacilli to be more prevalent in formula fed than in breast fed infants [10, 23]. A lactobacilli species, *L. Rhamnosus*, is however found to be more common in breast fed infants, when compared to formula fed and weaned infants [39].

Human breast milk stimulates growth and health of the neonate in several ways, as early breast milk contains large amounts of immunoglobulin A (IgA), immune cells, cytokines and growth factors [9], and the health promoting effect of breast feeding, thus, cannot be linked entirely to the influence on microbial colonization patterns. Non-absorbed oligosaccharides of breast milk are however generally considered to be prebiotic and to promote growth of beneficial lactic acid bacteria in the infant microbiota. Another functionality of human milk oligosaccharides is that they inhibit attachment of bacteria to the intestinal epithelium, due to their ability to act as receptor analogs of mucosal adhesion molecules in the GI tract [23, 40].

Several studies have indicated that the source of neonatal feeding influences later health status. A recent meta-study shows a significant protective effect of breast feeding on development of early onset IBD [41], and a birth-cohort study comprising more than 30.000 infants shows that breast fed infants have significantly lowered prevalence of obesity later in childhood [42]. Additionally evidence emerges for a protective effect of breast feeding against development of T1D and celiac disease, for the latter a significant protection is described, when the infant is breast fed through the time of gluten introduction in the diet [41, 43, 44].

Reduced diversity and antibiotics in early life

The general hypothesis that the lack of diversity in the human microbiota influences the long-term development of immune related diseases is supported by several epidemiological studies. Infants with an estimated reduction in microbial fecal diversity during the first year of life were shown in the large Danish long-term birth-cohort (the COPSAC cohort) study to have increased risk of developing allergic diseases in preschool age [45]. Recently, another study on the Danish COPSAC cohort has shown that maternal use of antibiotics during pregnancy significantly increases the risk of developing early childhood asthma [46]. A study show no effect of maternal antibiotics use during pregnancy on the subsequent neonate colonization of the intestinal mucosa, but a distinct change is observed in the microbial colonization pattern when antibiotics are administered postnatally to the infant [23].

Obesity

A study concluded that microbiota in infants born large for gestational age differed significantly compared to microbiota in infants born appropriate for gestatinal age. In this work *Proteobacteria* were found more often in high birth weight infants, whereas *Firmicutes* were more prevalent in babies with a normal birth weight. The high birth weight is known to be a risk factor for development of metabolic disturbances like diabetes and obesity later in life [47, 48].

In summary the reviewed studies indicate that the microbial colonization of the neonate mucosal tissues is a sequential and very likely a finely tuned process, which may be disturbed by events like birth mode and feeding source in neonatal life. The increasing prevalence of immune related diseases and the epidemiological data for mode of delivery, feeding mode and antibiotics use suggest that the ecology of the neonate GI tract can be skewed into an unfavorable bacterial diversity, which potentially disturbs the intestinal homeostasis and the maturation of the immune system. This distortion of the microbial intestinal environment is suggested by several epidemiologic studies to play a prominent role in the onset of IBD, celiac disease, T1D, obesity, asthma and allergic diseases.

NEONATAL IMMUNE MATURATION

At birth the fetus faces a dramatic transition from a sterile and protected life in the uterus to a radically distinct environment of microbial exposure and bacterial colonization in extra-uterine life. Maternal and environmental bacteria are transferred to the mucosal surfaces of the neonate during the passage through the birth canal and in early postnatal period. In order to avoid excessive immune responses in the neonate, the immediate colonization of mucosal surfaces requires efficient establishment of the intestinal barrier and a tight regulation of the immature immune response to establish tolerance towards commensals and food components and efficiently combat potential pathogenic bacteria.

ESTABLISHMENT OF INTESTINAL HOMEOSTASIS IN THE NEONATE

The intestinal epithelium makes up a physical and biochemical barrier between the bacteria in the gut lumen and the immune cells in the submocusal tissue. The establishment and maintenance of this barrier are highly important for the synergistic coexistence between trillions of bacteria in the GI tract and their mammalian hosts [49].

The physical barrier

The interior of the GI tract is lined by a monolayer of intestinal epithelial cells (IEC). With a surface area at least 200 times that of the skin, it makes up the largest surface area of the body in contact with the exterior environment. The surface of the ileum is entirely covered by villi, which are finger-like projections covered themselves by a brush-border of microvilli. The glandular invaginations between the villi are named crypts, and proliferating stem cells of the intestine and Paneth cells are harbored in the ileal crypts. The ‘villi-microvilli-crypt’ architecture of the small intestine accounts for the remarkably large surface area of the mammalian gut [50, 51]. IEC comprise different cell types involved in the establishment and maintenance of intestinal homeostasis. Enterocytes, the most predominant cell type of the epithelium, are absorptive cells taking up nutrients, fluid and ions from the intestinal lumen, while Paneth cells and goblet cells of the intestinal epithelium secrete a range of different antimicrobial peptides (AMP) and mucus, respectively. Furthermore enteroendocrine cells, secreting various hormones, are found in the monolayer of the GI tract. The intestinal epithelium further contains microfold cells (M-cells), which are cells overlying Peyer’s patches of the small intestine. M-cells perform endocytosis of various gut luminal antigens, thereby directing these to the lymphoid cells beneath the M-cells. As mentioned, intestinal stem cells additionally make up a small fraction of the epithelial monolayer [3, 9, 50].

The maturity of the neonate gut varies between man and mouse, and with gestational age. While human babies are born at term with a mature-like crypt-villus structure, the intestinal epithelium of the murine neonate is immature and flat and the intestinal crypts and the protruding villi develop as

late as 10 days after birth [50, 52]. The human neonate, especially if born premature, tends to have increased intestinal permeability during the postnatal period, and the barrier integrity is still developing at birth. During the early postnatal period the intestinal barrier matures and becomes more selectively permeable in the human premature infant and in rodent neonatal models. The intestinal integrity is established rapidly in early life following the introduction of oral feeding. It can however be influenced by a range of different factors like infant feeding, gestational age and postnatal age [53, 54].

Different physical factors of the innate immune system have been shown to influence intestinal homeostasis in adult mice and human, and might as well contribute to establishment of intestinal barrier integrity in the neonate. In the healthy intestinal epithelium a natural establishment of intercellular junctional protein complexes is seen, including tight junctions (TJs), between the IEC in the intestinal monolayer. These protein complexes enforce the epithelial barrier and the level of TJ protein gene expression has been used as a measure of intestinal epithelial integrity [55-57]. Dysfunctional formation of the TJ protein complex has been observed in several autoimmune diseases including T1D, celiac disease and IBD [56, 58, 59]. In Paper 1 of this thesis we present data showing that intestinal microbiota supported a tight regulation of TJ gene transcription in early postnatal life, which can be speculated to influence the establishment of the TJ protein complex and gut closure in the neonate intestine.

Goblet cells of the intestinal epithelium produce mucins, which are large extracellular proteins of complex and glycosylated oligosaccharides. These are secreted on the apical surface of the intestinal monolayer and hereby provide a dense gel like structure, the mucus layer, which physically separates the microbiota from the intestinal epithelium [9, 60]. Mice lacking the mucin protein MUC2 develop spontaneous colitis, thus, indicating that mucins are critical for the establishment of epithelial barrier integrity [61]. In a study of the microbial influence on mucin transcriptional regulation in neonate mice (Paper 2), we have described that colonization of the neonate increases transcription of various mucin genes at birth. Presence of lactobacilli in the neonate murine intestine further seems to lower transcription of mucin genes early in life [62].

Microbe-Enterocyte crosstalk

IEC actively guard the microbiota of the GI tract and communicate to the immune system upon receiving microbial stimuli. As the communication from the IEC direct the subsequent enrollment of local and systemic immunity, the IEC play a critical role in directing the early life immune system [63].

Conserved microbial structures of commensal and pathogenic bacteria are recognized by the IEC through pattern recognition receptors (PRRs). This microbe-to-IEC crosstalk induces the IEC to produce various chemokines and cytokines, which recruit immune cells to the submucosal tissues of

the GI tract and influences the immune responses of the recruited and present cells in the tissue, respectively [63-65]. Until recently intestinal epithelia were believed to be devoid of PPRs, but expression of these potent immune stimulatory receptors has been described on IEC in several studies during the past years [66-68]. The family of toll-like receptors (TLRs) is a well described group of transmembrane receptors that recognize a broad ragne of conserved microbial structures. The TLRs have various ligand specificities. Microbe recognition through the innate TLRs induces a conserved signaling cascade, which promotes transcription of various genes with a role in host defense. This includes genes encoding inflammatory cytokines and chemokines, and effector molecules like AMP and inducible nitric oxide synthase (iNOS) with an ability to directly kill the pathogenic bacteria [69, 70].

In an epithelial cell line study TLR2 and TLR4 have been shown to be constitutively expressed at the apical surface of IEC, and furthermore to traffic from the apical membrane to cytoplasmic compartments upon first recognition of conserved microbial structures, thus, introducing tolerance towards subsequent stimulation through TLR2 and TLR4 [67]. In neonate mice a strategy to avoid excessive immune stimulation from microbial stimulation of the intestinal TLRs was demonstrated in a study revealing lipopolysaccharide (LPS)-induced downregulation of TLR4 in neonatal primary IEC at the time of birth. The expression of this receptor is high in fetal IEC, but the postnatal downmodulation likely contributes to intestinal tolerance during early life colonization [64].

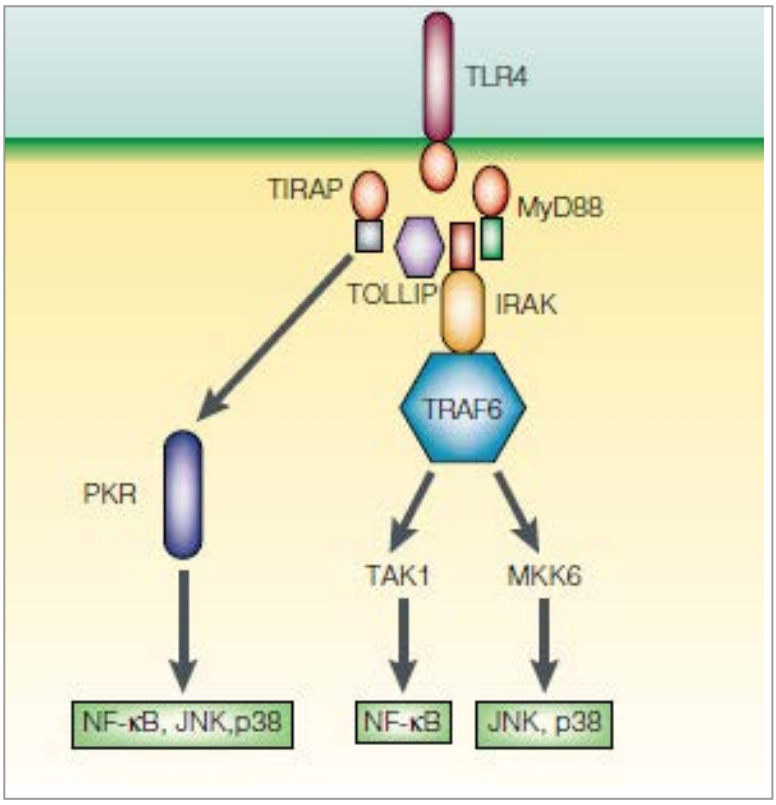

Figure 2: TLR expression on IEC. Strategies to avoid excessive immune responses in neonate mice involve the expression of TLRs on IEC. An important LPS-tolerizing mechanism in neonate IEC is the increased expression of microRNA-146a, which induces translational degradation of IRAK1. This inhibits the TLR signaling pathway, which is schematically shown here for the TLR4 signaling pathway, and induces tolerance to subsequent microbial stimuli [69]. Figure of TLR4 signaling pathway, modified from Medzhitov 2001 [70].

The decreased TLR sensitivity of murine IEC at birth has been shown to depend on an up-regulated expression of microRNA-146a (miR-146a). The increase of miR-146a expression induces a translational suppression and proteolytic degradation of the IL-1-associated kinase 1 (IRAK1), a TLR signaling protein of the conserved TLR signaling pathway (Figure 2), in the IEC. This inhibits TRL signaling and consequently induces early innate immune tolerance in the neonate. Importantly maintenance of IRAK1 suppression requires continues signaling through TLR4, and the suppression of IRAK1 is not seen in CS delivered mouse pups, which indicate the need for a continuoes and controlled microbial stimulation of TLR4 to induce miR-146a-induced mucosal tolerance [50, 64, 69]. Altogether the TLR studies in neonate mice and cell lines indicate that a controlled colonization induces tolerance through TLR recognition of conserved microbial structures, and that this tolerance might very well contribute to establishment of intestinal homeostasis in the neonate.

Antimicrobial peptides

The intestinal epithelial barrier plays a major role in host defense, however this monolayer of cells additionally adds to protection of the host by secreting various antimicrobial factors including AMP. These peptides are ancient cationic peptides, natural antibiotics, contributing to host defense and innate immunity in various organisms throughout the plant and animal kingdoms [9, 71]. In the neonate mouse the range of secreted AMP changes considerably during postnatal development, as was recently described [72]. The cathelicidin-related antimicrobial peptide (CRAMP) is expressed in the small intestine during the first two weeks of life and decreases with weaning and with maturation of the crypt-villus structure of the small intestine. Paneth cells, known to secrete large amounts of AMP in ileum, evolve in the intestinal epithelium after early neonatal period. They initiate a production of AMP including defensins and the C-type lectin regenerating islet-derived protein 3γ (Reg3γ), which replaces the production of CRAMP in early neonatal phase [50, 72]. The secretion of defensins accounts for the largest proportion of the secreted AMP in this phase and have been shown to be expressed independently of the nature of the intestinal microbiota, while Reg3γ expression is found to require intestinal colonization [73, 74]. As important effector molecules of the intestinal innate immunity AMP regulate and alter the community of commensal bacteria in the host intestine, however, also the microbes regulate the spectrum of AMP, as seen for Reg3γ. An important study by Salzman *et al.* [71] has shown that decreased or altered AMP production in mice results in significant changes in the microbiota composition along with modulations of the mucosal immune response and have further associated these changes with chronic intestinal inflammation.

Immunoglobulin A (IgA)

Secretory IgA in maternal breast milk is the first source of specific antibody mediated immune protection to the intestinal tract of the newborn. IgA is produced by plasma cells and is actively transported by the intestinal epithelium via the polymeric Ig receptor (pIgR). After transport of the secretory IgA across the epithelium, a proteolytic cleavage releases IgA from the polymeric receptor into the gut lumen [75]. In the lumen IgA shapes the microbial community and promotes intestinal homeostasis in the neonate by preventing translocation of commensals and pathogenic bacteria. In a neonate IgA-deficient mouse model it was shown that IgA-deficient mice have a significantly different microbiota at weaning than their IgA positive counterparts, and that this difference persists and magnifies into adulthood. The IgA deficient mice were further described to develop a gene expression profile associated with intestinal inflammation and to have a lowered intestinal integrity, as aerobic bacteria were found to be translocated from the gut to the mesenteric lymph nodes [76].

NEONATAL MATURATION OF THE IMMUNE SYSTEM

It is increasingly clear that interaction between early life microbiota and the developing immune system is highly important for later health status. Although the microbiota in adults and infants have been extensively studied, and variations in the microbiota have been linked to development of immune related diseases, numerous aspects of the mechanisms in the interplay between microbes and the developing cellular immune system are still unclear. Developmental maturation of neonate innate and adaptive immunity remains sparsely described and contradicting results are reported on infant immune responses.

The neonate human (and mouse) is particularly susceptible to inflammation and infectious diseases that only cause mild effects in the adult. Every year more than a million infant deaths within the first month of life are due to infectious causes [77]. The innate immune system of the neonate is generally considered to be impaired [78] and as described previously, specific mechanisms are demonstrated at mucosal surfaces in the neonate intestine to avoid excessive TLR-mediated immune stimulation towards commensal bacteria [64, 69]. The age-dependent decrease in TLR sensitivity can however also be seen as a part of the attenuation of the innate immune response that cause deficiencies in early life pathogen combat. As described earlier in this thesis, AMP, which are part of the innate defense in the neonate, are however readily produced and tightly regulated at intestinal mucosal surfaces in perinatal phase. It is furthermore well described that term born infants have large numbers of neutrophils in peripheral blood. Contradicting results have been reported about these phagocytic cells, and it seems that gestational age and the phagocytic capacity of the neutrophil-like cells are factors, which add to heterogeneity of results in studies of this cell group [79, 80].

Absence of specific, adaptive immunity is likely to contribute to the vulnerability of the newborn and low numbers of immune cells in the lymphoid tissues may be another important factor in this. T cell numbers are notably reduced in neonate mice compared to adult mice and various T cell immune responses are reported from the neonate T cell compartment [81, 82]. Though the neonate is generally considered to be poor or deficient with regard to the $CD4^+$ T helper 1 (T_H1) proinflammatory response, it has been reported that murine neonates are competent to mount a mature T_H1 inflammatory response under specific antigenic influence [83]. Strong inflammatory responses are described following injection of Cas-Br-M murine leukemia virus in neonate mice [84] and robust proinflammatory cytokine responses are mounted *in vitro* and *in vivo* in response to various TLR stimuli in neonate mice [85]. In human infants an adult-like T_H1 inflammatory response develops in response to injection of the *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) vaccine [86]. In contrast to earlier studies, describing attenuated or vague neonate immune responses [87], these studies propose that the neonate may be able to mount adult-like or even hyper innate responses upon microbial infection. It is suggested by some that the quantitative and qualitative deficiencies of the neonatal $CD4^+$ T cell pool are responsible for the plasticity of the neonate immune responses [83]. Still knowledge gaps on the cellular pathways and communication remain to be filled. This may introduce other groups of immune cells with a prominent role in tolerance establishment and infection combat of early life immunity.

Taken together, immune maturation of the neonate is highly influenced by the introduction of the neonate into an antigenic world. This demands a fast establishment of a tight epithelial barrier and an immune system able to combat pathogens and still remain tolerant towards the steadily increasing numbers of commensals in the human infant gut. The IEC monolayer guards the commensals of the gut and communicates with immune cells in the gut associated lymphoid tissue through PRRs and conserved signaling pathways as the TLR signaling pathway. In response to microbial stimulation specialized IEC secrete chemokines and cytokines. Additionally the intestinal epithelium barrier integrity is strengthened by secreted mucus and AMP from the IEC to the epithelial surface and by TJ complex formation. Innate and Adaptive immunity of the neonate are characterized as impaired or delayed compared to adult responses, but can mount strong proinflammatory responses under specific antigenic influence.

THE PERINATAL HEMATOPOIESIS

Hematopoiesis in the fetus and neonate differ from that in the adult. The main function of adult hematopoiesis is the production of new cells to balance the constant loss of hemic cells, while in fetal and neonatal state the extreme conditions of growth and fast physiologic changes demand an extraordinary supply of multipotential hematopoietic stem cells (HSC). The transition from a presumably sterile fetal life in the uterus to a life in the non-sterile environment further demands a critical need for antimicrobial action by immune cells, which is mirrored in the mobilization of cells and hematopoiesis in perinatal life [88].

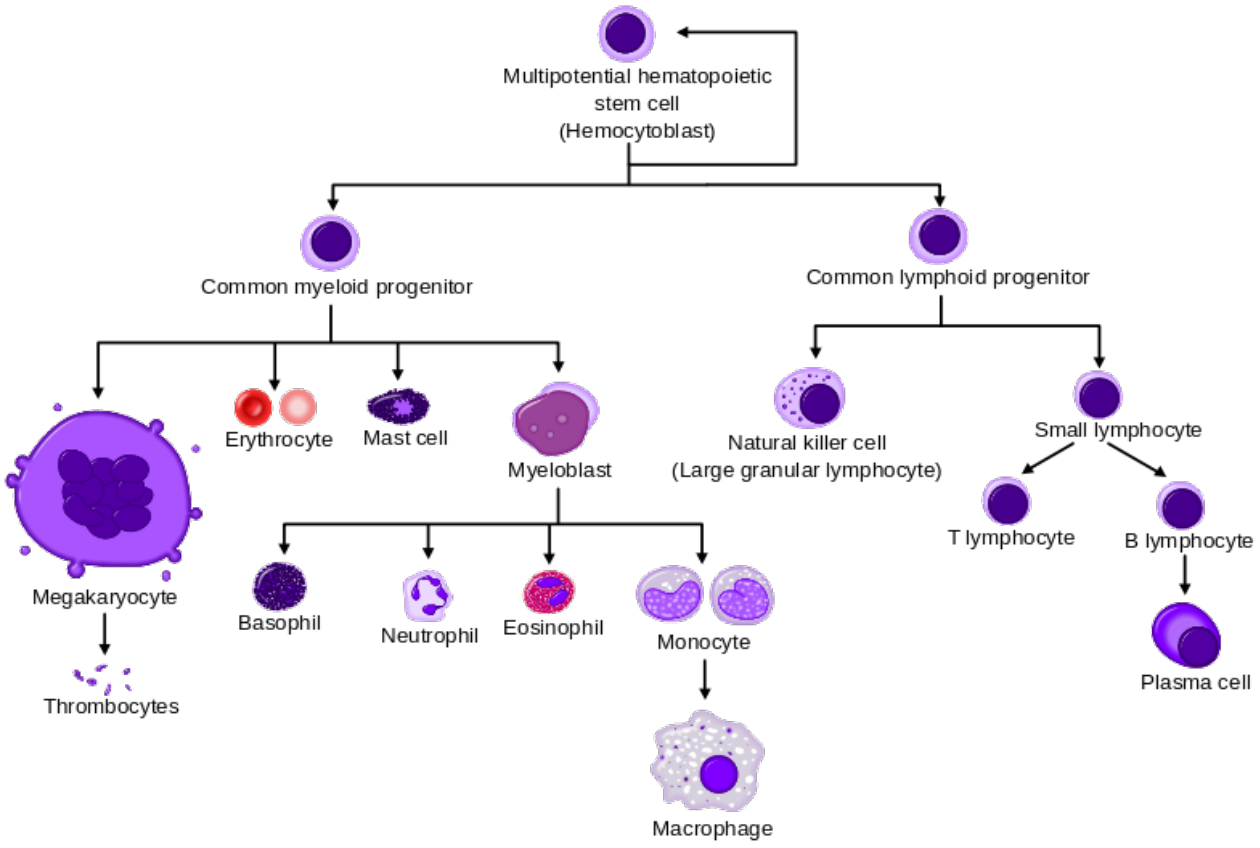

Figure 3. Hematopoietic stem cells give rise to all mature blood cells and tissues of the body. Erythrocytes, granulocytes and monocytes share the same myeloid progenitor [89].

HEMATOPOIETIC SITES AND MOBILIZATION

The bone marrow (BM) constitutes the primary site of hematopoiesis in adults, containing the majority of the developing HSC and hematopoietic progenitor cells (HPC), while liver and spleen are considered to be secondary hematopoietic organs in adults. These organs do however contribute significantly to blood cell development throughout life [90]. The proliferating HSC in adults are found primarily in the BM, while maturing cells of the myeloid lineages exit BM and enter the blood stream. Under steady-state conditions, only very low levels of HSC and HPC traffic in the

peripheral circulation, however, elevated levels of HSC and HPC can be mobilized to enter the blood stream upon influence of cytokines and other agents. Chemotherapy-induced mobilization of HSC and HPC to the blood stream is well described in cancer patients and is often enhanced by use of repeated stimulation with cytokines such as granulocyte colony-stimulating-factor (G-CSF), which in turn promotes release of membrane-bound stem cell factor (SCF) and proliferation of progenitor cells [91, 92].

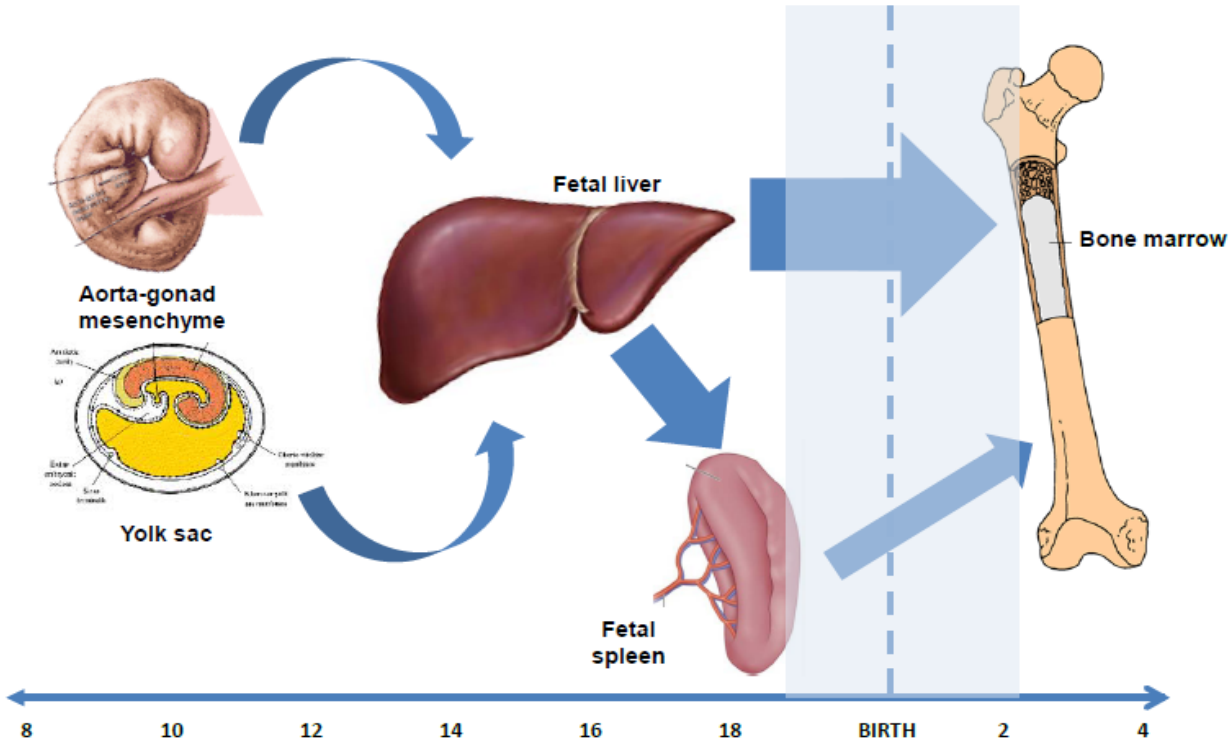

Figure 4: Reservoirs and migration of HSC during gestation and early postnatal life in mice. Blue arrows represent the blood borne migration of HSC. The size of the blue arrows correlates to the approximate numbers of HSC travelling from one organ to another. During gestation the HSC arise in the yolk sac and in the aorta-gonad mesenchyme, these organs seed the fetal liver with the HSC. At the time around birth (light blue area on figure) the HSC migrate to the BM. The fetal spleen is most likely continuously seeded with HSC during late gestation, and therefore makes up a prominent reservoir of HSC at the time of birth (Figure based on figure and text in [93]).

In mice HSC arise in the yolk sac and the aorta-gonad mesenchyme during fetal development and a blood borne distribution of HSC and HPC is responsible for the establishment of these cells in subsequent hematopoietic organs [94]. In late gestational age the primary concentration of HSC has changed to the liver, and at or near birth the cells migrate to the BM, where they reside throughout life. However, in the perinatal mouse the spleen constitutes another important hematopoietic organ

(see Figure 4) and even holds higher numbers of HSC in late gestational age and first days of life, than the developing BM [93, 95].

Circulating HSC from the fetal liver have been described as being constitutively present in the bloodstream and the spleen is generally believed to be seeded by these fetal liver-derived cells during late gestation and very early life [93], as illustrated in Figure 3. Discrepancy however characterizes the discussion of the migrating HSC to and from the spleen, as the spleen is described to be either gradually seeded by circulating HSC through entire perinatal phase or as being just a transient intermediate station for a wave of HSC passing through the spleen on their way from fetal liver to BM [90, 93, 95, 96]. Several studies describe the spleen as being a lifelong reservoir of low numbers of HSC and HPC with the capability to differentiate into myeloid and lymphoid lineage cells [95, 97, 98].

MYELOPEIOSIS AND GRANULOCYTOSIS IN THE NEONATE

HSC and HPC in the hematopoietic organs give rise to the common myeloid progenitor (Figure 4), which differentiate into immature myeloid cells (IMC) during myelopoiesis. The first steps of expansion and maturation of these myeloid progenitors take place in the hematopoietic organs and are controlled and induced by a range of soluble factors including cytokines as the granulocyte/macrophage colony-stimulating factor (GM-CSF) [99], G-CSF [100], IL-6[101] and SCF [102-104].

During steady state conditions IMC constitutively migrate into other peripheral tissues, where they differentiate into mature macrophages, dendritic cells (DC) and various granulocytes. Pathologic conditions, however, change the fate of some of the IMC, which expand and differentiate into myeloid derived suppressor cells (MDSC). MDSC of granulocytic and monocytic morphology have been shown to accumulate during a diverse range of pathologic conditions and to control or even suppress immune responses under these circumstances [103, 105]. Granulocytic MDSC are found to accumulate in large numbers in the tumor environment and in lymphoid tissues during cancer. Morphologically these cells resemble mature neutrophils and share several neutrophil characteristics. Although few characteristic have been described to differ between then, of these the ability of granulocytic MDSC to suppress T cell responses is the most important, however, controversy remains on the nature of the granulocytic MDSC and their relatedness to neutrophils [106, 107].

Neonate Granulocytosis

Neutrophils

Neutrophils, or polymorphonuclear leukocytes, are the most abundant leukocytes circulating the blood and make up a critical innate first line of defense against bacterial pathogens. Neutrophils are

continuously generated from activated and proliferating hematopoietic stem cells in the BM, and up to $2x10^{11}$ cells are generated per day in a normal adult human [108]. During steady state conditions only a small fraction of total neutrophils circulates the blood, whereas the majority is stored in the BM. Upon activation by infection the BM neutrophils are released to control the invading microbes in the periphery by phagocytosis, oxidative agents and formation of extracellular traps [108, 109]. The cytokine receptor CXCR2 is expressed on myeloid cells, and its ligands CXCL1 (KC) and CXCL2 (MIP-2) are expressed by endothelial cells of BM, monocytes and macrophages. The production of KC and MIP-2 by these cells can be readily upregulated when stimulated by G-CSF [110], which, thus, is a highly important regulator for tuning the production and mobilization of neutrophils to meet the increased need for these cells during infection [108, 109].

Murine and human neonates are characterized by a transient but pronounced increase in circulating granulocytes and granulocytic progenitor cells that accumulate in blood and reach their highest levels a few hours after birth. In the human and the murine neonate, the high neutrophil counts decreases and reaches the level of adults during the first week of life [77, 79]. The rise in granulocytosis correlates with a corresponding destruction of hematopoietic tissue in the perinatal liver. It has been suggested that the increasing neutrophil counts are induced by stress during labour, as the accumulation of mature and immature granulocytic cells in blood is paralleled by a sharp elevation of measured adrenaline, noradrenaline and dopamine in serum just after birth [111]. The accumulation of granulocytes in neonates has been linked to improved resistance toward infections, as impairments in the myeloid cell differentiation and neutrophil function at this time correlate with increased risk of infections. This is more pronounced in premature than in term infants, and the level of circulating neutrophils is characterized as notably lower in premature infants. The pronounced early life increase in neutrophilic count, described in term born infants, is absent in premature infants [79, 88, 111, 112].

Recent evidence has extended the functions of neutrophils and these cells are now recognized to produce a broad range of cytokines, extracellular traps (NETS) and effector molecules, which influences various immune responses in inflammation, infection and cancer [113]. Neutrophils are described to accumulate in the marginal zone of the human neonate spleen in the first days after birth. The marginal zone of the spleen surrounds the splenic white pulp and is characterized by the presence of B cells, which are specialized in T cell-independent immunoglobulin responses to circulating antigens. The colonizing neutrophils mobilize to the splenic marginal zone upon microbial colonization of the neonate gut and obtain a B cell-helper profile, which enhances the antimicrobial immunoglobulin defense of the B cells locally in the spleen marginal zone [114].

Myeloid Derived Suppressor Cells

MDSC constitute a heterogeneous group of innate myeloid cells characterized by their capacity to suppress T cell immunity. In mice they are generally characterized as $CD11b^{+}Gr\text{-}1^{+}$ cells and are found to accumulate in large numbers in lymphoid tissues during tumor-development and other pathological conditions [103, 106]. The MDSC consist of immature myeloid cells and precursors of macrophages, granulocytes and DCs, that have been prevented to differentiate into mature cells [103, 105].

MDSC have attracted much attention during the past decade, due to their immune suppressive potential of various T cell functions, and have primarily been described for their critical role in tumor immune escape mechanisms in tumor-bearing animals and cancer patients [115, 116]. A range of pathologic conditions like cancer, inflammation, sepsis and various autoimmune diseases are reported to inhibit the differentiation of IMC into mature myeloid cells [117-119]. This partial block in differentiation of the myeloid progenitor cells promotes an accumulation of the immature myeloid derived cells, which are in addition activated and promoted to proliferate in response to the tumor-environment, presence of pathogens or host-derived cytokines [105].

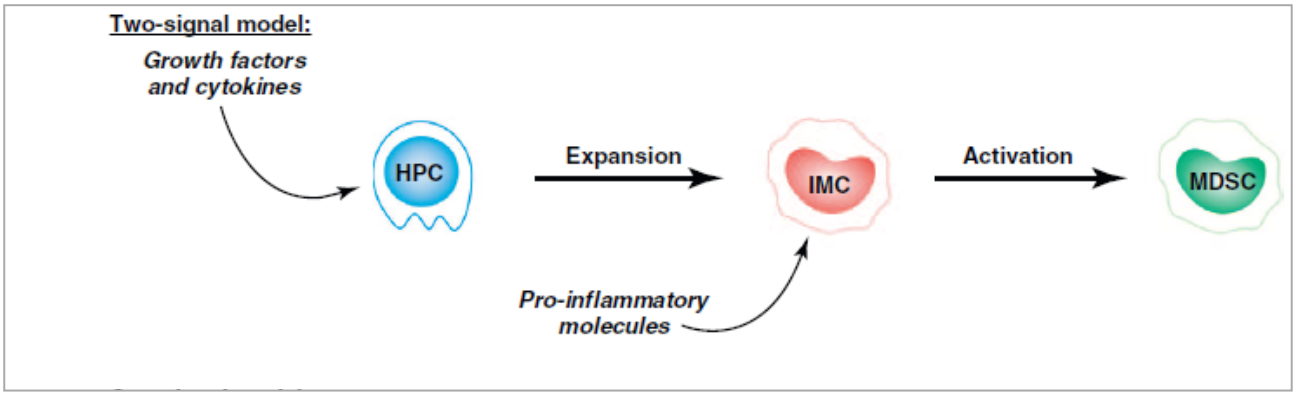

Figure 5: A proposed model for the expansion and activation of MDSC. Separate processes drive each step. The expansion step is driven by growth factors and cytokines and induces an expansion of the immature myeloid cells. An induction of the suppressive capacity of the myeloid cells however requires further stimulation of a strong pro-inflammatory signal from cytokines and TLR ligands (Modified figure from Condamine and Gabrilovich [105]).

A two-signal model is proposed for the accumulation and activation of MDSC, wherein the expansion of the MDSC group is separated in two processes driven by different signaling pathways. A schematized model of the IMC accumulation and differentiation into MDSC is shown in Figure 5 [105]. The expansion is thought to be induced by cytokines and growth factors, produced for example by tumors or inflamed tissues and will involve factors as GM-CSF, G-CSF and IL-6, which have been shown to play a role in accumulation of IMC and MDSC. The activation of the

accumulated cells requires a strong signal from pro-inflammatory cytokines like TNF [120], IFNγ, IL-6 and TLR ligands [117].

Historically MDSC have only been described with much attention during the past decade and were in the context of cancer characterized for the first time in the 1980's, as a group of cells, identified in the BM and spleen, contributing substantially to attenuation of anti-tumor defense in cancer patients [104]. A formerly unknown cell population with suppressive features was however described already in the late 1970's. These cells were named 'natural suppressor cells' and were found to be naturally occurring during the early maturation of lymphoid tissues in the neonate spleen. The early life splenocytes showed a remarkable capability to suppress *in vitro* immune responses in mixed leukocyte reactions and this observation was supported by an ability to induce tolerance towards different alloantigens introduced in the animal a few days after birth. The ease of tolerance induction and suppressive effect abruptly declined hereafter, suggesting that this condition was a 'short window of tolerance' based on a suppressive capability of myeloid cells in the lymphoid tissues of neonate mice [121].

MDSC subsets

Phenotypic, morphological and functional heterogeneity is the most common trait of the MDSC. The heterogeneity is believed to mirror a remarkable plasticity of this group of innate immune suppressive cells, which readily adapt to a diverse range of pathologic conditions. The heterogeneity of the MDSC, however, complicates the definition of how these cells influence immune regulation in disease and in the healthy organism [105].

In mice the co-expression of the markers CD11b and Gr-1, typically analyzed by use of flow cytometry, and the lack of markers typical for mature macrophages and DCs are the common characteristics of the MDSC [103]. The heterogeneous group of MDSC has a mixed morphology similar to granulocytes and monocytes, and recently several additional markers have been added to define the differing subsets of MDSC. The most prominent path of subset definition is based on the use of markers Ly6G and Ly6C, which are molecules preferentially found on the surface of granulocytes and monocytes, respectively. They belong to the Ly-6 family, which is a group of cell surface glycoproteins expressed on cells during early myeloid development from hematopoietic stem cells to lineage committed precursor cells [122]. The use of Ly6G and LY6C currently allows for a differentiation of MDSC into two dominating groups; a group of $CD11b^{+}Ly6G^{-}Ly6C^{high}$ cells with a monocytic morphology, and a group of $CD11b^{+}Ly6G^{+}Ly6C^{low}$ cells with a granulocytic morphology, which are commonly named monocytic MDSC (MO-MDSC) and granulocytic MDSC (G-MDSC), respectively [103, 123, 124].

In mice the heterogeneous MDSC were initially extensively studied by staining with the anti-Gr-1 monoclonal antibody (Ab), clone RB6-8C5, separating the $CD11b^{+}Gr1^{+}$ cells into two or three

cellular fractions based on the intensity of the Gr-1 mAb. A Gr-1^{high}, Gr-$1^{Intermediate}$ and Gr^{low} fraction of the cells have been recognized in several studies of murine tumor models. The identified subsets of high or low Gr-1 expression differ in their ability to suppress T-cell responses and can further be differentiated upon their morphologic differences. The Gr-1^{high} fraction mainly comprises immature and mature granulocytes and the Gr-1^{low} cellular subset comprises monocytes and immature myeloid cells [123, 125]. However, the use of the anti-Gr-1 mAb has been criticized for binding, though with different specificity, to both the Ly6G and Ly6C epitope, hereby complicating the definition of MDSC subsets [104, 124].

In humans phenotyping of MDSC has been less clearly defined. The MDSC have been described as cells expressing the myeloid surface markers CD33 and CD11b, and in some studies further shown to express typical granulocytic markers CD15 and CD66, and monocytic marker CD14 [123, 126].

Functionally distinct subsets of MDSC have been identified within the $CD11^{+}Gr\text{-}1^{+}$ cell group in mice, based on the immune suppressive mechanisms on various T cells functions. Interestingly the functional difference can be correlated to the described MDSC phenotypes of G-MDSC and MO-MDSC [105]. The MO-MDSC subset suppresses T cells via L-Arginine metabolism induced by upregulation of Arginase 1 (Arg1) and iNOS. Arg1 and iNOS use arginine in production of urea and nitric oxide (NO), respectively, and their upregulation depletes arginine from the microenvironment. This in turn induces loss of the CD3 chain in T cells and leads to inhibition of T cell proliferation [127]. An increase in iNOS production further leads to higher induction of NO, which are known to induce T cell apoptosis and to block T cell signaling pathways [105, 128]. The G-MDSC subset has been determined as the main MDSC subsets in the various tumor environments, and its suppressive activity is primarily conducted through production of high amounts of reactive oxygen species (ROS) produced by the NADPH oxidase complex. The produced nitric oxide (NO) reacts with the superoxide anion and produces peroxynitrite, which has been shown to inhibit CD8 T cells through a nitration of their T cell receptors [129]. This reaction results in altered recognition between the T cell receptor and the major-histo-compatibility (MHC) bound peptide. ROS are further known to promote programmed cell death and apoptosis [103, 105].

IMMUNOSUPPRESSIVE CELLS IN THE NEONATE

High levels of neutrophil-like MDSC have very recently been described in human neonate cord blood. The granular MDSC inhibit T cell responses and proliferation, and were further shown to control natural killer (NK) cells cytotoxicity, thus, suppressing innate and adaptive immune responses early in life. The neutrophil-like MDSC of this study were found only to be elevated in cord blood, when compared to MDSC in peripheral blood of newborns, children and adults [126]. Another study reveals accumulation of granulocytic MDSC in a murine tumor-model during gestation. They propose herein a shared mechanism between the feto-meaternal immune

suppression naturally occurring during gestation and the immune suppression introduced by MDSC in the tumor environment [130].

A study by Elahi and colleagues [131] showed a distinct immunosuppressive potential of $CD71^+$ erythroid cells in neonate mice and in human cord blood. These erythrocytes were able to diminish production of TNF and IL-6 from adult splenocytes after co-culture with the neonate cells. The suppression was mediated by the arginine metabolism and was shown to be closely linked to the presence of commesal microbiota. This study, thus, reveals distinct immunosuppression by a non-immune cellular subset in the lymphoid organs of neonate mice and human, and suggests that this mirrors a natural need for tolerance establishment in the neonate during the abrupt colonization with commensal microorganisms after birth.

Puga and colleagues in 2012 [114] showed that neutrophil-like cells colonize the marginal zone in the murine and human spleen concomitantly to postnatal microbial colonization and obtain a B cell-helper function. The neutrophil-B cell interaction in the splenic marginal zone enhances an innate T cell-independent antimicrobial Ig defense.

These recent studies have revealed new knowledge on the immune suppressive abilities of various cell types in the neonate. From these new findings a picture emerges of a complex regulation of immunity and tolerance in the newborn, which seems to involve new roles for granulocytic neutrophil-like cells and may also introduce non-immune cells with immunosuppressive features in the very early phase of life.

SCIENTIFIC MANUSCRIPTS

PAPER 1

"Neonatal Microbial Colonization in Mice Promotes Prolonged Dominance of $CD11b^+Gr\text{-}1^+$ Cells and Establishment of the $CD4^+$ T Cell Population in the spleen"

Matilde B. Kristensen, Stine B. Metzdorff, Anders Bergström, Dina S. M. Damlund, Lisbeth N. Fink, Tine R. Licht and Hanne Frøkiær

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NEONATAL MICROBIAL COLONIZATION IN MICE PROMOTES PROLONGED DOMINANCE OF CD11B$^+$GR-1$^+$ CELLS AND ESTABLISHMENT OF THE CD4$^+$ T CELL POPULATION IN THE SPLEEN

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Abbreviations: CONV; conventionally colonized · GF; germfree · MC; mono-colonized · GI; gastrointestinal · PND; post-natal-day · HSC; hematopoietic stem cells · FSC; forward scatter · SSC; side scatter

ABSTRACT

To assess the microbial influence on postnatal hematopoiesis, we examined the role of early life microbial colonization on the composition of leukocyte subsets in the neonatal spleen. A high number of $CD11b^{+}Gr\text{-}1^{+}$ splenocytes present perinatally was sustained for a longer period in conventionally colonized (CONV) mice than in mono-colonized (MC) and germfree (GF) mice, and the $CD4^{+}$ T cell population established faster in CONV mice. At the day of birth, compared to GF mice, the expression of *Cxcl2* was up-regulated and *Arg1* down-regulated in livers of CONV mice. This coincided with lower abundance of polylobed cells and hematopoietic tissue in the livers of neonate CONV mice compared to GF neonate mice. An earlier peak in the expression of the genes *Tjp1, Cdh1* and *JamA* in intestinal epithelia of neonate CONV mice compared to GF neonate mice indicated that accelerated closure of the epithelial barrier was related to the microbial colonization. In conclusion, we have identified an important microbiota-dependent neonatal hematopoietic event, which we suggest impacts the subsequent development of the T cell population in the murine spleen.

INTRODUCTION

During the past decades the prevalence of allergies and autoimmune diseases has increased dramatically in the western world [7], and it is generally accepted that the microbial environment is a key player in the development of these disorders [50, 132]. It is, however, still not clear how and when the intestinal microbiota influences the development of an immune system that is more prone to developing immune-related diseases as opposed to development of a healthy immune system.

In 2008, a meta-study concluded that delivery by caesarian section significantly increases the risk of developing childhood-onset type 1 diabetes [25], and a large long-term birth-cohort study of early life intestinal microbiota associates reduced fecal microbial diversity in early life with increased risk of later development of allergic diseases [45]. Recently, also maternal antibiotics use during pregnancy has been linked to an increased risk of early childhood asthma [46]. Altogether, these studies indicate that the microbiota in early life influences disease development. This is supported by several studies describing mode of delivery and neonatal feeding as highly influential on gut microbiota composition in early life as well as on permanent changes of the microbial community in the gastrointestinal (GI) tract [22, 133]. Hence, events during perinatal life, affecting the first microbes that inhabit the epithelium at mucosal sites, may be determining for the ‘set-point’ of the immune system.

Several studies have suggested a higher intestinal permeability in neonates than in adults. Intestinal integrity establishes rapidly and concomitantly to introduction of oral feeding in early life but can be influenced by infant feeding (breastfeeding vs. formula) and time of birth (preterm vs. term) [53, 134]. Recently, microbial products were shown to translocate from mucosal surfaces to central

lymphoid organs and specifically enhance systemic innate immune responses in mice [114, 135]. Though it remains speculative, the neonate intestine, due to its lowered integrity, could be particularly susceptible to translocation of commensal bacteria or microbial products across the intestinal barrier, which successively may influence the induction of mucosal and systemic tolerance in neonates.

We have recently reported that colonization with a complex microbiota, but not with a single bacterial strain, affected the adaptive immune development in neonatal mice, and that this response was accompanied by reduced proinflammatory cytokine gene expression in the intestine of conventionally colonized (CONV) animals [63]. A diverse microbiota has been shown to be indispensable for the adaptive immune system, as colonization with a conventional microbiota but not mono-colonization with *Escherichia coli* or lactobacilli supports development of oral tolerance in mice [136]. Additionally, germfree (GF) mice have been shown to have fewer regulatory T cells (Tregs) in lymphoid organs than CONV mice, and the regulating effect of the GF-derived Tregs was furthermore shown *in vitro* to be impaired compared to CONV Tregs [137]. Recently it was shown that postnatal microbial colonization plays a prominent role in the induction and establishment of a group of neutrophil-like cells with B cell-helper function in the marginal zone of the neonatal spleen, hereby promoting an antimicrobial immunoglobulin defense by interacting with B cells [114]. Thus, by influencing Tregs in lymphoid organs and production of immunoglobulins in spleens of newborns, it seems that the commensal microbiota holds a key role in priming and shaping of the early adaptive immune system. The mechanism behind this influence of postnatal colonization on the development of systemic immunity is however largely unknown.

The present study is based on the hypothesis that early microbial colonization of the GI tract leads to influx of microorganisms from the gut into circulation. We hypothesized that this affects the subsequent composition of spleen cells, and that this is of importance for establishing a well-balanced immune system. We examined the development of cell subsets in the spleens of pups born by GF dams, *Lactobacillus acidophilus* NCFM mono-colonized (MC) dams and CONV dams, and found that the establishment of the $CD4^+$ T cell pool in the spleen was accelerated by conventional microbiota. The largest difference between CONV and GF mice was seen in the very first days after birth, where the proportion of neutrophil-like cells positive for the markers $CD11b^+$ and $Gr\text{-}1^+$, naturally present as part of the perinatal pool of myeloid progenitor cells in spleen and liver, remained high during the first week in spleens of mice born by CONV dams while dropping rapidly after birth in spleens from GF mice. We suggest that the longer-lasting high number of $CD11b^+Gr\text{-}1^+$ cells is an important microbiota-dependent postnatal hematopoietic event that influences the subsequent development of adaptive immunity.

MATERIALS AND METHODS

PREPARATION OF BACTERIAL INOCULUM

L. acidophilus NCFM (Danisco, Copenhagen, Denmark) aliquots were prepared by inoculation in de Man, Rogosa and Sharpe broth (MRS) (Merck, Darmstadt, Germany) for anaerobic growth overnight at 37°C. The culture was harvested by centrifugation, washed in sterile PBS (Lonza, Basel, Schwitzerland) and diluted in PBS to $5x10^8$ CFU/ml. Plate counts were performed on MRS agar. The culture was frozen at -80°C until use as inoculums in colonization of adult mice (described below).

ANIMALS AND TISSUES

GF and CONV Swiss Webster mice, originally purchased from Taconic (Lille Skensved, Denmark), were bred and housed in sterile isolators or under specific pathogen-free conditions, respectively. GF mice were treated as previously described [63]. Eight female and 2 male GF mice were mono-colonized with *L. acidophilus* NCFM by applying $5x10^8$ CFU/ml in 0.5 ml PBS suspension orally and 0.5 ml to the abdominal skin. To confirm sterility and mono-colonization, respectively, fecal samples from GF and NCFM mice were cultured weekly on non-selective Luria-Bertani medium, under aerobic and anaerobic conditions. The day of birth was identified as Post-Natal-Day 1 (PND1), and four pups of each group were euthanized at PND 1, 2, 4, 7, 14 and 35 and spleens dissected. Furthermore, spleens from two GF and two CONV litters, taken by caesarian section at gestational day 19 (identified as PND-1) were dissected. Single cell suspensions were prepared of all spleens. Additionally, at PND2, 4 and 7, 2-3 spleens and livers from GF and CONV pups were formalin-fixed for preparation of tissue sections.

For gene expression analysis, liver and distal ileum from 8 CONV, 8 MC and 8 GF pups, delivered by 4 different dams of each group, were dissected and frozen in RNAlater (Qiagen, Hilden, Germany). All animal experiments were approved by the Danish Council for Animal Experimentation.

PREPARATION OF SPLENIC SINGLE CELL SUSPENSIONS

Spleen cells were washed and centrifuged in cooled RPMI 1640 with penicillin and streptomycin (Lonza). 5 minutes lysing of erythrocytes by suspension in 0.83% ice-cold ammonium-chloride preceded a final washing step and resuspension in RPMI. The number of viable cells was measured by Nucleocounter® NC-100™ (Chemometec, Allerød, Denmark).

IMMUNOSTAINING AND FLOW CYTOMETRY

Spleen cell surface antigens were analyzed by use of antibodies: Anti-CD3-PE clone 145-2C11, anti-CD4-APC clone RM 4-5, anti-CD8-APC clone 53-6.7, anti-CD11b-PECy7 clone M1/70, anti-Ly6G(Gr-1)-APC clone RB6-8C5 and anti-F4/80-FITC clone BM8 (all eBioscience, San Diego,

CA, USA) after blocking of FC antibody-binding by anti-CD16/CD32 (BD Biosciences, Franklin Lakes, NJ, USA) for 10 min. Cells were fixed in PBS with 2% methanol-free formaldehyde and analyzed within 1-3 days on a FACScanto flow cytometer (BD Biosciences). Data analyses and layouts were performed using FlowJo V10 (Tree Star, Ashland, OR, USA).

RNA-ISOLATION AND REAL-TIME QUANTITATIVE PCR (QPCR)

Ilea and livers kept in RNAlater were removed from storing solution and homogenized in RLT buffer from Qiagen (Hilden, Germany). RNA extraction, quantification, quality evaluation, reverse transcription, primer testing and validation and data analysis were carried out as previously described [62]. *Cxcl1*, *Cxcl2* and *Arg1* were analyzed in neonate GF and CONV livers with TaqMan® gene expression assays; Mn00433859_m1 (*Cxcl1)*, Mn00436450_m1 (*Cxcl2)*, Mn00475988_m1 (*Arg1)* and Mn006007939_s1 (*Actb)* (Life Technologies, Carlsbad, CA, USA). Tight junction genes *Cdh1*, *JamA* and *Tjp1* were analyzed in distal ileal tissues. The following primers were used; *Cdh1*-forward GTATCGGATTTGGAGGGACA, *Cdh1*-reverse CAGGACCAGGAGAAGAGTGC, *JamA*-forward GTTCCCATTGGAGTTGCTGT, *JamA*-reverse GGGAGAGGAGAAGCCAGAGT, *Tjp1*-forward GGTGACATTCAAGAAGGGGA (designed with NCBI PrimerBlast), *Tjp1*-reverse TCTCTTTCCGAGGCATTAGCA [138], *Actb*-forward GTCCACCTTCCAGCAGATGT and *Actb*-reverse GAAAGGGTGTAAAACGCAGC [62]. Fold-changes in gene expression were calculated as previously described [139].

EX VIVO STIMULATION OF SPLEEN CELLS

Production of MIP-2 was measured upon addition of *E. coli* Nissle 1917 (Multiplicity of infection (MOI) 4), *L. acidophilus* NCFM (MOI 1.5) or half the amount of each bacteria in a mixture to $5x10^5$ spleen cells in 0.1 ml RPMI. Supernatants were harvested after 20 hours (37 °C) and concentrations estimated using a commercial MIP-2 ELISA kit (R&D systems, Minneapolis, MN, USA).

H&E STAINING

Spleens and livers were fixed in 4% buffered formalin, embedded in paraffin and sliced into 5 µm sections. The tissues were mounted on SuperFrost Plus slides (Menzel-glaser, Braunschweig, Germany) and stained by H&E for evaluation of cellular composition.

STATISTICS

Statistics were performed with GraphPad Prism™V5.03 (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

THE MICROBIOTA AFFECTS THE DEVELOPMENTAL RATE OF $CD4^+$ T CELLS IN THE SPLEEN

The cellular composition of the neonatal spleen is vastly different from that of the adult spleen, as it contains only very few lymphocytes but a large proportion of fetal hematopoietic stem cells (HSC) [95]. The composition changes along with the maturation of the immune system [83]. To assess the influence of the microbiota on the cellular composition in the developing spleen, we measured the proportion of various leukocyte subsets in spleens of mice aged 4, 7 and 35 days born by CONV dams, GF dams and MC dams by use of flow cytometry (Figure 1A). In the period 7-35 days after birth the proportion of $CD3^+CD4^+$ T cells in spleens of all colonization groups increased from approximately 2% at PND7 to 16-25% of the total splenocytes on PND35. While no differences were seen at PND35 for the number of $CD3^+CD8^+$ cells in the three groups, the number of $CD3^+CD4^+$ cells was significantly higher in the CONV group (25.7%) as compared to the MC group (16.3%) and the GF group (19.0%). At later time points, this difference diminished, and no differences were seen between the CONV and GF T cell levels in adult mice (results not shown). There were no significant differences at any timepoint between the three colonization groups for $CD19^+$, $CD49b^+$ or $CD11c^+$ spleen cells (results not shown).

Remarkably, a large proportion of $CD11b^+$ cells (25.4% of all viable splenocytes) characterized spleens from PND4 CONV mice, while the $CD11b^+$ cells only constituted mean levels of 3.1 and 4.6 % in GF and MC PND4 mice, respectively (Figure 1A, right). The high proportion of $CD11b^+$ cells at PND4 diminished to 10.3% of total spleen cells at PND7 in CONV pups, but CONV spleens still held significantly higher levels of $CD11b^+$ cells than attained in MC pups.

The striking lack of effect of mono-colonization on the cellular development of the neonatal spleen prompted us to investigate, whether there is a need for polymicrobial stimulation to regulate the expression of chemokines involved in recruitment of $CD11b^+$ cells to the perinatal spleen. Consequently we isolated spleen cells from CONV PND4 mice and cultured with *L. acidophilus*, *E. coli* or a mixture containing half the concentration of each bacterium, to identify their ability to produce MIP-2 upon this stimulation. The splenocytes released markedly more MIP-2, when stimulated with *E. coli* and *L. acidophilus* in combination compared to stimulation with either of the bacteria in a monoculture (Figure 1B), supporting a requirement for polymicrobial stimulation for recruitment of $CD11b^+$ cells to the spleen.

Thus, the microbiota influenced the composition of the spleen both in the very early postnatal period, prolonging the presence of a large group of $CD11b^+$ cells and significantly increasing the levels of $CD4^+$ T cells during the later postnatal development of the CONV spleen. The nature and complexity of the microbiota furthermore strongly influenced the ability of spleen cells to produce MIP-2 important for recruitment and differentiation of $CD11b^+$ cells.

THE HIGH PROPORTION OF CD11B$^+$GR-1$^+$ CELLS IN THE NEONATAL MURINE SPLEEN IS MAINTAINED BY THE PRESENCE OF MICROBIOTA

Integrin CD11b is expressed on several leukocytes, including mature and differentiating monocytes and granulocytes. The CD11b$^+$ cells observed in neonatal mice therefore expectedly constitute a heterogeneous group of cells. CD11b is involved in inhibition of Toll-like receptor signaling [140] and subsets of neutrophils, characterized by high expression of CD11b and Gr-1, have been shown to hold regulatory properties and be involved in down-modulation of T cell responses [103, 114]. Thus, these cells might constitute a key regulator in establishment of the adaptive immune system. Accordingly, we stained splenocytes from newborn mice with anti-CD11b and anti-Gr-1. F4/80 was included as macrophage marker. The CD11b$^+$Gr-1$^+$ group represented by far the majority of the CD11b$^+$ cells described in fetal and neonate spleens (Figure 2A) and was negative for the macrophage marker F4/80 throughout the experiment. The CD11b$^+$Gr-1$^+$ cells comprised as much as 46-47% of total viable spleen cells in both groups at the day of birth (PND1) (Figure 2B) and were present in CONV as well as in GF spleens at Day 19 of gestation (PND-1) in comparable amounts (Figure 2C). As expected from the previous result for marker CD11b (Figure 1A), the presence of microbial colonization strongly influenced the ability to maintain the large group of CD11b$^+$Gr-1$^+$ cells in the neonatal spleen after the day of birth. As early as PND2 the level of CD11b$^+$Gr-1$^+$ cells in GF mice decreased to 15% while the level in the CONV spleens only decreased to 34% on PND2. This difference between the groups was maintained in 4 days old mice, as 24.5% of the cells of CONV spleens were CD11b$^+$Gr-1$^+$ cells compared to 3.1% of the cells in GF spleens (Figure 2A, B). Confirming the previous result for marker CD11b (Figure 1A), no difference was observed between the two groups on PND7. Importantly, we did not at any time point observe differences in the total number of cells in the spleens, which increased from below 10^6 cells at PND2 to around 10^7 at PND21 in both GF and colonized pups (Figure 2D).

Of note, splenocytes of neonate CONV and GF animals comprised at least three distinctly labeled subpopulations with regard to forward and side scatter (FSC and SSC) and surface markers CD11b and Gr-1, as shown in Figure 3A for CONV PND-1 spleen cells. At this early time point, the Gr-1^{High} and CD11b^{High} cells (P1) could be differentiated into two distinct groups based on size (FSC distribution); a FSC^{High} group and a FSC$^{Low\text{-}Int}$ group (Figure 3A, upper right panel). A group of cells described by Gr-1^{Int} and CD11b^{Int} (P2) had a SSC^{High}FSC^{Low} scatter profile (Figure 3A, lower right panel).

In GF spleens, a rapid shift from a FSC^{High}SSC^{High} group, present on PND-1, to a FSC^{Low}SSC^{High} group establishing on PND1, was seen (Figure 3B). In contrast, the FSC^{High}SSC^{High} and FSC^{Low}SSC^{High} group were both present at PND-1 in the CONV animals suggesting an influence of microbial colonization on immune development already during gestation. The two groups were well established in CONV spleens on PND7, while both groups were diminished to very low numbers at

PND4-7 in GF spleens (data not shown), this corresponded to the higher level of $CD11b^{+}Gr\text{-}1^{+}$ cells observed in spleens of CONV animals at this time (Figure 3B).

H&E staining of splenic tissues from PND2, 4 and 7 CONV mice (Figure 4) confirmed the presence of a large amount of cells with polylobed or ringed nuclei in CONV mice on Day 2 and 4 after birth; a morphology similar to mature and maturing neutrophils [104, 118]. Over the course of the first 7 days after birth, a distinct decrease in the level of cells with polylobed and ringed nuclei was seen in the spleen tissues.

Taken together, the $CD11b^{+}$ cells exhibited a phenotype of granulocytes, either conventional neutrophils or immature granulocytes, and constituted a significantly higher proportion of the spleen of CONV neonatal 2-4 days old mice compared to older mice and to GF mice of the same age.

EXPRESSION OF CXCL2 IS UP-REGULATED IN THE LIVER OF COLONIZED NEONATAL MICE

The bone marrow, spleen and liver cooperatively contribute to the hematopoietic homeostasis in the neonatal period. Perinatally, HSC present in the liver migrate to the bone marrow where they remain throughout life but a proportion of the hematopoietic liver cells migrate to the spleen or, upon circulation, homes to the liver [95, 141]. LPS stimulation of splenocytes results in a fast and transient up-regulation of *Cxcl1* (KC) and *Cxcl2* (MIP-2) expression [142] and importantly, a transient induction of MIP-2 *in vivo* leads to not only fast recruitment of polymorphonuclear leukocytes into the peripheral blood but also rapid activation of HSC and up-regulation of CD11b on these cells [91]. We therefore hypothesized that a difference in chemokine expression in the liver may reflect the systemic presence of microorganisms immediately after birth due to a permeable gut barrier that is, however, closed quickly supported by microbiota-assisted maturation of the gut epithelium [143]. To address this, we measured the expression of *Cxcl1* and *Cxcl2* in GF and CONV livers at PND1-2. As depicted in Figure 5, the expression of *Cxcl2* was significantly higher for pups of CONV dams than of GF dams on PND1. At PND2, no difference was seen. Expression of the *Cxcl1* in livers of GF and CONV neonate mice did not reveal any difference. GM-CSF and G-CSF likewise stimulate migration of neutrophils as well as the up-regulation of CD11b [91]. There was no measurable gene expression for these cytokines in the liver (data not shown).

H&E stains of neonatal CONV and GF liver tissues (Figure 6 A, B) revealed that livers of CONV PND2 contained ‘clusters’ of hematopoietic tissue at PND2, which decreased substantially over PND4 and 7 (Figure 6A, CONV2, 10x), and only very low numbers of granulocytes of mature or immature granulocytic morphology were present in CONV liver tissues at PND2, 4 and 7 (Figure 6A, 40x). A number of granulocytes with polylobed and ring-shaped nuclei tended to accumulate in vascular areas at PND7 in CONV liver tissues (Figure 6A, 40x, right). In the GF livers, the hematopoietic tissue was markedly more distinct and abundant on PND2 compared to livers of

CONV mice. Along with this, the number of granulocytes with polylobed and ring-shaped nuclei was at PND2 and PND4 substantially higher in the GF liver tissue compared to the CONV liver tissue of same age, and, most importantly, granulocytic cells were abundant around the portal veins in the GF livers on PND2 and PND4 (Figure 6B, 40x). At PND7, the granulocytic cells were found in substantially lower numbers in the tissues and lining the vessels of GF livers.

Cells of the $CD11b^{+}Gr\text{-}1^{+}$ phenotype as well as HSC are known to express arginase-1 (*Arg1*) [114, 144], and we consequently measured the expression of *Arg1* in livers of PND1 CONV and GF mice to support the observed differences in granulocyte and hematopoietic cellular content of the neonate livers. Interestingly, expression of *Arg1* was down-regulated in CONV mice as compared to GF mice (Figure 6C) indicative of a rapid drop in granulocytic cells and HSC in the CONV neonate liver on PND1.

In summary, a significantly higher transcriptional level of *Cxcl2* in PND1 livers of CONV compared to GF mice indicated systemic influence of microbiota on the mobilization and activation of polymorphonuclear leukocytes at the day of birth. Histology of CONV and GF livers revealed distinctly more hematopoietic tissue in GF livers than in CONV livers on PND2, concomitantly with an accumulation of cells with polylobed and ring-shaped nuclei in the tissue and surrounding the blood vessels of PND2 and PND4 GF liver tissues. Opposite this, very few cells with similar morphology were observed in CONV liver tissues at PND2 and PND4.

THE MICROBIOTA INFLUENCES THE REGULATION OF INTESTINAL TIGHT JUNCTION GENES IN THE NEONATAL MOUSE

To investigate the possible role of the microbiota on expression of genes involved in intestinal permeability we measured the expression of the tight junction genes; Tight junction protein 1 (*Tjp1 or Zo-1),* E-cadherin *(Cdh1)* and Junctional adhesion molecule A (*JamA)* in the distal ileal tissues of differently colonized mouse pups. The expression levels and the proteins encoded by these genes have previously been demonstrated to reflect the integrity of the intestinal barrier [55-57].

As shown for the expression of *Tjp1*, *JamA* and *Cdh1* (Figure 7); a higher relative expression of the genes in the ileum of CONV animals at PND1 as compared to the ileum of GF mice could be demonstrated. Moreover, the expression of all genes decreased from PND1 to PND6 in CONV intestinal tissue, while the expression increased from PND1 to 6 in the GF mice pups. In *L. acidophilus* MC ileal tissues all three genes were on PND1 measured to a transcriptional level in between the higher expression levels in CONV neonate intestine and lower levels of the GF intestinal tissue and did not change over time (data not shown).

Taken together, these data suggest a difference in the regulation and maturation between the colonized and non-colonized intestine, and hence that the postnatal microbiota influences the kinetics of the maturation of the intestinal barrier.

DISCUSSION

A key role of the microbiota for proper development of a well-balanced immune system is generally accepted, but an understanding of how this development is supported by the microbiota is lacking. Here, we focused on hematopoietic events in the perinatal period and showed that very early postnatal events in the hematopoietic system comprising the liver, spleen and bone marrow are greatly influenced by the presence of microorganisms, and that microbial colonization at birth accelerates the establishment of the $CD4^+$ T cell pool in the spleen during the first weeks of life in mice.

We demonstrated that the spleen of newborn mice, independently of the presence of microorganisms, contained high numbers of $CD11b^+Gr\text{-}1^+$ cells, which already at the day after birth (PND2), dropped dramatically. The $CD11b^+Gr\text{-}1^+$ cells decreased to the level of adult mice at PND4 in GF mice, while this number in pups of CONV mice decreased at a much slower rate and did not reach the adult level before 1-2 weeks of age. Whether the presence of these cells in the spleen is due to differentiation of HSC present in the spleen at birth or reflects an influx of cells from other organs cannot be finally concluded from the present study. However, a previous study of the perinatal hematopoiesis in mice concluded that a significant proportion of HSC and differentiating HSC from liver migrate to the spleen [95]. That the cells derive from the liver, rather than from proliferating HSC in the spleen, is supported by our microscopy data of the CONV spleen showing abundant neutrophil-like cells with a polylobed or ring-shaped nucleus but only sparse presence of hematopoietic tissue. Moreover, in the livers from GF and CONV mice the hematopoietic tissue was easily distinguished, but remarkably more abundant and distinct in the neonate GF livers. The neutrophil-like cells were frequently distributed in the tissue and in particular accumulated around the portal vein areas in the GF livers, while in livers from CONV mice the differentiating neutrophil-like cells were notably fewer around the portal veins and almost absent in the tissue. The absence of neutrophil-like cells may indicate a greater early life efflux of the immature neutrophil-like cells from the liver of newborn pups of CONV mice coinciding with an increased influx of these cells to the spleen at PND2-4. The significant reduction in the expression of *Arg1* in the liver of CONV mice at the day of birth further supports an efflux of neutrophil-like cells as the expression of *Arg1* has been ascribed as unique for differentiating, yet immature myeloid cells as well as neutrophils in early life [103, 114], and thus may reflect the abundance of neutrophil-like cells at this time point. Interestingly, in the spleen from fetuses taken from both CONV and GF mice 1 day prior to delivery, the proportions of $CD11b^+Gr\text{-}1^+$ cells were comparable and high (25-30%), confirming that during gestation this myeloid progenitor cell subset is abundant in the spleen [145].

Due to the rapid differentiation and migration of a vast number of hematopoietic cells early life represents an extremely complex and dynamic period, where many events take place concomitantly.

The diversity and dynamics of the $CD11b^{+}Gr\text{-}1^{+}$ cells present in the spleen during these days reflect this very well and suggest that both influx and differentiation, and perhaps also efflux of cells, take place in the spleen. The $CD11b^{+}Gr\text{-}1^{+}$ cells comprised at least three distinct subsets of cells that can be identified by their size and most likely represent different groups of differentiating cells of the myeloid lineage [146]. Especially around birth (PND-1 to PND1) we found dramatic changes. But while the cells of GF mice changed from FSC^{high} to FSC^{low} in this period, cells of CONV mice sustained both populations of $CD11b^{+}Gr\text{-}1^{+}$ splenocytes during the first days of life. Noteworthy, the three groups of $CD11^{+}Gr\text{-}1^{+}$ cells distinguished by size were already distinct in the fetal CONV spleen, suggesting an influence of the microbiota already during gestation. Regardless of this, the overall proportions of $CD11b^{+}Gr\text{-}1^{+}$ cells were similar and high (25-30%) in spleens from fetuses taken at the day before expected delivery (PND-1) from CONV and GF mice. This confirms other studies [145] showing that HSC establish in liver as well as the spleen during gestation. Importantly, this also indicates that an efflux of $CD11b^{+}Gr\text{-}1^{+}$ cells takes place in the liver at birth leading to a decrease in the $CD11b^{+}Gr\text{-}1^{+}$ population, which in the colonized mice is counteracted by an influx of similar cells into the spleen, most likely originating from the liver. *Cxcl2* (MIP-2) may hold dual roles; transient up-regulation leads to rapid up-regulation of CD11b on HSC and initiates their differentiation [91] and may furthermore recruit cells from one compartment to another. In the present study we found a transient up-regulation of *Cxcl2* expression at PND1 in the liver, and both stromal cells, endothelial and the leukocyte cells might be candidate producers of *Cxcl2*. Independently of the source, the up-regulated expression of *Cxcl2* could very well be a key event in both differentiation and recruitment of HSC from the liver.

Our results thus clearly demonstrate that microbial colonization impacts the perinatal hematopoiesis as well as the early development of adaptive immunity. It is however unclear how the colonizing microbes stimulate hematopoiesis, i.e. whether direct contact between HSC and microorganisms takes place and if, where the cells and the microbes encounter. Regardless of the site of encounter, the microbes must trespass the skin or epithelial barrier. The presence of bacteria in the spleen of healthy mice was recently reported by Puga and colleagues [114], hence translocation of bacteria from the GI tract into the blood circulation, and from here to the spleen, is plausible, in particular in the early postnatal period where the GI epithelium is immature. In this regard, we show that intestinal expression of the three genes *Tjp1, JamA* and *Cdh1* exhibited significantly different kinetics in the GF neonate intestine compared to the CONV neonate intestine from PND1 to 6, pointing to a microbiota dependent maturation and perhaps, closure of the GI epithelium during the early postnatal period. We speculate that a rapid maturation and closure of the GI epithelium in the presence of microorganisms may control the postnatal period in which uncontrolled microbial translocation is possible, thus limiting bacterial influx to the very first days of life. Of note, GI epithelium of the MC mice exhibited expression kinetics of the tight junction genes, which were

only slightly advanced compared to that of the GF mice. Thus, if this indicates a slower maturation and closure, more bacteria but only of one particular strain would translocate into circulation. Nevertheless, the proportion of $CD11b^+$ cells and $CD4^+$ T cells in the MC spleen at PND4, 7 and 35 was highly similar to the proportions in spleens of GF mice. Hence, we suggest that a single strain of bacteria is not enough to stimulate HSC differentiation into $CD11b^+Gr\text{-}1^+$ cells. This is supported by the far stronger MIP-2 response observed from neonate spleen cells stimulated with a mixture of Gram positive and Gram negative bacteria compared to the response upon stimulation with a single strain of bacteria.

$CD11b^+Gr\text{-}1^+$ cells comprise a heterogeneous group of myeloid derived cells, which includes differentiating (immature) cells as well as mature neutrophils [106, 117, 140] and share the lobed nuclei of mature neutrophils [104]. In mice, immature $CD11b^+Gr\text{-}1^+$ cells are often referred to as myeloid derived suppressor cells (MDSC) and represent a subset of cells known to expand in blood and lymphoid organs during cancer, inflammatory conditions and infection holding immunosuppressive properties [103, 118]. Based on expression of CD11b, Gr-1 and scatter distribution, we described a heterogeneous nature of these cells; comprising at least three distinct subpopulations in fetal and neonatal spleens, which differed between CONV and GF splenocytes, indicating a microbial influence on immune cell development already during gestation. With the well-established importance of a microbiota for proper development of a balanced immune system [3, 4] in mind, the observed prolonged presence of a high proportion of $CD11b^+Gr\text{-}1^+$, only in spleens of neonate mice born by dams with a diverse microbiota, might suggest a key role for these cells in proper development of the adaptive immunity. To establish this, however, requires assessment of the subpopulations of $CD4^+$ lymphocytes around PND7 to PND35, a task which is beyond the scope of the present study, where we aimed to describe the very early microbiota dependent postnatal cellular events.

In conclusion, we have presented data showing that the microbiota affects homeostatic events of importance for maturation of the splenic $CD4^+$ T cell pool, and that $CD11b^+Gr\text{-}1^+$ neutrophil-like cells are highly abundant in the neonatal spleens of CONV mice but not in GF or MC spleens. Even though we cannot from these data precisely pinpoint the mechanisms by which the $CD11b^+Gr\text{-}1^+$ cells are increased in the spleens of pups from CONV dams, the demonstration of the vast difference in the number of these cells PND2-7 strongly supports the key role of the microbiota from the very first period postpartum. We have thus identified an important perinatal microbiota-dependent event that may impact the subsequent population and polarization of T cells in the neonatal spleen in mice.

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DISCLOSURES

The authors declare no conflict of interest.

FIGURE LEGENDS

Figure 1. The establishment of the $CD3^+CD4^+$ population is accelerated by the microbiota and is preceded by dominance of $CD11b^+$ cells in the spleen of newborn CONV mice. (A) Time-dependent development of spleen cell populations at PND4, PND7 and PND35 in GF, MC and CONV mice, analyzed by flow cytometry for markers CD3, CD4, CD8 and CD11b. Data are expressed as percentages of total viable cells and analyzed by 2way-Anova with Bonferroni post test, ***$p<0.001$, *$p<0.05$, mean±SD, each point on graphs represents 3-7 individual experiments (n=3-7). (B) Release of MIP-2 from splenocytes isolated from CONV mice on PND4 and cultured with *L. acidophilus* NCFM, *E. coli* Nissle 1917 or a mixture of both bacteria, n=2, mean±SD.

Figure 2. Microbial colonization prolongs the predominance of $CD11b^+Gr\text{-}1^+$ cells in the neonate spleen of mice during the first week of life. (A) CONV and GF splenocytes stained for surface markers CD11b and Gr-1 on PND2, 4 and 7. Plots represent cellular percentages of all viable cells (B) Time course for $CD11b^+Gr\text{-}1^+$ splenocytes in CONV and GF mice PND1 to PND14, data are analyzed with 2way-Anova and Bonferroni post test, n=3-5, ***$p<0.001$, mean±SD. (C) Level of $CD11b^+Gr\text{-}1^+$ cells in spleens of fetal CONV and GF mice (PND-1). Columns present mean of two individual experiments in each group, each contains pooled spleens from 12 pups, mean±SD. (D) Time course of the absolute number of viable cells in spleens of CONV and GF mice aged PND1 to PND21, n=2-5, mean±SD.

Figure 3. Perinatal $CD11b^+Gr\text{-}1^+$ spleen cells comprise 3 distinct subpopulations differing in size and brightness of markers CD11b and Gr-1. (A) Freshly isolated CONV splenocytes stained with anti-CD11b and anti-Gr-1. Additionally is shown scatter distribution of the gated cells in populations P1 and P2 revealing two sub-populations of differing size in P1. (B) $CD11b^+Gr\text{-}1^+$ spleen cells (PND-1 and PND1) with different appearance depending on microbial colonization.

Figure 4. The number of granulocytes in the neonatal spleen is gradually reduced during the first 7 days of life. H&E stained tissue sections of CONV spleens. Arrows indicate polylobed or ring-

shaped nuclei representative of mature or maturing granulocytes in the spleen tissue. Magnification, x40. Data are representative of 2-3 independent experiments with similar results.

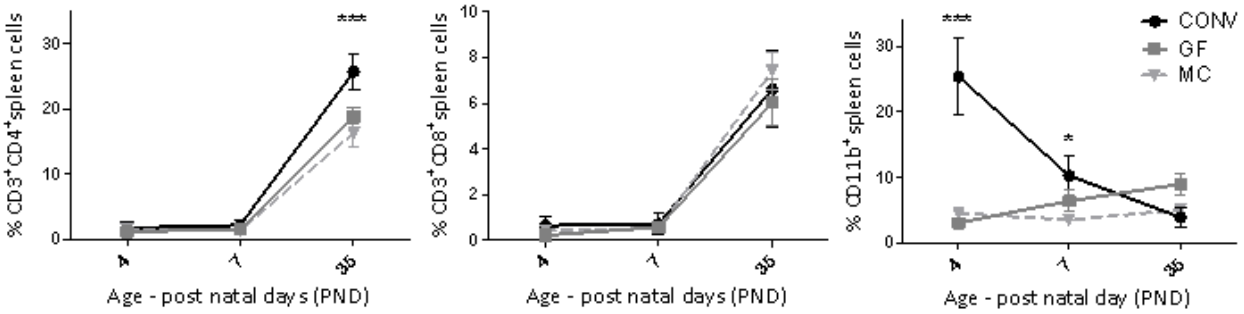
Figure 5. Microbial colonization is associated with a higher *Cxcl*2 and lower *Arg 1* expression in the liver at PND1. Expression of *Cxcl1 and Cxcl2* in livers of CONV and GF PND1 and PND2 mice determined by qPCR, n=5-10 mice per group/day, significant difference is between the expression in GF and CONV livers of the same age, data are analyzed with 1way-Anova and Bonferroni post test, **P<0.01.

Figure 6. Microbial colonization leads to reduced level of neutrophil-like cells in livers of 2-4 days old mice. H&E staining of neonatal liver tissue sections from CONV (A) and GF (B) mice at PND2, 4 and 7. Neutrophil-like cells accumulate around blood vessels at PND2 and 4 in liver tissues of GF mice, while these cells are largely absent from CONV liver tissues at the same age. Inserts depict cellular content around portal vein areas of liver tissues. Two livers of each day and colonization were dissected and stained. The presented images are representative of all stains of the particular day and colonization, 10x and 40x magnification. (C) Expression of *Arg1* determined by qPCR in livers of CONV and GF mice on PND1, n=7-9, data are analyzed with Student's t-test (unpaired, two-tailed), *p<0.05.

Figure 7. Microbial colonization increases expression of the tight junction (TJ) genes *Tjp1*, *Cdh1* and *JamA* in ileum on the day of birth (PND1). Tight junction gene expression determined by qPCR in CONV, MC and GF mouse pups on PND1 and PND6. n=8, data are analyzed with 1way-Anova and Bonferroni post test, *p<0.05, ***p<0.001.

Figure 1

A


% CD3+CD4+spleen cells
***
Age - post natal days (PND)
% CD3+CD8+spleen cells
Age - post natal days (PND)
% CD11b+spleen cells
***
*
CONV
GF
MC
Age - post natal day (PND)

B

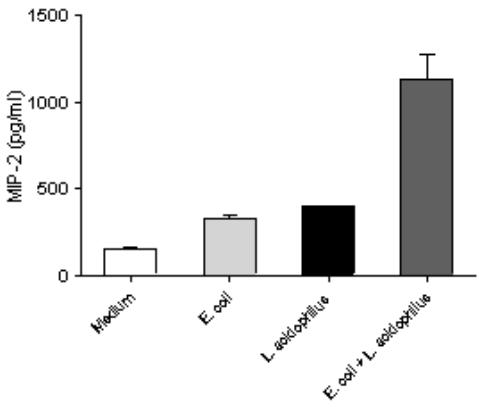
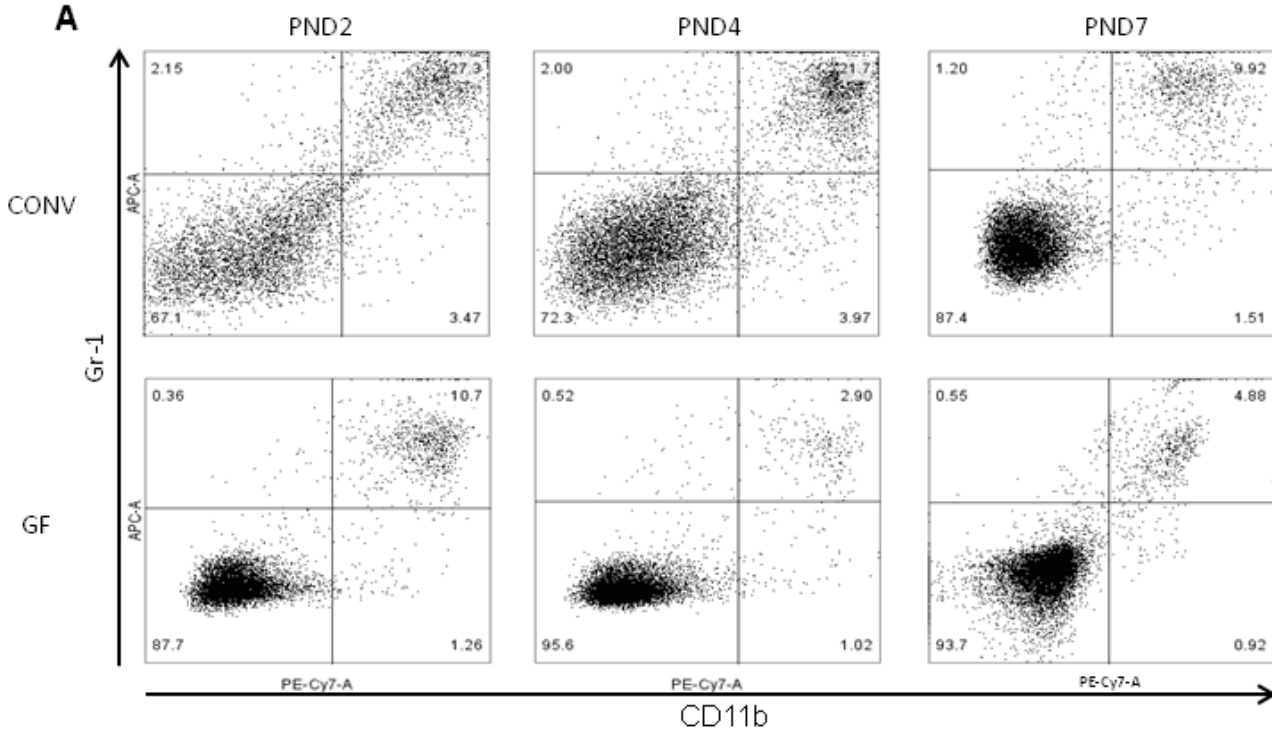
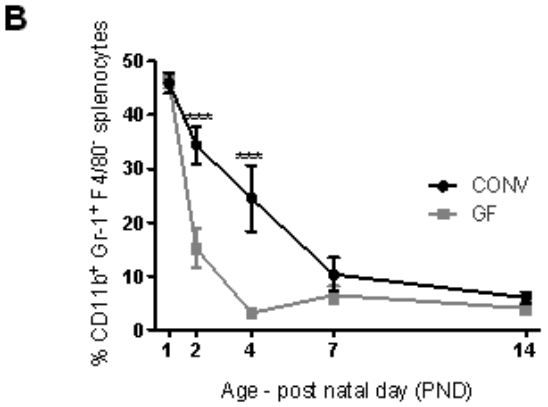
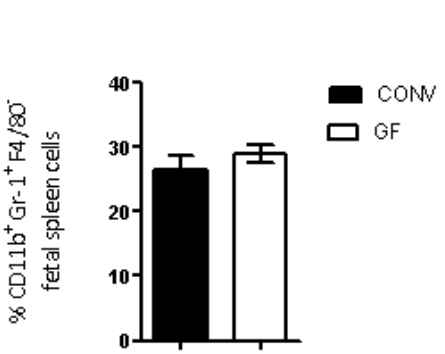

MIP-2 (pg/ml)
1500
1000
500
0
Medium
E.coli
L.acidophilus
E.coli + L.acidophilus

Figure 2


A
PND2
PND4
PND7
CONV
GF
Gr-1
APC-A
PE-Cy7-A
CD11b
2.15
27.3
67.1
3.47
2.00
21.7
72.3
3.97
1.20
9.92
87.4
1.51
0.36
10.7
87.7
1.26
0.52
2.90
95.6
1.02
0.55
4.88
93.7
0.92


B
% CD11b+ Gr-1+ F4/80- splenocytes
CONV
GF
Age - post natal day (PND)


C
% CD11b+ Gr-1+ F4/80- fetal spleen cells
CONV
GF

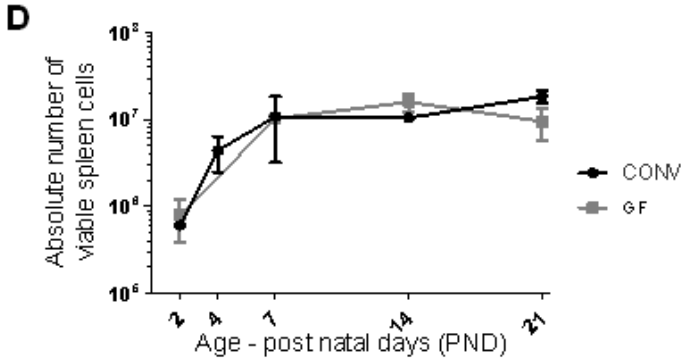
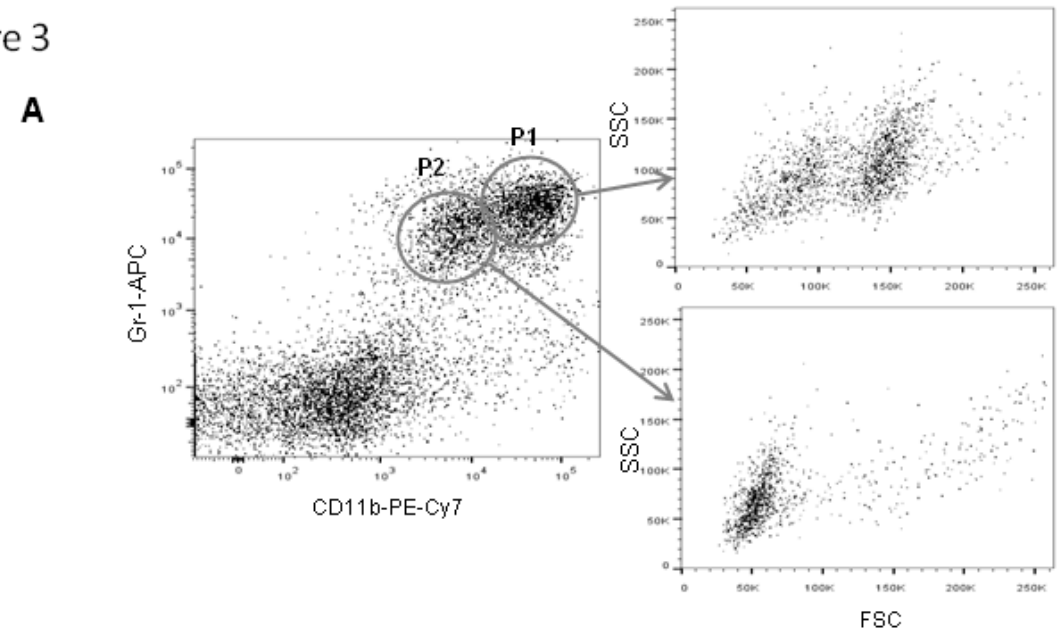

D
Absolute number of viable spleen cells
CONV
GF
Age - post natal days (PND)

Figure 3


A
P1
P2
Gr-1-APC
CD11b-PE-Cy7
SSC
SSC
FSC

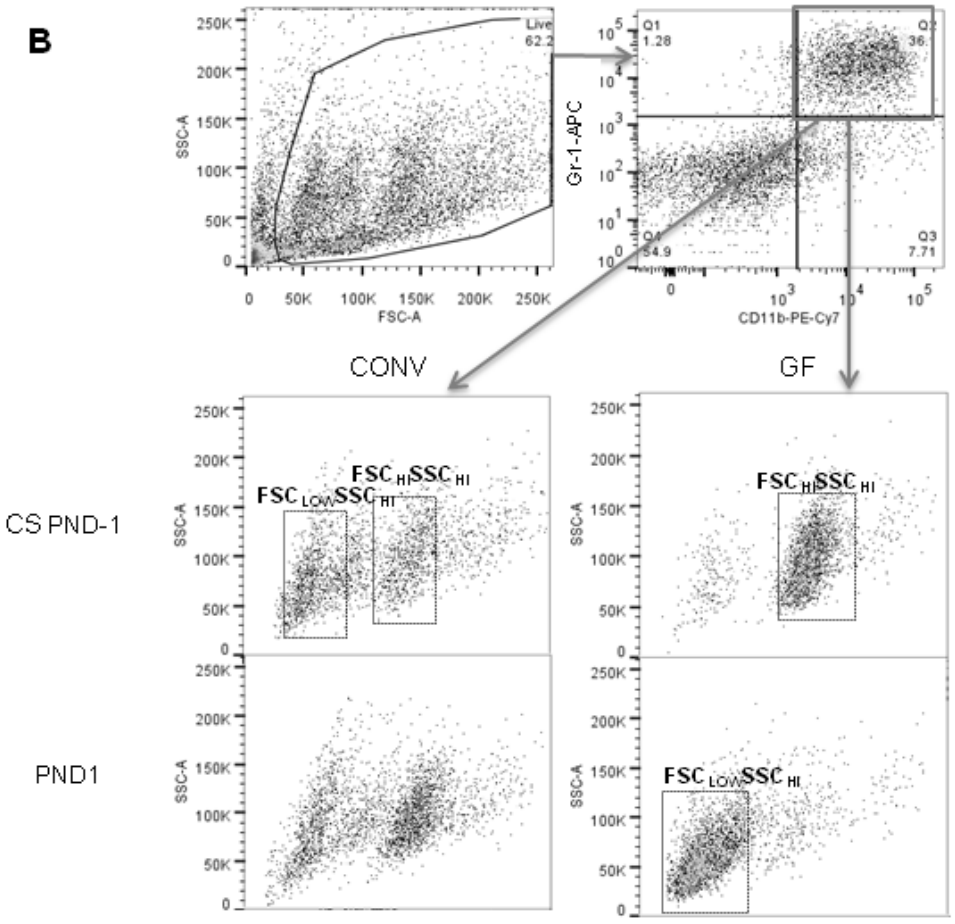

B
Live
62.2
SSC-A
FSC-A
Q1
1.28
Q2
36
Q4
54.9
Q3
7.71
Gr-1-APC
CD11b-PE-Cy7
CONV
GF
CS PND-1
PND1
FSC HI SSC HI
FSC LOW SSC HI
FSC HI SSC HI
FSC LOW SSC HI

Figure 4

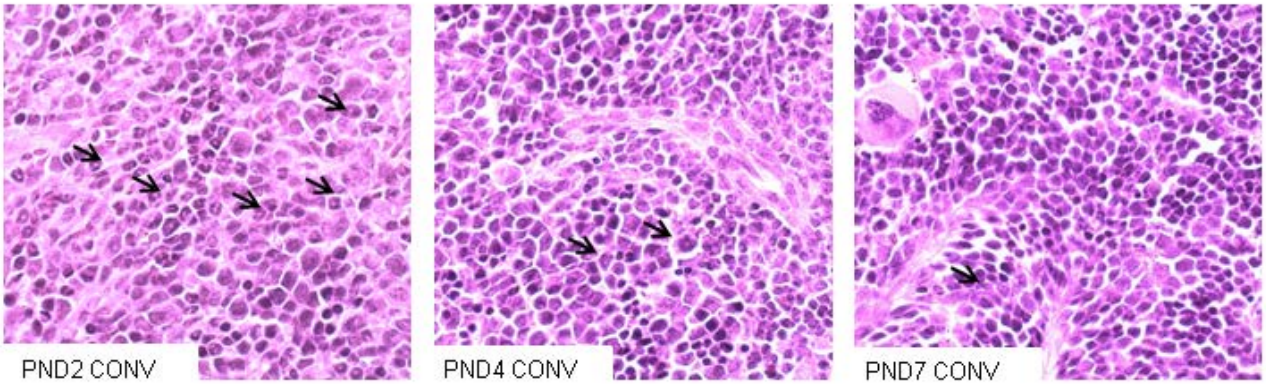


Figure 5

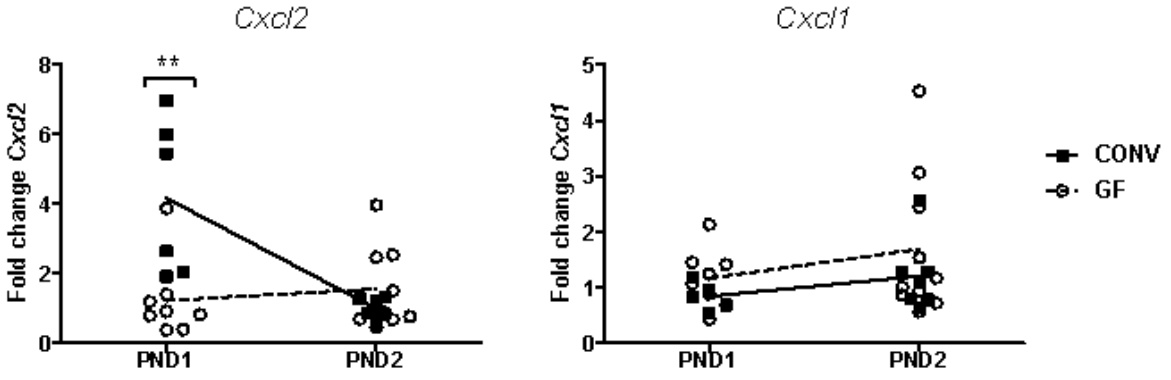


Figure 6

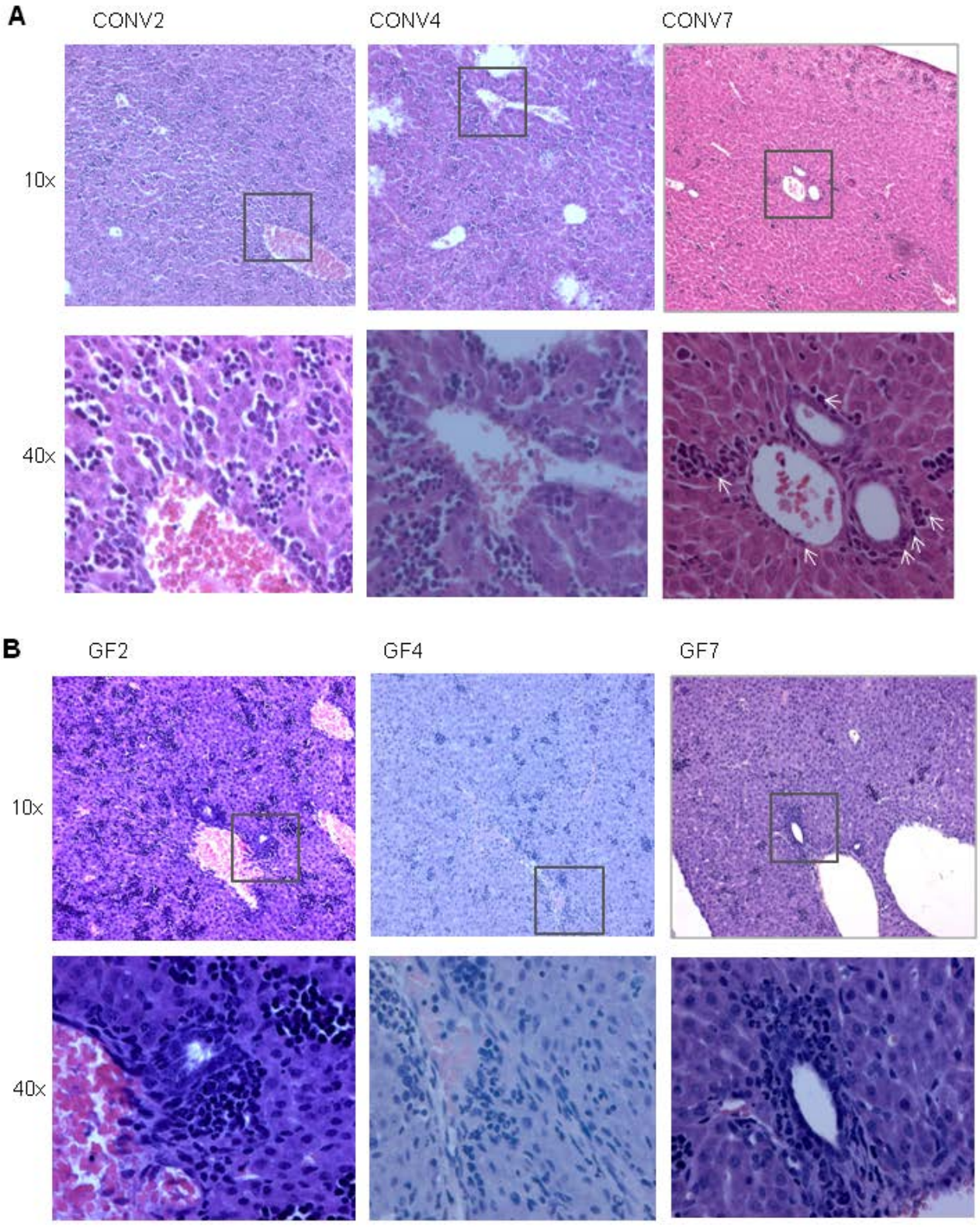


Figure 6C

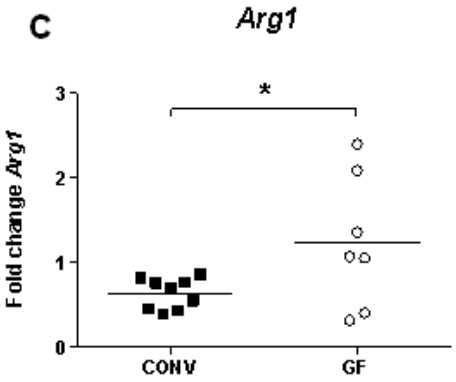
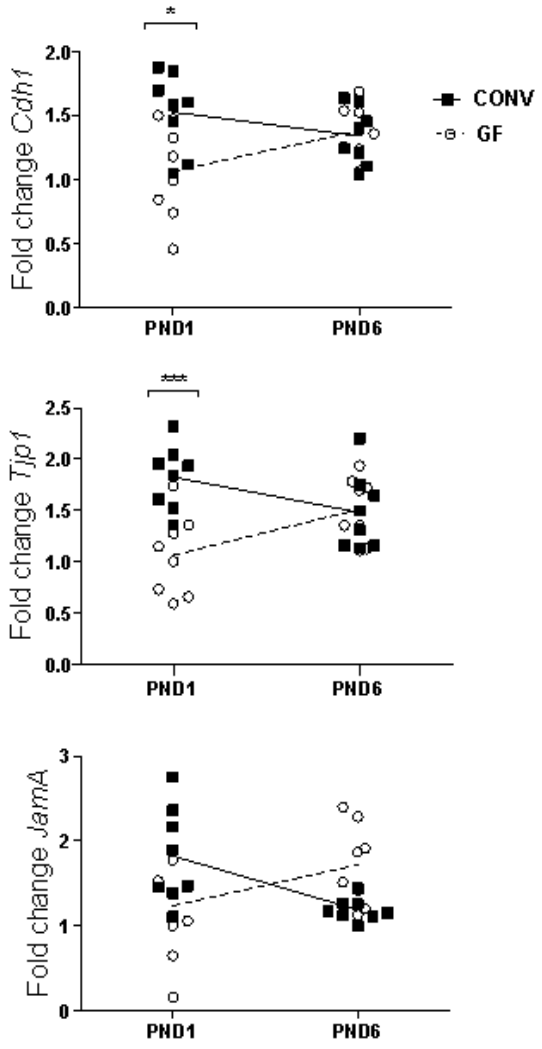


Figure 7



PAPER 2

"Nature of bacterial colonization influences transcription of mucin genes in mice during the first week of life"

Anders Bergström, Matilde B. Kristensen, Martin I. Bahl, Stine B. Metzdorff, Lisbeth N. Fink, Hanne Frøkiær and Tine R. Licht

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BMC Research Notes

RESEARCH ARTICLE Open Access

Nature of bacterial colonization influences transcription of mucin genes in mice during the first week of life

Anders Bergström[1*], Matilde B Kristensen[1], Martin I Bahl[1], Stine B Metzdorff[2], Lisbeth N Fink[3], Hanne Frøkiær[2] and Tine R Licht[1]

Abstract

Background: Postnatal regulation of the small intestinal mucus layer is potentially important in the development of adult gut functionality. We hypothesized that the nature of bacterial colonization affects mucus gene regulation in early life.
We thus analyzed the influence of the presence of a conventional microbiota as well as two selected monocolonizing bacterial strains on the transcription of murine genes involved in mucus layer development during the first week of life.
Mouse pups (N = 8/group) from differently colonized dams: Germ-free (GF), conventional specific pathogen free (SPF), monocolonized with either *Lactobacillus acidophilus* NCFM (*Lb*) or *Escherichia coli* Nissle (*Ec*) were analyzed by qPCR on isolated ileal tissue sections from postnatal days 1 and 6 (PND1, PND6) after birth with respect to: (i) transcription of specific genes involved in mucus production (*Muc1-4*, *Tff3*) and (ii) amounts of 16S rRNA of *Lactobacillus* and *E. coli*. Quantification of 16S rRNA genes was performed to obtain a measure for amounts of colonized bacteria.

Results: We found a microbiota-independent transcriptional increase of all five mucus genes from PND1 to PND6. Furthermore, the relative level of transcription of certain mucus genes on PND1 was increased by the presence of bacteria. This was observed for *Tff3* in the SPF, *Ec,* and *Lb* groups; for *Muc2* in SPF; and for *Muc3* and *Muc4* in *Ec* and *Lb*, respectively.
Detection of bacterial 16S rRNA genes levels above the qPCR detection level occurred only on PND6 and only for some of the colonized animals. On PND6, we found significantly lower levels of *Muc1*, *Muc2* and *Muc4* gene transcription for *Lb* animals with detectable *Lactobacillus* levels as compared to animals with *Lactobacillus* levels below the detection limit.

Conclusions: In summary, our data show that development of the expression of genes encoding secreted (*Muc2/Tff3*) and membrane-bound (*Muc1/Muc3/Muc4*) mucus regulatory proteins, respectively, is distinct and that the onset of this development may be accelerated by specific groups of bacteria present or absent at the mucosal site.

Keywords: Germ free mice, Monocolonized, qPCR, LinRegPCR, Postnatal transcription onset, Probiotics, *Lactobacillus acidophilus* NCFM, *Escherichia coli* Nissle, 16S rRNA

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Background

The interplay between the microbiota of the gut and the intestinal mucus layer in early life is important in the development of the epithelial barrier as part of the innate immune defense [1]. The first weeks and months after birth are believed to be crucial for establishment of the gut microbiota and consequently for the health and integrity of the epithelium throughout life [2,3]. In this period, a development regulated by endogenous factors such as hormones, in parallel with gene regulation caused by the microorganisms present in the gut, takes place [4,5]. The presence and composition of the microbiota has been shown to be directly involved in the regulation of gene transcription in the intestinal epithelium, including the mucin genes, *Muc1-4* and the trefoil factor *Tff3* [4,6].

In the human intestines, MUC1-4 are the most prevalent [6] of the different mucin gene transcripts described to date [1,7,8]. In the gastrointestinal tract, specific mucins show coordinated expression and localization with the viscosity regulating trefoil factors (TFF's), in particular TFF3 [1]. Epithelial linings contain both membrane-bound (MUC1, MUC3, MUC4) and secreted gel-forming mucins (MUC2) expressing highly specific oligosaccharide side chains, which are important in relation to filtering the entry of various moieties e.g. bacteria and food to the underlying tissue. The membrane-bound mucins act as cell-surface receptors and sensors, mediating signals to trigger cell proliferation, apoptosis, differentiation and specific secretions, when relevant [1]. The four human mucin genes (*MUC1-4*) all share a fairly high degree of sequence, distribution and functional homology to the mouse mucin genes *Muc1-4* [9-12].

As facultative anaerobes, lactobacilli and *E. coli* strains have been recognized as successful early life colonizers of the sterile gastro-intestinal tract [13,14]. Strains of *Lactobacillus acidophilus* are known to stimulate transcription of mucin genes *in vitro* [15,16]. Moreover administrations of probiotic lactobacilli and bifidobacteria have been shown to increase ileal gene and protein levels of *Muc3* in adult rats [17] and cell cultures [16], respectively. Certain *E. coli* strains have been associated with increased production of *MUC2*, *MUC3* and *MUC4* in human ileal cells [18].

In order to elucidate the role of microbial colonization in the postnatal regulation of *Muc1-4* and *Tff3*, we investigated the expression of these genes in mouse ileal segments isolated at the first day after birth (PND1) and six days after birth (PND6), respectively, from specific pathogen free, conventionally bred mice (SPF), mice monocolonized with either *Lactobacillus acidophilus* NCFM (*Lb*) [19] or *E. coli* Nissle (*Ec*) [20,21], and from germ free (GF) mice [15,22]. Specifically, samples were collected and analyzed at PND1 and PND6 to examine the immediate postnatal effects, which are relevant for immune system priming [22,23]. Quantification of bacterial 16S rRNA gene levels was performed to obtain a measure of bacterial colonization levels in the different animal groups on PND1 and PND6.

Results and discussion

qPCR

We introduced several new primers in this study, all scoring successfully on our validation criteria. LinRegPCR [24,25] was utilized for qPCR analysis, as it enables individual PCR efficiencies to be calculated. The standard curve assumption, that in all samples the PCR efficiency/amplicon, based on one "representative" DNA sample is constant, is replaced by an assumption-free method based on linear regression in the exponential phase of the fluorescence of the actual individual samples analyzed [24]. Further, by including in the subsequent calculation of average efficiency/amplicon, only successful samples within 5 % of the mean efficiency/amplicon, contributions from diverging samples to the final results are excluded.

We tested the choice of reference gene, but interestingly found no significant difference in the results between beta-actin [26,27], neuroplastin (Genevestigator recommendation) nor the geometrical mean of them both.

Effect of time and bacterial colonization on regulation of Muc1-4 and Tff3 transcription

In GF mice, *Muc1-4* and *Tff3*, all showed statistically significant increases in transcription from PND1 to PND6, indicating that this event occurs during the first postnatal week independently of the presence of microbes (Figure 1). For certain mucin genes, presence of bacteria in the colonized animals correlated with an increased relative abundance of transcripts on PND1 compared to transcription levels of the same genes in GF mice. This was particularly evident for the genes *Muc2* and *Tff3*. Increased transcription on PND1 of *Tff3* was observed in conventional pups (SPF) as well as in pups of dams' monocolonized with either *Lb* or *Ec*, while for *Muc2*, this was observed only in presence of a full microbiota (SPF). For *Muc3* in *Ec* and *Muc4* in *Lb*, a higher level of transcription was observed on PND1 than in GF pups, indicating that *E. coli* and *Lactobacillus* may specifically stimulate transcription of these genes immediately after birth (Figure 1).

The higher level of *Muc2* and *Tff3* transcriptions at PND1, both encoding secreted proteins with goblet cell origin [28], indicates that the presence of bacteria affects gene transcription onset in these exocytotic cells. While both gene products play protective roles during gut inflammatory conditions, at sites of epithelial damage [18,29-34] and during postnatal development [35,36],

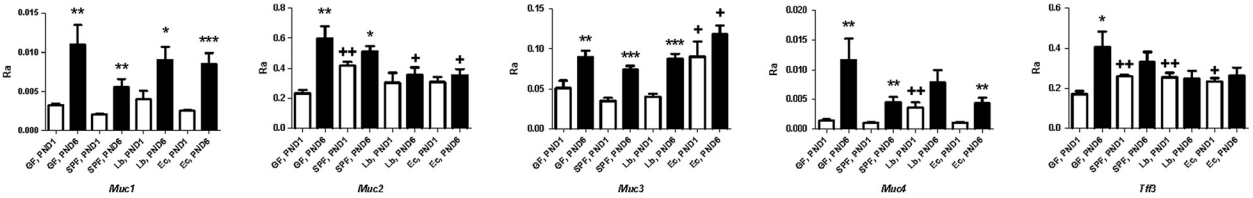

Figure 1 Comparison of mucin gene expression between the four animal groups on PND1 and PND6. Transcription of *Muc1*, *Muc2*, *Muc3*, *Muc4* and *Tff3* on postnatal days (PND) 1 and 6 for pups in groups: GF, SPF, *Lb* and *Ec*. Each column represents the average relative abundance (Ra) (See Methods) of 8 animals. Error bars show SEM values. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to PND1 (for the same group) $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$, $^{+++}$ $p < 0.001$ relative to GF (for the same day).

Muc2, unlike Tff3, polymerizes into a protective gel-like structure [1]. Based on the obtained results, it is however not possible to determine whether there is a connection between this difference in functionality and the corresponding gene regulation.

Previously, we demonstrated how microbiota affects ileal gene expression of a number of immune related genes (specific cytokines and chemokines) during the first week after birth [23]. As seen for *Muc2* in the present study, and also for a number of Toll-like receptor signaling pathway related genes such as *Tlr2/4*, *Irak1* and the chemokine *Cxcl2*, encoding MIP-2, the presence of a full microbiota was required to influence gene expression on PND1, which was only to a limited degree affected by monocolonization with either *Lactobacilli* or *E. coli* [23].

Increased transcription of *Muc3* and *Muc4* on PND1 was observed in *Ec* and *Lb* pups, respectively, but not in SPF (Figure 1). Although specific probiotic bacteria, including *Lactobacillus acidophilus* NCFM [15], *Lactobacillus rhamnosus* [17], *Bifidobacterium bifidum* [17], *Lactobacillus plantarum* [16,17] as well as two atypical, enteropathogenic *E.coli* strains [18], have previously been shown to stimulate mucin gene expression, this study is to our knowledge the first to address such effects at a very early stage of life. *Muc1* transcription levels were in this study apparently not affected by the presence of bacteria.

Bacterial 16S rRNA abundance on PND1 and PND6

None of the PND1 samples contained *Lactobacillus* or *E. coli* in amounts above the qPCR detection limit (DL), in any of the four animal groups (Table 1). This was expected, since only partial bacterial colonization is achieved so short after birth. On PND6, 5/8 pups in both the *Lb* and SPF groups, respectively, were positively above the *Lactobacillus* 16S rRNA DL, while 8/8 and 0/8 in the *Ec* and SPF groups, respectively, were colonized above the *E.coli* DL. These observations corresponded to differences in N_0 values (See Methods) of >300-fold for *Lactobacillus* and >160-fold for *E.coli*. This shows that bacterial levels in the ileal sections increased between PND1 and PND6 after birth, although the employed procedure did not allow detection of bacterial 16S rRNA in all pups. Culture-based techniques have shown that the gut mucosal surfaces in newborn mice follow a rather conserved colonization pattern [37]. In particular, lactobacilli colonize within the first 1–2 days after birth, whereas coliforms are normally not quantifiable in the mucosal layers until approximately 9 days after birth [14]. These results are thus consistent with findings in the SPF group in this study. It is however important to note, that the current analysis was performed on whole intestinal sections, including both luminal contents and mucosal surfaces, whereas the other studies referred to were based on analysis of mucosal surfaces only.

There was a significantly lower level of transcripts ($p < 0.05$) of *Muc1*, *Muc2* and *Muc4* in the pups with detectable amounts of lactobacilli on PND6 in the *Lb* group than in pups with colonization below the detection limit (Figure 2). In other words, colonization with relatively high levels of *Lactobacilli* in the pups had a negative effect on mucin gene transcription on PND6. For *Muc2*, pups colonized with *Lactobacillus* below the detection limit in the *Lb* group were indeed comparable to GF pups.

It has been established by others that degradation of mucin in adult rats [38] as well as gene expression of *Muc1-4* and *Tff3* in six week old mice [6], is different

Table 1 16S rRNA measured presence vs. absence of all 4 animal groups on each of days PND1 and PND6

	PND1		PND6	
	Lactobacillus 16S	*E. coli* 16S	*Lactobacillus* 16S	*E. coli* 16S
GF	0/8	0/8	0/8	0/8
SPF	0/8	0/8	5/8	0/8
Ec	0/8	0/8	0/8	8/8
Lb	0/8	0/8	5/8	0/8

The fraction denotes number of samples significantly above detection limit (DL) of the total number (N = 8 in each group).

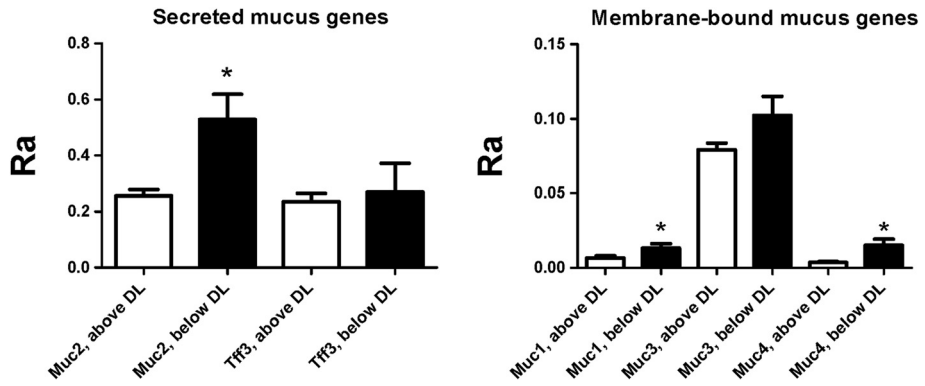

Figure 2 Difference in mucin gene expression between pups with detectable and undetectable *Lactobacillus* 16S in *Lb*. Comparison of *Muc1*, *Muc2*, *Muc3*, *Muc4* and *Tff3* transcription on PND6 between pups in the *Lb* group, where *Lactobacillus* 16S could be detected and could not be detected, respectively. Each column represents the average relative abundance (Ra) of 5/8 (above DL) or 3/8 (below DL) animals. Error bars show SEM values. DL = Detection Limit. * $p < 0.05$, denotes significant difference between detectably colonized and not colonized.

when comparing GF and conventional animals. Clearly, gene regulation induced by the colonizing microbiota is a complex and continuous process occurring throughout the first weeks of life, and as a more stable and adult-like microbiota is probably not achieved until the end of weaning process at approximately 21 days after birth [39], the expression of the mucus regulating genes may change not only in newborn animals, but also later in life in response to periodic changes in the microbiota

Conclusions

In this manuscript, we show distinct differences between the expression patterns of secreted (*Muc2*/*Tff3*) and membrane-bound (*Muc1*/*Muc3*/*Muc4*) mucus-regulatory genes in the very first days after birth. Presence of a full microbiota (SPF) increased the relative level of transcription of *Muc2* and *Tff3*, which implies the two corresponding secreted gene products, Muc2 and Tff3, to play protective roles in the postnatal intestinal layer development. The immediate activation of *Muc2*/*Tff3* transcription may provide a coating of the new born ileal epithelial layer, allowing only passage of certain substances or organisms.

Methods

Animal experiments

GF Swiss Webster mice and SPF mice, containing conventional microbiota, were purchased from Taconic (Lille Skensved, Denmark) and kept in GF isolators or under specific pathogen-free conditions, respectively [22]. Fecal samples from GF mice, taken at sampling i.e. once a week, were cultivated on non-selective LB medium and under aerobic and anaerobic conditions to confirm sterility of isolators. For breeding, pairs of female mice were housed with one male until plugs were observed. Mono-colonization of pregnant mice with *Ec* and *Lb* was performed 7 days after mating by applying $5x10^8$ CFU ml^{-1} in 0.5 ml PBS suspension orally using a pipette and 0.5 ml to the abdominal skin. *Lb* was grown anaerobically in de Man, Rogosa, and Sharpe broth (MRS, Merck, Darmstadt, Germany) and *Ec* aerobically in Luria-Bertani broth (LB, Merck) overnight at 37°C. The cultures were harvested, washed twice in sterile phosphate-buffered saline (PBS) (Lonza, Basel, Switzerland), re-suspended in 1/50 of the original culture volume and frozen at −80 °C until use. Prior to administering bacteria to the mice, *Ec* suspensions were diluted tenfold in PBS immediately to obtain $5x10^8$ CFU ml^{-1}. *Lb* suspensions were not diluted. Four litters spontaneously delivered from 4 different mothers in each group; SPF, GF, *Lb* and *Ec*, were used for the experiment. At post-natal days 1 and 6, the pups were put down and the distal ileum (segment from cecum and 3 cm up) was removed from the small intestine of two pups per litter and frozen in RNAlater (Qiagen, Hilden, Germany). No separation of mucosal from luminal content was performed.

Ethics

The mouse experiment was performed under a license to Department of Microbiology, National Food Institute, from the Danish Council for Animal Experimentation.

RNA isolation

Tissues were removed from RNAlater and homogenized by a rotor strator in RLT buffer (Qiagen). RNA from tissue homogenate was extracted using RNeasy Mini Kit from Qiagen following the supplier's protocol.

Primer design and validation

A list of all primers used in this study is presented in Table 2. All primers found in references were initially checked with the Net Primer Software (http://www.

Table 2 Primers used for qPCR

Primer name	Fwd (5´-3´)	Rev (5´-3´)	Amplicon size	Reference
Muc1	TCGTCTATTTCCTTGCCCTG	ATTACCTGCCGAAACCTCCT	185	This study
Muc2	CCCAGAAGGGACTGTGTATG	TTGTGTTCGCTCTTGGTCAG	276	Modified from [44]
Muc3	TGGTCAACTGCGAGAATGGA	TACGCTCTCCACCAGTTCCT	98	Modified from [6]
Muc4	GTCTCCCATCACGGTTCAGT	TGTCATTCCACACTCCCAGA	280	This study
Tff3	CTCTGTCACATCGGAGCAGTGT	TGAAGCACCAGGGCACATT	77	[45]
Neuroplastin	CGCTGCTCAGAACGAACCAAGAA	CTTACGGGTGGCAGTGAGTT	160	Modified from [46]
Beta-actin	GTCCACCTTCCAGCAGATGT	GAAAGGGTGTAAAACGCAGC	117	This study
Lactobacillus 16S rRNA	AGCAGTAGGGAATCTTCCA[a]	CACCGCTACACATGGAG[b]	341	[a] [47][b] [48]
E. coli 16S rRNA	CATGCCGCGTGTATGAAGAA	CGGGTAACGTCAATGAGCAAA	96	[49]

premierbiosoft.com/netprimer/index.html). Primers not scoring a rating of at least 90 % were not accepted and new primers were then designed with NCBI's primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and the quality was again verified until satisfaction with the Net Primer Software. All newly designed primers were designed to span exon junctions to avoid amplification of genomic DNA. The specificity of all primers was evaluated *in silico* using nucleotide BLAST, (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Quantitative PCR (qPCR)

Isolated ileal RNA was reverse transcribed into cDNA using SuperScript® VILO™ cDNA Synthesis Kit from Invitrogen, Denmark. After verifying the quality of the cDNA by spectroscopy ($A_{260}/A_{280} = 1.8 \pm 10$ %) measured on a NanoDrop ND-1000 Spectrophotometer (Saveen Werner, Limhamn, Sweden), it was used as template in quantitative real-time PCR using the ABI prism 7900HT from Applied Biosystems. All cDNA concentrations were within the range of 90-100 ng μl^{-1}. The amplification reactions were carried out in a total volume of 20 μl containing 10 μl master mix (2x PerfeCTa™ SYBR® Green SuperMix, ROX from Quanta Biosciences ™), 0.4 μl of each primer (10 μM), 2 μl template cDNA, and 7.2 μl nuclease-free water (Qiagen GmbH, Germany) purified for PCR. The amplification program consisted of one cycle of 50 °C for 2 min; one cycle of 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min; and finally one cycle of dissociation curve analysis for assessing the amplification products (95 °C for 15 s, 60 °C for 20s and increasing ramp rate by 2 % until 95 °C for 15 s). These conditions were selected based on preliminary qPCR experiments on target DNA with similar concentrations (100 ng μl^{-1}). Samples of all amplification products were further subjected to gel electrophoresis in 2 % agarose, followed by ethidium bromide staining in order to verify amplicon sizes.

qPCR setup

Three separate qPCR experiments on ileal cDNA were performed; 1) and 2) were separate replications of relative quantifications on mucus gene transcription (*Muc1-4* and *Tff3*) with selected reference genes (see next paragraph) and 3) on presence or absence of specific bacterial 16S rRNA analysis (*Lactobacillus*, *E.coli*).

qPCR data analysis

All qPCR analysis was performed with the freely available LinRegPCR tool developed by Ruijter et al. [24,25]. The raw fluorescence data were exported from the ABI prism 7900HT SDS-software, and the LinRegPCR program was used to estimate baselines and individual PCR efficiencies in order to calculate output as target starting concentration, expressed in arbitrary fluorescence units N_0, for each PCR sample by the formula $N_0 = \text{threshold} / (\text{Eff}_{\text{mean}}^{\text{Ct}})$, where Eff_{mean} denotes the optimal PCR mean efficiency/amplicon, threshold the optimal "cutoff" in the exponential region and C_t is the number, where each PCR sample exceeds this threshold. Samples with no amplification, baseline error, too much noise or without plateau were automatically excluded by the LinRegPCR software. Subsequently, for each amplicon the average of all remaining, successful samples within 5 % of the mean value of all successful samples/amplicon were used in the calculation of Eff_{mean} for each amplicon. All N_0-values were calculated as means of double qPCR determinations.

For relative quantification of mucus gene transcripts, two different eukaryotic reference genes were used namely beta-actin [40] and neuroplastin, the latter suggested by the Genevestigator software (https://www.genevestigator.com) [41,42] based on microarray data on similar organism (*M. musculus*) and tissue (ileum). We used the geometric mean of the two reference genes as previously suggested [43]. Normalization to relevant reference gene expression was then calculated according to the formula:

$Ra = Ratio = N_0^{Sample} / N_0^{Reference}$ and averaged over the two qPCR experiments.

Unspecific amplification of 16S rRNA bacterial genes from GF mice was used to specify detection limits for specific amplifications (*Lactobacillus*, *E .coli*). Cutoffs for presence of either bacterium were defined by at least 5 C_t-values difference from the GF samples. No normalization to reference genes and thus relative quantification was used for the 16S analysis, since the purpose was only to determine presence vs. absence of detectable bacteria.

Statistics

All statistics was performed with GraphPad Prism 5. One-way ANOVA followed by Dunnett's *post hoc* test with GF as control group and Student's *t*-test was used to compare mucus gene expression between the four animal groups and development from PND1 to PND6, respectively. P-values lower than $p = 0.05$ were considered statistically significant. Welch's correction for unequal variances was applied, when necessary.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AB performed the qPCR experiments, including cDNA syntheses, data interpretation and statistical analysis, and wrote the manuscript. MBK and SBM performed the animal experiments, including isolation of ileal tissue and RNA purification. TRL, HF and LNF conceived of the study setup and participated in its design and coordination. TRL, MBK, HF and MIB contributed to data analysis and interpretation as well as preparation of the manuscript. All authors read and approved the final manuscript.

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PAPER 3

"*Establishment of tolerance to commensal bacteria requires a complex microbiota and is accompanied by decreased intestinal chemokine expression*"

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Establishment of tolerance to commensal bacteria requires a complex microbiota and is accompanied by decreased intestinal chemokine expression

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[1]*Center for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark, Lyngby;* [2]*Department of Basic Sciences and Environment, Faculty of Life Sciences, University of Copenhagen, Frederiksberg; and* [3]*Department of Toxicology and Risk Assessment and Department of Microbiology, National Food Institute, Technical University of Denmark, Søborg, Denmark*

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Fink LN, Metzdorff SB, Zeuthen LH, Nellemann C, Kristensen MB, Licht TR, Frøkiær H. Establishment of tolerance to commensal bacteria requires a complex microbiota and is accompanied by decreased intestinal chemokine expression. *Am J Physiol Gastrointest Liver Physiol* 302: G55–G65, 2012. First published September 29, 2011; doi:10.1152/ajpgi.00428.2010.—Intricate regulation of tolerance to the intestinal commensal microbiota acquired at birth is critical. We hypothesized that epithelial cell tolerance toward early gram-positive and gram-negative colonizing bacteria is established immediately after birth, as has previously been shown for endotoxin. Gene expression in the intestine of mouse pups born to dams that were either colonized with a conventional microbiota or monocolonized (*Lactobacillus acidophilus* or *Eschericia coli*) or germ free was examined on *day 1* and *day 6* after birth. Intestinal epithelial cells from all groups of pups were stimulated ex vivo with *L. acidophilus* and *E. coli* to assess tolerance establishment. Intestine from pups exposed to a conventional microbiota displayed lower expression of *Ccl2*, *Ccl3*, *Cxcl1*, *Cxcl2*, and *Tslp* than germ-free mice, whereas genes encoding proteins in Toll-like receptor signaling pathways and cytokines were upregulated. When comparing pups on *day 1* and *day 6* after birth, a specific change in gene expression pattern was evident in all groups of mice. Tolerance to ex vivo stimulation with *E. coli* was only established in conventional animals. Colonization of the intestine was reflected in the spleen displaying downregulation of *Cxcl2* compared with germ-free animals on *day 1* after birth. Colonization reduced the expression of genes involved in antigen presentation in the intestine-draining mesenteric lymph nodes, but not in the popliteal lymph nodes, as evidenced by gene expression on *day 23* after birth. We propose that microbial detection systems in the intestine are upregulated by colonization with a diverse microbiota, whereas expression of proinflammatory chemokines is reduced to avoid excess recruitment of immune cells to the maturing intestine.

mucosal immunology; epithelial cells; chemokines; neonatal

THE INTESTINAL MICROBIOTA represents a large load of foreign antigen that must be tolerated by the immune system. Remarkably, this microbiota plays a crucial role in immunogenesis, with specific microbial constituents currently being assigned different roles (7, 17). The establishment of tolerance to microbial stimuli after birth must therefore be accompanied by appropriate signals necessary for the layout of a functioning immune system. Despite progress in mapping the metagenome of the intestinal system, conflicting reports exist on the bacteria necessary to shape the immune system and maintain tolerance. Segmented filamentous bacteria (7) and purified microbe-associated molecular pattern molecules such as a *Bacteroides fragilis* polysaccharide (17) have been shown to be sufficient to establish normal numbers of T cell subsets in the intestine and in the spleen, respectively. In contrast, a full conventional microbiota is required for induction of oral tolerance to food antigens (24), whereas little is known about which signals are required for establishment of tolerance to the microbiota itself.

Colonization of adult germ-free animals rapidly infers maturation of the compromised immune system, locally in the intestine as well as systemically (10, 17). In the natural setting, where colonization occurs at birth, this colonization-induced maturation is accompanied by age-dependent maturation of the intestine and lymphoid tissues. Intestinal epithelial cells (IECs) are sentinels of the bacterial microbiota of the gut and communicate with the immune system via secretion of cytokines and chemokines. As IEC chemokines recruit myeloid and lymphoid cells to the gut, they are crucial in directing the early maturation of both the local and systemic immune system (9). IECs exposed to microbe-associated molecular patterns become insensitive to further bacterial stimuli (14, 32), indicating a critical mechanism in maintaining noninflammatory conditions in the gut. Part of the response of epithelial cell lines to TLR2 and TLR4 ligands is transfer of Toll-like receptors (TLRs) from the apical to the cytosolic compartment, causing tolerance to subsequent ligand challenges (2, 23). In vivo, the intracellular TLR4 receptors are responsive, and the major LPS-tolerizing mechanisms in neonate intestinal epithelial cells have been shown to be microRNA-induced ubiquitination and degradation of IRAK-1 (5, 14) and LPS-dependent inhibition of p38 MAPK via NF-κB regulated MAPK phosphatase-1 (32). Certain IEC-secreted factors, such as cathelin-related antimicrobial peptide (19), are not regulated by colonizing bacteria, but levels secreted evolve with age. Hence, although it is well established that microbiota-enterocyte interactions play a key role in maturation of the gut immune system, few studies have addressed the chemokine and cytokine response of IECs to stimulation at time of birth and the consequences for immune maturation (14).

Lactobacilli and *Escherichia coli* strains are as facultative anaerobic microorganisms successful early colonizers of the sterile gastrointestinal tract (27). Single strains of these bacteria are insufficient to establish tolerance to oral antigens (a process involving both the innate and the adaptive parts of the immune system) in adult animals (24). We hypothesized that in

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contrast, to prevent excessive inflammation in the immature intestine, exposure to either of these bacteria (originating from the monocolonized mothers) would render IECs from the pups nonresponsive to subsequent ex vivo stimulation by the same bacteria. We used the strains *Lactobacillus acidophilus* NCFM and *E. coli* Nissle, which are potent modulators of enterocyte responses in vitro (34). To characterize the initial response to these intestinal bacteria, we measured the expression of cytokine, chemokine, and pattern recognition signaling genes. We demonstrate that only conventional colonization induces IEC tolerance to subsequent ex vivo stimulation, that this tolerance induction was concomitant with increased intestinal TLR2 expression, with reduced expression of chemokines and thymic stromal lymphopoietin (TSLP) in the intestine and in the spleen, and that this was followed by reduced expression of genes involved in antigen presentation in mesenteric lymph nodes (MLN).

MATERIALS AND METHODS

Animal experiment. Germ-free and conventional Swiss Webster mice were purchased from Taconic (Lille Skensved, Denmark) and kept in germ-free isolators or housed under specific pathogen-free conditions, respectively. Absence of bacteria in the germ-free mice was confirmed by cultivation of fecal samples. Six sets of two germ-free females and one male were housed together until plugs were observed. Similarly, two breeding sets were set up for conventionally colonized mice. Monocolonization of female mice with *E. coli* Nissle and *L. acidophilus* NCFM was performed 7 days after mating by applying 5×10^8 CFU/ml in 0.5 ml PBS suspension orally and 0.5 ml to the abdominal skin. Four litters spontaneously delivered from the four dams in each group [conventional, germ-free, and monocolonized (*E. coli* Nissle or *L. acidophilus* NCFM)] were used for the experiment. On the morning of the night when pups were born, *postnatal day 1* (PND1), and on *postnatal day 6* (PND6), four pups per litter were euthanized. Spleen and a segment of the distal ileum (3 cm from cecum and up) from two pups per litter were dissected and frozen in RNAlater (Qiagen, Hilden, Germany). The small intestines from two other pups were used for determination of gene expression in isolated epithelial cells. IECs were isolated as described below for ex vivo studies, immediately pelleted. and resuspended in RNAlater for storage at −80°. LPS content in stomachs from PND6 pups was determined by the Pyrochrome kit (Associates of Cape Cod, East Falmouth, MA). From two conventional and two germ-free pups from separate litters, MLN, and popliteal lymph nodes (PLN) were dissected at *postnatal day 23* (PND23) and frozen in RNAlater. The mouse experiment was performed under a license to Department of Microbiology, National Food Institute, from the Danish Council for Animal Experimentation (Dyreforsøgstilsynet).

Preparation of bacterial suspensions. L. acidophilus NCFM was grown anaerobically in de Man, Rogosa, and Sharpe broth (MRS, Merck, Darmstadt, Germany) and *E. coli* Nissle aerobically in Luria-Bertani broth (LB, Merck) overnight at 37°C. The cultures were harvested, washed two times in sterile phosphate-buffered saline (PBS, Lonza, Basel, Switzerland), resuspended in PBS and frozen at −80°C. For use in ex vivo experiments, bacteria were killed by a 40-min UV exposure prior to freezing. The endotoxin concentration in *L. acidophilus* NCFM preparations were <0.10 EU/ml measured by limulus amoebocyte assay (Associates of Cape Cod).

Isolation of epithelial cells for ex vivo stimulation. At PND6, epithelial cells were isolated for ex vivo stimulation studies from small intestines of two to three pups per litter. The small intestines were placed in Hanks' buffered saline (HBSS, Lonza), opened longitudinally, and cut in small pieces. The epithelial cells were detached from the underlying tissue by incubation in fresh HBSS containing 2 mM EDTA at 37°C for 10 min. Residual tissue was removed by use of a 70-μm filter. Cells were washed in cold PBS and resuspended in culture medium [RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% (vol/vol) heat-inactivated FCS; all from Lonza]. Cells were seeded in 48-well tissue culture plates (Nunc, Roskilde, Denmark) at 4×10^5 cells per 500 μl per well. To each well was added either 50 μl of culture medium (unstimulated), *L. acidophilus* NCFM, or *E. coli* Nissle suspensions, to a final concentration of 30 μg/ml. The IECs were stimulated for 2 h at 37°C in 5% CO_2 and subsequently frozen in RNAlater (Qiagen). The purity of IECs was assessed by staining for the leukocyte marker CD45 (phycoerythrin-labeled rat anti-mouse CD45 purchased from Abcam, Cambridge, UK) by flow cytometry. IECs contained 1.3 ± 0.4% $CD45^+$ cells compared with 0.5 ± 0.3% $CD45^+$ cells in IECs stained with an isotype control antibody (IgG2b). Viability and cell numbers of IECs were determined by propidium iodide exclusion using a NucleoCounter (Chemometec, Allerød, Denmark). IECs were >70% viable after isolation.

RNA isolation and amplification. Cell samples were spun at 3,000 *g*, 5 min at 4°C to remove RNAlater. Tissue was removed from RNAlater and homogenized by use of a rotor stator in RLT buffer (Qiagen). RNA from cell pellet and tissue homogenate was extracted by using the RNeasy Mini Kit from Qiagen following the supplier's protocol. The quantity and purity of extracted RNA was evaluated by Nanodrop spectroscopy (Wilmington, DE). cDNA was produced from ~500 ng total RNA by using High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Gene expression analysis by real-time PCR. A custom TaqMan Low Density Array with 24 TaqMan Gene Expression Assays (Applied Biosystems, Table 1) was used for gene expression analysis of terminal ileum and intestinal epithelial cells of mouse pups born to dams either conventionally colonized, monocolonized with *L. acidophilus* or *E. coli*, or germ free, on PND1 and PND6. An inventoried TaqMan Mouse Immune Array (Part Number 4367786, Applied Biosystems) containing 90 TaqMan Gene Expression Assays of genes known to have implications on the immune response was used for expression analysis of lymph nodes on PND23. The 24 genes on the custom arrays (Table 1) were chosen on the basis of in vitro studies (34) and preliminary comparisons of germ-free and colonized mice by using the Immune Array. PCR was performed as previously described (34). Briefly, to each cDNA sample (50 ng RNA in 50 μl) was added 50 μl TaqMan Universal PCR Master Mix (Applied Biosystems). Arrays were run in standard mode by using the 7900HT Fast Real-time PCR system (Applied Biosystems). Single gene expression of *Cxcl2*, *Il6*, *Il10*, *Tnf*, and *Actb* was analyzed in IECs and *Cxcl2* and *Actb* in spleen. For each sample, 2 μl cDNA (3 ng/μl) was amplified in duplicates by using universal fast thermal cycling parameters (Applied Biosystems) and TaqMan Fast Universal PCR Master (Applied Biosystems) in a total reaction volume of 10 μl. Fold changes in gene expression were calculated by the comparative cycle threshold (C_T) method. The expression of target genes was normalized to a reference gene [$\Delta C_T = C_T(\text{target}) - C_T(\text{reference})$]. We compared *Actb* and 18S rRNA, which gave comparable results, and chose to use *Actb* as reference gene. For tissue samples and isolated IEC, the average gene expression of germ-free PND1 mice was used as calibrator. For ex vivo stimulated IECs, unstimulated germ-free cells were used as calibrator. Fold change in gene expression was calculated as $2^{-\Delta\Delta CT}$ where $\Delta\Delta C_T = \Delta C_T(\text{sample}) - \Delta C_T(\text{calibrator})$.

Data analysis. GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA) was used to perform two-way ANOVA on all four treatment groups with Bonferroni posttest, except for data in Figs. 4 and 5, which were analyzed by Student's *t*-test. Although fold increase is plotted in gene expression experiments, statistical analysis was performed on ΔC_T values [$\Delta C_T = C_T(\text{target}) - C_T(\text{reference})$] since these are assumed normally distributed as opposed to the fold change values. Principal component analysis (PCA) was performed on

Table 1. *Gene expression assays used for intestinal samples on PND1 and PND6*

Gene Symbol	Gene Name	Protein	Assay ID
Reference genes			
Actb	**actin, beta, cytoplasmic**	**β-Actin**	**Mm00607939_s1**
18S	eukaryotic 18S rRNA		Hs99999901_s1
Leukocyte marker			
Ptprc	protein tyrosine phosphatase, receptor type, C	CD45	Mm00448463_m1
Ccl2	chemokine (C-C motif) ligand 2	MCP-1	Mm00441242_m1
Ccl3	chemokine (C-C motif) ligand 3	MIP-1α	Mm00441258_m1
Cxcl1	chemokine (C-X-C motif) ligand 1	KC	Mm00433859_m1
Cxcl2	**chemokine (C-X-C motif) ligand 2**	**MIP-2**	**Mm00436450_m1**
Ccl19	chemokine (C-C motif) ligand 19	CCL19	Mm00839967_g1
Cytokines			
Il10	**interleukin 10**	**IL-10**	**Mm00439616_m1**
Il7	interleukin 7	IL-7	Mm00434291_m1
Il6	**interleukin 6**	**IL-6**	**Mm00446190_m1**
Tgfb1	transforming growth factor, beta 1	TGF-β	Mm03024053_m1
Tnf	**tumor necrosis factor**	**TNF-α**	**Mm00443258_m1**
Tslp	thymic stromal lymphopoietin	TSLP	Mm00498739_m1
Signaling			
Nfkb1	nuclear factor of kappa light chain gene enhancer in B-cells 1	NFκκ1	Mm00476361_m1
Nfkb2	nuclear factor of kappa light chain gene enhancer in B-cells 2	NFκκ2	Mm00479807_m1
Ikbkb	inhibitor of kappaB kinase beta	IKKβ	Mm00833995_m1
Irak1	interleukin-1 receptor-associated kinase 1	IRAK1	Mm00434254_m1
Tollip	Toll interacting protein	Tollip	Mm00445841_m1
Stat4		STAT4	Mm01257238_g1
Pattern recognition receptors			
Clec7a	c-type lectin domain family 7, member a	Dectin-1	Mm00490960_m1
Tlr2	Toll-like receptor 2	TLR2	Mm00442346_m1
Tlr4	Toll-like receptor 4	TLR4	Mm00445274_m1
Ly96	lymphocyte antigen 96	MD-2	Mm00444223_m1

Genes measured by TaqMan low-density array and by TaqMan gene expression assays (in bold, used with ex vivo stimulated cells). PND1 and PND6, *postnatal days 1* and *6*, respectively.

$-(\Delta C_T)$ values using Latentix 2.00 (Latent5 Aps, Copenhagen, Denmark). In PCA plots, similar location (top/bottom or left/right in the plots) of a gene symbol and a sample marker therefore indicates low ΔC_T values, i.e., high relative expression of that particular gene in the sample. Values were normalized by subtracting the mean and dividing by the standard deviation (autoscaling) prior to modeling. *Il6* was excluded from all models because its expression was not detected in 25% of the samples.

RESULTS

The presence of a conventional microbiota, but not L. acidophilus or E. coli, affects intestinal expression of cytokines, chemokines, and signaling molecules in neonatal mice. Microorganisms are involved in the recruitment of immune cells to the gut. A too vigorous influx and activation of neutrophils in the newly colonized gut may, however, be hazardous, leading to acute inflammation and tissue necrosis in the epithelium. Mechanisms that initially help avoiding too rigorous responses are needed to establish tolerance to gut microbes. Moreover, in this period the gut epithelium develops rapidly, in particular in the presence of microorganisms (18). Accordingly, we investigated the effect of colonization on *day 1* and *day 6* postnatally on gene expression of a number of chemokines and other genes involved in recruitment and activation of immune cells as well as genes involved in microbial recognition and response. A conventional microbiota caused significant alterations in intestinal gene expression of a subset of the measured cytokine and chemokine genes on PND1 and PND6, whereas monoassociation with *E. coli* or *L. acidophilus* did not, compared with pups remaining germ-free (Fig. 1*A*, monoassociated groups not shown when not significantly different from germ-free). On PND1, *Cxcl2* and *Tslp* displayed a lower expression in conventionally colonized pups than in germ-free pups. In contrast, on PND6, *Tslp* expression was higher in conventionally colonized pups than in germ-free pups. The genes encoding CCL19 [a chemoattractant for lymphocytes and dendritic cells (11)] and TNF-α were upregulated in conventionally colonized intestines compared with the sterile intestines, but only on PND6. Monocolonization with *L. acidophilus* was the only treatment causing a lower expression of *Il7* compared with germ-free conditions. Gene expression of most of the assayed receptor genes and signaling molecules was only significantly altered by a conventional microbiota (Fig. 1*B*, monoassociated groups not shown) and, except for *Tlr2*, only on PND1. Only expression of *Clec7* and *Stat4* displayed a different pattern, being downregulated by *E. coli* monocolonization, compared with no colonization. Intestinal gene expression of *Tlr2*, *Tlr4*, *Ptprc*, *Ikbkb*, *Nfkb1*, *Nfkb2*, *Ly96*, and *Irak1* was increased by conventionalization compared with germ-free mouse pups. Expression of *Ccl19*, *Tslp*, *Tnf*, *Tlr2/4, Ptprc*, *Ikbkb*, *Nfkb1/2*, *Ly96*, and *Irak1* was furthermore increased with age, probably due to growth of epithelial tissue after birth.

When the individual variation is large relative to the average colonization-induced differences, it can be useful to employ PCA for interpretation of data. In this way, we investigated correlations between all genes measured in the individual samples on PND1. In the PCA score plot (Fig. 2*A*) single animals are indicated as colored "sample markers." The variables measured (in this case expression of genes) are indicated as gene symbols in the corresponding loading plot. If a sample

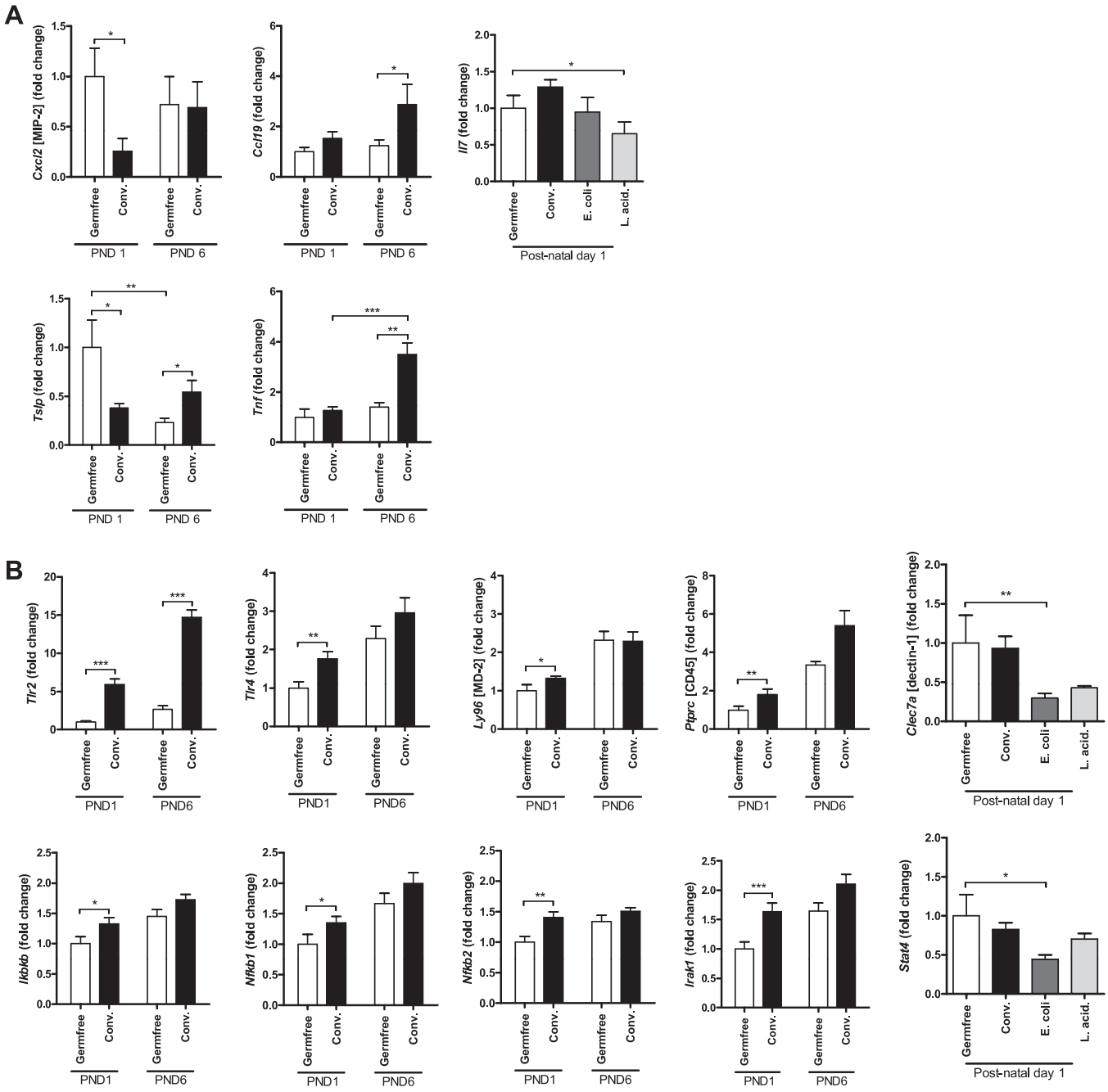

Fig. 1. Expression of chemokines and cytokines in the newly colonized intestine is only substantially altered by a conventional microbiota (Conv.). Expression of 22 genes (Table 1) was measured on the day of birth (PND1) and 5 days later (PND6) in terminal ileal samples of mouse pups born to dams maintained germ free (Germfree) or colonized 7 days after mating with *Lactobacillus acidophilus* NCFM (L. acid.), *Escherichia coli* Nissle, or a conventional microbiota. All groups were included in 2-way ANOVA analyses, but monocolonization groups are only depicted if significantly different from germ free. *A*: significantly altered expression of cytokine and chemokine genes on PND1 and PND6 in conventionally colonized pups compared with germ-free pups. Gene expression at PND1 was different from PND6 for *Tslp* (germ-free) and *Tnf* (conventional). For *Il7*, PND1 gene expression in all colonization groups is shown (no colonization-dependent difference in the expression of this gene was seen on PND6). Data were normalized to *Actb* and then to Germfree PND1, which was defined to 1. Data depicted are means and SE of 6–8 samples (2 pups from each of 3–4 litters). $^{**}P < 0.01$, $^{*}P < 0.05$ compared with Germfree PND1 or Germfree PND6, $^{***}P < 0.001$ for *Tnf* compared with conventional PND1. *B*: significantly altered expression of receptor and signaling molecule genes on PND1 and PND6 in conventionally colonized pups compared with germ-free pups. For *Clec7a* and *Stat4*, PND1 gene expression in all colonization groups is shown (no colonization-dependent difference in the expression of these genes was seen on PND6). All genes were significantly differently expressed at PND1 compared with PND6 ($P < 0.0001$ in 2-way ANOVA including all groups). Data were normalized to *Actb* and then to Germfree PND1, which was defined to 1. Data depicted are means and SE of 6–8 samples (2 pups from each of 3–4 litters). $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$ compared with Germfree PND1 or Germfree PND6.

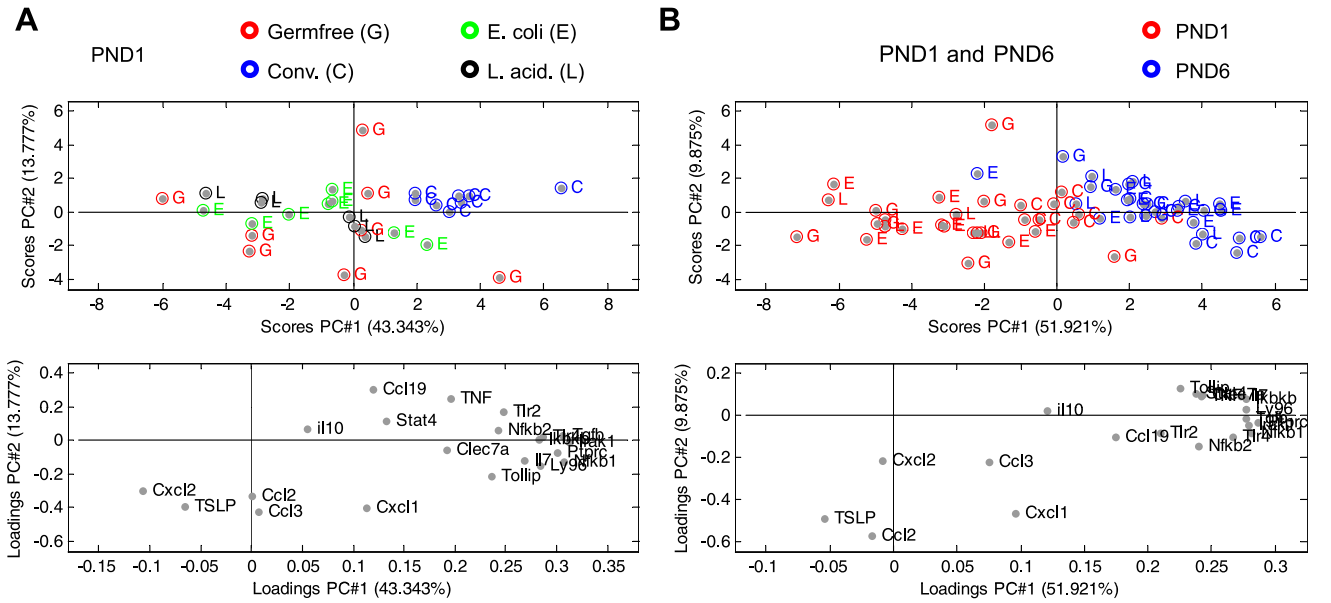

Fig. 2. Samples from pups exposed to a conventional microbiota group distinctly from mono- and noncolonized samples when expression of all measured genes is analyzed together. Gene expression data were obtained as in Fig. 1 and used to generate principal component analysis (PCA) models revealing the main sources of variation in the data set. Plots show scores (samples, *top*) and loadings (gene symbols, *bottom*) for a PCA model of PND1 ileum sample data (*A*) and of PND1 and PND6 ileum samples modeled together (*B*). Each mouse pup is represented by a colored marker. When a marker thus representing a single sample is in the same area of the plot as a gene symbol, this gene was highly expressed in the sample. When markers representing individual samples are close to each other, their expression pattern of all the measured genes was similar. Gene symbols close to each other display similar expression patterns across all samples. A gene symbol far from the origin of the plot displays high variation in expression between samples. Percentages on the plot axes indicate how much of the variation in the data set is described by 1 principal component (PC). Only PC1 and PC2 are shown. *A*: expression of the main part of the investigated genes is strongly affected by the conventional microbiota compared with germ-free and monocolonized animal samples. *B*: when assessing PND1 and PND6 samples together, the time factor appears to be more important than colonization.

marker is located in a area of the score plot that corresponds to a gene symbol in the loading plot, this sample has a high expression of that particular gene. In our analysis, samples from conventionally colonized mice clustered to the right, similarly to most receptor and cytokine genes (Fig. 2*A*). This indicates that these samples were very similar and displayed a high expression of especially *Il10*, *Stat4*, *Nfkb1/2*, *Clec7a*, *Ccl19*, *Tnf*, and *Tlr2* compared with samples from non- and monocolonized animals. In contrast, the majority of the samples from germ-free mice were found in the lower part of the plot, similarly to *Tslp*, *Ccl2*, *Ccl3*, *Cxcl1*, and *Cxcl2* gene symbols, indicating a high expression of this group of genes in germ-free pups. Samples from monoassociated animals were distributed in a pattern resembling samples from germ-free mice. The same grouping of genes and samples was evident when samples of isolated IECs were analyzed (data not shown). When intestinal samples from both PND1 and PND6 were included in a PCA model, PND1 sample markers were found segregating to the left and PND6 samples in the upper right of the plot (Fig. 2*B*), showing that the expression of *Ccl19*, *Tlr2/4*, *Tollip*, *Nfkb2*, and most other genes increased with age in all groups. Again, *Tslp*, *Ccl2*, *Ccl3*, *Cxcl1*, and *Cxcl2* clustered separately from the majority of genes, indicating a different age-dependent induction of these genes (lower expression with higher age). Again, the same pattern was seen when isolated IEC were analyzed (data not shown). Taken together, our data demonstrate that in particular genes encoding chemokines involved in recruitment of neutrophils and monocytes were downregulated by colonization with a conventional microbiota immediately after birth (PND1), followed by a significant upregulation of CCL19, which are involved in recruitment of dendritic cells and lymphocytes, key players in the adaptive immune response, and of TSLP, which modulates dendritic cells to become inducers of tolerance (31) at PND6.

Only a conventional microbiota induces tolerance toward restimulation. To elucidate how neonatal exposure to single bacteria or a full microbiota influences IEC responsiveness, we studied the expression of four cytokines and chemokines in IECs isolated from differentially colonized mice at PND6 and stimulated ex vivo with *E. coli* or *L. acidophilus* (Fig. 3). Ex vivo exposure to *E. coli* significantly increased the expression of *Cxcl2*, *Tnf*, *Il6*, and *Il10* in IEC from germ-free mice (Fig. 2*A*). In contrast, in unstimulated IECs from *E. coli* monoassociated mice, gene transcription was at the same high level as in *E. coli*-stimulated cells (Fig. 3*A*). This could be interpreted as induction of endotoxin tolerance; however, it probably rather indicates a high baseline gene transcription unmodifiable by bacteria added ex vivo. Endotoxin present in the gastrointestinal tract of the *E. coli*-associated mouse pups may cause this high baseline activation. Consistent with this, we measured the endotoxin content in stomachs of mice at PND6 and found high levels in stomachs of *E. coli*-exposed animals (1,942 ± 302 EU/stomach, $n = 3$), low levels in conventional mice (2.6 ± 1.9 EU/stomach), and no detectable endotoxin in stomachs of germ-free animals or *L. acidophilus*-associated animals.

IECs isolated from conventionally and *L. acidophilus*-associated pups responded similarly to cells from germ-free animals to ex vivo *L. acidophilus* stimulation with increased *Il10* expression and upregulated all cytokine and chemokine genes in response to *E. coli* stimulation (Fig. 3*B*). Expression of

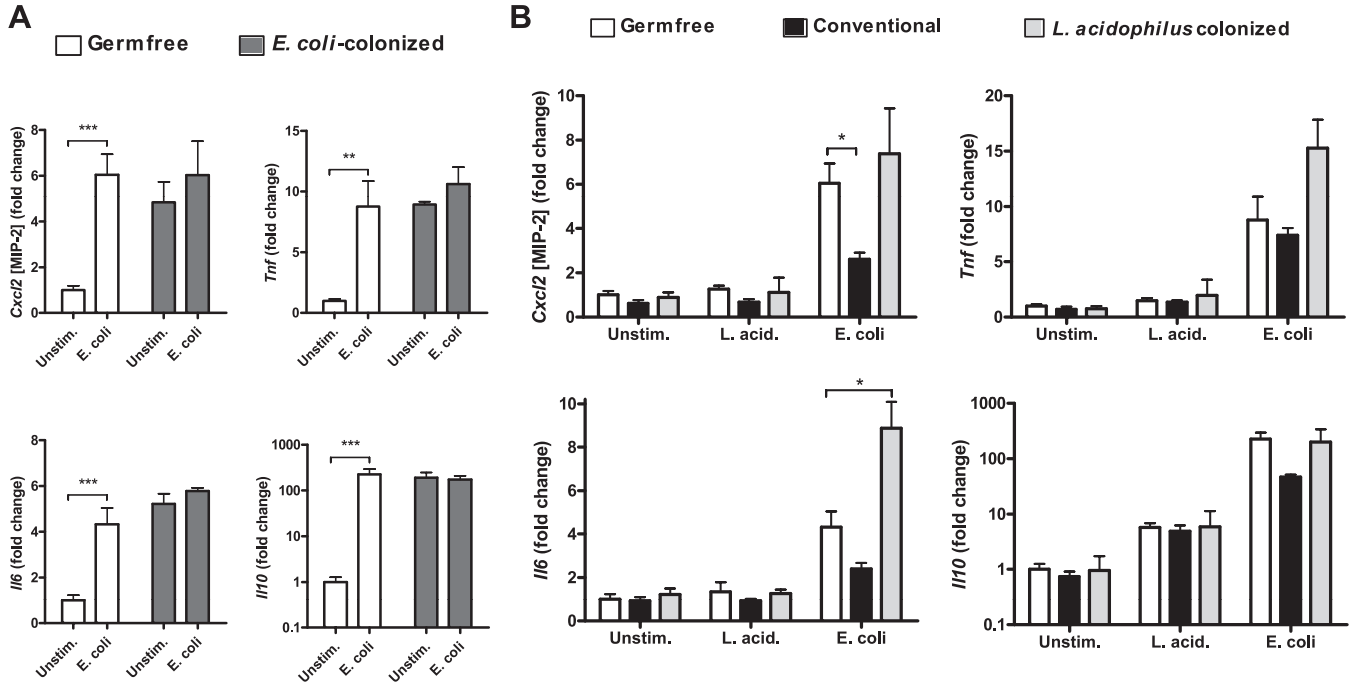

Fig. 3. A conventional microbiota induces *E. coli* hyporesponsiveness whereas *L. acidophilus*-monoassociated mice exert hyperresponsiveness toward *E. coli*. Isolated intestinal epithelial cells (IECs) from neonate mice at PND6 were stimulated for 2 h with *L. acidophilus* NCFM or *E. coli* Nissle. Mothers were either conventional, germ-free, or monocolonized with *L. acidophilus* NCFM or *E. coli* Nissle. *A*: cytokine gene expression response of IECs from germ-free and *E. coli*-colonized pups. *B*: cytokine gene expression response of IECs from germ-free, conventionally colonized, and *L. acidophilus*-colonized pups. Data were normalized to *Actb* and then to Germfree unstimulated (Unstim.), which was defined to 1. Data depicted are means and SE of 3–4 independent experiments with cells pooled from 2–3 pups. $***P < 0.001$, $**P < 0.01$, $*P < 0.05$ compared with IECs from germ-free mice.

Cxcl2 was significantly blunted in response to *E. coli* stimulation in IECs from conventional mice (~3-fold increase with stimulation) compared with IECs from germ-free mice (~6-fold increase with stimulation), suggesting that endotoxin tolerance was induced upon acquisition of a normal microbiota. We have previously measured the concentration of secreted CXCL2 18 h after stimulation of epithelial cells by ELISA and could confirm a close relationship between the gene expression and the secreted protein (34). IL-6 and TNF-α induction by *E. coli* was not significantly blunted in cells from conventionally colonized animals. Enterocytes from pups of mice monocolonized with *L. acidophilus* responded just as potently to *L. acidophilus* and *E. coli* stimulation as IECs from germ-free mice, suggesting that no tolerance to gram-positive or gram-negative bacteria was induced in this group. The data even indicate that *L. acidophilus* colonization induced hyperresponsiveness toward endotoxin stimulation, since *E. coli*-induced *Il6* expression was higher in cells from *L. acidophilus*-associated pups than in germ-free animals.

Cxcl2 expression is lower in the spleen of conventional and E. coli-associated mice on the day of birth compared with germ-free animals. The immature gut is believed to lack integrity, and we speculated that IECs in a period shortly after birth may be bypassed by bacteria translocating to other tissues and that the early response to intestinal colonization may therefore not be limited to IECs. Consequently, we assayed the expression of *Cxcl2* in the spleens from germ-free, monocolonized, and conventionally colonized mouse pups on PND1, since neutrophils are recruited to the spleen during pathogen translocation in mice (21), probably because of upregulation of *Cxcl2*. In addition, *Cxcl2* expression can serve as a measure of NF-κB activation (14). Surprisingly, no activation reminiscent of inflammation in the spleen was observed. Instead, expression of *Cxcl2* was decreased in the spleen of mouse pups born to mothers harboring a conventional microbiota compared with germ-free pups (Fig. 4*A*), and, in contrast to the ileal response (Fig. 4*B*), monoassociation with *E. coli* also reduced *Cxcl2* expression in the spleen. Although purely speculative, our data may indicate that bacterial translocation takes place immedi-

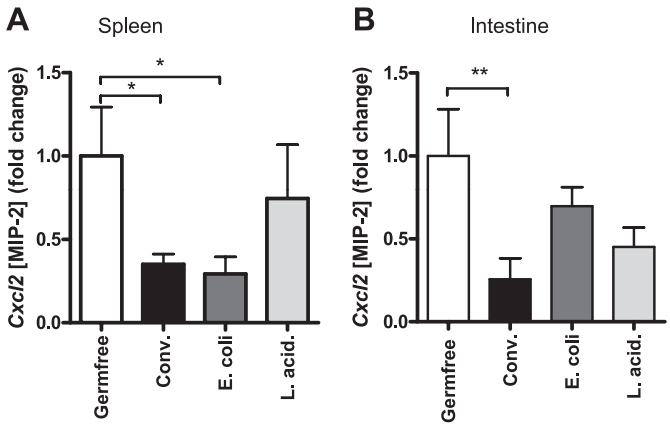

Fig. 4. Expression of *Cxcl2* is reduced in the spleen of conventionally colonized and *E. coli*-colonized mouse pups on the day of birth compared with germ-free mice. Expression of *Cxcl2* was measured on the day of birth in splenic (*A*) and ileal (*B*) samples of mouse pups born to dams maintained germ free or colonized with *Lactobacillus acidophilus* NCFM, *Escherichia coli* Nissle, or a conventional microbiota. Data were normalized to *Actb* and then to Germfree, which was defined to 1. Data depicted are means and SE of 6–8 samples. $**P < 0.01$, $*P < 0.05$ compared with germ-free mice.

ately after birth and that mechanisms exist that help tolerating the presence of bacteria in the spleen.

MLNs of conventionally colonized mice display reduced gene expression of genes involved in antigen presentation compared with PLNs. Immune cell tolerance to the intestinal microbiota is believed to occur through regulatory priming of lamina propria and MLN dendritic cells by TSLP produced by epithelial cells (25) and through provision of retinoic acid to $CD103^+$ dendritic cells that induce regulatory T cells (9). It is not known how intestinal microbes themselves participate in maintaining tolerance, although there is some evidence of bacteria inducing TSLP (35), and bacteria may regulate the retinoic acid available for Treg priming. Assessing expression of our panel of genes in intestinal tissue and spleen at weaning (3 wk of age) revealed no differences between germ-free and conventionally colonized animals (data not shown). We therefore aimed to detect whether conventional colonization induced a distinctive tolerogenic environment in MLN. We dissected MLN and PLN from germ-free and conventionally colonized animals on *day 23* and screened for expression of 90 immune system-related genes. Of these, *Ctla4*, *Cd40*, *Cd80*, *Smad7*, and *Selp* were selectively downregulated in MLN in conventionally colonized compared with germ-free mice (Fig. 5*A*) and were unchanged by colonization in PLN. These are all genes involved in antigen-specific responses, whose downregulation perhaps contributes to the ability to establish oral tolerance. *H2-Ea* and *Stat6* were highly upregulated in both MLN and PLN in conventional mice compared with germ-free mice (Fig. 5*B*), showing that the gut microbiota influences the expression of specific genes systemically.

DISCUSSION

The purpose of this study was to assess whether monocolonization with gram-negative or gram-positive bacteria at birth induces the same pattern of gene expression in IECs as a conventional microbiota, and whether IECs of colonized mice become nonresponsive to the bacterial colonizers.

A conventional microbiota is known to promote tolerance not only to commensal bacteria, but also locally and systemically to food antigens. We observed that early exposure to single strains of *E. coli* and *L. acidophilus* stimulated IECs modestly, whereas a conventional microbiota induced a shift in expression of cytokine and TLR-signaling related genes that set conventional animals apart from germ-free and monoassociated animals both on PND1 and PND6. However, expression of cytokine and TLR-signaling genes was to some extent increased in all animal groups with increased time after birth (Fig. 2*B*), indicating that colonization with a conventional microbiota accelerated the development of the intestine and further matured IECs compared with germ-free conditions.

Lotz et al. (14) have reported that although isolated primary cells from late gestational fetuses respond readily to LPS stimulation by producing KC (CXCL1) and MIP-2 (CXCL2), the LPS response in IECs from conventionally colonized newborn mice is blunted after a surge of chemokine production only hours after birth, suggesting acquisition of postnatal LPS

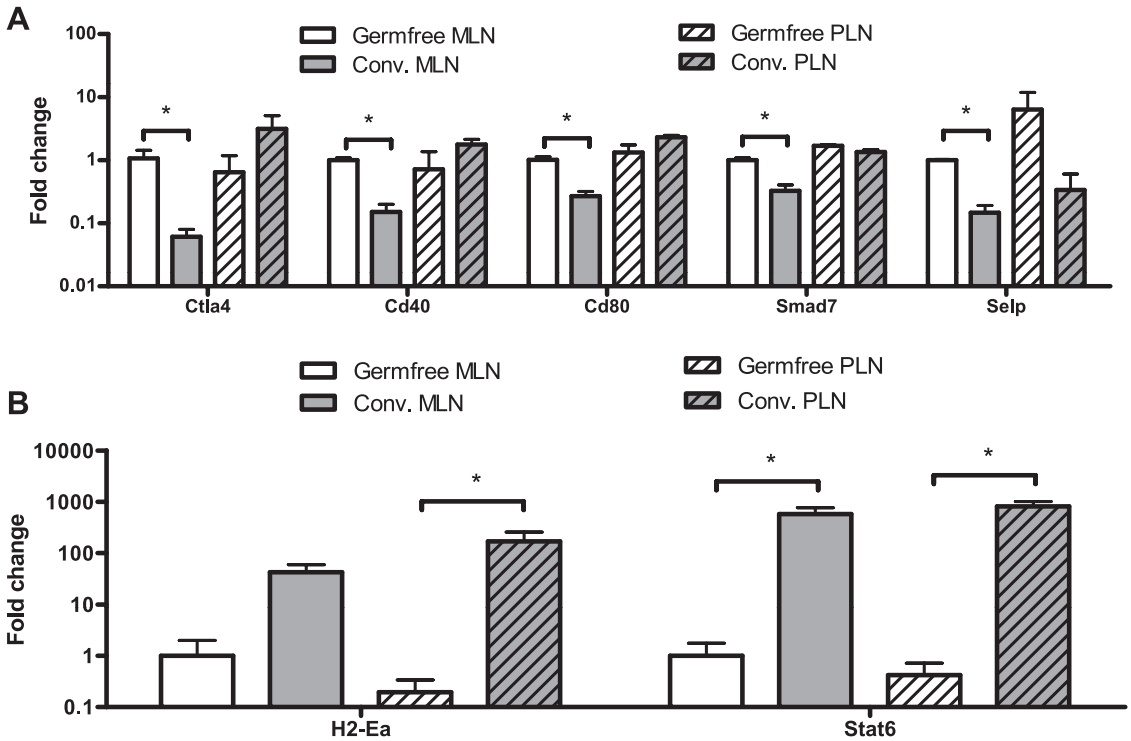

Fig. 5. Expression of genes involved in antigen presentation and T cell activation at weaning is differentially changed in mesenteric (MLN) and popliteal lymph nodes (PLN) of colonized mice compared with germ-free mice. Expression of 90 genes related to immune function was measured at weaning (PND23) in MLNs and PLNs of pups born to dams maintained germ free or colonized with a conventional microbiota. *A*: genes displaying downregulation in MLN but not in PLN of conventional pups compared with germ-free pups. *B*: genes upregulated in both MLN and PLN of pups with a conventional microbiota compared with germ-free pups. Data were normalized to *Actb* and then to Germfree MLN, which was defined to 1. Data depicted are means and SE of 2 mice. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with the same tissue in germ-free mice.

tolerance. This is the only study to date addressing LPS tolerance in neonate primary cells, and it implies that engagement of TLR4 with LPS changes the IEC responsiveness just as published with several immortalized cell lines (1, 4), although through degradation of IRAK-1 (14). We wanted to assess whether tolerance can also be induced to intact gram-negative and gram-positive bacteria in newborn mice. A conventional microbiota induced *E. coli* (i.e., LPS) tolerance, measured as a lower *Cxcl2* gene expression response in conventional IECs compared with germ-free IECs. This difference was not significant for *Il6* or *Tnf* expression, perhaps because CXCL2 is a major chemokine secreted by IEC and its induction is therefore more sensitive to regulation, but the significance of this discrepancy is unknown. No tolerance was induced in gnotobiotic mice only colonized by *L. acidophilus*. On the contrary, cells from this group of mice responded more strongly than the germ-free group by *Il6* expression, which may indicate that monocolonization with certain bacteria, including lactobacilli, may halt the development of intestinal tolerance in some respects. In IECs from *E. coli* monoassociated pups, cytokine and chemokine gene expression was increased to a level nonmodifiable by ex vivo stimulation. This high nonstimulated ex vivo response may reflect an increased sensitivity to culturing after high-dosage LPS exposure in vivo or a high load of LPS present in the isolated IECs "overcoming" tolerance and is in line with the findings that only acquiring a conventional microbiota substantially modulates intestinal cytokine gene expression (Figs. 1 and 2). Although our previous work has demonstrated the importance of assessing gene expression over time in in vitro stimulated cells, because of scarcity of isolated IECs we only measured the ex vivo gene expression after 2 h of stimulation (33, 34). However, when the response of cells from differently colonized mice stimulated with the same bacteria differs, this may indicate differences between the cells, e.g., in expression of pattern recognition receptors.

The differences in gene expression patterns between conventionally and *E. coli*-associated animals exclude the possibility that LPS alone can regulate the intestinal immune maturation guided by IEC chemokines. Importantly, different parts of the immune system have been shown to require stimulation from specific microbes to attain full function. This is true for $CD4^+$ Th cells, which are induced in the spleen by a *Bacteroides* zwitterionic polysaccharide (17), and specifically for $CD4^+$ Th17 cells, which require the presence of segmented filamentous bacteria to reach normal numbers (7, 10). In the ileum, segmented filamentous bacteria (present in a conventional microbiota) changed the expression of more genes than lactobacilli or bifidobacteria alone (29), which corresponds to our observations.

Increased expression of *Tlr4* and especially *Tlr2* was found in mice harboring a conventional microbiota compared with germ-free mice. An upregulation of TLR2 in vivo has been demonstrated to be strongly involved in epithelial repair and integrity of the gut barrier (3, 12), and an intact intestine is believed to be of major importance for the regulation of local and systemic immunity. Hence, establishment of integrity of the epithelial barrier may be one of the major achievements of the first-arriving members of the conventional microbiota. Upregulation of TLR4 has also been reported in newly colonized adult animals (7). Downstream signaling molecules in the TLR signaling cascade were slightly upregulated, perhaps indicating a more rapid growth of the gut epithelium in the conventionalized mice. Also the genes encoding TNF-α, CCL19, and other genes involved in recruitment of cells of the adaptive immune system were upregulated at PND6 in mice with a conventional microbiota compared with germ-free mice (Fig. 1).

IL-7 is a cytokine involved in B and T cell development and may thus play an important role in the maturation of the gut immune system. Whether the lower expression in *L. acidophilus*-associated animals has any significance cannot be concluded alone from the presented data.

The group composed of *Ccl2*, *Ccl3*, *Cxcl1*, *Cxcl2*, and *Tslp* were seemingly regulated independently of all other genes (Fig. 2, *A* and *B*). Our data on the expression of *Tslp* suggest that the amount of TSLP drops shortly after birth and then increases again and that the microbiota speeds up this maturation process. TSLP plays an important role in maintaining a tolerant state in the gut and promotes Th2 responses (31). One possible explanation for the drop in *Tslp* expression early after birth could be that it delays antibody responses against the beneficial microbiota. This is, however, purely speculative.

Downregulation of the four chemokines may indicate that in particular recruitment of cells of the innate immune system should not be too vigorous during the very early colonization. This may be an important first step in establishing tolerance toward the gut microbiota. Overall, our analysis indicates that intestinal transcriptional regulation of TSLP and the major neutrophil and macrophage attractant chemokines is fundamentally different from transcriptional regulation of the assayed cytokines, receptors, and transcription factors in the early postnatal period. This dichotomy may indicate that mechanisms specialized to cope with the dramatic change from fetus to individual organisms exist, to allow the presence of the establishing microbiota.

We and others (14, 34) have previously demonstrated that *Cxcl2* expression is lower in IECs in late gestational fetuses than on PND1. However, unlike Lotz et al. (14), we did not observe a higher *Cxcl2* expression on PND 1 compared with PND6 selectively in conventional animals, but in all groups (Fig. 2*B*), and *Cxcl2* was downregulated both with increasing age and by colonization. The higher chemokine expression in young, germ-free animals may indicate that proinflammatory chemokines are necessary to recruit resident phagocytic cells in the fetal state both in the spleen and in the mucosa but are downregulated by the microbiota after birth. This may be due to a postnatal degradation of IRAK-1 protein as shown by Lotz et al., and our finding that *Irak1* expression increased with age indicates the importance of a constant expression of this gene for a rapid change in response during, e.g., virus infection. Perhaps representing an important prenatally recruited population, resident chemokine (C-X3-C motif) receptor 1-positive intestinal macrophages have been shown to be crucial for the induction of tolerance to food antigens (8, 14).

The increased CD45 expression with colonization and age may indicate recruitment of intraepithelial lymphocytes. On PND6, $<1\%$ of isolated IECs was CD45 positive (data not shown). Because isolated IECs (data not shown) display the same gene expression pattern as the intact intestine tissue (Fig. 2, *A* and *B*), our data mainly describe how colonization impacts on cells in the epithelial lining, although we cannot exclude

that early colonization also affects the lamina propria immune cell compartment (20).

Stat4 and *Clec7* were the only genes expressed to a lower degree in *E. coli*-exposed intestine than in the germ-free intestine on PND1. The Dectin-1 encoding gene *Clec7* is also downregulated by *E. coli* in IECs in vitro (34), probably excluding an effect of *E. coli* colonization on the number of dendritic cells in the intestinal mucosa [Dectin-1 may be used as a dendritic cell marker in some tissues but it cannot differentiate dendritic cell from IECs (26, 34)].

Downregulation of *Cxcl2* expression in the spleen of conventionally colonized animals already within the first 8 h after birth accompanied the reduced expression of this chemokine in the intestine. This finding suggests that the systemic immune system participates in tolerance induction to the commensal microbiota, preventing excessive inflammation very early in life. An overview of the most important findings is shown in Fig. 6.

At 3 wk of age, when a more complex microbiota is establishing itself because of consumption of solid food (28), the gene expression of cytokines, chemokines, and signaling molecules in ileal tissue and spleen did not differ between conventional and germ-free animals (data not shown). Hereby our study underlines that, after PND1, the microbiota primarily speeds up the maturation process during the first weeks of life and that differences in gene expression in germ-free animals colonized at a certain age does not necessarily reflect the normal development of the symbiotic relationship between host and microbiota. In contrast, gene expression in the intestinal-draining MLNs from conventionalized animals differed from that of the distant PLN cells at weaning. The tolerogenic nature of MLNs, which continuously sample the commensal microbes arriving as "cargo" of intestinal dendritic cells (16), was reflected in the downregulation of *Cd40*, *Cd80* (T cell costimulatory molecules), and *Smad7* (inhibitor of immune regulatory TGF-β signaling). *Ctla4* was also selectively downregulated in MLNs, perhaps indicating a reduced need for T cells to evade stimulatory signals when local antigen-presenting cells display an anti-inflammatory phenotype. Finally, *Selp* was downregulated in MLNs by microbial colonization. P-selectin, encoded by *Selp*, recruits neutrophils to inflammatory sites, a process that may require regulation for the immune system not to overreact to the commensal microbiota. The selective decrease in expression of antigen-presenting relevant genes in MLN may reflect that MLN cells should rarely mount a strong immune response to commensal microorganisms, whereas the presence of bacteria in PLN would indicate infection.

Strikingly, histocompatibility 2, class II antigen E alpha (H2-Ea, the mouse homologue of HLA-DR) expression was 40-fold higher in MLN and 800-fold higher in PLN in conventional mice compared with germ-free mice at weaning. The number of MHC-II molecules has previously been shown to increase in the kidneys of recently colonized germ-free mice (6), but such a strong systemic increase of MHC-II expression with colonization has not been reported before.

STAT6 is the primary signaling molecule responding to ligation of the IL-4 and IL-13 receptor, and its activation is

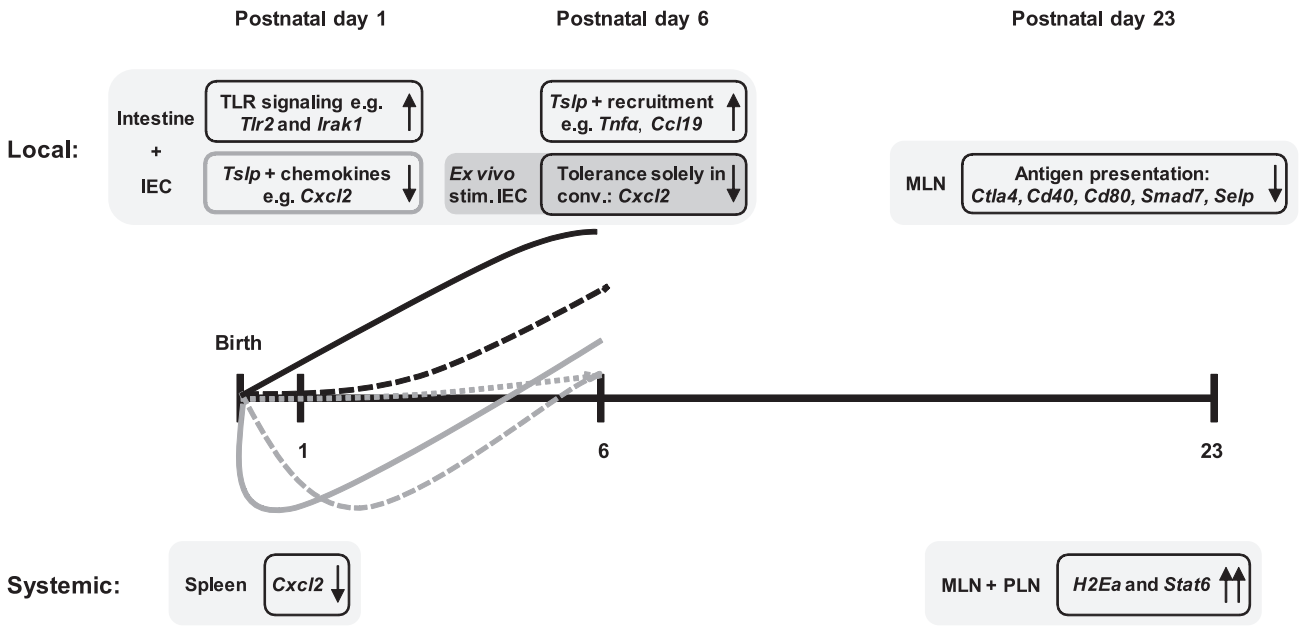

Fig. 6. Summary diagram encapsulating the major findings of the study. Arrows indicate levels of gene expression in tissue from conventionally colonized pups compared with germ free. In conventionalized mice, the drop in expression of the chemokine gene, *Cxcl2* at PND1 coincides with a drop in *Cxcl2* expression in the spleen at *day 1* and with a lack of *Cxcl2* responsiveness in PND6 gut epithelial cells to ex vitro stimulation with *E. coli*. (Stim.IEC) At weaning (*postnatal day 23*), specific genes involved in antigen presentation were downregulated in the gut-associated MLN of conventional pups compared with germ free, but not in the distant PLN, whereas the expression of *H2Ea* and *Stat6* was strongly upregulated in both types of lymph nodes, indicating that the microbiota also affects the systemic immune system. The time-course curves indicate a suggested model for development in intestinal gene expression: For many genes a microbiota is dispensable for expression, but the microbiota accelerates the increase in their expression (black lines, solid line: conventional mice; dashed line, germ-free mice). For a number of genes (chemokine and *Tslp*) the development of expression undergoes 2 phases: initially a decrease in the expression, followed by an increase in expression (gray lines). This development seems to be microbiota dependent, and the drop in expression in the germ-free mice may occur later (dashed line) or may not take place at all (dotted line).

required for Th2 polarization (22). *Stat6* expression was increased more than 500-fold in both MLN and PLN of colonized mice, compared with germ-free pups that expressed hardly any *Stat6*. Downregulation of *Stat6* was found in the ileum of adult conventionalized mice by Gaboriau-Routhiau et al. (7), but high *Stat6* expression in lymph nodes has not previously been correlated with the presence of a microbiota. On the contrary, IL-4 responses have been shown to be conserved under germ-free conditions (30), so the role of more *Stat6* transcripts is unknown. Of note, STAT6 has been shown to play a key role in the regulation of inflammation through NF-κB and to be involved in the protection against sepsis (13). Interestingly, IL-4 and IL-13, both signaling through STAT6, are the only cytokines able to limit IEC chemokine secretion (15). Thus the upregulation of STAT6 in MLN and PLN may constitute an important regulatory mechanism to avoid vigorous responses to commensal microorganisms. In addition, *H2Ea* (MHC-II) and especially *Stat6* expression may serve as markers for early systemic immune maturation enhanced by colonization.

A major limitation of the present study is that we did not confirm differences in mRNA levels on the protein level. Accordingly, we cannot conclude that upregulated genes actually result in functional proteins. However, we have previously demonstrated that *Cxcl2* gene expression is reflected in secreted protein in in vitro stimulated germ-free IECs (34). As regards the genes encoding for CCL2, CCL3, CXCL1, CXCL2, and TSLP exhibiting a downregulated expression in conventionalized mice compared with germ-free, we may expect that microorganisms are involved in a specific suppression of the transcription of these genes. The suppression of *Cxcl2* expression took place both in the gut as well as in the spleen, but whether the suppression in the spleen was indirectly mediated or a consequence of microorganisms present in the spleen due to translocation from the gut remains to be established. Reduced protein expression of this important chemokine, as a consequence of conventional colonization, also remains to be confirmed in future studies, in vivo in the intestine and spleen as well as ex vivo in bacteria-stimulated IECs. Likewise, investigation of an extended number of cytokines may add to the understanding of the postnatal events in the intestine. In conclusion, a complex microbiota, but not single bacterial strains, reduced the gene expression of proinflammatory chemokines in vivo and induced tolerance toward *E. coli* stimulation ex vivo. Conventional colonization was also required for a MLN-specific tolerogenic gene expression profile to develop. Overall, our data support the importance of a diverse microbiota for mucosal as well as systemic immune tolerance development.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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PAPER 4

"*Lactobacillus acidophilus* induces a slow but more sustained chemokine and cytokine response in naïve foetal enterocytes compared to commensal *Escherichia coli*"

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Lactobacillus acidophilus induces a slow but more sustained chemokine and cytokine response in naïve foetal enterocytes compared to commensal *Escherichia coli*

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Abstract

Background: The first exposure to microorganisms at mucosal surfaces is critical for immune maturation and gut health. Facultative anaerobic bacteria are the first to colonise the infant gut, and the impact of these bacteria on intestinal epithelial cells (IEC) may be determinant for how the immune system subsequently tolerates gut bacteria.

Results: To mirror the influence of the very first bacterial stimuli on infant IEC, we isolated IEC from mouse foetuses at gestational day 19 and from germfree neonates. IEC were stimulated with gut-derived bacteria, Gram-negative *Escherichia coli* Nissle and Gram-positive *Lactobacillus acidophilus* NCFM, and expression of genes important for immune regulation was measured together with cytokine production. *E. coli* Nissle and *L. acidophilus* NCFM strongly induced chemokines and cytokines, but with different kinetics, and only *E. coli* Nissle induced down-regulation of Toll-like receptor 4 and up-regulation of Toll-like receptor 2. The sensitivity to stimulation was similar before and after birth in germ-free IEC, although Toll-like receptor 2 expression was higher before birth than immediately after.

Conclusions: In conclusion, IEC isolated before gut colonisation occurs at birth, are highly responsive to stimulation with gut commensals, with *L. acidophilus* NCFM inducing a slower, but more sustained response than *E. coli* Nissle. *E. coli* may induce intestinal tolerance through very rapid up-regulation of chemokine and cytokine genes and down-regulation of Toll-like receptor 4, while regulating also responsiveness to Gram-positive bacteria.

Background

The human gastrointestinal (GI) tract, the largest surface area of the body in contact with the environment, is lined by a single layer of intestinal epithelial cells (IEC). In adults, the GI tract is colonised by more than 10^{14} microorganisms comprising more than 500 different phylotypes [1]. The gut microbiota is pivotal for the development and maintenance of intestinal immunological homeostasis. The intestinal epithelium plays key roles in maintaining this immune homeostasis in the gut as an active player in maintaining tolerance to the microbiota and food antigens as well as in pathogen combat.

The GI tract of the foetal baby is sterile, but colonisation starts immediately after birth with bacteria from the mother and the environment and, within a few days, it is colonised by numerous bacterial species. These pioneer bacteria have been shown to modulate gene expression in IEC including genes involved in metabolism, absorption, barrier function and IEC maturation [2]. Colonisation at birth by facultative anaerobes, such as enterobacteria, coliforms, lactobacilli and streptococci, creates a reducing environment during the first week of life enabling colonisation by strict anaerobes including bifidobacteria, bacteroides, clostridia and eubacteria [3]. This microbial colonisation contributes to recruitment of immune cells to the GI tract and may

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furthermore be a major contributor to establishment of the systemic immune system [4,5]. Thus colonisation in early infancy is crucial in relation to the final composition of the permanent microbiota in adults and also in inducing intestinal and immunological maturation.

IEC sense commensals through expression of pattern recognition receptors (PRRs) recognising conserved microbial structures. The IEC respond by secreting a wide range of chemokines that recruit immune cells to the GI tract, and cytokines that affect the immune cells scattered in the GI tract including DC, macrophages and lymphocytes [6-9]. Due to the heavy bacterial antigen load in the lumen, the expression of PRRs is tightly regulated in IEC. IEC express Toll-like receptor (TLR) 1-9 [10], nucleotide-binding oligomerisation domain (NOD) 1 and NOD2 [11]. However, contradicting data from cell line studies on the expression of TLRs in IEC exist. Several reports demonstrate that IEC are non-responsive towards lipopolysaccharide (LPS) and express no or very low levels of TLR4 [12,13], while other groups have reported the presence of TLR4 [10,14,15]. This discrepancy may be explained by the finding that IEC gain a cross-hyporesponsive phenotype after stimulation with either LPS or lipoteichoic acid due to decreased signalling through TLR2 and TLR4 [10]. Cario et al. elegantly demonstrated that both TLR2 and TLR4 are constitutively expressed apically in an IEC cell line but traffic to cytoplasmic compartments after ligand stimulation [14]. IEC isolated from intestinal tissue express *Tlr2* and *Tlr4* mRNA but at low levels both in humans [16] and mice [17]. Knowledge on IEC responses to microbe-associated molecular patterns (MAMPs) is to a large extent based on cell line studies as cell lines are naïve to MAMP stimulation. However, cell lines may not entirely reflect IEC responses at birth.

Besides playing a role in the recruitment and maturation of immune cells in the GI tract, the bacteria colonising the sterile gut probably induce tolerance dependently on TLR-activation [18]. In this respect, the MAMPs present in the first-coming species might be crucial in tolerance development. It was recently demonstrated that, although both foetal and neonatal IEC express the TLR4/MD-2 receptor complex, they differ dramatically in their responsiveness to LPS, and it was suggested that intestinal bacterial colonisation in the newborn is facilitated by postnatal establishment of IEC tolerance towards LPS stimulation [19]. Moreover, IEC help maintaining the specialised intestinal tolerogenic environment through secretion of different mediators, such as thymic stromal lymphopoietin (TSLP) and transforming growth factor (TGF)-β and commensals differentially affect TSLP and TGF-β production [20,21]. Thereby the composition of the microbiota indirectly affects immune cells through effects on IEC.

We hypothesized that the very first bacteria encountered by naive IEC influence the signal molecules released to the gut environment, and that Gram-positive (G^+) commensals prime IEC differently from Gram-negative (G^-) commensals and LPS. Moreover, the developmental state of the IEC may play a role in their responsiveness, and this study is the first to compare foetal and neonatal germfree murine IEC responsiveness to G^+ (*Lactobacillus acidophilus*) and G^- (*Escherichia coli*) commensals *in vitro*. We present indices that the type of bacterial stimulus indeed affects gene expression in naïve primary IEC, thus suggesting an important role of the first postnatal bacteria for immune cell recruitment and tolerance induction in the GI tract.

Results

L. acidophilus and *E. coli* strongly induce chemokine gene expression in foetal primary epithelial cells in vitro

In the first days of life, recruitment of immune cells to the gut is probably one of the most important aspects of gut immune maturation. In this respect, IEC play a pivotal role by secreting chemokines attracting specific immune cells. We speculated that the composition of the gut microbiota affects this maturation process by affecting the chemokine expression in IEC, and therefore studied how expression of a set of chemokines in foetal near-term IEC was affected by *in vitro* bacterial stimulation with two gut-derived commensals (Figure 1). As representatives of gut G^+ and G^- commensals we chose *E. coli* Nissle and *L. acidophilus* NCFM [22] as these strains in earlier studies were found to be potent stimulators of epithelial cell lines [21]. *E. coli* was most potent in up-regulating *Cxcl1*, *Cxcl2*, *Ccl2* and *Ccl3* encoding keratinocyte-derived chemokine (KC), macrophage-inflammatory protein (MIP)-2, monocyte chemoattractant protein (MCP)-1 and MIP-1α respectively. Generally, chemokine expression induced by *E. coli* did not increase from 2 to 4 h, whereas induction of *Ccl3* (encoding MIP-1α) by *L. acidophilus* reached transcription levels induced by *E. coli* only at 4 h.

L. acidophilus and *E. coli* up-regulate gene expression of pro-inflammatory and regulatory cytokines in foetal epithelial cells in vitro

As opposed to chemokine production, cytokine secretion by primary IEC upon bacterial stimulation via their PRRs remains poorly described. Hence we evaluated how *L. acidophilus* and *E. coli* modulate the cytokine environment upon engagement of PRRs *in vitro* in foetal IEC (Figure 2). *L. acidophilus* and *E. coli* induced expression of interleukin (*Il*)*6*, *Il10* and tumour necrosis factor (*Tnf*) in a dose-dependent manner. *E. coli* and LPS induced *Il6*, *Il10* and *Tnf* faster than *L. acidophilus*. After 4 h of stimulation, the expression of *Il6* and *Tnf*

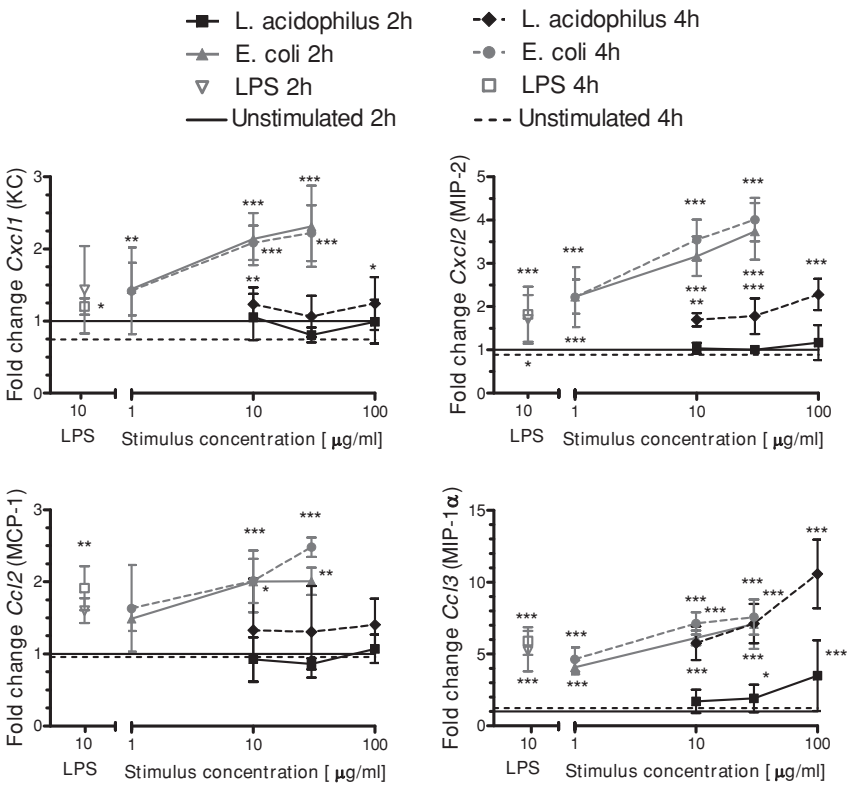

Figure 1 *L. acidophilus* and *E. coli* strongly induce chemokine expression in foetal primary epithelial cells in vitro. Epithelial cells from foetuses at Day-1 were stimulated for 2 h and 4 h with LPS (10 μg/ml), *L. acidophilus* NCFM (10, 30 and 100 μg/ml) or *E. coli* Nissle (1, 10, 30 μg/ml). Gene expression was measured by RT-PCR. Symbols indicate mean fold increase and SD of 4 independent experiments with cells pooled from 6-10 pups. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ compared to "unstimulated cells". Data were normalised to *Actb* and then to the average of "unstimulated cells 2 h" from the 4 experiments, which was defined to 1.

induced by *L. acidophilus* reached levels induced by *E. coli*. Induction of *Il10* by *E. coli* peaked at 2 h while *Il10* induction by *L. acidophilus* was strongest at 4 h. These differences might imply differences in the kinetics of the two distinct PRR signalling pathways, with signalling through TLR4 being faster than signalling through TLR2. TGF-β and TSLP are known to be secreted by IEC and to induce a tolerogenic DC phenotype [21], hence we also looked at transcription of genes encoding TSLP and TGF-β1. Expression of *Tgfb1* was not significantly changed upon *in vitro* stimulation with either bacteria (data not shown), while low concentrations of *E. coli* modestly up-regulated expression of *Tslp* and the highest concentration of *L. acidophilus* down-regulated expression of *Tslp* at 4 h.

Foetal epithelial cells produce cytokines upon in vitro stimulation with Gram-positive and Gram-negative commensals with different kinetics

As cytokine production by IEC has been reported to be low [17], we wished to validate the high increases in expression of *Il6*, *Tnf* and *Il10* by measuring protein secretion from *in vitro* stimulated foetal IEC by enzyme-linked immunosorbent assay (ELISA) at 2 h, 4 h and 18 h of culture (Figure 3). We also measured MIP-2, known to be secreted by IEC [19]. Production of the four proteins correlated well with the transcription levels shown in Figure 1 and 2, as expression of all genes in IEC stimulated with *L. acidophilus* was increasing from 2 h to 4 h, and protein concentrations after 18 h culture were highest for these cells. For *E. coli*-stimulated cells, gene expression was increasing (*Il6*), maintained (*Tnf* and *Cxcl2*) and decreasing (*Il10*) over time and, accordingly, protein levels after 18 h were higher, unchanged, slightly higher and lower for IL-6, TNF-α , MIP-2 and IL-10, respectively, than at the early time points. The chemokine MIP-2 was produced in the highest amounts, but also significant amounts of the three cytokines were produced. The difference between the bacteria again points towards a later induction of certain immunological markers by *L. acidophilus* compared to *E. coli*. LPS induced a higher IL-10 production than *E. coli* after 18 h, perhaps reflecting that LPS-induced expression of *Il10* was not

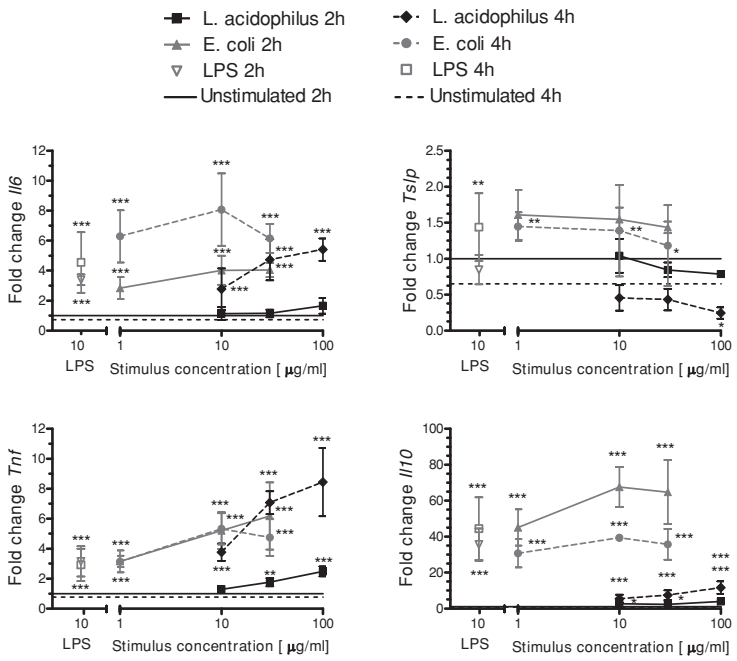

Figure 2 *L. acidophilus* and *E. coli* up-regulate expression of pro-inflammatory and regulatory cytokines in foetal epithelial cells in vitro. Epithelial cells from foetuses at Day-1 were stimulated for 2 h and 4 h with LPS (10 μg/ml), *L. acidophilus* NCFM (10, 30 and 100 μg/ml) or *E. coli* Nissle (1, 10, 30 μg/ml). Gene expression was measured by RT-PCR. Symbols indicate mean fold increase and SD of 4 independent experiments with cells pooled from 6-10 pups. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ compared to "unstimulated cells". Data were normalised to *Actb* and then to the average of "unstimulated cells 2 h" from the 4 experiments, which was defined to 1.

decreasing from 2 h to 4 h as was *E. coli*-induced *Il10* expression.

E. coli is more potent than *L. acidophilus* in up-regulating *Tlr2*, *Nfkb1*, and *Nfkb2* gene expression and in down-regulating expression of *Tlr4* and *Clec7a* genes in foetal epithelial cells

When IEC sense bacteria, the first and primary interaction is between PRRs and their bacterial ligands. Signalling through the different cascades downstream of PRRs (MyD88 pathway or TIR-domain-containing adapter-inducing interferon-β (TRIF) pathway) then activates transcription of effector genes including genes encoding cytokines and chemokines. Hence, we evaluated the expression of genes encoding TLR2, TLR4, Dectin-1 and MD-2 in near-term foetal IEC after *in vitro* stimulation with *E. coli* Nissle and *L. acidophilus* NCFM. As depicted in Figure 4, *E. coli* (and pure LPS), even at a low concentration (1 g/ml), down-regulated expression of *Tlr4* significantly. On the contrary, *Tlr2* expression was strongly enhanced by *E. coli* with strongest effects after 4 h stimulation. Although their transcripts were detected in IEC, no changes in expression of the genes encoding MD-2 (Ly96), IRAK1, IKKβ or Tollip, all recognised to be important regulators of TLR4 signalling were observed (data not shown). Dectin-1 is a PRR known to recognise fungal β-1,3 and β-1,6 linked glucans, which in a Ca^{2+} independent manner enhances phagocytosis [23]. Dectin-1 is expressed by DC, monocytes, neutrophils, macrophages and in Caco2 IEC [24], but has not previously been studied in primary IEC. As shown in Figure 4, *Clec7a* encoding Dectin-1 was significantly down-regulated after 4 h stimulation with both LPS and *E. coli*. The down-stream signalling cascade after TLR activation involves nuclear factor (NF)κB. Both *Nfkb1* and *Nfkb2* were significantly up-regulated at 4 h upon *E. coli* stimulation, but this was not seen after stimulation with *L. acidophilus*.

Age dependent gut maturation does not influence early responses of epithelial cells towards Gram-positive and Gram-negative commensals

To unravel how age influences the IEC responsiveness independently of the microbiota we studied expression of 5 selected genes after *in vitro* stimulation with *L. acidophilus* and *E. coli* in primary IEC isolated from germfree mice at Day-1, post-natal day (PND)1 and PND6 (Figure 5). By keeping the mice germfree the only fluctuations in gene expression observed would be an effect of immune maturation with age. Interestingly, a significant transient drop in expression of *Tlr2* was observed at PND1 in unstimulated IEC and *L.*

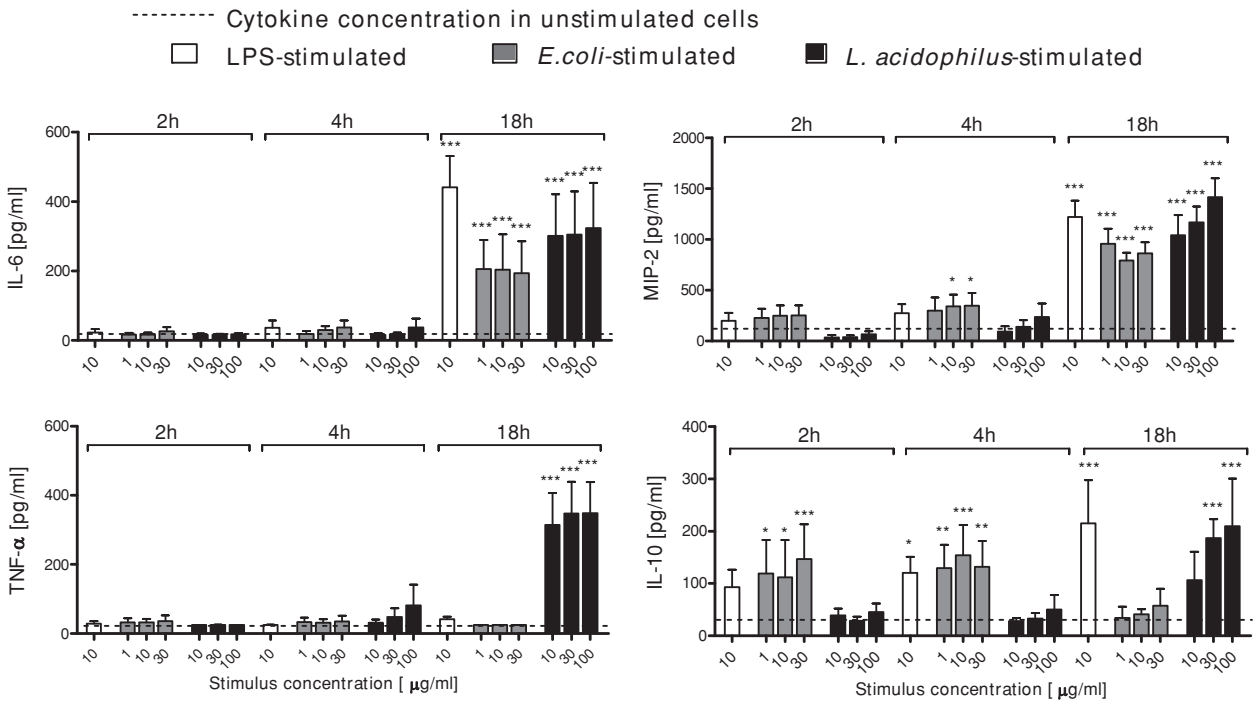

Figure 3 Foetal epithelial cells produce cytokines upon in vitro stimulation with G^+ and G^- commensals with different kinetics. Cytokine and chemokine production in epithelial cells from foetuses at Day-1 after 2 h, 4 h and 18 h stimulation with LPS (10 μg/ml), *L. acidophilus* NCFM (10, 30 and 100 μg/ml) or *E. coli* Nissle (1, 10, 30 μg/ml) measured by ELISA. Symbols indicate mean and SD of 4 independent experiments with cells pooled from 6-10 pups. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ compared to "unstimulated cells" represented by dotted lines.

acidophilus stimulated IEC. This could be a mechanism that allows the G^+ microbiota to establish at birth. The lower *Tlr2* expression was accompanied by a decreased expression of *Il10* and *Tnf* and an increased expression of *Cxcl2* on PND1 in unstimulated cells. However, age did not significantly influence the response towards *L. acidophilus* and *E. coli*.

Discussion

We here demonstrate that *L. acidophilus* NCFM and *E. coli* Nissle potently induce pro-inflammatory genes, a number of tolerance related genes, as well as genes involved in recruitment of immune cells to the GI tract. Although G^- bacterial stimulation impacts faster than G^+ bacteria, G^+ bacterial stimulation elicits a more sustained response giving rise to higher production of cytokines and chemokines. Moreover, *E. coli* induces transcription of *Tlr2*, a receptor for many G^+ bacteria, and down-regulates transcription of the LPS receptor, TLR4. *Tlr2* expression was reduced immediately after birth independently of stimulation, but responses to stimulation were similar in IEC isolated from germ-free mice before and after birth.

Upon *E. coli* stimulation, foetal IEC expressed genes encoding the anti-inflammatory cytokines IL-10 and TSLP, and the chemokines KC, MIP-2 and MCP-1 potently and rapidly when compared to *L. acidophilus* stimulation. Relating the transcription data to the amount of protein measured in 18 h culture supernatant suggests a fast and transient up-regulation of cytokine and chemokine production induced by *E. coli* compared to a slower and more sustained up-regulation induced by *L. acidophilus*. This could be interpreted as fast induction of endotoxin tolerance during the culture period by *E. coli*. In line with our earlier studies in Caco2 cells [21], the two distinct bacteria differentially affected *Tslp* expression: *E. coli* enhanced *Tslp* expression, whereas *L. acidophilus* slightly reduced it. Production of TSLP by IEC is pivotal in maintaining gut homeostasis and indices on lower expression of *Tslp* in primary IEC from Crohn's disease patients than in healthy individuals have been reported [20]. The first microbial stimuli in neonate life probably affect TSLP production, which impacts on DC in the gut and thereby immune homeostasis. Pro- and anti-inflammatory gene expression as well as *Tlr4* downregulation induced by *E. coli* may be indispensable for balancing the immune system in the gut and, as LPS exhibited similar effects, other G^- bacteria may have the same role.

Epithelial cell lines have been shown to develop a cross hypo-responsive phenotype after exposure to TLR2 and TLR4 ligands, probably due to altered

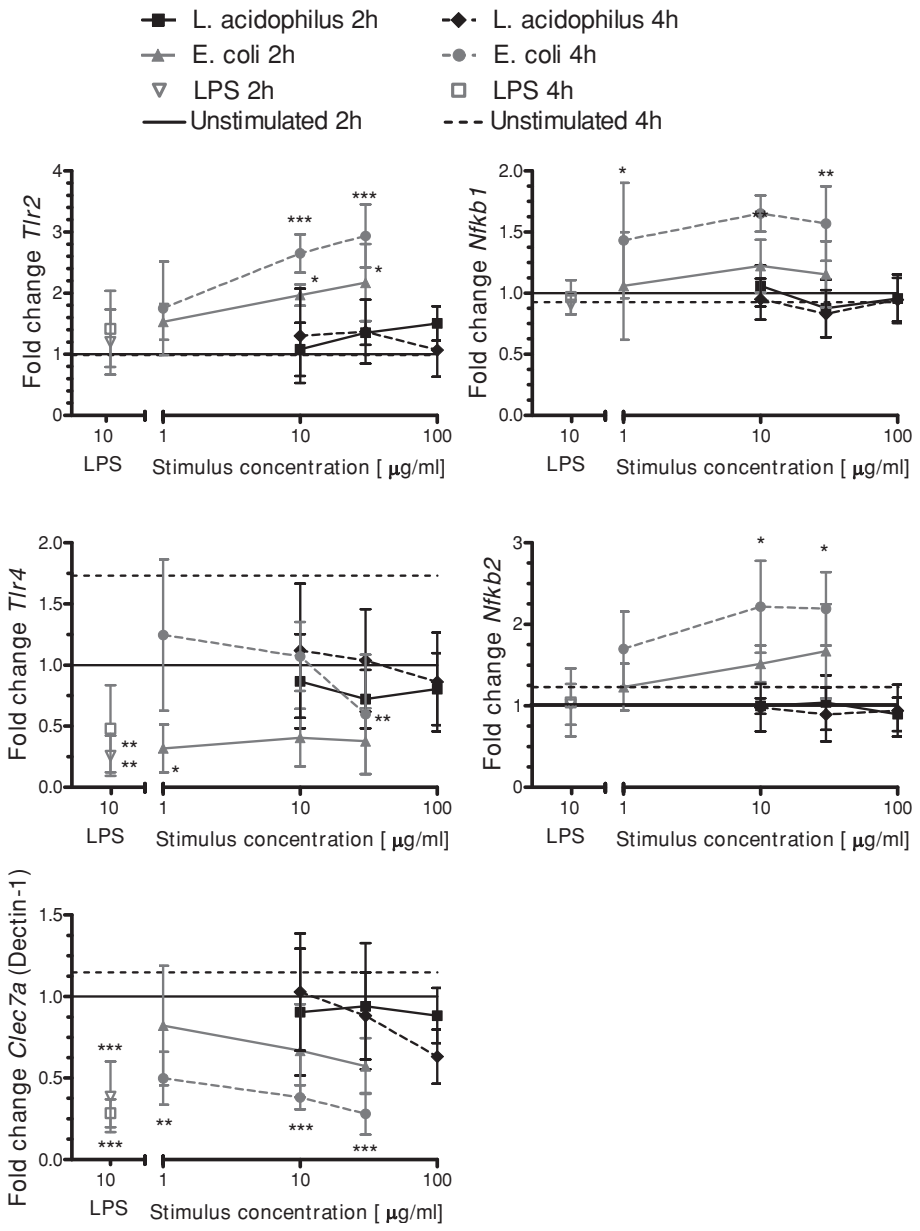

Figure 4 *E. coli* is more potent than *L. acidophilus* in up-regulating Tlr2, Nfkb1, and Nfkb2 expression and in down-regulating expression of Tlr4 and Clec7a in foetal epithelial cells. Epithelial cells from foetuses at Day-1 were stimulated for 2 h and 4 h with LPS (10 μg/ml), *L. acidophilus* NCFM (10, 30 and 100 μg/ml) or *E. coli* Nissle (1, 10, 30 μg/ml). Gene expression was measured by RT-PCR. Symbols indicate mean and SD of 4 independent experiments with cells pooled from 6-10 pups. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ compared to "unstimulated cells". Data were normalised to *Actb* and then to the average of "unstimulated cells 2 h" from the 4 experiments, which was defined to 1.

signalling through TLR2 and TLR4 [10]. However, no studies have reported how these PRRs are regulated in primary IEC from a sterile gut upon first microbial encounters. We found that *E. coli* induced up-regulation of *Tlr2* and down-regulation of *Tlr4*. It could be postulated that if the pioneer bacteria colonizing the sterile gut is a G$^-$strain, LPS will down-regulate expression of TLR4 to enable LPS tolerance to be established. However, at 4 h the down-regulation seen at 2 h already approached the basal expression level (non-stimulated). This points towards a transient down-regulation of TLR4 by G$^-$ bacteria-derived MAMPs, underlining that the expression of PRRs is tightly regulated in a dynamic fashion in order to initiate a quick response. As expression of *Tlr2* was induced by *E. coli*, G$^-$ commensals may induce cross hyper-responsiveness

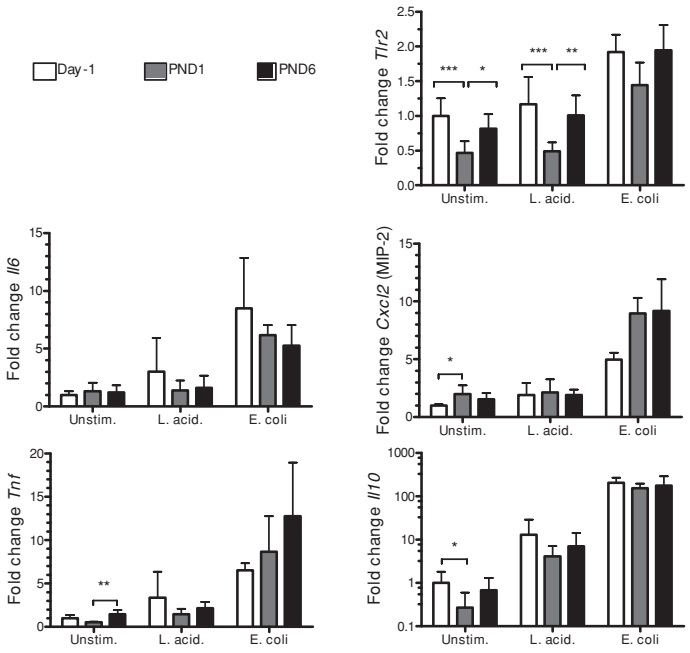

Figure 5 Age dependent gut maturation does not influence early responses of epithelial cells towards G+ and G- commensals. Epithelial cells from foetuses at Day-1 and neonates at post natal day (PND) 1 and PND6 from germfree mothers stimulated for 2 h with *L. acidophilus* NCFM or *E. coli* Nissle. Bars indicate mean and SD of 4 independent experiments with cells pooled from 6-10 pups (Day-1) or 2-3 pups (PND1 and PND6). ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. Data were normalised to *Actb* and then to the average of "Day -1 unstimulated" from the 4 experiments, which was defined to 1.

towards G^+ commensals in naïve primary IEC, which has not been reported before. Interestingly, *Clec7a* was down-regulated by *E. coli*, also pointing towards a yet undescribed cross-regulation of responsiveness to intestinal microorganisms. The induction of tolerance towards both G^+ and G^- MAMPs has been shown to depend on up-regulation of Tollip, which results in reduced phosphorylation of IL-1 receptor associated kinase (IRAK) and hence reduced NF-κB activation in both primary and immortalized IEC [10,25]. We measured expression of the genes encoding NFκB1, NFκB2, IKKβ (the IκB kinase), Tollip, and IRAK1. However, only *E. coli* induced up-regulation of *Nfkb1* and *Nfkb2* while expression of the other signalling proteins was not modulated by *in vitro* stimulation. Regulation of signalling relies on phosphorylation of the gene products, protein-protein interactions and protein translocation. Hence, transcriptional regulation is presumably more relevant for the responder cytokine and chemokine genes reported here.

During the first days of life, IEC develop and mature and crypts are formed. It has been demonstrated that germfree rats have impaired formation of crypt cells suggesting that the microbiota supports IEC growth and maturation [26]. *E. coli* Nissle, but not LPS, up-regulated *Tlr2* expression, which may indicate a role for commensals in establishing intestinal integrity [27]. The fact that *E. coli* Nissle changed the expression of more genes and acted more potently than *E. coli*-derived LPS reflects that intact *E. coli* does not, as opposed to LPS, exclusively signal through TLR4. Lotz et al. [19] report higher secretion of MIP-2 and KC upon *in vitro* LPS stimulation at day -1 before birth compared with PND1 and PND6 in mice harbouring a conventional microbiota due to tolerance acquisition at birth. However, their study does not take into account that age might influence IEC maturation stage. In order to evaluate how IEC develop with age we studied the IEC responsiveness in germfree pups at Day-1, PND1 and PND6. Except for a transient drop in *Tlr2* expression at PND1, we did not find strong age-dependent differences in the IEC response. The drop in *Tlr2* expression at PND1 may allow G^+ commensals to colonise the gut without concomitant danger signals. Based on these findings, foetal IEC isolated from conventional mice represent an attractive supplement to polarised IEC cell line models for comparison of commensal bacteria as they are naïve to stimulation while being physiologically immature IEC and susceptible to tolerance induction. However, as IEC responses are clearly dose-dependent, *in vivo* experiments are still required to reveal the extent of contact between IEC and bacteria or bacterial components.

Conclusions

Overall, our data confirm the hypothesis that the concomitant induction of chemokines, pro- and anti-inflammatory cytokines in enterocytes by the first-coming bacteria is indeed genus dependent. We conclude that *E. coli* and LPS may induce LPS-tolerance partly through very rapid and potent up-regulation of chemokine and cytokine genes and down-regulation of *Tlr4*, whereas stimulation by *L. acidophilus* Gram-positive commensals may be potentiated by the up-regulation of *Tlr2* by Gram-negative bacteria.

Methods

Preparation of UV-killed bacteria

L. acidophilus NCFM was grown anaerobically in de Man, Rogosa, and Sharpe broth (Merck, Darmstadt, Germany) and *E. coli* Nissle aerobically in Luria-Bertani broth (Merck) overnight at 37 C. The cultures were harvested, washed twice in sterile phosphate-buffered saline (PBS) (Lonza, Basel, Switzerland) and re-suspended in 1/10 the growth volume of PBS. The bacteria used for *in vitro* stimulation were killed by a 40-min exposure to UV-light and stored at -80 C, as we from earlier studies have concluded that live and UV-killed bacteria elicit similar responses in epithelial cell lines [21]. Concentration was determined by lyophilisation. Endotoxin levels in *L. acidophilus* NCFM preparations were determined with the Pyrochrome kit (Ass. of Cape Cod, East Falmouth, MA, USA) to < 0.10 EU/ml in the highest concentration of stimuli used in cell culture experiments.

Animal experiments

Conventional and germfree Swiss Webster mice were purchased from Taconic (Lille Skensved, Denmark), and housed under either specific pathogen-free conditions or in germfree isolators (as previously described [28]). Absence of colonising bacteria in germfree mice was confirmed by cultivation of faecal samples. Foetal IEC were isolated from foetuses derived from 4 conventional mothers. Caesarean section was performed on full-term pregnant females at gestation day 19 (referred to as Day -1), foetuses were killed immediately, and subsequently the small intestine was removed. Cells were pooled from 6-10 foetuses. Small intestinal tissue of neonatal mice was obtained from spontaneously delivered pups from germfree mothers at PND1 and PND6. Cells were pooled from 2-3 pups.

Isolation of primary epithelial cells

The small intestine was placed in Hanks buffered saline (HBSS, Lonza) and cut into small pieces. The epithelial cells were detached from the underlying tissue by incubation in fresh HBSS containing 2mM EDTA at 37°C for 10 minutes with vigorously shaking every 3 minutes. Residual tissue was removed by passing the suspension through a 70μm filter. Cells were subsequently washed in cold PBS and re-suspended in culture medium (RPMI 1640 supplemented with 100 U/ml penicillin, 100μg/ml streptomycin, 2 mM L-glutamine, 10% (v/v) heat-inactivated FCS, all from Lonza). Cells were seeded in 48-well tissue culture plates (Nunc, Roskilde, Denmark) at $4x10^5$cells/500 μl/well. Fifty μl/well of bacteria or LPS O26:B6 (Sigma-Aldrich, St. Louis, MO, USA) were then added to obtain final concentrations of 1, 10, 30 or 100 μg/ml as indicated. The stimulus concentrations were chosen based on optimization experiments showing that smaller amounts of *E. coli* Nissle than *L. acidophilus* NCFM were required for stimulation of IEC. The cells were incubated for 2 h, 4 h or 18 h at 37°C in 5% CO_2 and subsequently harvested by centrifugation and frozen in RNAlater (Qiagen, Hilden, Germany). The purity of the IEC was checked by staining for the lymphocyte marker CD45 (PE-labelled rat anti-mouse CD45 purchased from Abcam, Cambridge, UK) by flow cytometry. IEC contained 0.8 ± 0.4% $CD45^+$ cells at PND6 (staining with a matched isotype antibody (IgG2b) subtracted). Viability and cell numbers of IEC were determined by staining the cell nuclei with propidium iodide before and after cell lysis (reagents from Chemometec, Allerød, Denmark) and analysed with NucleoCounter (Chemometec). Viability of the IEC was evaluated during culture. We found that 23.5 ± 5.0% of freshly purified IEC were dead, 45.3 ± 2.9% after 2 h, 65.7 ± 6.5% after 4 h, 62.3 ± 6.6% after 7 h and 73.3 ± 7.6% after 24 h of culture.

RNA purification and amplification

Samples were spun at 3000 *g*, 5 min, 4°C to remove RNAlater. RNA was extracted from the cell pellet using Mini Kit from Qiagen following the supplier's protocol for animal cells. The quantity and purity of extracted RNA was evaluated by Nanodrop spectroscopy (Thermo Scientific, Wilmington, DE, USA). cDNA was produced from app. 500 ng total RNA using High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Foster City, CA, USA) according to the instructions of the manufacturer.

Gene expression analysis by real-time polymerase chain reaction

For quantitative real-time polymerase chain reaction (RT-PCR), TaqMan Arrays (384-well Micro Fluidic Cards) were designed with the 20 TaqMan Gene Expression Assays (Applied Biosystems) listed in Table 1, permitting 8 randomized samples tested in duplicates on each card. The genes studied were chosen based on experiments comparing expression of more

than 90 immune-related genes in intestinal cells of germfree and conventionally colonized mice (unpublished data). Genes with changed expression were included in the present study, as they were suspected to be affected by bacterial stimuli. To each cDNA sample (50 ng RNA in 50 μl) was added 50 μl TaqMan Universal PCR Master Mix (Applied Biosystems). Samples were mixed and loaded on the cards, which were centrifuged at 300 *g*, 1 min, 4°C and sealed. The PCR amplification was performed in standard mode using 7900HT Fast Real-time PCR system (Applied Biosystems). Additionally, single gene expression of *Cxcl2*, *Tnf*, *Il10*, *Il6* and *Actb* was analysed (TaqMan Gene Expression Assays listed in Table 1). For each sample, 2 μl cDNA (3 ng/μl) was amplified in duplicates under universal fast thermal cycling parameters (Applied Biosystems) using TaqMan Fast Universal PCR Master Mix (Applied Biosystems) in a total reaction volume of 10 μl. Relative quantification (fold increase) was calculated by the comparative C_T method. Briefly, C_T is the threshold cycle, which is the cycle number where the amplified target reaches the defined threshold. The expression is normalised to the expression of a reference gene [C_T = C_T(target)-C_T(reference)]. We evaluated *Actb* and 18S rRNA, which gave comparable results and chose to use *Actb* as reference gene. The efficiency of the PCR assays was tested by serial dilution of samples for 12 of the 24 genes on the TLDA arrays and was close to 100% (curve slopes between 3.3 and 3.4). Amplification specificity was similar for reference and target genes. The specificity of the assays was ensured by choosing intron-spanning TaqMan probes. In each dataset, a specific group of samples was used as calibrator (indicated in figure legends). Comparative gene expression was calculated as [C_T = C_T(target)- C_T(calibrator))] and fold change (2^{-CT}) values were plotted. Since C_T(calibrator) = 0, Fold change = 1 for the calibrator group.

Cytokine quantification in culture supernatants

The production of MIP-2, IL-10, -6, TNF-α was analysed using commercially available ELISA kits (R & D systems, Minneapolis, MN, USA).

Statistical analysis

GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA, USA) was used to perform two-way ANOVA with Bonferroni post-test. Although fold change is plotted in gene expression experiments, statistical analysis was performed on $C_T$values as these are

Table 1 Genes measured by Taqman low density array and (if bold also) by Taqman gene expression assays

Gene	Gene name	Protein	Assay ID
House keeping genes			
Actb	**actin, beta, cytoplasmic**	**β-Actin**	**Mm00607939_s1**
18S	eukaryotic 18S rRNA	-	Hs99999901_s1
Chemokines			
Ccl2	chemokine (C-C motif) ligand 2	MCP-1	Mm00441242_m1
Ccl3	chemokine (C-C motif) ligand 3	MIP-1α	Mm00441258_m1
Cxcl1	chemokine (C-X-C motif) ligand 1	KC	Mm00433859_m1
Cxcl2	**chemokine (C-X-C motif) ligand 2**	**MIP-2**	**Mm00436450_m1**
Cytokines			
Il10	**interleukin 10**	**IL-10**	**Mm00439616_m1**
Il6	**interleukin 6**	**IL-6**	**Mm00446190_m1**
Tgfb1	transforming growth factor, beta 1	TGF-β	Mm03024053_m1
Tnf	**tumor necrosis factor**	**TNF-α**	**Mm00443258_m1**
Tslp	thymic stromal lymphopoietin	TSLP	Mm00498739_m1
Regulation			
Nfkb1	nuclear factor of kappa light chain gene enhancer in B-cells 1	NFκB1	Mm00476361_m1
Nfkb2	nuclear factor of kappa light chain gene enhancer in B-cells 2	NFκB2	Mm00479807_m1
Ikbkb	inhibitor of kappaB kinase beta	IKKβ	Mm00833995_m1
Irak1	interleukin-1 receptor-associated kinase 1	IRAK1	Mm00434254_m1
Tollip	toll interacting protein	Tollip	Mm00445841_m1
Pattern recognition receptors			
Clec7a	c-type lectin domain family 7, member a	Dectin-1	Mm00490960_m1
Tlr2	**toll-like receptor 2**	**TLR2**	**Mm00442346_m1**
Tlr4	toll-like receptor 4	TLR4	Mm00445274_m1
Ly96	lymphocyte antigen 96	MD-2	Mm00444223_m1

assumed normally distributed as opposed to the fold change values.

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Authors' contributions
LHZ participated in the study, performed data analysis and wrote the manuscript. LNF and SBM designed and performed the study, carried out statistical analyses and edited the manuscript. MBK, TRL and CN participated in cell experiments and expression studies. HF participated in the design of the study and edited the manuscript. All authors read and approved the final manuscript.

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DISCUSSION

The aim of this PhD was to characterize the influence of early life microbiota on intestinal homeostasis and immune system maturation in the neonate by use of a germfree mouse model. Fetal, neonate and weaned mice, colonized at birth with maternal conventional microbiota, mono-colonized with *L. acidophilus* or *E. coli* or kept germfree, were compared for analyses of an age-dependent effect of colonization on cellular immune modulation and intestinal homeostasis.

The work of the PhD study are presented in four manuscripts (Paper 1-4), which covers various subjects from the microbial influence on splenic cellular development in the neonate mouse and early life hematopoiesis in the murine spleen and liver (Paper 1) to expression analyses of genes encoding mucins, TJ proteins, chemokines, cytokines and proteins of the TLR signaling pathway in in the intestinal tissue, liver and spleen of fetal and neonatal mice with differing colonization (Paper 2, 3 and 4).

A vast body of evidence supports the importance of a controlled sequential but yet suitably diverse colonization of the maturing neonate intestine for development of a balanced immune system and for establishment of intestinal homeostasis. Major knowledge gaps, however, remain about the mechanisms of microbial support of the developing homeostatic immune system.

In Paper 1 we described the presence of high levels of $CD11b^{+}Gr\text{-}1^{+}$ cells (47%) in the spleen at the day of birth. The high proportions of $CD11b^{+}Gr\text{-}1^{+}$ cells were present independently of microbial colonization of the neonate mouse. The proportion declined during the first week of life, but interestingly, this decline happened at a much higher rate in GF than in CONV neonate mice. Conventional colonization, thus, sustained the presence of $CD11b^{+}Gr\text{-}1^{+}$ cells in the CONV spleen during the first week of life. H&E staining of splenic tissues confirmed the presence of high levels of granular neutrophil-like cells with polylobed nuclei in the CONV neonate spleen, however also cells with ringshaped nuclei were scattered around in the tissue. Interestingly, microscopy of the corresponding neonate livers in the first week of life revealed markedly more hematopoietic tissue in the GF neonate livers compared to their CONV counterparts. In the GF livers granulocytic cells were scattered around in the tissue and tended to accumulate around the portal veins, while very few granulocytic cells were distinguished in the CONV livers during the first days of life.

HSC and HPC mobilize from the neonatal liver during perinatal phase to seed the spleen and BM. A characteristic rise in circulating neutrophil-like cells has been described in the peripheral blood stream of term born human infants and mice and is described to reach the highest level a few hours after birth. This neonate granulocytosis seems to be a part of the normal hematopoietic events in the neonate and gradually stabilizes, and reaches the level of adults during the first week of life [88, 111, 112]. As $CD11b^{+}Gr\text{-}1^{+}$ cells are naturally present as part of the hematopoietic progenitor cell pool [103], the dynamic mobilization of cells between the hematopoietic organs in the neonate,

which seemed markedly delayed or diminished in GF livers and spleens, indicated that colonization influenced hematopoietic events in the neonate. Assuming that the large proportion of $CD11b^{+}Gr\text{-}1^{+}$ cells, we observed in the neonate spleen of CONV and GF neonate mice, was rather immature myeloid cells or HPC of granular morphology, than mature neutrophils, these cells might be seeding the spleen as part of the natural hematopoietic accumulation of granular progenitor cells brought to the spleen as a wave of HPC on their way from liver to BM.

MIP-2 is a well described chemokine for recruitment of granulocytes during inflammatory conditions. Pelus *et al.* [91] describes that a transient induciton of MIP-2 *in vivo* induces a fast recruiment of polymorphnuclar leukocytes, activation of HSC and further an upregulation of CD11b on these cells. As we described a significantly higher expression of the MIP-2 encoding gene (*Cxcl2*) in livers of CONV mouse pups on the day of birth, when compared to GF counterparts, this supported the microscopy data and indicated that a stronger mobilization of hematopoietic tissue may take place in livers of CONV neonates compared to GF neonates. Paper 3, however, describes a significant reduction of the expression of *Cxcl2* in spleens and intstine of CONV mouse pups at the day of birth, compared to GF counterparts. These results can be seen as contradicting the increase in *Cxcl2* expression of CONV neonate livers as described in Paper 1. Yet the transient nature of the MIP-2 induction and the plural role of this chemokine may explain the opposing results obtained in spleen, intestine and liver of PND1 CONV neonate mice. Activation and mobilization of HSC in the neonate liver may take place cocomitantly to a lowered gene expression of *Cxcl2* in the spleen and intestine. For the latter tissues a reduced *Cxcl2* expression and subsequently lowered secretion of MIP-2 may be part of local tolerance establishment and avoidance of excessive immune responses from recruited phagocytic cells in this tissue. With the rapidly advancing number of intestinal microbes in mind and, thus, potential microbial stimulation, this mechanism may be important for maintenance of intestinal homeostasis in the newborn.

The FSC SSC distribution of the $CD11b^{+}Gr\text{-}1^{+}$ neonate spleen cells depicted in Paper 1 (Figure 3) revealed a heterogeneous distribution of this group in early life, containing at least three distinct but highly granular groups that visibly differed in size. This underlines that the neonatal spleen is not populated solely by mature neutrophils, or a homogeneous group of immature progenitor cells, but more likely harbors myeloid cells of differing maturation status, which are committed to the granulocytic lineage. The distribution of the heterogeneous $CD11b^{+}Gr\text{-}1^{+}$ cell group, being highly dominant in the neonate spleen, differs between the CONV and the GF mouse pup, as indicated in Figure 3B of Paper 1 (the original version of this figure is shown here as Figure 6 for clarity of discussion). The differing time course decline of the $CD11b^{+}Gr\text{-}1^{+}$ cells in the CONV and the GF spleens indicated that the presence of microbial colonization of the neonate mouse is involved in the accumulation and maintenance of these cells. While the subgroups of the CONV $CD11b^{+}Gr\text{-}1^{+}$ cells were maintained from before birth till at least PND4, a more dynamic shift between subgroups of

CD11b^{+}Gr-1^{+} cells were seen in the GF spleens for the same age. Considering the two-signal model proposed by Condamine and Gabrilovich [105], and with the reservation that this model is purely speculative, it is tempting to employ the two-signal model to elaborate on the differences between GF and CONV splenic CD11b^{+}Gr-1^{+} cellular subsets.

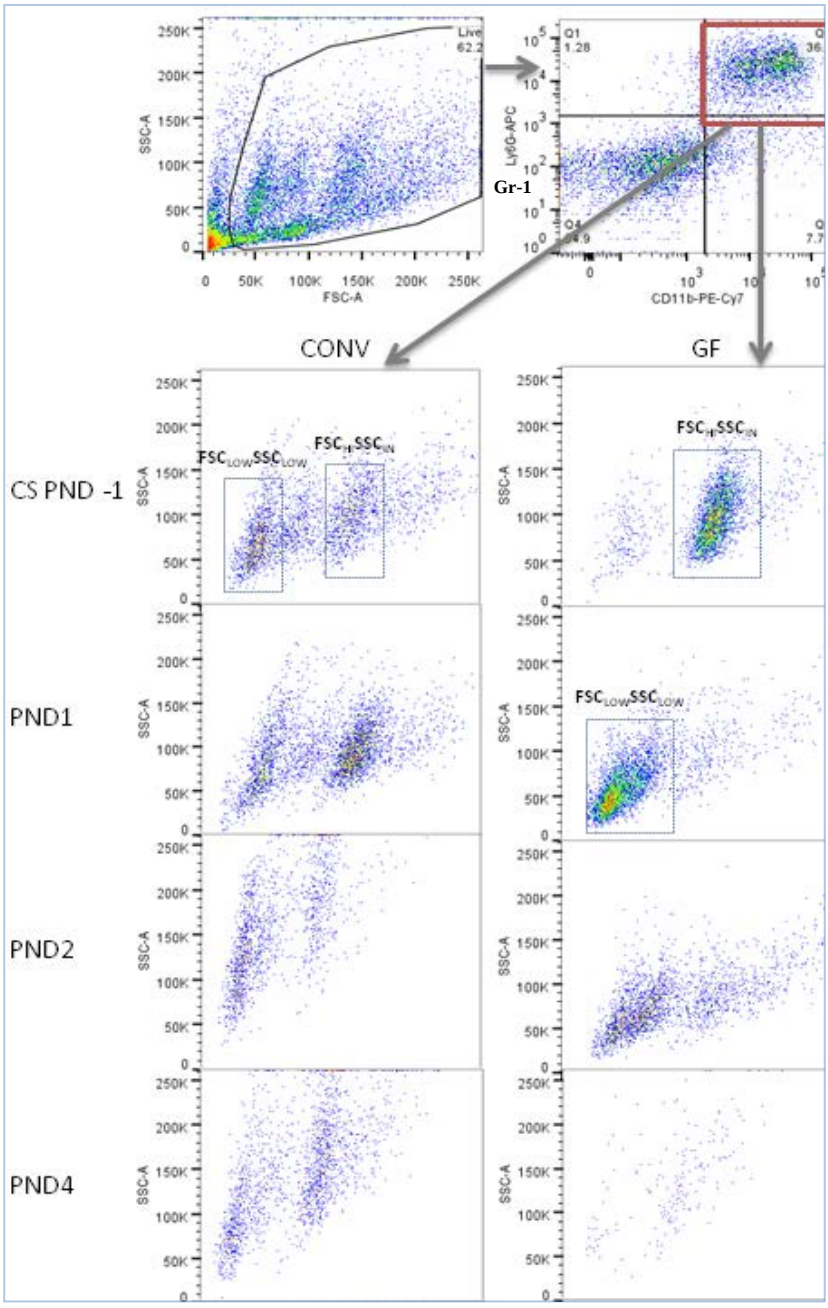

Figure 6: Flow cytometric analyses of the CD11b^{+}Gr-1^{+} cells in spleens of CONV and GF mouse pups from the day before birth (PND-1) to PND4. The spleen cells were gated first for viability and subsequently the group of CD11b^{+}Gr-1^{+} cells, shown in the red square, was analyzed with regard to their FSC SSC distribution. For details on animals, flow cytometric markers and gating see Paper 1 of this thesis.

Conditions for accumulation of the granular CD11b^{+}Gr-1^{+} cells are most likely present in the neonate mouse pup at birth independently of colonization, as the same proportion of CD11b^{+}Gr-1^{+} cells were measured by flow cytometry in both groups at this time, as described in Paper 1. More specifically the factors involved in the cellular accumulation could be a late gestational-birth induced increase in chemokines as GM-CSF and G-CSF, which are described as highly influential

on accumulation of HSC and HPC in humans and mice [91]. A differing ability for the GF and CONV splenic tissue to continuously support the presence of the $CD11b^{+}Gr\text{-}1^{+}$ cells was however indicated by the flow cytometric analyses of these cells (Figure 6), which in GF mice presented a dynamic turnover of $CD11b^{+}Gr\text{-}1^{+}$ cell groups during early life. Though speculative, it is tempting to suggest that presence of microbially induced pro-inflammatory cytokines in the CONV neonate very early in life may promote a subsequent activation and differentiation of the $CD11b^{+}Gr\text{-}1^{+}$ cells, which is not induced in the GF pup, due to lack of proper antigenic stimulation.

We have in our work not shown a higher pro-inflammatory potential of CONV neonate spleen cells, however, we have shown, in Paper 3, that CONV mice on the day of birth have an increased intestinal expression of genes encoding cytokines and proteins in the TLR signaling pathways, when compared to GF and MC neonate intestines. It should be emphasized that these results cannot interpret on the local cytokine environment in the spleen; however, it suggests that it is reasonable that the conventional microbiota induces a locally increased production of cytokines and microbe triggered signaling pathways in the local tissue relative to the induction caused by MC or GF intestinal tissue.

Recently several studies, briefly reviewed in this thesis, have shown that various cell groups identified in the neonate human or mouse are involved in immune regulation and suppression in early life. Among these, the description of granular MDSC in cord blood of human and mice suggests a role for neutrophil-like cells in early life immune regulation. Additionally, the described B helper-cell profile neutrophils and the $CD71^{+}$ erythroid cells were shown to be markedly promoted by the presence of neonate commensal colonization. These studies show that varying cellular subsets in the newborn have roles in early life immune regulation, and furthermore describe the immunosuppressive potential to depend on microbial colonization.

Whether the high proportions of $CD11b^{+}Gr\text{-}1^{+}$ cells in spleens of GF and CONV neonates are all potential immunosuppressive myeloid cells, mature neutrophils or, what is more likely, a mix of both these groups, is not revealed by the present work. Distribution of these cells in liver and spleen, their morphologic and phenotypic relatedness to MDSC, the time of arrival (birth) and the influence of the intestinal microbiota on the maturation of these $CD11b^{+}Gr\text{-}1^{+}$ cells are indicative of a potential role for these cells in early life immune regulation.

This assumption however, is relatively weak, if it was not supported by detailed descriptions made by scientists in the late 1970es of immune suppressive cells in newborn mice [121], and a study by Greifenberg *et al.* [117] describing $CD11b^{+}Gr\text{-}1^{+}$ cell subsets with strong immunosuppressive properties in the spleens of healthy adult mice, although the adult mice are described to harbor notably lower levels of neutrophil-like $CD11b^{+}Gr\text{-}1^{+}$ cells than the neonate mouse.

As indicated by the flow cytometric analyses in Figure 6 the $CD11b^{+}Gr\text{-}1^{+}$ subgroups vary between GF and CONV neonate spleens already before the mouse is born. This suggests a role of microbiota on these cells already during gestation and adds to the increasing evidence [15] of an influence of maternal and fetal microbiota on the unborn fetus.

With the newest findings on neutrophil-like MDSC in human cord blood, B cell helper neutrophils and $CD71^{+}$ erythrocytes in neonates, it seems likely that the neonate immune system orchestrate a range of different cell types, which serve as immune regulatory cells during the critical time of colonization and tolerance establishment just around birth.

CONCLUSION

- The expression of genes encoding epithelial tight junction proteins, held a delayed expression profile in GF intestinal tissue, when compared to regulation of these genes in the CONV intestinal tissue. This indicates microbial promotion of intestinal barrier establishment.
- A dominating group of $CD11b^{+}Gr\text{-}1^{+}$ cells were described in the neonate spleen on the day of birth independently of the nature of microbiota
- Conventional colonization of the newborn mouse prolongs the prominent presence of $CD11b^{+}Gr\text{-}1^{+}$ cells in the spleen of the neonate mouse, while in GF neonates the $CD11b^{+}Gr\text{-}1^{+}$ spleen cells are rapidly turned over.
- A heterogenous nature was demonstrated of the $CD11b^{+}Gr\text{-}1^{+}$ cells, showing that microbiota influenced presence and turnover rate of the various $CD11b^{+}Gr\text{-}1^{+}$ subgroups.
- Microbial colonization influenced hematopoietic mobilization of liver tissue in the neonate
- Gene expression profiles of intestinal epithelium revealed that tolerance establishment requires a complex conventional microbiota.

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